

## Chapter I. Isolation of Natural Products as Anticancer Drugs

### I.1 Introduction

Human beings have relied on natural products as a resource of drugs for thousands of years. Plant-based drugs have formed the basis of traditional medicine systems that have been used for centuries in many countries such as Egypt, China and India.<sup>1</sup>

Today plant-based drugs continue to play an essential role in health care. It has been estimated by the World Health Organization that 80% of the population of the world rely mainly on traditional medicines for their primary health care.<sup>2</sup> Natural products also play an important role in the health care of the remaining 20% people of the world, who mainly reside in developed countries. Currently at least 119 chemicals, derived from 90 plant species, can be considered as important drugs in one or more countries.<sup>3</sup> Studies in 1993 showed that plant-derived drugs represent about 25% of the American prescription drug market, and over 50% of the most prescribed drugs in the US had a natural product either as the drug or as the starting point in the synthesis or design of the agent.<sup>4</sup>

There are more than 250,000 species of higher plants in the world, and almost every plant species has a unique collection of secondary constituents distributed throughout its tissues. A proportion of these metabolites are likely to respond positively to an appropriate bioassay, however only a small percentage of them have been investigated for their potential value as drugs. In addition, much of the marine and microbial world is still unexplored, and there are plenty of bioactive compounds awaiting

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<sup>1</sup> Balandrin, N. F.; Kinghorn, A.D.; Farnsworth, N. R.; *Human Medicinal Agents from Plants*; Kinghorn, A. D.; Balandrin, N. F., Eds., ACS Symposium Series 534, **1993**, 2-12.

<sup>2</sup> Farnsworth, N.R.; Akerele, O.; Bingel, A.S.; Soejarto, D.D.; Guo, Z. *Bull. WHO*, **1985**, *63*, 965-972.

<sup>3</sup> Arvigo, R.; Balick, M.; *Rainforest Remedies*, Lotus Press, Twin Lakes **1993**.

<sup>4</sup> Grifo, F.; Newman, D. J.; Fairfield, A. S.; Bhattacharya, B.; Grupenhoff, J. T. *The Origin of Prescription Drugs*, Grifo, F. and Rosenthal, J. Eds., Island Press, Washington D.C **1997**: p 131

discovery in these two worlds. Besides their direct medicinal application, natural products can also serve as pharmacophores for the design, synthesis or semi-synthesis of novel substances for medical uses. The discovery of natural products is also important as a means to further refine systems of plant classification.

## **I.2 Natural Products as Anticancer Drugs.**

Cancer continues to be a great threat to human life. It causes the second highest mortality rate in the US, and every year about 1 million new cases of cancer are diagnosed in this country. Nearly one out of every four Americans will develop cancer during his or her life. The number of cancer deaths continued to increase from 1973 to 1990. In 1990 about 510,000 Americans died of cancer.<sup>5</sup> It is estimated that from 1970 to 1995, the US government has spent a total of approximately 30 billion dollars through the National Cancer Institute (NCI) on devising improved treatments for cancer.<sup>6</sup>

In the past twenty years, there has been a lot of progress in the war against cancer. Advances in cellular biology and molecular biology have helped us in understanding different mechanisms of this disease. More and more anticancer drugs and vaccines have been developed. Natural products have contributed significantly to the development of anticancer drugs. According to a recent review,<sup>7</sup> among the 79 FDA approved anticancer drugs and vaccines from 1983-2002, 9 of them were directly from the isolation of natural products and 21 of them were natural product derivatives. Also among the 39 synthetic

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<sup>5</sup> Cooper, G. M. *The Cancer Book*. Jones and Bartlett Publishers, Boston, MA **1993**, 7

<sup>6</sup> Pezzuto, J. M. Plant-derived anticancer agents. *Biochem. Pharmacol.* **1997**, 53, 121-133

<sup>7</sup> Cragg, G. M.; Newman, D. J. Snader, K. M. Natural Products as Sources of New Drugs over the Period 1981-2002. *J. Nat. Prod.* **2003**, 66, 1022-37

anticancer drugs, 13 of them were based on a pharmacophore originated from natural compounds.

### **I.3 The ICBG and NCDDG Programs.**

Two research programs support our studies of bioactive natural products, the ICBG and the NCDDG program.

The International Cooperative Biodiversity Group Program (ICBG program) was funded by the National Institutes of Health (NIH). This program focuses on the plants from two regions: the South American country Suriname (formerly Dutch Guiana) and the African country Madagascar. Both countries have previously been determined to be strategically important for biodiversity. The program has diverse goals in addition to those of natural product isolation or drug discovery. These additional goals include the development of alternative uses for natural resources, education, and economic development for the people of these countries. The research program at Virginia Tech focuses on the isolation and characterization of bioactive compounds, including those with anticancer, anti-malarial and anti-mycobacterial activities.

The National Cooperative Drug Development Group (NCDDG) Program was funded by the National Cancer Institute (NCI). This program focuses on the development of novel natural or synthetic compounds as anticancer agents. The work at Virginia Tech is primarily concerned with the isolation of new natural products with novel mechanisms of action. The extracts of these studies are drawn from the NCI repository of natural extracts and include both marine and plant extracts.

#### **I.4 The Bioassay Guided Isolation of Natural Products.**

The discovery of natural drugs is guided by bioassay. Bioassay plays a very important role in every step of the discovery program. First it can be used to detect the bioactivity of the crude extracts and thus guide the selection of extracts for further study. In the isolation steps the bioassay will guide the fraction of a crude sample towards the pure isolated compound. For these purposes, bioassay must be rapid, simple, reliable, reproducible and most important, predictive. It should also model a living organism well. Unfortunately, no bioassay can meet all of the above criteria. *In vivo* testing (such as on rats) can provide more valid data than *in vitro* cellular testing; however, animal testing is complicated, slow and expensive, and is normally only used on pure compounds that have demonstrated *in vitro* activity.

Currently there are a large number of available bioassay systems in the area of anticancer drugs, divided into two groups; cellular assays and cell-free assays. Cellular assays utilize intact cells (yeast cells, mammalian cells, etc.) while cell-free assays utilize isolated systems (enzymes, DNA fragments, etc.) for bioactivity study. These cell-free assays are usually mechanism-based, with a key enzyme or other biomolecule as the target.

Cytotoxicity assays are very commonly used in cellular assays. Since cytotoxicity is an activity that is consistent with anticancer activity, the major advantage of cytotoxicity assays is that all potential mechanisms of cellular proliferation can be monitored simultaneously. Thus, the search for new anti-cancer reagents in the past has been primarily focused on extracts showing cytotoxicity to one or two cell lines. The approach has been fruitful and led to the discovery of paclitaxel, among many other

compounds. Cytotoxicity-based assays are normally reported as  $IC_{50}$  values (the concentration of a sample that can inhibit 50% growth of a target cell in a single cell line). The cell line employed in the cytotoxicity assay of our group is the A2780 human ovarian cancer cell line. The A2780 assay is a general cytotoxicity assay, which means that in many cases the active compound will simply be toxic, and thus will not be suitable for drug use.

The use of cell-free mechanism-based assays is a second approach to drug discovery. These assays utilize isolated assay systems (cellular receptor, enzyme, etc.) to test the bioactivity. Basically these assays are designed to test the unknown extract, fraction, or pure compound in comparison to known antitumor agents in mechanisms that have been clearly delineated. Mechanism-based assays are very selective and sensitive and also reproducible. An important advantage of these assays is that once a lead compound is discovered, its mechanism of action is already known, and lead optimization can thus be carried out more efficiently. Because of these advantages, several mechanism-based assays are currently employed in the NCDDG project, such as assays for inhibitors of Akt-kinase, Myt-1 kinase and DNA polymerase  $\beta$  (pol- $\beta$ ) assay. If a novel compound is found with a similar effect to a known specific compound, it can be classified to the specific mechanistic class. This approach can lead to a more systematic method to discover new anticancer drugs.

## **I.5 Mechanism Based Bioassays Employed in the NCDDG Program.**

### **I.5.1. The Akt-kinase Bioassay.**

Akt (protein kinase B), a serine/threonine kinase, is a critical enzyme in signal transduction pathways involved in cell proliferation, apoptosis and angiogenesis. Akt kinase, together with another kinase (p53), play opposing roles in signaling pathways that determine cell survival.<sup>8</sup> In mammalian cells three forms of the Akt enzyme (Akt-a, b, g or Akt-1, 2, 3) are reported that exhibit a high degree of homology, but differ slightly in the localization of their regulatory phosphorylation sites. The principal role of Akt is to facilitate growth factor-mediated cell survival and to block apoptotic cell death, which is achieved by phosphorylating several pro-apoptotic factors.<sup>9</sup> For example, Akt-a is expressed to various degrees in breast cancer cell lines and is important in estrogen-stimulated growth. Treatment of multiple cancer cell lines with the Akt inhibitors could result in reduced survival of both drug resistant and drug sensitive cells.<sup>10</sup> Therefore, searching for Akt-inhibitors from natural products could be a useful method for anti-cancer drug development.

### **I.5.2 Myt-1 Kinase Bioassay.**

Myt1 kinase belongs to a unique class of dual-specificity kinases (DSKs). Myt1 kinase phosphorylates adjacent threonine-14 (T14) and tyrosine-15 (Y15) residues in Cdk/Cyclin complexes (Cdc2-kinase), which is a key modulator enzyme for the timing of cell to enter mitosis stage. The activation of Cdc2 at the G2-M transition is triggered by

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<sup>8</sup> Sabbatini P; McCormick, F. Phosphoinositide 3-OH kinase (PI3K) and PKB/Akt delay the onset of p53-mediated, transcriptionally dependent apoptosis. *J. Biol. Chem.* **1999**, 274, 24263-269.

<sup>9</sup> Mayo, L. D.; Donner, D. B. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proc. Nat. Acad. Sci.* **2001**, 98, 11598-607.

<sup>10</sup> Kozikowski A. P.; Sun, H.; Brognard, J.; Dennis P. A novel PI analogs selectively block activation of the pro-survival serine/threonine kinase Akt. *J. Am. Chem. Soc.* **2003**, 125, 1144-49.

dephosphorylation at Y15 and the level of dephosphorylation at Y15 is controlled by two protein kinases, Wee1 and Myt-1, which act in opposite ways to control the activity of Cdc2.<sup>11,12</sup> Inhibitory phosphorylation of Cdc2 by Myt-1 kinase is important for the activity of Cdc2.<sup>13,14</sup> Inhibition of Myt-1 kinase would cause the premature activation of Cdc2, which would lead to mitotic catastrophe and cell death. Thus, inhibition of Myt-1 kinase might be a new way of cancer treatment.

The Myt1 kinase assays were carried out by our collaborator Ms. Marni Brisson in Dr. John Lazo's group at the University of Pittsburgh.

### **I.5.3 DNA Polymerase $\beta$ (Pol- $\beta$ ) Bioassay.**

The DNA polymerase  $\beta$  (pol- $\beta$ ) assay was developed to aid in the search of natural products as DNA polymerase  $\beta$  inhibitors. The bio-function of the enzyme DNA polymerase beta (pol- $\beta$ ) is to repair the DNA damage inflicted on DNA in tumor cells by antitumor agents, such as bleomycin and cis-platin.<sup>15</sup> This enzyme repairs single nucleotide gaps in DNA which are produced by the base excision repair pathway of mammalian cells. It was found that cancer cell lines with overexpressed pol- $\beta$  displayed a decreased sensitivity to cancer chemotherapeutics and DNA-damaging agents such as

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<sup>11</sup> Mueller, P. R.; Coleman, T. R.; Kumagai, A.; Dunphy, W. G. Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. *Science* **1995**, *270*, 86-91.

<sup>12</sup> Wells, N.J., Watanabe, N., Tokusumi, T., Jiang, W., Verdecia, M.A. and Hunter, T. The C-terminal domain of the Cdc2 inhibitory kinase Myt1 interacts with Cdc2 complexes and is required for inhibition of G(2)/M progression. *J. Cell Science* **1999**, *112*, 3361-73.

<sup>13</sup> Liu, F., Stanton, J.J., Wu, Z. and Piwnicka-Worms, H. The human Myt1 kinase preferentially phosphorylates Cdc2 on threonine 14 and localizes to the endoplasmic reticulum and Golgi complex. *Mol. Cell. Biol.* **1997**, *17*, 571-579.

<sup>14</sup> Booher, R.N., Holman, P.S. and Fattaey, A. Human Myt1 is a cell cycle-regulated kinase that inhibits Cdc2 but not Cdk2 activity. *J. Biol. Chem.* **1997**, *272*, 22300-305.

<sup>15</sup> Hoffmann, J. S.; Pillaire, M. J.; Garcia-Estefania, D.; Lapalu, S.; Villani, G. In vitro bypass replication of the cisplatin-d(GpG) lesion by calf thymus DNA polymerase  $\beta$  and human immunodeficiency virus type I reverse transcriptase is highly mutagenic. *J. Biol. Chem.* **1996**, *26*, 271-279.

cisplatin and mechlorethamine.<sup>16,17</sup> Thus, inhibitors of this enzyme may block this repair and thereby enhance the activity of therapeutically employed DNA damaging agents.

The pol- $\beta$  assay was carried out by our collaborator Dr. Gao Zhijie in Dr. Sidney Hecht's group at the University of Virginia.

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<sup>16</sup> Canitrot, Y.; Cazaux, C.; Frechet, M.; Bouayadi, K.; Lesca, C.; Salles, B.; Hoffmann, J. S., Overexpression of DNA polymerase  $\beta$  in cell results in a mutator phenotype and a decreased sensitivity to anticancer drugs. *Proc. Nat. Acad. Sci.* **1998**, *95*, 12586-12594.

<sup>17</sup> Moynihan, K.; Elion, G. B.; Ali-Osman, F.; Marcelli, S.; Keir, S.; Bigner, D. D.; Friedman, H. S. Enhancement of melphalan activity by inhibition of DNA polymerase- $\alpha$  and DNA polymerase- $\beta$ . *Cancer Chemo. Pharm.* **1996**, *38*, 349-354.

## Chapter II. Isolation of bioactive compounds from *Cryptocarya crassifolia*

### II.1 Introduction.

As part of our ICBG program to isolate bioactive antitumor compounds from terrestrial plants, methanol extracts of the fruit and bark of a southern African laureate tree, *Cryptocarya crassifolia* (Lauraceae) from Madagascar, were found to display weak biological activity versus the A2780 mammalian cell line. A number of bioactive compounds including two known caryalactones (**2.1**) and (**2.2**), and two known flavonoids (**2.14**) and (**2.15**) were isolated. All the compounds were characterized by spectral analysis and comparison with the published literature data.

### II.2 Chemical and biological investigation of *Cryptocarya crassifolia*.

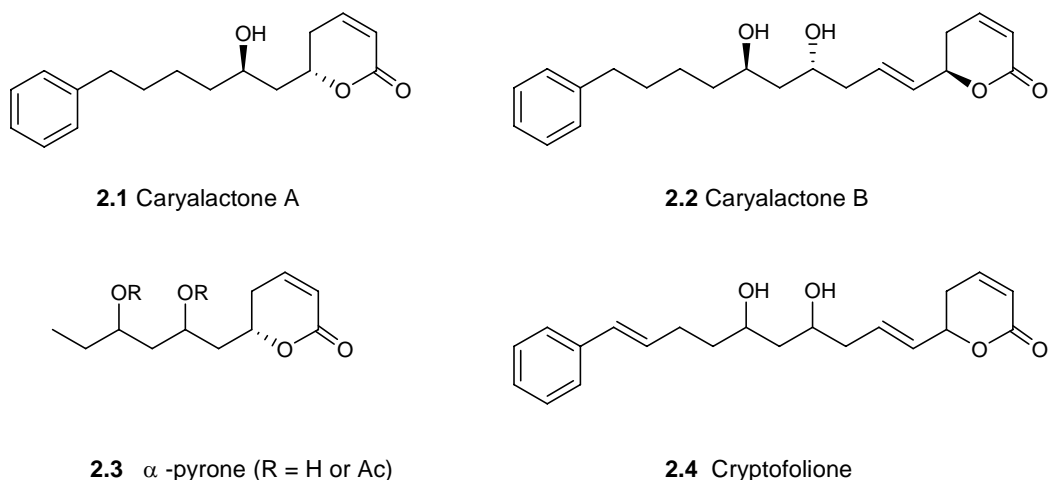
A large number of research studies have been carried out on different *Cryptocarya* plants, with more than 30 types of compounds reported. These compounds include cryptocaryalactones, terpenoids, steroidal alkaloids and flavonoids, etc. The plant *Cryptocarya crassifolia* was also called *Ravensara crassifolia* in some references.<sup>18,19</sup> It is a laureate tree up to 18-20 m high growing mainly in the eastern region of Madagascar. The genus *Ravensara* is endemic to Madagascar and plants of this genus have been used in traditional medicine as treatment of some skin diseases. In 2001, Raelison *et al.* studied the stem bark of this plant and isolated two weakly anti-fungal active caryalactones, compounds **2.1** and **2.2** (Figure 2-1).<sup>20</sup>

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<sup>18</sup> Queiroz, E. F.; Wolfender, J. L.; Raelison, G.; Hostettmann, K., Determination of the absolute configuration of 6-alkylated -pyrones from *Ravensara crassifolia* by LC-NMR. *Phytochem. Anal.* **2003**, *14*, 34-45.

<sup>19</sup> Chandrasekhar, S.; Narsihmulu, C.; Sultana, S. S.; Reddy, M. S., The first stereoselective total synthesis of (6S)-5,6-dihydro-6-[(2R)-2-hydroxy-6-phenylhexyl]-2H-pyran-2-one. *Tetrahedron Lett.* **2004**, *45*, 9299-9301.

<sup>20</sup> Raelison, G. E.; Terreaux, C.; Queiroz, E. F.; Zsila, F.; Simonyi, M.; Antus, S.; Randriantsoa, A.; Hostettmann, K., Absolute configuration of two new 6-alkylated- $\alpha$ -pyrones (2H-pyran-2-ones) from *Ravensara crassifolia*. *Helv. Chim. Acta.* **2001**, *84*, 3470-3476



**Figure 2-1.** Caryalactones from *Cryptocarya* species.

### II.3 Structure and bioactivities of cryptocaryalactones.

Cryptocaryalactones (also called caryalactones) are  $\alpha$ -pyrone (**2.3**) derivatives which are found in almost all the *Cryptocarya* plants (Figure 2-1). More than 130 different cryptocaryalactones have been reported. The indigenous *Cryptocarya* plant growing in southern Africa, *Cryptocarya lactifolia*, is considered the richest  $\alpha$ -pyrone source among the higher plants.<sup>21</sup> The most commonly seen cryptocaryalactone, cryptofolione (**2.4**), is abundant in the bark of *Cryptocarya myrtifolia*, comprising up to 0.9% wt based on the mass of dry material. Generally cryptocaryalactones contain a linear polyketide chain with a 2-pyrone ring at one end and a *trans*-styrenyl group at the other end. A literature search indicated that no significant bioactivities of cryptocaryalactones against human tumor cells have been reported. The antifungal or antimicrobial activities of these compounds have been investigated, but the results

<sup>21</sup> Drewes, S. E.; Sehlapelo, B. M.; Horn, M., 5,6-Dihydro- $\alpha$ -pyrones and two bicyclic tetrahydro- $\alpha$ -pyrone derivatives from *Cryptocarya latifolia*. *Phytochemistry* **1995**, 38, 1427-1430.

showed they were not active enough to be lead compounds.<sup>20,22</sup> Previous studies on cryptocaryalactones were mainly carried out because of a phytochemical interest in *Cryptocarya* plants, rather than because of pharmaceutical interest in active compounds.

The configurations of the hydroxyl groups in cryptocaryalactones do not follow any defined pattern, since several natural diastereomers have been reported for most caryalactones. For example, the (6*R*,2*S*) (**2.5**) or (6*S*,2*R*) (**2.6**) and (6*R*,2*R*) (**2.7**) isomers of the known cryptocaryalactone D have been reported as natural products, and the absolute stereochemistry of each chiral center was determined by making conventional Mosher esters derivatives.<sup>23,24,25,26</sup> These three isomers, together with the synthetic (6*S*,2*S*) isomer (**2.8**),<sup>27</sup> all have significantly different optical rotation values (Figure 2-2). Interestingly, the two enantiomers **2.7** and **2.8** did not show exactly opposite optical rotation values in the literature, perhaps because of impurities in the isolated natural compound **2.7**.

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<sup>22</sup> Raharivelomanana, P. J.; Terrom, G. P.; Bianchini, J. P.; Coulanges, P., Study of the antimicrobial action of various essential oils extracted from Malagasy plants. II: Lauraceae. *Arch. Inst. Past. Madagascar*. **1989**, *56*, 261-265.

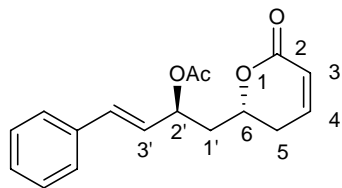
<sup>23</sup> Govindachari, T. R.; Parthasarathy, P. C. and Modi, J. D. *Indian. J. Chem.* **1972**, *10*, 149-153.

<sup>24</sup> Spencer, G. F., England, R. E. and Wolf, R. B., *Phytochemistry*, **1984**, *23*, 2499-2512.

<sup>25</sup> Drewes, S. E., Horn, M. M. and Scott-Shaw, R.,  $\alpha$ -Pyrone and their derivatives from two *Cryptocarya* species. *Phytochemistry*, **1995**, *40*, 321-3.

<sup>26</sup> Drewes, S. E.; Horn, M. M.; Ramesar, N. S.; Ferreira, D.; Nel, R. J.; Hutchings, A., Absolute configurations of all four stereoisomers of cryptocaryalactone and deacetylcryptocaryalactone. *Phytochemistry* **1998**, *49*, 1683-1687

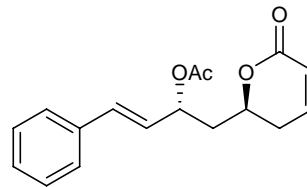
<sup>27</sup> Meyer, H. H., Synthesis of cryptocaryalactone, *Lieb. Ann. Chem.* **1984**, 977-981.



**2.5** Caryalactone (6*R*,2'*S*)

Source: *Cryptocarya bourdelloni*

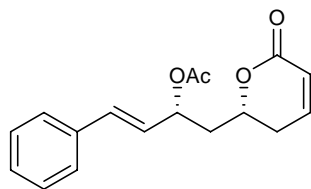
$[\alpha]_D = +19^0$



**2.6** Caryalactone (6*S*,2'*R*)

Source: *Cryptocarya moschata*

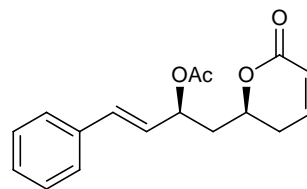
$[\alpha]_D = -20^0$



**2.7** Caryalactone (6*R*, 2'*R*)

Source: *Cryptocarya wylei*

$[\alpha]_D = +62^0$



**2.8** Caryalactone (6*S*, 2'*S*)

Source: Synthetic

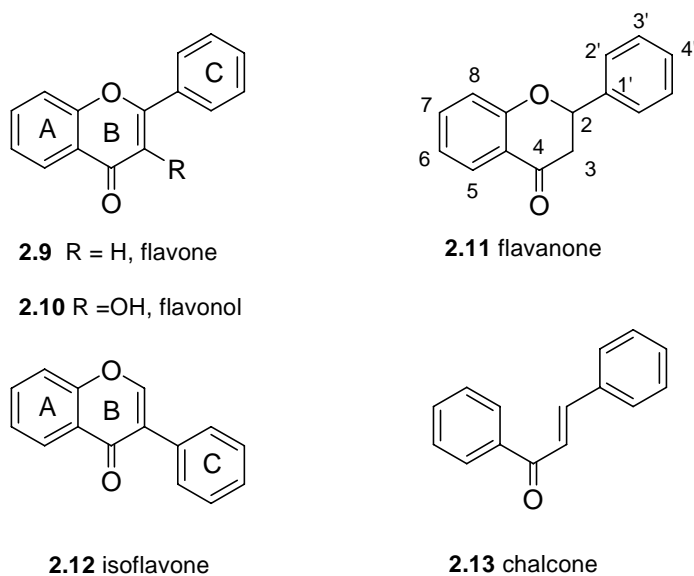
$[\alpha]_D = -75^0$

**Figure 2-2** Optical rotations of natural and synthetic diastereomers of caryalactones

## II.4 Introduction to the structure and bioactivities of flavonoids.

Flavonoids are among the most widely distributed natural products in the plant world. They are also among the earliest natural compounds that have been studied. Flavonoids are present in almost all kinds of terrestrial plants, and today more than 2000 flavonoid compounds have been reported. Because of the complexity of the flavonoid family, it is not possible to show all the structures here. Generally flavonoids can be divided into flavones (**2.9**), flavanols (**2.10**), flavanones (**2.11**), isoflavones (**2.12**), and chalcones (**2.13**) (Figure 2-3). Flavonoids usually have a three-ring system consisting of a cinnamoyl-based B,C-ring and a benzenoid A-ring. All three rings can be substituted with

hydroxyl groups, methoxyl groups, or other substituents, leading to a large number of possible structures.



**Figure 2-3** Five major types of flavonoids

The bioactivities of flavonoids are also very broad, and include anti-bacterial, anti-malarial, and anti-fungal activities.<sup>28,29,30,31</sup> Some flavonoid compounds have been used as supplemental medicines or vitamins for a long time. For example, catechin, an important medicinal component in green tea, was shown to be helpful in the treatment of viral hepatitis.<sup>32</sup> It also appears to prevent oxidative damage to the heart, kidney, lungs, and spleen. Preliminary studies on animals show that catechin prevents oxidative damage

<sup>28</sup> Nkunya, M. H.; Waibel, R.; Achenbach, H. Antimalarials and other constituents of plants of the Genus *Uvaria*. Three flavonoids from the stem bark of the antimalarial *Uvaria dependens*. *Phytochemistry* **1993**, *34*, 853-6.

<sup>29</sup> Bizagos, E.; Crance, J. M.; Passagot, J.; Deloince, R. Effect of antiviral substances on hepatitis A virus replication in vitro. *J. Med. Virol.* **1987**, *22*, 57-66.

<sup>30</sup> Murakami, N.; Mostaqul, H. M.; Tamura, S.; Itagaki, S.; Horii, T.; Kobayashi, M. New anti-malarial flavonol glycoside from *hydrangeae dulcis folium* *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2445-2447

<sup>31</sup> Roy, R.; Pandey, V. B.; Singh, U. P.; Prithiviraj, B. Antifungal activity of the flavonoids from *Clerodendron infortunatum* roots. *Fitoterapia* **1996**, *67*, 473-474.

<sup>32</sup> Nicole A; Fasel-Felley, J.; Perrissoud, D.; Frei, P. C. Influence of palmitoyl-3-catechin and heptyl-3-catechin on the leucocyte migration inhibition test carried out in the presence of PPD and hepatitis B surface antigen (HBsAg). *Int. J. Immunopharm.* **1985**, *7*, 87-92.

to blood as well. Some other flavonoid drugs, such as rutin<sup>33</sup> (blood-pressure-reducing drug) and nevadensin<sup>34</sup> (anti-inflammatory drug and antioxidant) also play important roles in the medicinal area. A lot of flavonoid compounds, such as quercetin and rutin, have been found to have antitumor activities due to the inhibition of DNA-topoisomerase I.<sup>35</sup> However, their activities were not potent enough for these compounds to be anticancer drugs.

## II.5 UV spectral analysis of flavonoids.

UV spectroscopy has become a major technique for the structural analysis of flavonoids for two reasons. First, the UV spectra of different types of flavones are usually different, and thus these spectra can be used to identify the structure class. Second, the information from the UV spectra of flavonoids can be considerably enhanced by the use of certain UV-shift reagents. The commonly used UV-shift reagents are aluminum chloride (AlCl<sub>3</sub>), sodium methoxide (NaOMe), sodium acetate (NaOAc), and boric acid (H<sub>3</sub>BO<sub>3</sub>). The preparation and use of these reagents has been described by Mabry *et al.* in 1970.<sup>36</sup>

The UV-spectra of most flavonoids consist of two major absorption bands, one of which occurs in the range of 245-285 nm (Band II), the other in the range of 300-380 nm (Band I). Usually Band II has stronger intensity than Band I for most known flavonoids except chalcones, which have a relatively stronger intensity in Band I than in Band II.

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<sup>33</sup> Ahmad, M.; Gilani, A. H.; Aftab, K.; Ahmad, V. U. Effects of kaempferol-3-O-rutinoside on rat blood pressure. *Phytother. Res.* **1993**, *7*, 314-316.

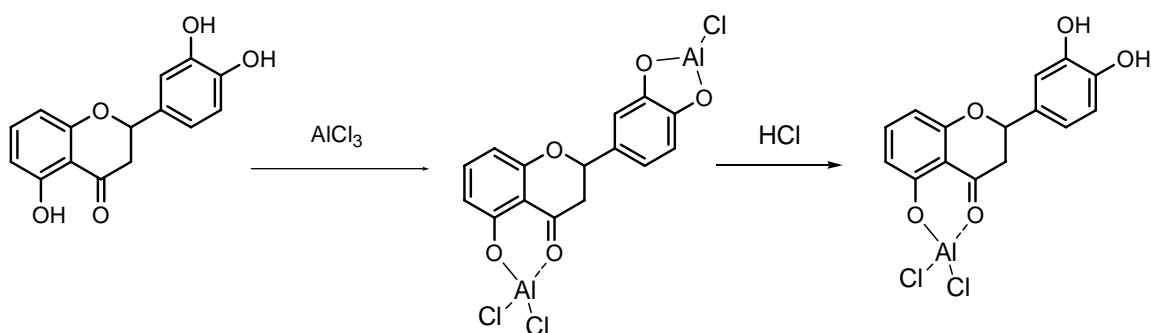
<sup>34</sup> Suksamrarn, A.; Poomsing, P.; Aroonrerk, N.; Punjanon, T.; Suksamrarn, S.; Kongkun, S. Antimycobacterial and antioxidant flavones from *Limnophila geoffrayi*. *Arch. Pharm. Res.* **2003**, *26*, 816-820.

<sup>35</sup> Jacobasch, G.; Raab, B.; Pforte, H.; Salomon, A. Anticancer formulations with flavonols or flavonoids. *Ger. Offen.* **1999**, *4*

<sup>36</sup> Mabry, T. J. and Harborne, J. B., In *The Systematic Identification of Flavonoids*. **1970**, Springer, New York, Heidelberg, Berlin.

Theoretically Band II absorption can be considered as originating from the A-ring and Band I could be considered as originating from the C-ring.<sup>37</sup>

When a flavonoid compound has a hydroxyl group at C-5 on the A-ring, it can form a stable complex with  $\text{Al}^{3+}$  ion, and this complex is stable to hydrochloric acid. Although sometimes *ortho*-dihydroxyl groups in flavonoids can also form complexes with  $\text{Al}^{3+}$  ion, these complexes can be destroyed by hydrochloric acid (Figure 2-4). In the UV-spectrum of  $\text{Al}^{3+}$  chelated 5-hydroxy-4-keto flavonoids, both absorption Band II and Band I will be shifted to longer wavelength by 35-60 nm. Aluminum chloride is thus commonly used to examine the presence of C-5 hydroxyl groups in an unknown flavonoid compound.



**Figure 2-4.** Stable complex of  $\text{Al}^{3+}$  chelated flavonoids

All hydroxyl groups on a flavonoid nucleus can be easily deprotonated by a strong base such as NaOMe. When a flavonoid compound has a hydroxyl group on the C-4' position of the C-ring, treatment with NaOMe will shift the UV absorption Band I to longer wavelength by 40-60 nm without reducing intensity. Absorption Band II will not be affected by this treatment. For a flavanol (**2.9**) type compound that has a hydroxyl

<sup>37</sup> Harborne, J. B. In *The Flavonoids*. 1975, Chapman & Hall, London.

groups at C-3 of the B ring, treatment with NaOMe will also shift the UV absorption Band I to longer wavelength by 40-60 nm, but the intensity of Band I will decrease considerably. Therefore, the shift reagent sodium methoxide is commonly used to examine the presence of C-3 or C-4' hydroxyl group of a flavonoid compound. These two shift reagents, sodium methoxide and aluminum chloride, will be used frequently in our structural elucidation of flavonoid-related compounds.<sup>38</sup>

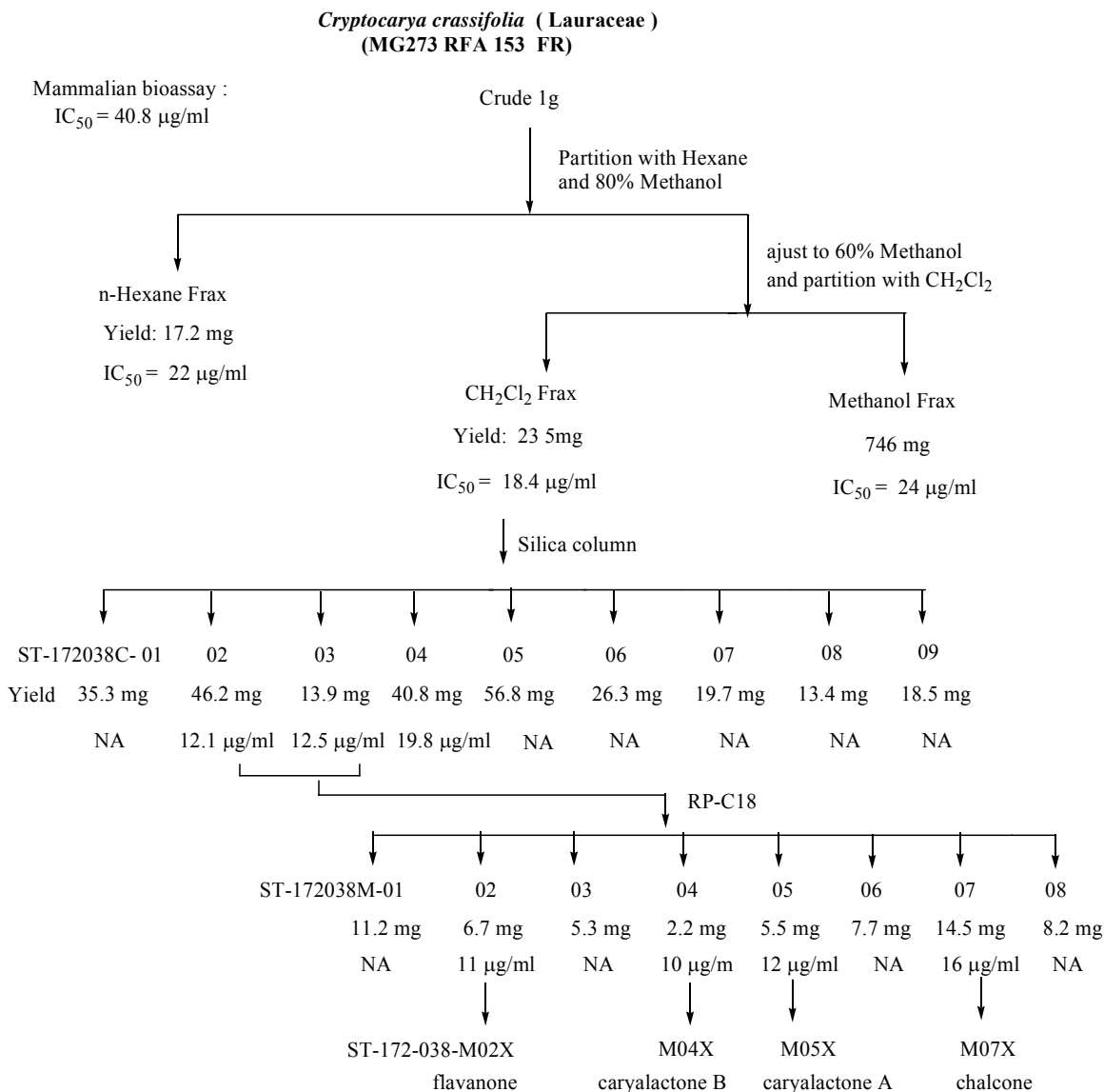
## II.6 Results and Discussion.

An extract of the fruit of the plant *Cryptocarya crassifolia* was found to display weak bioactivity against the A2780 human ovarian cancer cell lines ( $IC_{50} = 40.8 \mu\text{g/mL}$ ). The crude extract (1 g) was partitioned between 80% aqueous MeOH and hexanes (Scheme 2-1). The aqueous MeOH fraction was then diluted to 60% MeOH with water and extracted with  $\text{CH}_2\text{Cl}_2$ . The  $\text{CH}_2\text{Cl}_2$  fraction was found to be the most active fraction after bioassay. This fraction was then subjected to silica column chromatography with elution with a gradient of  $\text{CHCl}_3$  to  $\text{CHCl}_3/\text{MeOH}$  (1:1). The two most active fractions were further separated by reverse phase C-18 chromatography (aqueous MeOH gradient from 60% to 100% MeOH) which gave four major active compounds, numbered as ST-172038-M02, M04, M05, M07. Each fraction was further purified by RP-C18 HPLC to give pure compound ST-172038-M02X, -M04X, -M05X, and -M07X.

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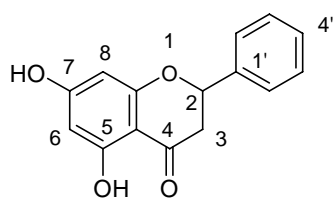
<sup>38</sup> See Chapter 3

**Scheme 2-1:** Isolation tree of the fruit extract of *Cryptocarya crassifolia*

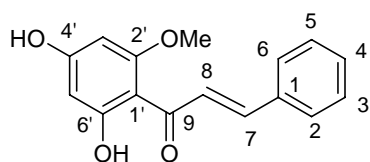


Compound ST-172038-M02X was isolated as an optically inactive yellow solid. Its UV spectrum indicated that it was a flavanone by the presence of characteristic absorption bands at 275 nm (band II) and 324 nm (band I). Its <sup>1</sup>H NMR spectrum also showed the characteristics of a flavanone compound with one proton at δ 5.24 (dd, *J* = 11.0 and 5.5 Hz, H-2) and a pair of methylene protons at δ 2.80 (dd, *J* = 17.0 and 5.5 Hz,

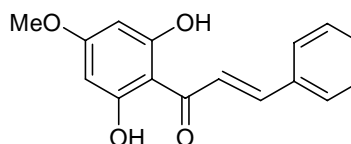
H-<sub>3a</sub>) and  $\delta$  3.21 (dd,  $J = 17.0$  and  $5.5$  Hz, H-<sub>3b</sub>). A low resolution FAB-MS indicated a molecular weight of 256.1, consistent with a composition C<sub>15</sub>H<sub>12</sub>O<sub>4</sub>. The presence of two hydroxyl groups on C-5 and C-7 of the A-ring was evidenced by <sup>1</sup>H NMR signals for a downfield phenolic proton at 12.05 ppm (C-5 OH) and two aromatic proton signals at  $\delta$  6.01 (2H, overlapped, H-6 and H-8). These facts suggested that compound ST-172038-M02X was the common flavanone, ( $\pm$ )-pinocembrin (**2.14**), (5,7-dihydroxy-flavanone) (Figure 2-5). The <sup>13</sup>C NMR data of ST-172038-M02X also matched literature data (Table 2.1).<sup>39</sup> This flavanone **2.14** was synthesized by Rosenmund in 1928,<sup>40</sup> and also isolated from a *Pinus* plant in 1948.<sup>41,42</sup>



**2.14** Pinocembrin



**2.15** Cardamonin



**2.16** 4'-Methoxy-2',6'-dihydroxy-chalcone

**Figure 2-5** Structure of the flavonoids isolated from the fruit extract.

<sup>39</sup> Miyakado, M.; Kato, T.; Ohno, N.; Mabry, T. J. Pinocembrin and eudesmol from *Hymenoclea monogyra* and *Baccharis glutinosa*. *Phytochemistry* **1976**, *15*, 846-852.

<sup>40</sup> Rosenmund, K. W.; Rosenmund, M. Synthesis of naringenin and phloretin. *Chem. Ber.* **1928**, *61B*, 2608-12.

<sup>41</sup> Lindstedt, G. Constituents of pine heartwood. IX. Heartwood of *Pinus montana*. *Acta. Chem. Scand.* **1949**, *3*, 755-758.

<sup>42</sup> Narasimhachari, N.; Seshadri, T. R. A new effect of hydrogen bond formation. Chelation and stability of flavanones. *Proc. Indian. Acad. Sci.* **1948**, *27A*, 223-39.

**Table 2-1** Comparison of the  $^{13}\text{C}$  NMR data of ST-172-038-M02X with literature data

	ST-172038-M02X <sup>a</sup>	Pinocembrin <sup>b</sup> (Lit) <sup>39</sup>		ST-172038-M02X	Pinocembrin
C-2	78.6	78.4	C-9	162.8	162.7
C-3	42.5	42.2	C-10	102.1	101.9
C-4	196.0	195.8	C-1'	138.2	138.0
C-5	163.7	163.6	C-2',6'	126.8	126.5
C-6	96.4	96.1	C-3',5'	128.5	128.5
C-7	166.9	166.6	C-4'	128.6	128.5
C-8	95.2	95.1			

<sup>a</sup> DMSO-*d*<sub>6</sub> 100 MHz, <sup>b</sup> DMSO-*d*<sub>6</sub> 75 MHz.

Compound ST-172038-M07X was also isolated as a yellow solid. Its UV spectrum indicated it was a chalcone derivative by its characteristic very strong absorption Band I at 350 nm and a weak absorption Band II at 285 nm, (appearing as a shoulder on Band I). Its  $^1\text{H}$  NMR spectrum in  $\text{CDCl}_3$  also showed characteristic signals of chalcone type compounds, with a pair of *trans*-coupled vinyl protons at  $\delta$  7.72 (d,  $J$  = 15.5 Hz, H-8) and 7.87 (d,  $J$  = 15.5 Hz, H-7). Low resolution FABMS gave a molecular weight of 270.1, consistent with the composition of  $\text{C}_{16}\text{H}_{14}\text{O}_4$ . The presence of two hydroxyl groups and one methoxyl group on the A ring was evidenced by the two *meta*-coupled aromatic proton signals at  $\delta$  6.02 (d,  $J$  = 2.0 Hz, H-3') and  $\delta$  5.94 (d,  $J$  = 2.0 Hz, H-5') and one methyl signal at  $\delta$  3.48 (s). Comparison of its  $^{13}\text{C}$  NMR data with literature data of two known compounds, 2'-methoxy-4',6'-dihydroxy-chalcone (**2.15**) (also called cardamonin)<sup>43,44,45</sup> and 4'-methoxy-2',6'-dihydroxy-chalcone (**2.16**)<sup>46</sup> indicated that the

<sup>43</sup> Itokawa, H.; Morita, M.; Mihashi, S. Phenolic compounds from the rhizomes of *Alpinia speciosa*. *Phytochemistry* **1981**, *20*, 2503-2506

<sup>44</sup> Krishna, B. M. and Chaganty, R. B; Cardamonin and alpinetin from the seeds of *Alpinia speciosa*. *Phytochemistry* **1973**, *12*, 238-242.

<sup>45</sup> Bheemasankara, R. C.; Namosiva, R. T.; Suryaprakasam S. Cardamonin and alpinetin from the seeds of *Amomum subulatum*, *Planta Med.* **1976**, *29*, 391-2.

$^{13}\text{C}$  NMR data of M07X matched better with that of cardamonin (**2.15**) (Table 2-2), since the carbon signals for C-2' and C-6' of chalcone **2.16** overlapped together. Therefore, the methoxy group was placed on C-2' and compound ST-172038-M07X was determined as the known compound, cardamonin (**2.15**).

**Table 2-2** Comparison of NMR data of ST-172038-M07X with literature.<sup>44,46</sup>

	ST-172038- M07X <sup>a</sup>	Cadamonin <b>2.15</b> <sup>b</sup> (Lit) <sup>44</sup>	Chalcone <b>2.16</b> <sup>b</sup> (Lit) <sup>46</sup>	ST-172038-M07X <sup>c</sup> $\delta_{\text{H}}$	Cadamonin (Lit) <sup>44</sup> $\delta_{\text{H}}$
	$\delta_{\text{C}}$	$\delta_{\text{C}}$	$\delta_{\text{C}}$		
C-1	136.1	136.5	136.5		
C-2, 6	128.4	129.0	129.1	7.37 (m)	7.37 (m)
C-3, 5	129.9	129.7	129.8	7.34 (m)	7.33 (m)
C-4	130.2	130.7	130.9		
C-7	143.6	144.3	142.9	7.87 (d, $J = 15.5$ )	7.85 (d, $J = 15.8$ )
C-8	127.6	127.6	128.4	7.72 (d, $J = 15.5$ )	7.71 (d, $J = 15.8$ )
C-9	193.2	192.8	193.4		
C-1'	107.2	106.4	106.2		
C-2'	167.2	168.3	165.4		
C-3'	91.8	92.3	94.6	6.02 (d, $J = 2.0$ )	6.03 (d, $J = 2.0$ )
C-4'	166.3	165.8	167.2		
C-5'	96.8	97.0	94.6	5.94 (d, $J = 2.0$ )	5.91 (d, $J = 2.0$ )
C-6'	164.4	164.3	165.4		
OMe	56.0	56.3	55.8	3.48 (s)	3.48 (s)

<sup>a</sup> CDCl<sub>3</sub> 125 MHz, <sup>b</sup> CDCl<sub>3</sub> 100 MHz, <sup>c</sup> CDCl<sub>3</sub> 500 MHz

Compounds ST-172038-M04X and M05X were both identified as caryalactones. Their NMR spectra both showed signals for an  $\alpha,\beta$ -unsaturated lactone ring with two adjacent vinyl protons (viewed by COSY) at  $\delta$  6.00 (1H, dd,  $J = 9.5$  and 1.5Hz) and  $\delta$  6.94 (1H, m) and a lactone carbonyl carbon signal at  $\delta$  164.4. The  $^{13}\text{C}$  NMR data of these

<sup>46</sup> Shimomura, H.; Sashida, Y.; Mimaki, Y.; Oohara, M. and Fukai, Y., A Chalcone derivative from the bark of *Lindera umbellata*. *Phytochemistry* **1988**, 27, 3937-9.

two compounds matched very well with the data of the two caryalactones **2.1** and **2.2** that Raelison *et al.* had previously reported from the bark of this plant (Table 2.3). The optical rotation values of these two compounds also matched Raelison's values.<sup>20</sup> Therefore, both these two compounds were identified as known compounds.

**Table 2-3** Comparison of the <sup>13</sup>C-NMR data of two caryalactones with literature data.

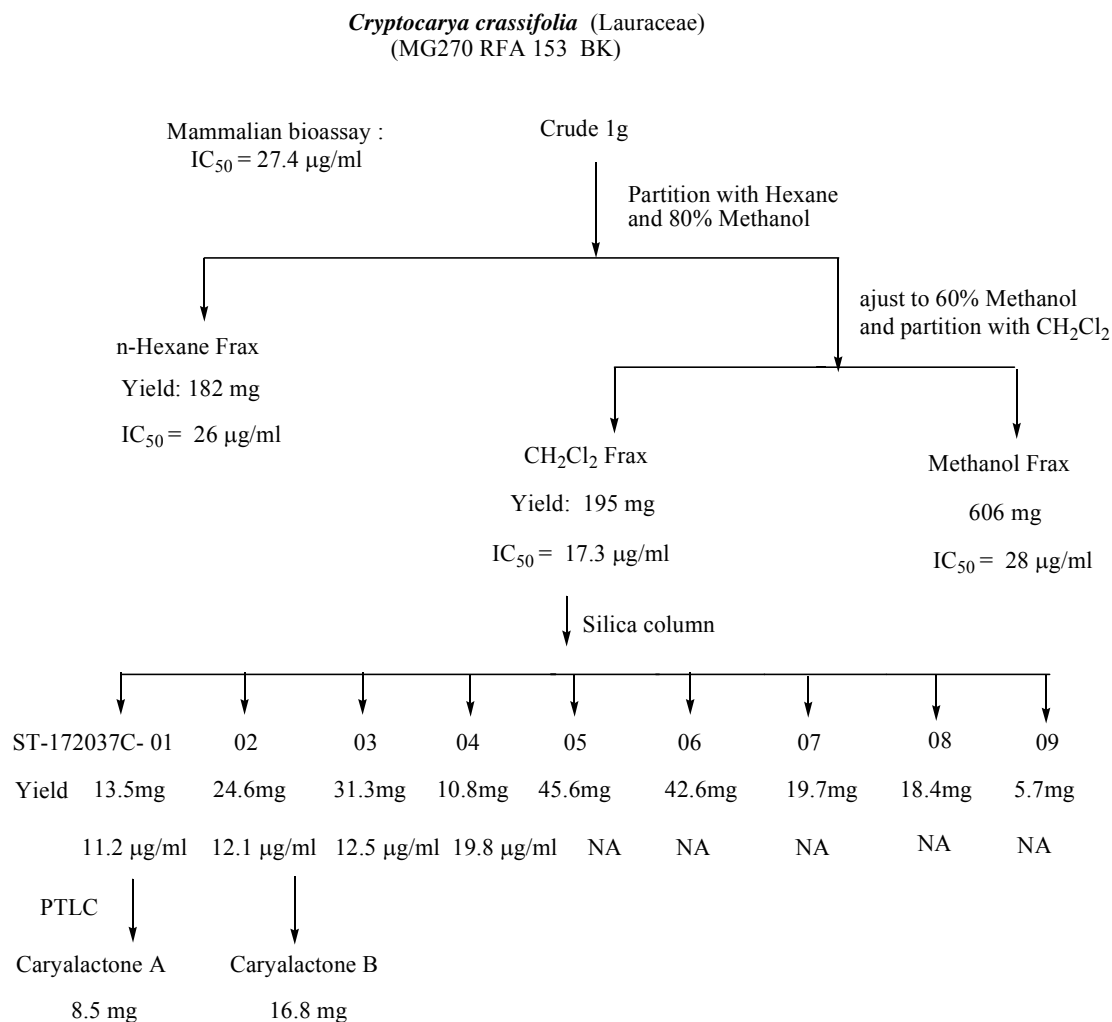
	ST-172038-M05X <sup>a</sup>	Caryalactone-A <sup>a</sup>	ST-172038-M04X <sup>a</sup>	Caryalactone-B <sup>a</sup>
C-2	164.5	164.6	164.2	164.3
C-3	121.4	120.9	121.6	121.0
C-4	145.4	145.8	144.9	144.8
C-5	29.9	29.8	29.8	29.7
C-6	75.1	74.9	77.9	77.5
C-1'	42.2	42.0	129.9	129.6
C-2'	67.2	66.8	131.4	131.4
C-3'	37.8	37.6	40.4	40.3
C-4'	25.2	25.2	68.3	68.3
C-5'	31.4	31.2	42.0	42.0
C-6'	35.9	35.7	69.3	69.1
C-7'			37.3	37.2
C-8'			25.5	25.4
C-9'			31.8	31.3
C-10'			35.9	35.8
C-1''	142.4	142.3	142.5	142.4
C-2'',6''	128.3	128.1	128.3	128.2
C-3'',5''	128.4	128.2	128.4	128.3
C-4''	125.7	125.5	125.7	125.6

<sup>a</sup> CDCl<sub>3</sub> 125 MHz

The bark extract was also examined by the A2780 assay and it also showed weak activity (IC<sub>50</sub> = 27.4 μg/mL). However, isolation work indicated that the same two caryalactones were present in this extract also (Scheme 2-2). Since Raelison, *et al.* have

studied this extract already, there was no point in fully investigating all the isolated compounds. This extract was thus dropped.<sup>47</sup>

**Scheme 2-2.** Isolation tree of the bark extract of *Cryptocarya crassifolia*



**II.7 Experimental Section.**

**General procedures.** Preparative thin layer chromatography (PTLC) plates (silica gel 20 × 20 cm, 1000 microns) were obtained from Analtech Inc. Reverse phase HPLC was

<sup>47</sup> The carylactone structure was deduced independently by NMR analysis before we found Raelison's publication, which used the plant name *Ravensara crassifolia* instead of *Cryptocarya crassifolia*.

carried on Varian Dynamax RP-C18 HPLC column with aqueous MeOH as mobile phase. Isolation progress was monitored by analytical TLC and visualized with phosphomolybdic acid spray.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were obtained on a JEOL-500 MHz NMR spectrometer in  $\text{CDCl}_3$ . Mass spectra (LR-FABMS) were determined by the analytical services staff in the Department of Chemistry at Virginia Polytechnic Institute and State University..

**Plant extraction.** The plant material was collected from Madagascar by our collaborators of ICBG program. Voucher specimens are deposited at the Missouri Botanical Garden, St. Louis, Missouri.

**Isolation of bioactive constituents.** The fruit extract of *Cryptocarya crassifolia* (1 g) was partitioned between 80% aqueous MeOH (200 mL) and hexanes ( $2 \times 100$  mL). The aqueous MeOH fraction was then diluted to 60% MeOH with water and extracted with  $\text{CH}_2\text{Cl}_2$  ( $3 \times 50$  mL). All of the fractions were then dried by rotary evaporation. The  $\text{CH}_2\text{Cl}_2$  fraction (235 mg) was determined as the most active fraction after A2780 bioassay. This fraction was then subjected silica column chromatography with  $\text{CHCl}_3$ ,  $\text{CHCl}_3/\text{MeOH}$  (100:1),  $\text{CHCl}_3/\text{MeOH}$  (97:3),  $\text{CHCl}_3/\text{MeOH}$  (95:5),  $\text{CHCl}_3/\text{MeOH}$  (92:8),  $\text{CHCl}_3/\text{MeOH}$  (85:15),  $\text{CHCl}_3/\text{MeOH}$  (80:20),  $\text{CHCl}_3/\text{MeOH}$  (75:25),  $\text{CHCl}_3/\text{MeOH}$  (50:50) and MeOH, yielding 9 fractions. The most two active fractions were adjacent to each other, and were combined. The combined fraction was subjected to reverse phase C-18 chromatography with a gradient of 80% aqueous MeOH to 100% MeOH to give four active fractions, ST-172038-M02, M04, M05 and M07. Each fraction was further

purified by RP-C18 HPLC with elution with 75% to 85% aqueous MeOH to yield four pure compounds ( $\pm$ )-pinocembrin **2.14** (6.7 mg), cardamonin **2.15** (2.2 mg), caryalactone-A **2.1** (5.5 mg) and caryalactone-B **2.2** (14.5 mg).

The bark extract of *Cryptocarya crassifolia* was fractionated in the same way. Crude extract (1 g) was partitioned between 80% aqueous MeOH (200 mL) and hexanes (2  $\times$  100 mL). The aqueous MeOH fraction was then diluted to 60% MeOH with water and extracted with CH<sub>2</sub>Cl<sub>2</sub> (3  $\times$  50 mL). All of the fractions were then dried by rotary evaporation and tested by A2780 bioassay. The most active fraction, the CH<sub>2</sub>Cl<sub>2</sub> fraction (195 mg) was then subjected to silica column chromatography with CHCl<sub>3</sub>/MeOH (100:1), CHCl<sub>3</sub>/MeOH (97:3), CHCl<sub>3</sub>/MeOH (95:5), CHCl<sub>3</sub>/MeOH (92:8), CHCl<sub>3</sub>/MeOH (85:15), CHCl<sub>3</sub>/MeOH (80:20), CHCl<sub>3</sub>/MeOH (75:25), CHCl<sub>3</sub>/MeOH (50:50) and 100% MeOH to yield 9 fractions. The two most active fractions were directly purified by preparative TLC with elution with 5% MeOH in CHCl<sub>3</sub> and gave caryalactone-A **2.1** (8.5 mg) and caryalactone-B **2.2** (15.5 mg).

**( $\pm$ )-Pinocembrin (2.14):** Yellow crystals, UV  $\lambda_{\max}$  (MeOH) nm (log  $\epsilon$ ): 208 (4.64), 275 (4.43), 324 (3.32). <sup>1</sup>H NMR:  $\delta$  12.05 (1H, s, 5-OH), 7.37-7.32 (5H, overlapped, aromatics), 6.02 (2H, overlapped, H-6 and H-8), 5.24 (1H, dd,  $J$  = 11.0 and 5.5 Hz, H-2), 3.21 (1H, dd,  $J$  = 17.0 and 5.5 Hz, H-<sub>3a</sub>), 2.80 (1H, dd,  $J$  = 17.0 and 11.0 Hz, H-<sub>3b</sub>), <sup>13</sup>C-NMR:  $\delta$  196.2, 166.4, 163.9, 163.1, 138.5, 128.8, 128.5, 126.5, 103.2, 96.7, 95.5, 79.1, 43.2 ppm. LR-FABMS:  $m/z$  = 257.1 (C<sub>15</sub>H<sub>12</sub>O<sub>4</sub>, M+H).

**Cardamonin (2.15):** Yellow crystals, UV:  $\lambda_{\max}$  (MeOH) nm (log  $\epsilon$ ): 207 (4.04), 289 (2.73), 341 (4.84).  $^1\text{H}$  NMR:  $\delta$  7.87 (1H, d,  $J = 15.5$  Hz), 7.72 (1H, d,  $J = 15.5$  Hz), 7.37-7.32 (5H, overlapped, aromatics), 6.02 (1H, d,  $J = 2.0$  Hz) and 5.94 (1H, d,  $J = 2.0$  Hz), 3.48 (3H, s).  $^{13}\text{C}$  NMR: (see Table 2.2). LR-FABMS:  $m/z = 271.1$  ( $\text{C}_{16}\text{H}_{14}\text{O}_4$ , M+H).

**(6S)-5,6-Dihydro-6-[(2R)-2-hydroxy-6-phenylhexyl]-2H-pyran-2-one (2.1):** Yellow powder,  $[\alpha]_{\text{D}} = -62^\circ$  ( $c = 0.4$ ,  $\text{CHCl}_3$ ). UV  $\lambda_{\max}$  (MeOH): nm (log  $\epsilon$ ): 208 (4.24), 256 (2.63).  $^1\text{H}$  NMR:  $\delta$  7.25-7.17 (5H, m, overlapped, aromatics), 6.85 (1H, m, H-4), 5.98 (1H, dd,  $J = 9.5, 2.0$  Hz, H-3), 4.71 (1H, m, H-6), 3.96 (1H, m, H-2'), 2.61 (2H, t,  $J = 7.0$  Hz, H-6'), 2.30 (2H, m, H-5), 1.80 (1H, dd,  $J = 15.5, 7.0$  Hz, H-1<sub>a</sub>), 1.76 (1H, dd,  $J = 15.5, 7.0$  Hz, H-1<sub>b</sub>), 1.59-1.34 (6H, m, overlapped, H-3',4',5').  $^{13}\text{C}$  NMR: see Table 2.3; LR-FABMS:  $m/z = 275.2$  ( $\text{C}_{17}\text{H}_{22}\text{O}_3$ , M+H).

**(6R)-6-[(4R,6R)-4,6-Dihydroxy-10-phenyldec-1-enyl]-5,6-dihydro-2H-pyran-2-one (2.2):** Yellow powder.  $[\alpha]_{\text{D}} = +72^\circ$  ( $c = 0.5$ ,  $\text{CHCl}_3$ ). UV:  $\lambda_{\max}$  (MeOH) nm (log  $\epsilon$ ): 208 (4.53), 256 (2.68).  $^1\text{H}$ -NMR:  $\delta$  7.25-7.17 (5H, m, overlapped, aromatics), 6.87 (1H, m, H-4), 6.04 (1H, dd,  $J = 9.5, 2.0$  Hz, H-3), 5.87 (1H, dt,  $J = 15.5, 8.0$  Hz, H-2'), 5.68 (1H, dd,  $J = 15.5, 7.0$  Hz, H-1'), 4.90 (1H, m, H-6), 4.01 (1H, m, H-6'), 3.91 (1H, m, H-4'), 2.62 (2H, t,  $J = 7.0$  Hz, H-10'), 2.43 (2H, m, H-5'), 2.28 (2H, m, H-3), 1.62-1.33 (8H, overlapped, H-5', 7', 8', 9').  $^{13}\text{C}$  NMR data: see Table 2.3; LR-FABMS:  $m/z = 345.2$  ( $\text{C}_{21}\text{H}_{28}\text{O}_4$ , M+H).

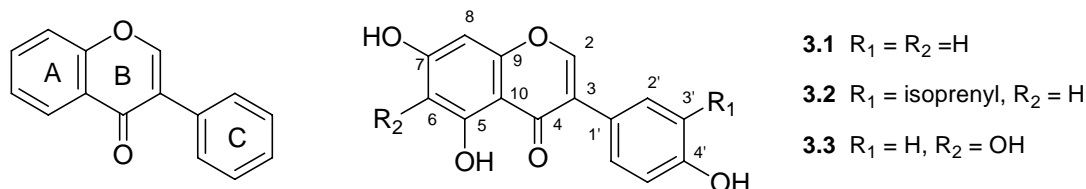
## Chapter III. Purification and Characterization of Isoflavones From A Lotus Plant.

### III.1 Introduction to Isoflavones.

An Egyptian lotus plant, *Lotus polyphyllus*, was investigated by one of the previous members of the Kingston group, Dr. Maged Abdel-Kader, who continued his research in the natural product area in Egypt. The initial sample collection, extraction and open column separation work on this extract were all carried out by Dr. Maged Abdel-Kader, who fractionated the CH<sub>2</sub>Cl<sub>2</sub> fraction of this extract by chromatography on silica gel and elution with CHCl<sub>3</sub>/MeOH gradient. This yielded eight fractions of increasing polarity (#MSA-01 to 08). The final purification of these fractions by TLC and HPLC and structure elucidation were done by the current author. Two new isoflavones as well as several known compounds were separated and characterized by NMR and MS.

As we have indicated in chapter II, isoflavones are isomers of flavones in the flavonoid family. The structural difference between an isoflavone and a flavone is that the C-phenyl ring of an isoflavone is connected at C-3 instead of C-2 position of the B-ring (Figure 3-1). In this structure, the C-ring is not well conjugated with the A-B rings because it is not in the  $\beta$  position of the  $\alpha,\beta$  enone on B-ring. Therefore, the UV spectrum of an isoflavone is not like that of a flavone. It typically has a strong Band II at 260-280 nm and a weak Band I at 290-310 nm. When the number of hydroxyl groups on the A-ring increases, the relative intensity of Band I will decrease and sometimes it will appear as a small shoulder on Band II. The most commonly seen isoflavones from Nature are genisteins (**3.1**), erythrinins (**3.2**) and calycosins (**3.3**). Derivatives of some of these

compounds have shown good anti-malarial, anti-bacterial or anti-HIV activities,<sup>48,49,50</sup> however, no clinical trials of any of them have been reported.



**Figure 3-1** General structure of isoflavones

### III.2 Results and discussion

The eight fractions, numbered from MSA-01 to MSA-08, were tested for cytotoxicity by the A2780 bioassay. Unfortunately, they showed poor activities ( $IC_{50} > 20 \mu\text{g/ml}$ ). Among these fractions, MSA-01, 03, 04, 05, 06 were found to be simple 4-hydroxy-*trans*-cinnamic acid esters, and they will not be discussed here. Fractions MSA-02, 07, and 08 were found to be flavonoids by their characteristic UV-absorptions.

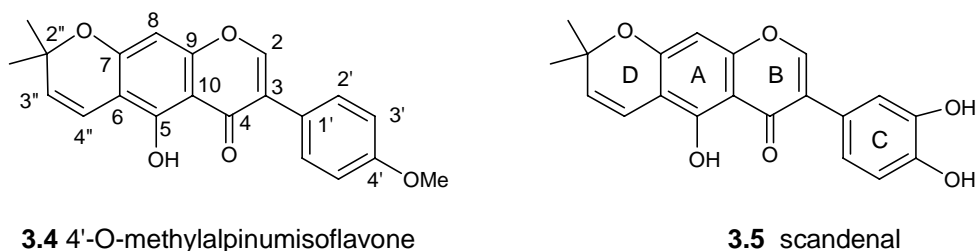
Fraction MSA-02 (22 mg received from Dr. Adel-Kader) was purified by preparative TLC on silica gel to give pure compound MSA-02X (15 mg). The UV spectrum of MSA-02X in MeOH showed a major peak at 274 nm with a 358 nm shoulder peak. A CI-MS experiment showed a molecular ion peak at  $m/z = 351.2 (M+H)^+$  which suggested a formula of  $C_{21}H_{19}O_5$ . Its  $^1\text{H-NMR}$  spectrum in  $\text{CDCl}_3$  showed peaks for two methyl groups at  $\delta$  1.48 (6H, br, s), one methoxyl group at  $\delta$  3.45 (3H, s), a pair of vinyl protons at  $\delta$  5.61 (d,  $J = 8.0$  Hz) and 6.70 (d,  $J = 8.0$  Hz), one aromatic proton at  $\delta$  6.40

<sup>48</sup> Anjala, C. W.; Juma, B. F.; Bojase, G.; Gashe, B. A.; Majinda, R. T., Isoflavones from the root bark of *Piscidia erythrina*. *Planta Med.* **2002**, *68*, 78-99.

<sup>49</sup> Kobayashi, M.; Mahmud, T.; Yoshioka, N.; Shibuya, H.; Kitagawa, I. An isoflavone glycoside from the stem of *Euphorbia hirta*. as antimalarial compound. *Chem.Pharm. Bull.* **1997**, *45*, 1615-1619.

<sup>50</sup> Yin, S.; Fan, C. Q.; Wang, Y.; Dong, L.; Yue, J. M. A prenylated isoflavone from *Pouzolzia indica*: Its in vitro antimicrobial activity and cytotoxic evaluation. *Bioorg. Med. Chem.* **2004**, *12*, 367-369.

(s), four aromatic protons in a A<sub>2</sub>B<sub>2</sub> spin system at  $\delta$  6.98 (2H, d,  $J = 8.5$  Hz) and  $\delta$  7.54 (2H, d,  $J = 8.5$  Hz), one sharp proton signal at  $\delta$  7.89 (s), and one downfield phenolic proton at  $\delta$  12.91 (s). The very sharp proton signal at  $\delta$  7.89 (s) was correlated to a <sup>13</sup>C NMR signal at  $\delta$  152.3, and the proton signal at  $\delta$  6.40 (s) was correlated to a <sup>13</sup>C NMR signal at  $\delta$  100.4 (from HSQC). These characteristic correlations indicated that this compound was a 5,7,4'-trihydroxyl-isoflavone (genistein) derivative. The <sup>1</sup>H NMR data of MSA-02X matched well with that of a known isoflavone, 4'-*O*-methyl-alpinumisoflavone (**3.4**), which was reported by Khalid and Waterman in 1983.<sup>51</sup> Since no <sup>13</sup>C NMR data was provided in the original paper, the partial structures of the A, B, and D rings in the isoflavone skeleton were confirmed by comparison of the <sup>1</sup>H and <sup>13</sup>C NMR data with literature data of another known isoflavone, scandenal (**3.5**)<sup>52</sup> (Table 3.1). These data matched well, which suggested that MSA-02X shares the same partial structure as scandenal for the A, B and D rings. Therefore, MSA-02X was determined as the known compound, 4'-*O*-methyl-alpinumisoflavone.



**Figure 3-2** Structures of MSA-02X and scandenal

<sup>51</sup> Khalid, S. A.; Waterman, P. G. Thonningine A and thonningine B: two 3-phenylcoumarins from the seeds of *Millettia thonningii*., *Phytochemistry* **1983**, 22, 1001-1008..

<sup>52</sup> Mahabusarakam, W; Deachathai, S.; Phongpaichit, S.; Jansakul, C. and Taylor, W. C. A benzyl and isoflavone derivatives from *Derris scandens*., *Phytochemistry* **2004**, 65, 1185-1194.

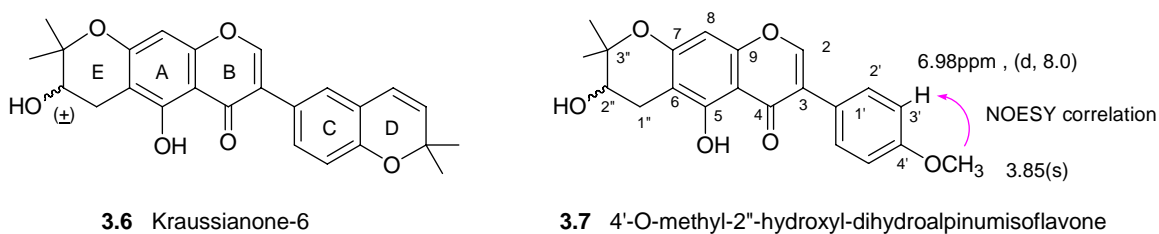
**Table 3-1** NMR data of A,B, and D rings of MSA-02X and alpinumisoflavone.<sup>51</sup>

C#	Scandenal <sup>a</sup>	MSA-02X <sup>a</sup>	Scandenal	MSA-02X
C-2	152.9	152.4	7.89 (s)	7.89 (s)
C-3	122.5	122.9		
C-4	180.7	181.0		
C-5	158.8	159.6		
C-6	106.2	106.1		
C-7	160.1	159.9		
C-8	95.3	96.8	6.36 (s)	6.39 (s)
C-9	159.5	159.9		
C-10	106.4	106.1		
C-2''	78.4	78.4		
C-3''	128.6	127.5	5.65 (d, <i>J</i> = 8.0)	5.61 (d, d, <i>J</i> = 8.0)
C-4''	115.6	114.7	6.74 (d, <i>J</i> = 8.0)	6.70 (d, <i>J</i> = 8.0)
C-2'' 2Me	28.5	28.3	1.49 (s)	1.48 (s)

<sup>a</sup> CDCl<sub>3</sub> 125 MHz

Fraction MSA-08 was a minor fraction with only 2.2 mg obtained. It was purified by HPLC on a C-18 column with elution by 90% aqueous MeOH and yielded pure compound MSA-08X (1.5 mg). Its <sup>1</sup>H NMR spectrum in CDCl<sub>3</sub> was similar to that of MSA-02X, with signals for 2 methyl groups at δ 1.40 (s), and 1.42 (s), one methoxyl group at δ 3.84 (s), one methine proton at δ 3.88 (m), a pair of gem-coupled methylene protons at δ 2.80 (d, *J* = 16.0 and 7.0 Hz) and δ 3.03 (dd, *J* = 17.0 and 5.5 Hz), one aromatic protons at δ 6.41 (s), four aromatic protons in a A<sub>2</sub>B<sub>2</sub> spin system at δ 6.98 (2H, d, *J* = 8.5 Hz) and δ 7.45 (2H, d, *J* = 8.5 Hz), one proton at δ 7.95 (s) and one phenolic proton at δ 13.16 (s). High resolution FABMS indicated a composition of C<sub>21</sub>H<sub>20</sub>O<sub>6</sub> from its molecular ion peak at *m/z* = 369.1335 (M+H). Thus, MSA-08X might be a hydrated derivative of MSA-02X. Comparison of the <sup>1</sup>H and <sup>13</sup>C NMR data with those of the known isoflavone kraussianone-6 (**3.6**) suggested that MSA-08X shares the same partial

structure as kraussianone-6 on the A, B and E rings (Table 3.2).<sup>53</sup> The difference between kraussianone-6 and MSA-08 is that the latter has a methyl group ( $\delta_{\text{H}}$  3.84,  $\delta_{\text{C}}$  58.0) on the C-4' position of the C ring in the place of the pyran ring of kraussianone-6. The position of this methyl group was determined by a 1-D NOESY experiment. Irradiation of the methyl signal at 3.84 ppm showed clear positive NOE enhancement of the two *ortho*-protons at  $\delta$  6.98 (H-3') (Figure 3-3). Therefore, the structure of MSA-08X was assigned as (3.7). A literature search showed that this compound was a new analog of 2''-hydroxyl-dihydroalpinumisoflavone,<sup>54</sup> so it was named as 4'-*O*-methyl-2''-hydroxyl-dihydroalpinumisoflavone (3.7). Because the amount of pure compound 3.7 was too small (1.5 mg), the stereochemistry of C-2'' hydroxyl group was not identified.



**Figure 3-3.** Structure of kraussianone-6 and MSA-08X

<sup>53</sup> Drewes, S. E.; Horn, M. M.; Khan, F.; Munro, Q. Q.; Dhlamini, J. T.; Rakuambo, C.; Meyer, J. M. Minor pyranoisoflavones from *Eriosema kraussianum*: activity, structure, and chemical reaction studies. *Phytochemistry* **2004**, *65*, 1955-1967.

<sup>54</sup> Tanaka, H.; Tanaka, T.; Etoh, H.; Watanabe, N.; Ahmad, M.; Qurashi, I.; Khand, M. R. Two new isoflavones from *Erythrina suberosa* var. *glabrescences*. *Heterocycles* **1998**, *48*, 2661-2667.

**Table 3-2** NMR data of MSA-08X and Kraussianone-6.

Carbon #	Kraussianone-6 <sup>a</sup>	MSA-08X <sup>b</sup>		Kraussianone-6	MSA-08X
C-2	155.3	153.1	H-2	7.94 (s)	7.95 (s)
C-3	122.9	123.3			
C-4	182.2	181.0			
C-5	160.1	160.3			
C-6	104.2	104.6			
C-7	160.3	158.8			
C-8	97.2	97.6	H-8	6.45 (s)	6.42 (s)
C-9	156.1	154.5			
C-10	104.9	104.8			
C-1''	25.7	25.5	H-1a''	2.75 (dd, <i>J</i> =17, 6.8)	2.80 (dd, <i>J</i> =17.0, 7.0)
C-2''	68.8	68.6	H-1b''	2.97 (dd, <i>J</i> =17, 5.4)	3.03 (dd, <i>J</i> =17.0, 5.5)
C-3''	79.1	78.6	H-2''	3.84 (br)	3.86 (br, m)
3''-Me	23.8	24.2	Me	1.37 (s)	1.40 (s)
3''-Me	25.9	26.5	Me	1.41 (s)	1.42 (s)

\* <sup>a</sup> CDCl<sub>3</sub>, 100MHz, <sup>b</sup> CDCl<sub>3</sub>, 125MHz

Fraction MSA-07 (8.5 mg received) was purified by preparative TLC on silica gel followed by HPLC on a C-18 reverse phase column with elution by 90% aqueous MeOH. A total of 6 mg pure compound MSA-07X was obtained. High resolution FABMS indicated its composition to be C<sub>21</sub>H<sub>20</sub>O<sub>6</sub>, the same as that of MSA-08X (*m/z* = 369.1335 M+H, calculated for 369.1338). Thus, MSA-07X is an isomer of MSA-08X. Its UV spectrum in MeOH showed a major peak at 264 nm with a 285 nm shoulder peak. And its <sup>1</sup>H NMR spectrum showed signals for 2 methyl group at δ 1.24 (s), 1.38 (s), one methoxy group at δ 3.84 (s), one methine proton at δ 4.78 (dd, *J* = 9.0 and 8.0 Hz), a pair of gem-coupled methylene protons at δ 3.20 (d, *J* = 17.0 and 8.0 Hz) and 3.24 (dd, *J* = 17.0 and 9.0 Hz), one aromatic proton at δ 6.40 (s), four aromatic protons in an A<sub>2</sub>B<sub>2</sub> spin system at δ 6.98 (2H, d, *J* = 8.5 Hz) and δ 7.42 (2H, d, *J* = 8.5 Hz), one proton at δ 7.85 (s), and one phenolic proton at δ 13.15 (br, s). The sharp singlet at 7.85 ppm correlated to a <sup>13</sup>C

NMR signal at  $\delta$  152.1 and the proton at  $\delta$  6.32 correlated to a  $^{13}\text{C}$  NMR signal at  $\delta$  94.4 (viewed by HSQC). These findings indicated this compound was also a 5,7,4'-trihydroxy-isoflavone. The presence of a phenolic hydroxyl group at C-5 was also proved by an  $\text{AlCl}_3$ -UV-shift reagent test. The UV-absorption band II was shifted from 265 nm to 285 nm when 5%  $\text{AlCl}_3$  in anhydrous MeOH was added to the MeOH solution of MSA-07X.

The  $^1\text{H}$  NMR differences between MSA-08X and 07X were mainly in the shift of the methine proton signal at  $\delta$  4.78 (1H, dd,  $J = 9.0$  and  $8.0$  Hz) and the gem-coupled methylene protons at  $\delta$  3.20 and 3.24. These facts suggested that MSA-07X has a benzofuran ring instead of benzo-pyran ring. Comparison of the  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of MSA-07X with literature data of the known isoflavone, ulexin-D (**3.8**),<sup>55</sup> indicated that they both share the same partial structure of their A, B and E rings (Table 3-3). The methoxyl signal at 3.84 ppm was assigned to the C-4' position of C-ring by a 1-D NOESY experiment. Irradiation of the methyl signal at 3.84 ppm showed clear NOE enhancement of the two *ortho*-protons at 6.98 ppm (H-3'). Also, the UV-absorption band I at 389 nm of MSA-07 (in MeOH) did not shift when MSA-07 was treated with 5% NaOMe in MeOH, supporting the conclusion that the phenol hydroxyl group on C-4' was methylated. A structure search in the literature indicated that compound MSA-07 is a new analog of erythrinin-C (**3.9**),<sup>56, 57</sup> so we named it as 4'-*O*-methyl-erythrinin-C (**3.10**).

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<sup>55</sup> Maximo, P.; Lourenco, A.; Feio, S. S.; Roseiro, J. C. Flavonoids from *Ulex airensis* and *Ulex europaeus* ssp. *europaeus* *J. Nat. Prod.* **2002**, *65*, 175-178.

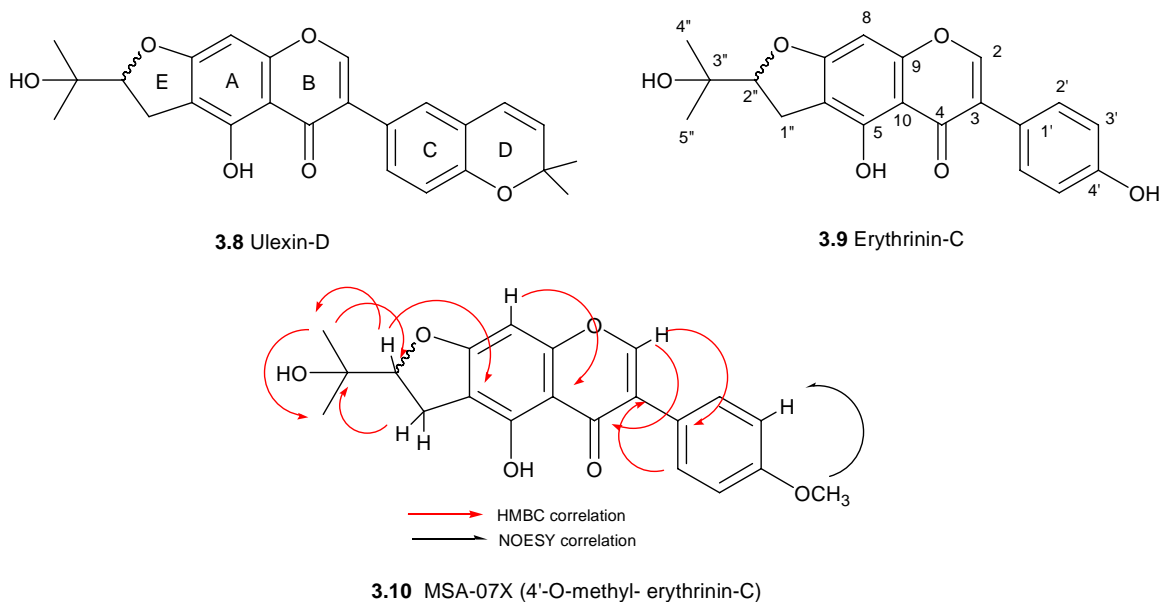
<sup>56</sup> Tanaka, H.; Tanaka, T.; Etoh, H.; Watanabe, N.; Ahmad, M.; Qurashi, I.; Khand, M. R. Two new isoflavones from *Erythrina suberosa* var. *glabrescences*. *Heterocycles* **1998**, *48*, 2661-2667.

<sup>57</sup> Deshpande, V. H.; Pendse, A. D.; Pendse, Ratna. Erythrinins A, B and C, three new isoflavones from the bark of *Erythrina variegata*. *Indian J. Chem.* **1977**, *15B*, 205-7.

**Table 3-3**  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of MSA-07X and ulexin-D.

$\delta_{\text{C}}$	Ulexin-D <sup>a</sup>	MSA-07X <sup>b</sup>	$\delta_{\text{H}}$	Ulexin-D	MSA-07X
C-2	152.7	152.1	H-2	7.84 (s)	8.10 (s)
C-3	123.7	123.7	H-6	6.37 (s)	6.40 (s)
C-4	181.7	180.9			
C-5	163.4	163.7	H-1a''	3.12 (dd, $J=15.7, 8.1$ )	3.20 (dd, $J=16, 8.0$ )
C-6	107.2	106.2	H-1b''	3.20 (dd, $J=15.7, 9.4$ )	3.24 (dd, $J=16.0, 9.2$ )
C-7	158.4	158.2	H-2''	4.79 (t, $J=8.7$ )	4.78 (t, $J=8.5$ )
C-8	93.3	94.4	H-4''	1.24 (s)	1.20 (s)
C-9	158.4	159.9	H-5''	1.36 (s)	1.38 (s)
C-10	106.7	106.1			
C-1''	26.7	27.0			
C-2''	71.8	71.9			
C-3''	91.9	91.6			
C-4''	23.8	24.2			
C-5''	25.8	26.0			

<sup>a</sup>  $\text{CDCl}_3$ , 125MHz. <sup>b</sup>  $\text{CDCl}_3$ , 100MHz

**Figure 3-4.** Structure of erythrinin-C and MSA-07X

The optical rotation value of MSA-07X is  $[\alpha]_D^{23} = -2.4^\circ$  (MeOH), while that of erythrinin-C isolated by Tanaka *et al.* was  $[\alpha]_D^{25} = -7.8^\circ$  (MeOH).<sup>57</sup> It is not clear whether the observed difference in rotation is due to one or both samples being partial racemates, or to difficulties in making an accurate determination of optical rotation on small quantities of compound. The absolute stereochemistry and optical purity of erythrinin-C were not established by Tanaka *et al.*, or by this work.

### III.3 Experimental Section.

**General methods.** Preparative thin layer chromatography (PTLC) plates (silica gel 20 × 20 cm, 1000 micros) were from Analtech Inc. Reverse phase HPLC was carried on Varian Dynamax RP-C18 HPLC column and MeOH/water as mobile phase. <sup>1</sup>H and <sup>13</sup>C NMR spectra were obtained on a JEOL-500 MHz spectrometer. High resolution FABMS were determined by the analytical services staff of Virginia Polytechnic Institute and State University.

**4'-O-Methyl-alpinumisoflavone (3.4):** Crude MSA-02 (22 mg) was purified on preparative silica TLC with 5% MeOH in CHCl<sub>3</sub>. Compound **3.4** (15 mg) was obtained as yellow crystals. UV  $\lambda_{\max}$  (MeOH) nm (log  $\epsilon$ ): 205 (4.54), 275 (4.34), 358 (3.13); <sup>1</sup>H NMR:  $\delta$  1.48 (6H, 2CH<sub>3</sub> overlapped), 3.45 (s), 5.58 (d,  $J = 8.0$  Hz), 6.40 (s), 6.67 (d,  $J = 8.0$  Hz), 6.98 (2H, d,  $J = 8.5$  Hz), 7.54 (2H, d,  $J = 8.5$  Hz), 7.89 (s), 12.89 (br, s). <sup>13</sup>C NMR: 181.2, 162.7, 159.9, 159.6, 158.8, 152.3, 130.2, 127.6, 124.0, 122.9, 114.7, 114.3, 100.7, 94.4, 78.5, 55.7, 28.3; CI-MS:  $m/z = 351.2$  (M+H); C<sub>21</sub>H<sub>18</sub>O<sub>5</sub>.

**4'-O-Methyl-2"-hydroxyl-dihydroalpinumisoflavone (3.7):** Crude MSA-08 (2.2 mg) was purified by reverse phase HPLC on a Varian Dynamax RP-C18 column eluted with 90% aq. MeOH and yielded 1.5 mg of pure compound **3.7** as white powder.  $[\alpha]_D^{23} = -4.3^\circ$  (MeOH); UV  $\lambda_{\max}$  (MeOH) nm (log  $\epsilon$ ): 205 (4.68), 269 (4.56), 354 (3.25);  $^1\text{H}$  NMR  $\delta$  13.16 (s), 7.45 (2H, d,  $J = 8.5$  Hz), 6.98 (2H, d,  $J = 8.5$  Hz), 6.41 (1H, s), 3.88 (1H, m), 3.84 (3H, s), 3.03 (1H, dd,  $J = 17.0$  and 5.5 Hz), 2.80 (1H, d,  $J = 16.0$  and 7.0 Hz), 1.42 (3H, s), 1.40 (3H, s);  $^{13}\text{C}$  NMR: 181.0, 161.8, 160.3, 159.4, 159.0, 158.4, 154.5, 152.1, 130.4, 124.0, 123.6, 114.2, 106.3, 98.2, 78.6, 68.6, 55.5, 27.5, 26.3, 24.2; HR-FABMS:  $m/z = 369.1343$  (M+H); calculated for  $\text{C}_{21}\text{H}_{20}\text{O}_6$ ,  $m/z = 369.1338$ ,  $\delta = 1.4$  ppm.

**4'-O-Methyl-erythrinin-C (3.10):** Crude MSA-07 (8.5 mg) was purified by preparative TLC on silica gel with elution with 15% MeOH in  $\text{CHCl}_3$ . The partially purified product (6.0 mg) was further purified by reverse phase HPLC on a Varian Dynamax RP-C18 column eluted with 80% aq. MeOH and yielded 5.5 mg of pure compound **3.10** as a pale yellow powder.  $[\alpha]_D^{23} = -2.4^\circ$  (MeOH); UV  $\lambda_{\max}$  (MeOH) nm (log  $\epsilon$ ): 208 (4.52), 265 (4.44), 385 (2.78);  $^1\text{H}$  NMR  $\delta$  13.15 (br, s), 7.85 (1H, s), 7.42 (2H, d,  $J = 8.5$  Hz), 6.98 (2H, d,  $J = 8.5$  Hz), 6.40 (1H, s),  $\delta$  4.78 (dd,  $J = 9.0$  and 8.0 Hz), 3.24 (dd,  $J = 17.0$  and 9.0 Hz), 3.20 (d,  $J = 17.0$  and 8.0 Hz), 1.38 (3H, s), 1.24 (3H, s);  $^{13}\text{C}$  NMR: 180.9, 166.3, 160.4, 159.9, 158.2, 154.5, 152.8, 152.1, 130.3, 123.7, 123.0, 114.2, 106.1, 100.5, 94.4, 91.6, 71.9, 55.5, 27.0, 26.0, 24.2; HR-FABMS:  $m/z = 369.1335$  (M+H); calculated for  $\text{C}_{21}\text{H}_{20}\text{O}_6$ ,  $m/z = 369.1338$ ,  $\delta = -0.1$  ppm.

## Chapter IV. Isolation of Cytotoxic Cardenolides from a *Brexiella* sp.

### IV.1 Introduction.

As part of our ICBG program to isolate bioactive antitumor compounds from terrestrial plants, ethanol extracts from the leaves and bark of a *Brexiella* sp. plant (Celestraceae) were found to display significant biological activity versus A2780 mammalian cell lines. Two known cardenolides were isolated and found to be responsible for the bioactivities. Both compounds were characterized by spectral analysis and comparison to known literature data.

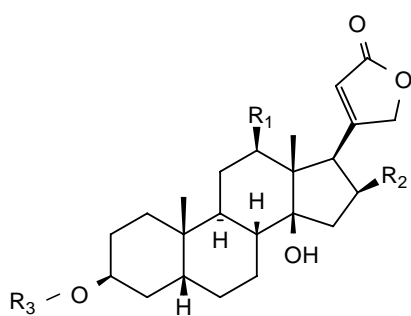
### IV.2 Structure and Basic Properties of Cardenolides.

Cardenolides (also called cardeno-glycosides) are steroid saponins with a specific  $\alpha,\beta$ -unsaturated lactone linked at the C-17  $\beta$  position of the steroid skeleton and saccharides linked at the C-3 position (Figure 4-1). The name “cardenolide” came from their strong heart stimulant effect that could be used to improve cardiac contractility in the treatment of congestive heart failure. Cardenolides are widely found in the seeds, leaves and stems of plants in the Scrophulariaceae, Apocynaceae, Liliaceae and Asclepiadaceae families. Today there are more than 400 cardenolide derivatives reported from terrestrial plants as well as from the bodies of some insects. The first cardenolide, digitaline (**4.1**) was isolated from a purple herb *Digitalis purpurea* as early as 1869 by Nativelle.<sup>58</sup> However, in 1935 Stoll *et al.* reinvestigated this plant and found that

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<sup>58</sup> For earliest reports of the isolation of cardenolides, see: a). Stoll, A.; Angliker, E.; Barfuss, F.; Kussmaul, W.; Renz, J. Cardiac glycosides. XXVII. Separation and analysis of cardiac glycosides by chromatography on silica. *Helv. Chim. Acta.* **1951**, *34*, 1460-1467. b). Stoll, A.; Kreis, W. Glucosides of *Digitalis lanata*. *Helv. Chim. Acta.* **1934**, *17*, 790-3. c). Stoll, A.; Suter, E.; Kreis, W.; Bussemaker, B. B.; Hofmann, A. Heart-activating substances of squill. scillaren, I. Heart glucosides. *Helv. Chim. Acta.* **1933**, *16* 703-33.

digitaline was actually an enzyme-hydrolyzed secondary metabolite of this plant.<sup>59</sup> Further isolation after deactivation of the plant enzyme gave a number of original cardenoglycosides such as purpurea glycoside-A (4.2) and purpurea glycoside-B (4.3) (Figure 4-2). Most of the reported cardenolides have very good cytotoxic activities ( $IC_{50} < 0.5 \mu\text{g/mL}$ ) in different cell lines. However, due to their strong toxicity and the side effect of life-threatening cardiac arrhythmias, cardenolides are not suitable for use as antitumor drugs and have a low therapeutic index in the clinical treatment of heart disease. Today the most frequently used cardenolide type drugs are cedilanid (4.4) and digoxine (4.5).



4.1  $R_1=OH, R_2=H, R_3=H$

4.2  $R_1=H, R_2=H, R_3 = \beta\text{-D-glucopyranosyl-(1-4)-O-}\beta\text{-digitoxosyl-(1-4)-O-}\beta\text{-digitoxosyl}$

4.3  $R_1=H, R_2=OH, R_3 = \beta\text{-D-glucopyranosyl-(1-4)-O-3-O-acetyl-}\beta\text{-digitoxosyl-(1-4)-O-3-O-acetyl-}\beta\text{-digitoxosyl-(1-4)-}\beta\text{-digitoxosyl}$

4.4  $R_1=OH, R_2=H, R_3 = \beta\text{-D-glucopyranosyl-(1-4)-O-3-O-acetyl-}\beta\text{-digitoxosyl-(1-4)-O-}\beta\text{-digitoxosyl-(1-4)-}\beta\text{-digitoxosyl}$

4.5  $R_1=H, R_2=H, R_3 = \beta\text{-D-digitoxosyl-(1-4)-O-}\beta\text{-digitoxosyl-(1-4)-O-}\beta\text{-digitoxosyl}$

**Figure 4-1** Structure of cardenoglycosides

The bioactivity of the cardenolides mainly comes from their unsaturated lactone ring.<sup>60</sup> Cardenolides act through inhibition of  $\text{Na}^+, \text{K}^+$ -ATPase,<sup>61</sup> a cell membrane

<sup>59</sup> Stoll, A. and Kreis, W., The original glucosides of *Digitalis*. *Helv. Chim. Acta.* **1933**, *16*, 1049-53.

<sup>60</sup> Van-Quaquebeke, E.; Simon, G.; Andre A.; Dewelle J.; Yazidi M. E.; Bruyneel F.; Tuti, J.; Nacoulma, O.; Guissou, P.; Decaestecker C., Identification of a novel cardenolide (2"-oxovoroscharin) from *Calotropis procera* and the hemisynthesis of novel derivatives displaying potent in vitro antitumor activities and high in vivo tolerance: structure-activity relationship analyses. *J. Med. Chem.* **2005**, *48*, 849-56.

<sup>61</sup> Florkiewicz, R. Z.; Anchin, J.; Baird, A., The inhibition of fibroblast growth factor-2 export by cardenolides implies a novel function for the catalytic subunit of  $\text{Na}^+, \text{K}^+$ -ATPase. *J. Biol. Chem.* **1998**, *273*, 544-551.

enzyme which uses the energy released by ATP hydrolysis to promote the outward transport of  $\text{Na}^+$  ions and the inward transport of  $\text{K}^+$  ions.<sup>62</sup> Cardenolides inhibit its activity and consequently produce a positive response in the heart. The binding ability of cardenolides to  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase depends not only on the unsaturated lactone ring but also on the saccharide units at the C-3 position.<sup>63</sup> Increasing the number of sugar units that are linked on its C-3 position increases their binding activity and water solubility, and also decreases their toxicity.

The sugar arrangement in naturally occurring cardenolides is usually a terminal glucose unit at the end with deoxy sugar units in the middle. The deoxy sugars could be 6-deoxy-hexapyranoses such as rhamnose or fucose, or 2,6-di-desoxy-hexapyranoses such as digitoxose, thevetose or sarmentose. These different types of sugars and different linkages among them lead to a large number of cardenolide derivatives. The terminal sugar can be lost during the collection and isolation process. These secondary cardenolides, generally considered to be artifacts, show less activity than the primary ones.

The plant species *Brexiella* has not been previously subjected to phytochemical studies. It is a member of the higher plant family Celastraceae, which is widely distributed in the territories of Somalia, Djibouti, South and North Yemen, Kenya, Madagascar, Tanzania and down to south eastern Africa. A large number of compound

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<sup>62</sup> Thomas, R.; Gray, P.; Andrews, J. Digitalis: Its mode of action, receptor, and structure-activity relationships. *Adv. Drug Res.* **1990**, *19*, 312-362

<sup>63</sup> Farr, C. D.; Burd, C.; Tabet, M. R.; Wang, X.; Welsh, W. J.; Ball, W. J., Three-dimensional quantitative structure-activity relationship study of the inhibition of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase by cardio-tonic steroids using comparative molecular field analysis. *Biochemistry* **2002**, *41*, 1137-1148.

types have been identified in this plant family, including forty alkaloids, phenolic glycosides, tannins, terpenoids.<sup>64</sup>

### **IV.3 Isolation of Cardenolides from *Brexiella* sp. (Celestraceae).**

#### **Results and discussion.**

As part of our ongoing program to isolate anticancer compounds from terrestrial plants, the ethanol extract from the bark of a *Brexiella* sp. (MG1815) was found to display significant biological activity, with  $IC_{50} = 8.5 \mu\text{g/mL}$  against the A2780 mammalian cell lines. A sample of this extract (400 mg) was partitioned between aqueous 80% MeOH and hexanes (Scheme 4-1). The aqueous MeOH fraction was then diluted with water (to 60% MeOH) and extracted with dichloromethane. Each fraction was subjected to solvent removal by rotary evaporation and bioassay; the dried aqueous MeOH fraction was found to be the most active fraction. Since a lot of phenolic compounds such as tannins have previously been reported in this plant family, this aqueous MeOH fraction was then subjected to a test for phenols with  $\text{FeCl}_3/\text{K}_2\text{Fe}(\text{CN})_4$ , which gave a positive result. Therefore, this fraction was first detanninized by polyamide column chromatography (aqueous MeOH gradient from 50% to 100% MeOH followed with 20% ammonia in MeOH). The most active fraction (12 mg) was found to be the 50% MeOH fraction, and this fraction was then subjected to RP-18 solid phase extraction (aqueous MeOH gradient) and re-purified on C-18 HPLC to afford 0.5 mg of compound **A** with good activity ( $IC_{50} = 0.13 \mu\text{g/mL}$ ). However the amount of sample was too small to fully characterize its structure, and the depletion of crude extract from the bark

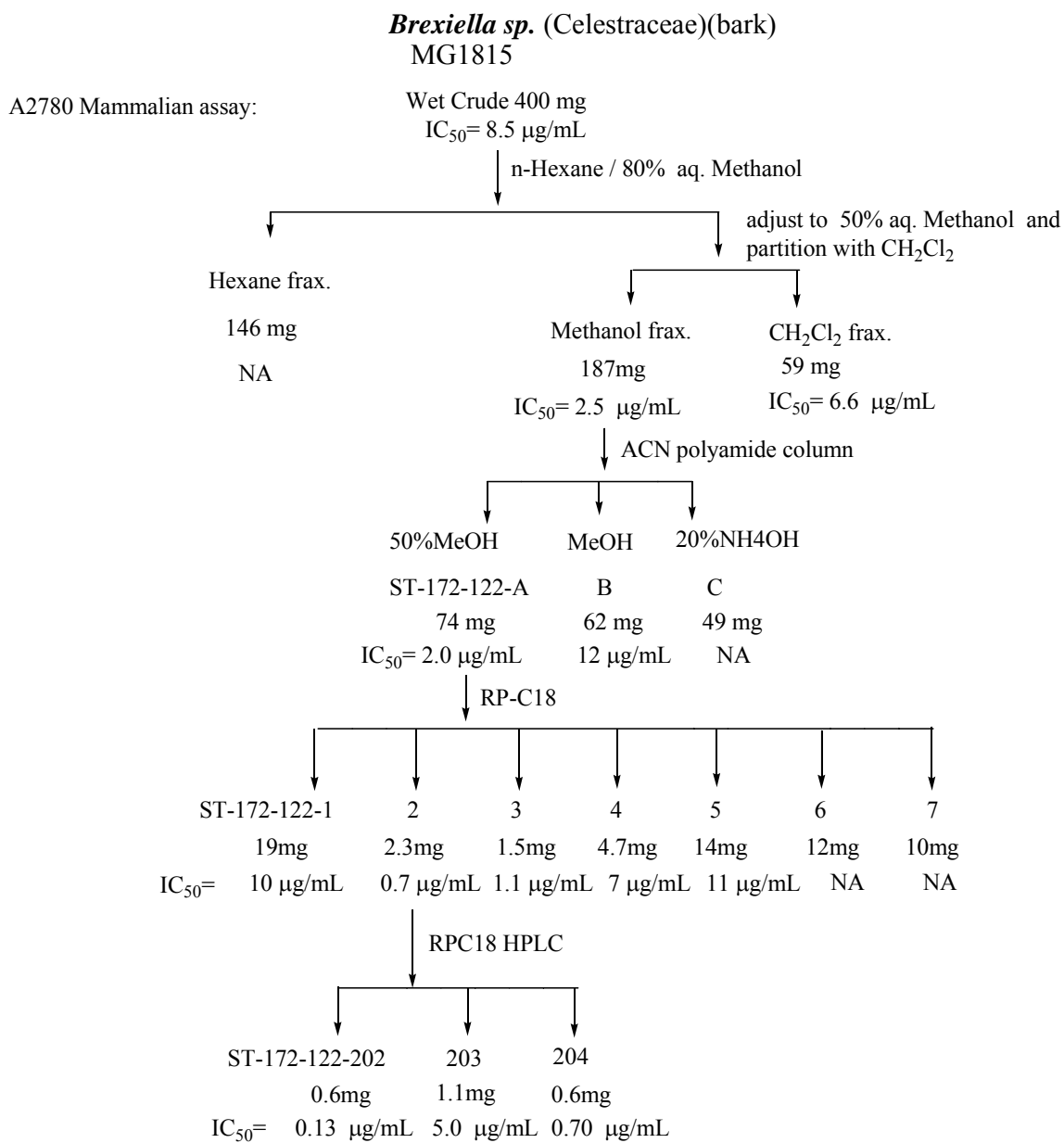
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<sup>64</sup> Elmi, A. S. The chewing of khat in Somalia. *J. Ethnopharm.* **1983**, 8, 163-168

(MG1815) led us to investigate the leaf extract (MG1817) of the same plant and see whether compound **A** could be obtained from it.

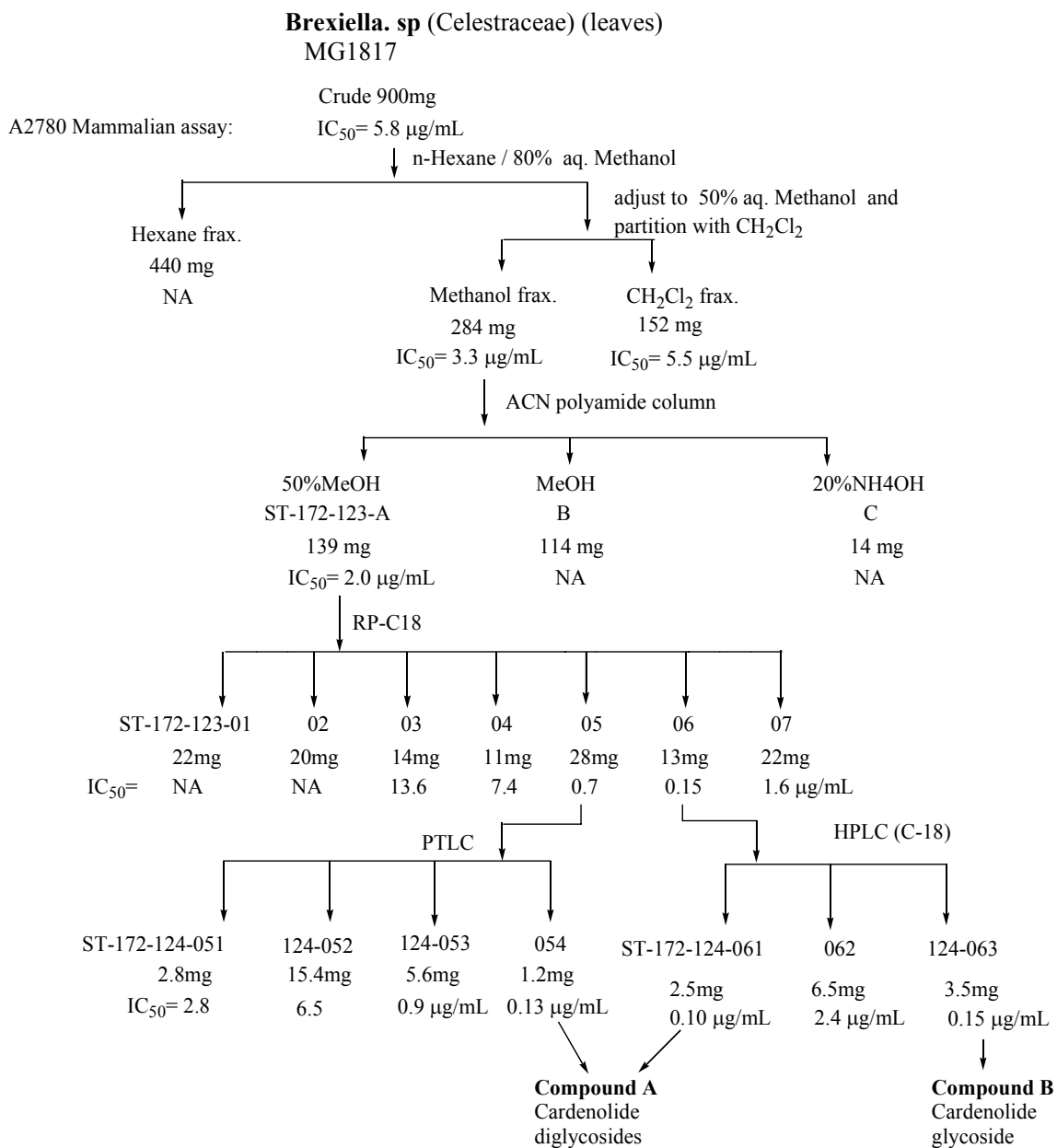
The extract of leaves *Brexiella* sp. (MG1817) (900 mg) was found to display even better biological activity against the A2780 mammalian cell lines ( $IC_{50} = 5.8 \mu\text{g/mL}$ ) (Scheme-2). Liquid partition again gave the aqueous MeOH fraction as the most active fraction. This fraction was also detanninized by polyamide column chromatography (aqueous MeOH gradient from 50% to 100% MeOH followed with 20% ammonia in MeOH). The most active fraction (139 mg) remained the 50% MeOH fraction, and this fraction then subjected to RP-18 solid phase extraction (aqueous MeOH gradient) and re-purified on C-18 HPLC to afford a total of 3.7 mg of compound **A** ( $IC_{50} = 0.13 \mu\text{g/mL}$ ) as well as a second active compound **B** (3.5 mg,  $IC_{50} = 0.15 \mu\text{g/mL}$ ).

**Scheme 4-1.** Isolation of cardenolides from the bark extract of *Brexiella* sp. (Celestraceae)



**Compound A**

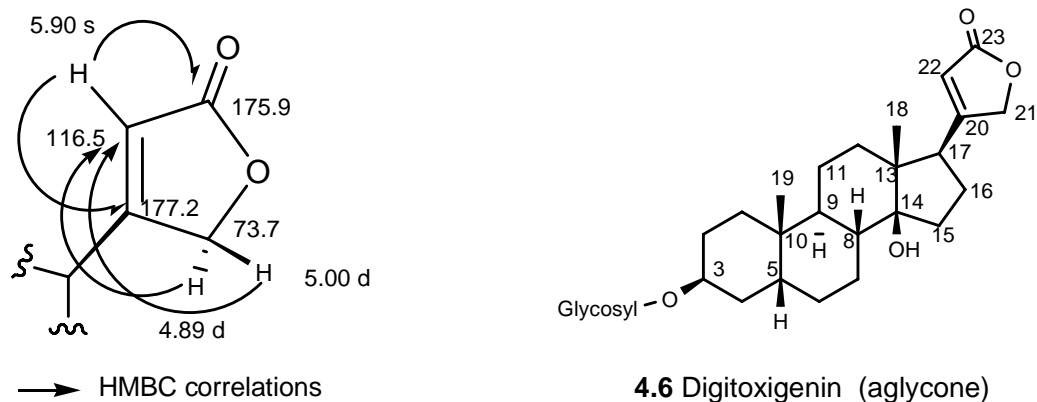
**Scheme 4-2.** Isolation of cardenolides from the leaf extract of *Brexiella* sp.



#### IV.4 Structure Elucidation of Compounds A and B.

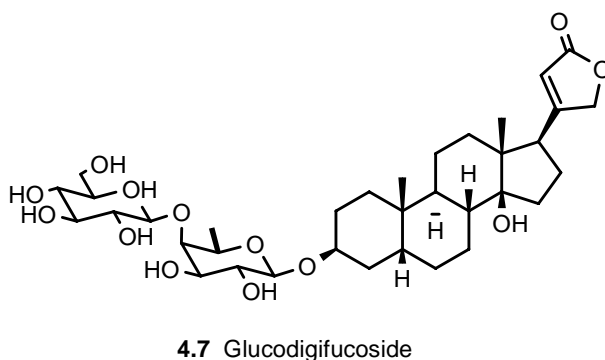
The  $^1\text{H}$  NMR spectrum of compound **A** (in  $\text{CD}_3\text{OD}$ ) displayed signals for three methyl groups  $\delta$  0.89 (s), 1.02 (s), 1.68 (d,  $J = 7.5$  Hz), a number of sugar protons ( $\delta$  3.5 - 4.5, most overlapped with the solvent signal), and two anomeric protons  $\delta$  4.33 (d,  $J = 8.5$  Hz), 4.31 (d,  $J = 8.5$  Hz). This suggested that compound **A** might be a steroid saponin with two sugar units, one of which was 6-deoxy sugar. Furthermore, a very sharp singlet at  $\delta$  5.90 indicated the presence of a vinylic proton, and two germ-coupled protons at  $\delta$  5.00 (d,  $J = 18.5$  Hz) and 4.89 (d,  $J = 18.5$  Hz) were also observed, which were characteristic of the unsaturated lactone ring of a cardenolide. The  $J$  coupling values of 8.5 Hz for the two anomeric protons indicated that these two sugars were both connected by a  $\beta$ -linkage.  $^{13}\text{C}$  NMR and DEPT experiments showed the presence of 35 carbon signals with 3 methyl, 11 methylene, 16 methine, and 5 quaternary carbons. COSY, HMBC and HMQC experiments were carried out in both pyridine- $d_5$  and MeOH- $d_4$  to clarify the structure.

Compound **A** has a molecular formula of  $\text{C}_{35}\text{H}_{54}\text{O}_{13}$  as determined by HRFABMS. The partial structure of the unsaturated  $\gamma$ -lactone ring was determined by the 2-D NMR (HSQC and HMBC) experiments as shown in Figure 4-2. The vinyl proton ( $\delta$  5.90, s) showed HMBC correlation to both the carbon signal at 177.2 and 175.9. The two strongly coupled protons at  $\delta$  5.00 (d,  $J = 18.5$  Hz) and 4.89 (d,  $J = 18.5$  Hz) also showed HMBC correlations to the carbon signal at  $\delta$  177.2 and 116.5. The two bridgehead methyl signals assigned by HSQC with  $\delta_{\text{H}}$  0.89 (s), 1.02 (s) and  $\delta_{\text{C}}$  18.5, 14.5 ppm and a quaternary oxygenated carbon signal at  $\delta$  85.1 matched well with the characteristics of a digitoxigenin (**4.6**) type of cardenolide.



**Figure 4-2** Important HMBC and NOESY correlations observed for compound A

Because of the severe overlap of methylene proton signals in the  $^1\text{H}$  NMR spectrum from 1.5-2.0 ppm, the framework of the cardienolide aglycone of compound A was built up mainly from its  $^{13}\text{C}$  NMR spectrum. Since the carbon data of digitoxigenin aglycones have been published, a comparison of  $^{13}\text{C}$  NMR spectrum of compound A with that of a known digitoxigenin diglycoside derivative, glucodigifucoside (**4.7**) (Figure 4-3) was possible. This comparison indicated that compound A and glucodigifucoside (**4.7**) both shared a common steroid skeleton (Table 4-1).



**Figure 4-3** Structure of glucodigifucoside

**Table 4-1.** Comparison of  $^{13}\text{C}$  and  $^1\text{H}$  NMR data of compound A and glucodigifucoside<sup>65</sup>

Carbon	Glucodigi-Fucoside <sup>a</sup>	Compound A <sup>a</sup>		Glucodigi-Fucoside	Compound A
C-1	30.1	29.7	C-13	50.1	49.8
C-2	27.0	26.7	C-14	85.5	85.2
C-3	75.0	75.0	C-15	32.4	32.1
C-4	30.2	29.9	C-16	26.8	26.5
C-5	35.3	35.0	C-17	51.1	50.8
C-6	26.5	26.5	C-18	15.4	15.1
C-7	21.4	21.2	C-19	23.1	22.7
C-8	41.7	41.4	C-20	177.6	177.2
C-9	35.9	35.6	C-21	74.4	73.7
C-10	36.5	36.2	C-22	116.8	116.5
C-11	21.6	21.4	C-23	176.3	175.9
C-12	40.0	39.8			
Proton					
H-3	4.03 (m)	4.00 (m)	H-22	5.92	5.90 (s)
H-15	2.20 (m)	2.19 (m)	H-1'	4.33 (d, 8.0)	4.31(d, 8.0)
H-16	2.20 (m)	2.19 (m)	H-1''	4.38 (d, 8.0)	4.36(d, 8.0)
H-17	2.85 (m)	2.83 (dd)	18-CH <sub>3</sub>	0.91 (s)	0.89 (s)
H-21-a	4.94 (dd, 18.4, 1.7)	4.89 (dd, 18.2, 1.5)	19-CH <sub>3</sub>	1.03 (s)	1.02 (s)
H-21-b	5.02 (dd)	5.00 (dd)			

<sup>a</sup>CD<sub>3</sub>OD, 100 MHz

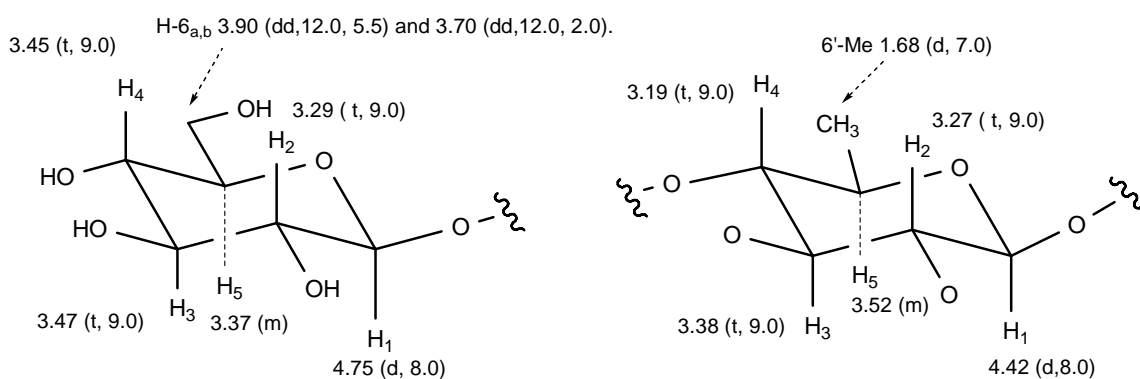
The chemical shift of the C-5 methine at 35.0 ppm and of C-10 at 36.2 ppm indicated that the A-B ring of digitoxigenin skeleton was cis-fused, because if it were A-B trans, the chemical shifts of C-5 and C-10 should be around 31.5 and 40.8 ppm.<sup>66,67</sup> A NOESY experiment also support the digitoxinin skeleton with the NOESY correlation of 18-methyl group at  $\delta$  0.89 (s) to H-5 at  $\delta$  1.65 (m), and of the 19 methyl group at 1.02ppm (s) to H-8 at  $\delta$  1.78 (dd,  $J$  = 11.5 and 1.3 Hz).

<sup>65</sup> Castro, B. F.; Dias, S. F. J.; Howarth, O.; Braga, O. A., Complete  $^1\text{H}$  and  $^{13}\text{C}$  assignments of the *Digitalis lanata* cardenolides, glucodigifucoside and glucogitoroside by 1D and 2D NMR. *Magn. Res. Chem.* **1997**, *35*, 899-903.

<sup>66</sup> Yadava, R. N., A new cardenolide from the seeds of *Prosopis spicigera*. *Fitoterapia* **1999**, *70*, 284-286.

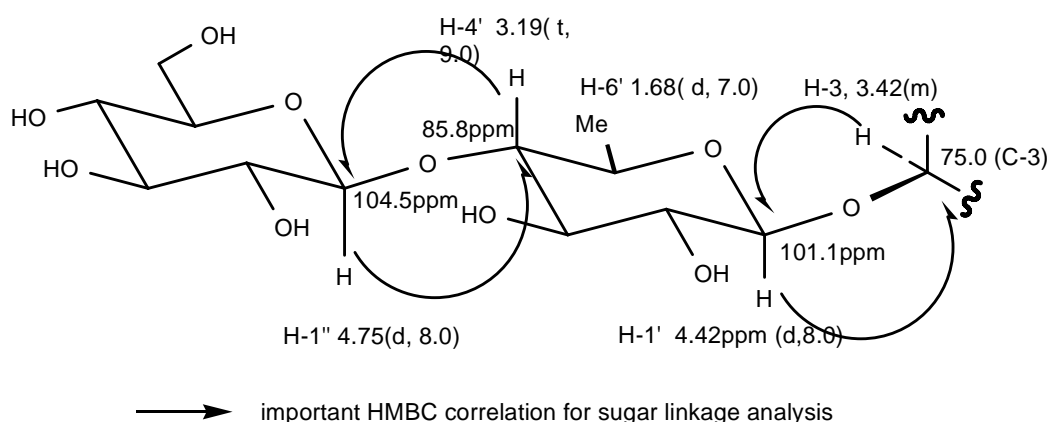
<sup>67</sup> Sun, K.; Li, X., Progress in studies on chemical constituents and pharmacological effect of *Semen lepidii* and *Semen descurainiae*. *Zhongcaoyao* **2002**, *33*, s3-s5

The two glycol-units of compound A were identified by a COSY experiment in pyridine- $d_5$  instead of  $CD_3OD$  to eliminate the overlap of solvent signal. The two anomeric protons appeared at  $\delta$  4.75 (d,  $J = 8.0$  Hz) and 4.42 (d,  $J = 8.0$  Hz) in this solvent. In addition to the COSY experiment, a 1D-TOCSY experiment was also carried out by irradiating the two anomeric protons. When the anomeric proton at  $\delta$  4.42 was irradiated, the TOCSY spectrum gave a spin system with  $\delta$  4.75 (d, H-1'',  $J = 8.0$  Hz), 3.29 (dd, H-2'',  $J = 9.0$  and  $8.0$  Hz), 3.47 (t, H-3'',  $J = 9.0$  Hz), 3.45 (t, H-4'',  $J = 9.0$  Hz), 3.37 (m, H-5''), 3.90 (dd, H-6a'',  $J = 12.0$  and  $5.5$  Hz) and 3.70 (dd, H-6b'',  $J = 12.0$  and  $2.0$  Hz). By this coupling pattern the sugar was identified as glucopyranose. When the anomeric proton at  $\delta$  4.42 was irradiated, the TOCSY spectrum revealed another spin system with  $\delta$  4.42 (d, H-1',  $J = 8.0$  Hz), 3.27 (dd, H-2',  $J = 9.0$  and  $8.0$  Hz), 3.38 (t, H-3',  $J = 9.0$  Hz), 3.19 (t, H-4',  $J = 9.0$  Hz), 3.52 (m, H-5'), 1.68 (d, 6-Me,  $J = 7.0$  Hz), and the sugar was identified as quinovose (6-deoxy-glucopyranose) by these values. The assigned sugar structures were shown in Figure 4-4 below.



**Figure 4-4** Structures of two saccharides from TOCSY experiment.

The arrangement of these two sugars was also elucidated by an HMBC experiment in pyridine-*d*<sub>5</sub> (indicated in Figure 4-5). H-3 at  $\delta$  3.42 (m) showed a clear HMBC correlation to the anomeric carbon C-1' ( $\delta$  101.3) of the quinovose. Also, H-4' of the quinovose  $\delta$  3.19 showed an HMBC correlation to the anomeric carbon C-1'' ( $\delta$  104.5) of the glucopyranose. Therefore, the sugar linkage was determined as  $\beta$ -glucopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -quinovoside. Connecting the digitoxinin aglycone with the sugar part gave the complete structure of compound **A** as the known compound, digitoxigenin glucodigigulomethyloside (**4.8**) (Figure 4-5).

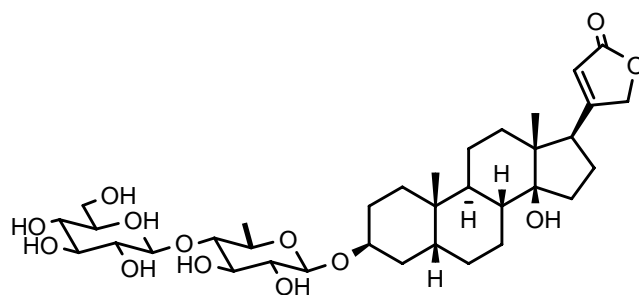


**Figure 4-5** Determination of sugar linkage by HMBC

Glucodigigulomethyloside was isolated by Makarevich in large quantities from the seeds of *Cheiranthus allionii* (Cruciferae). Its structure was deduced in 1975 by enzymatic hydrolysis and comparison of the *R<sub>f</sub>* value of the aglycone with known compounds.<sup>68</sup> Since the original reference did not give NMR data and clear structural

<sup>68</sup> Makarevich, I. F.; Kolesnikov, D. D.; Kovalev, I. P.; Gordienko, V. G.; Kabanov, V. S. Cardiac glycosides of *Cheiranthus allionii*. XI. *Khim. Prir. Soed.* **1975**, *11*, 754-8.

identification, it is useful to record and study the 1-D and 2-D NMR spectra of this compound again, which gave a clear structural elucidation.



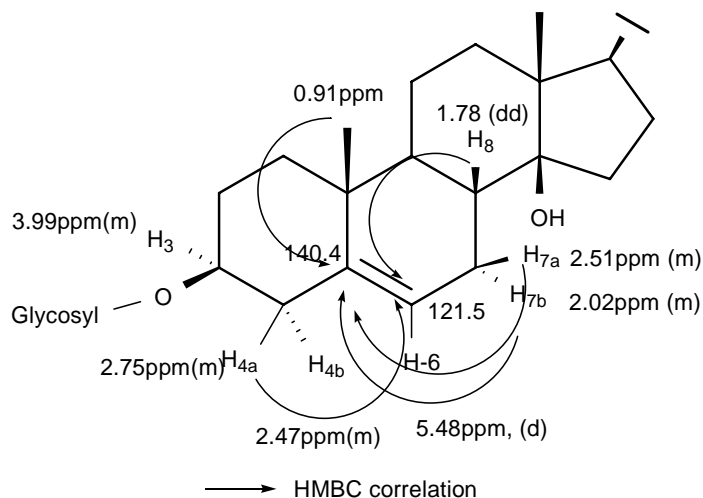
4.8 Glucodigigulomethyloside

**Figure 4-6** Complete structure of Compound **A** (Glucodigigulomethyloside).

The structure of compound **B** was elucidated by the same strategy as compound **A**. HR-FABMS showed a molecular formula of  $C_{29}H_{42}O_9$ . Compound **B** also had a similar  $^1H$  NMR to that of compound **A**, with one sharp singlet vinyl proton ( $\delta$  5.90), two gem-coupled protons at  $\delta$  5.04 (d,  $J = 18.5$  Hz) and 4.89 (d,  $J = 18.5$  Hz) and two bridged methyl groups at  $\delta$  0.91 (s) and  $\delta$  1.07 (s); but has only one  $\beta$ -linked sugar with one anomeric proton signal at  $\delta$  4.37 (d,  $J = 7.8$  Hz). It also had one more vinyl proton at  $\delta$  5.48 (m).  $^{13}C$  NMR and DEPT spectrum showed that compound **B** had 6 quaternary carbon, 11 methine, 10 methylene, and 2 methyl carbon signals. Comparison of the  $^{13}C$  NMR spectrum of compound **B** with that of compound **A** showed two more  $sp^2$  carbons at  $\delta$  140.4 (quaternary) and  $\delta$  121.5 (methine). Therefore, compound **B** has an additional double bond in its aglycone skeleton.

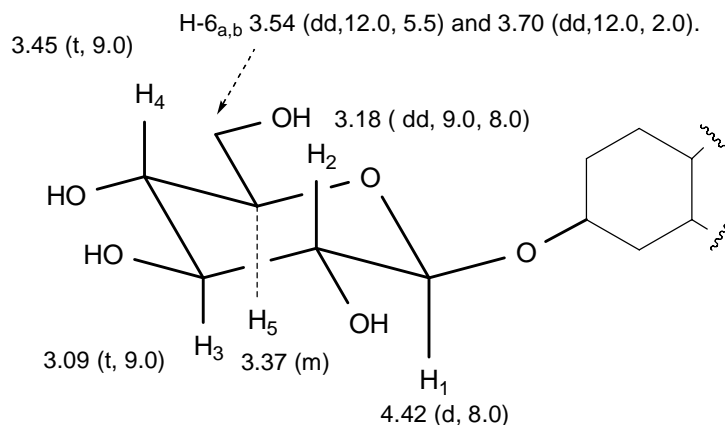
The position of the double bond was determined as  $\Delta^{5-6}$  by COSY, HSQC and HMBC experiments. The vinylic proton at  $\delta$  5.48 only showed COSY correlation with two protons at  $\delta$  2.52 (m) and 2.02 (m), and these two protons were found on the same

carbon at  $\delta$  32.5 (by HSQC) and they also showed COSY correlation to the proton on C-8, a characteristic bridgehead proton at  $\delta$  1.78 (dd,  $J = 16.0$  and  $2.0$  Hz). Therefore, these two protons were on C-7 and the vinylic proton at  $\delta$  5.48 was assigned to C-6 ( $\delta$  121.5). Further HMBC experiments confirmed this skeleton, as shown in Figure 4-7.



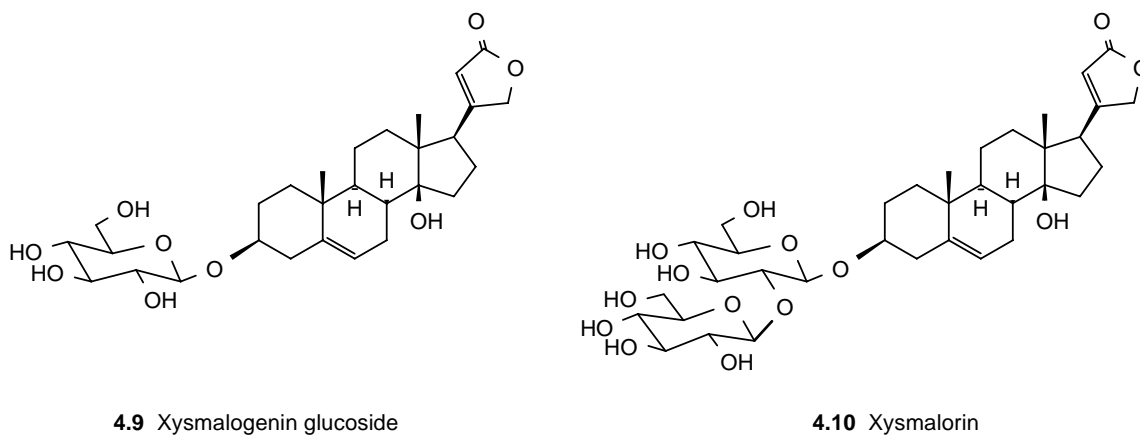
**Figure 4-7.** Important HMBC correlations of Compound **B**

The sugar part of compound **B** was again determined by a 1D-TOCSY experiment in  $C_6D_5N$ , which gave a spin system with  $\delta$  4.42 (d, H-1',  $J = 8.0$  Hz), 3.18 (dd, H-2',  $J = 9.0$  and  $8.0$  Hz), 3.09 (t, H-3',  $J = 9.0$  Hz), 3.45 (t, H-4',  $J = 9.0$  Hz), 3.37 (m, H-5'), 3.54 (dd, H-6a',  $J = 12.0$  and  $5.5$  Hz) and 3.70 (dd, H-6b',  $J = 12.0$  and  $2.0$  Hz). The sugar was identified as glucopyranose by its proton coupling pattern.



**Figure 4-8.**  $\beta$ -Glucopyranose unit from TOCSY and COSY experiment.

The complete structure of compound **B** was thus identified as xysmalogenin- $\beta$ -glucoside (**4.9**) (Figure 4-9). This compound was previously reported by Reichstein in 1967.<sup>69</sup> Since no  $^{13}\text{C}$  NMR data were reported in the original reference, the  $^{13}\text{C}$  NMR data of compound **B** were compared with those of another known xysmalogenin-diglucoside, xysmalorin (**4.10**),<sup>70</sup> which matched well on the aglycone part (Table 4-2). This also supported the conclusion that compound **B** was xysmalogenin glycoside.



**Figure 4-9.** Structure of compound **B** and xysmalorin

<sup>69</sup> Reichstein, P.; Kaufmann, H.; Stoecklin, W.; Reichstein, T., Glycosides and aglycons from *Glossostelma carsoni* roots, *Helv. Chim. Acta.* **1967**, *50*, 2114-38.

<sup>70</sup> Ghorbani, M.; Kaloga, M.; Frey, H. H.; Mayer, G. and Eich, E., Phytochemical reinvestigation of *Xysmalobium undulatum* (Uzara), *Planta Med.* **1997**, *63*, 343-6.

**Table 4-2.** Comparison of  $^{13}\text{C}$  NMR data of compound B and xysmalorin<sup>70</sup>

Carbon	Xysmalorin <sup>a</sup>	Compound B <sup>b</sup>		Xysmalorin	Compound B
C-1	36.9	36.7	C-13	49.4	49.8
C-2	29.4	29.3	C-14	84.0	85.1
C-3	78.1	78.4	C-15	32.8	32.5
C-4	38.1	38.9	C-16	26.2	26.3
C-5	139.7	140.4	C-17	50.5	50.8
C-6	121.6	121.5	C-18	15.7	14.8
C-7	26.9	26.9	C-19	19.4	18.7
C-8	37.4	37.7	C-20	176.4	176.5
C-9	46.9	47.3	C-21	73.3	73.8
C-10	36.9	37.1	C-22	116.5	116.6
C-11	20.8	20.9	C-23	174.0	174.5
C-12	38.5	38.9			

<sup>a</sup>CD<sub>3</sub>OD, 100 MHz, <sup>b</sup>CD<sub>3</sub>OD, 125 MHz

#### IV.5 Experimental Section.

**General Experimental Procedures.** The isolation process was monitored by Whatman MK-RPC-18 TLC plates. Polyamide columns were packed with ECOCHROM polyamide material. Reverse phase C-18 chromatography was carried out on a Horizon-400 flash column chromatograph with Biotage RPC-18 flash columns. Reverse phase HPLC was carried out on a Varian Dynamax RP-C18 HPLC column with MeOH/water as mobile phase.  $^1\text{H}$  and  $^{13}\text{C}$  NMR data were recorded on a JEOL 500MHz NMR instrument, and all  $J$  values are given in Hertz. High resolution FABMS were determined by the analytical service group staff of Virginia Polytechnic Institute and State University.

**Plant material.** The bark and leaves of a *Brexiella* sp. (Celestraceae) were collected in Madagascar by our ICBG collaborators as MG1682 and MG1684.

**Isolation process.** The plant extract (900 mg) was dissolved in 90% aq. MeOH and extracted with hexane. The MeOH fraction was diluted with water to a composition of 50% MeOH in water; this was then partitioned with CH<sub>2</sub>Cl<sub>2</sub>. The fractions were then evaporated to dryness; bioassay revealed that the aqueous MeOH fraction (284 mg) was the most active fraction. This fraction was then chromatographed over a polyamide column using a MeOH/water gradient (50% MeOH in water to pure MeOH, then 20% aq. ammonia in MeOH). The most active fraction (139 mg) was eluted with 50% MeOH, and this was then subjected to RP-18 flash column chromatography with an aqueous MeOH gradient to yield seven fractions. The two most active fractions were re-purified on C-18 HPLC to afford a total of 3.7 mg of compound **A** with IC<sub>50</sub> = 0.13 µg/mL as well as 3.5 mg of compound **B** with IC<sub>50</sub> = 0.15 µg/mL.

**Digitoxigenin-gluco digigulomethyloside (4.8):** Colorless solid. <sup>1</sup>H NMR (CD<sub>3</sub>OD): δ 5.90 (1H, s), 5.02 (1H, d, *J* = 18.5), 4.90 (1H, d, *J* = 18.5), 4.35 (1H, d, *J* = 7.8), 4.31 (1H, d, *J* = 7.8), 3.86 (1H, dd, *J* = 12.0 and 2.0), 3.66 (1H, dd, *J* = 12.0 and 5.0), 3.34 (1H, t, *J* = 9.0), 3.33-3.30 (6H, overlapped by solvent signal), 3.29 (dd, *J* = 9.0 and 8.0), 2.83 (1H, m), 2.19 (1H, m), 1.87-1.49 (27H, m, overlapped), 1.34 (3H, d, *J* = 7.5), 1.32-1.27 (2H, m), 1.02 (3H, s), 0.89 (3H, s). <sup>13</sup>C NMR (CD<sub>3</sub>OD): (Table 4-1). HRFABMS: *m/z* = 683.3658 (M+H)<sup>+</sup>; calculated for C<sub>35</sub>H<sub>55</sub>O<sub>13</sub>, *m/z* = 683.3643, Δ = 2.4 ppm.

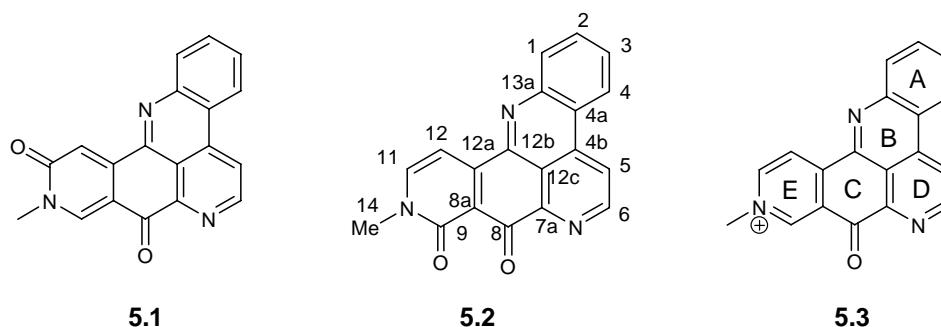
**Xysmalogenin-gluco pyranoside (4.9):** Colorless solid. <sup>1</sup>H NMR (CD<sub>3</sub>OD): δ 5.90 (s), 5.45 (m), 5.04 (d, *J* = 18.5), 4.89 (d, *J* = 18.5), 4.38 (d, *J* = 7.8), 3.85 (dd, *J* = 12.0 and 2.0), 3.62 (dd, *J* = 12.0 and 5.0), 3.59 (t, *J* = 9.0), 3.29 (dd, *J* = 9.0 and 8.0), 3.15 (t, *J* =

9.0), 2.86 (dd,  $J = 9.6$  and  $5.5$ ), 2.26-2.15 (m, overlapped, 8H), 1.88-1.85 (m, overlapped, 5H), 1.61-1.48 (m, overlapped, 9H), 1.38-1.15 (m, 4H), 1.07 (s, 3H), 0.91 (s, 3H).  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ ):  $\delta$  176.4, 174.5, 140.4, 121.5, 116.6, 101.1, 85.1, 78.4, 76.8, 75.6, 74.0, 73.8, 70.3, 61.4, 51.2, 50.8, 48.0, 47.3, 38.9, 38.2, 37.7, 37.1, 32.5, 29.3, 26.9, 26.3, 20.9, 18.6, 14.8. HRFABMS:  $m/z = 535.2916$  (M+H); calculated for  $\text{C}_{29}\text{H}_{43}\text{O}_9$ ,  $m/z = 535.2907$ ,  $\Delta = 1.7$  ppm.

## Chapter V. Isolation of Pyridoacridine Alkaloids As Akt Kinase Inhibitors

### V.1 Introduction.

Marine organisms have provided a wide variety of natural products with novel structures.<sup>71</sup> Good examples of these structures are the polycyclic aromatic pyridoacridine alkaloids, amphimedine (**5.1**) and neoamphimedine (**5.2**). As part of our NCDDG program of searching for bioactive compounds from marine organisms, the crude extract of the marine sponge *Petrosia* sp. (Petrosiidae) showed weak inhibitory activity against the A2780 cancer cell line as well as an inhibitor of the enzyme Akt kinase. Bioassay-directed fractionation led to the isolation of two known pyridoacridine alkaloids, amphimedine (**5.1**) and neoamphimedine (**5.2**) from the CH<sub>2</sub>Cl<sub>2</sub> fraction of the crude extract. The structures were elucidated by 2D-NMR experiments as well as by comparison of their <sup>1</sup>H and <sup>13</sup>C NMR data with the literature. Both compounds showed weak cytotoxicity to the A2780 mammalian cell line as well as weak activities as inhibitors of the enzyme Akt kinase.



**Figure 5.1** Structure of amphimedine, neoamphimedine and deoxyamphimedine

<sup>71</sup> a) Blunt, J. W.; Copp, B. R.; Munro, M. H.; Northcote, P. T.; Prinsep, M. R. Marine natural products. *Nat. Prod. Rep.* **2004**, *21*, 1-49. b) Berlinck, R. G. S.; Hajdu, E.; Rocha, R. M.; Oliveira, J. H. H.; Hernandez, I. L. C.; Selegim, M. H. R.; Granato, A. C.; Almeida, E. V. R.; Nunez, C. V., Challenges and Rewards of Research in Marine Natural Products Chemistry in Brazil. *J. Nat. Prod.* **2004**, *67*, 510-522.

## V.2 Chemical And Biological Investigation of Pyridoacridine Alkaloids.

In 1983, two research groups, lead by Schmitz and Shoolery, reported a novel marine alkaloid from the pacific sponge *Amphimedon* sp. and named it amphimedine (**5.1**). This compound was the first example of a new class of marine polycyclic alkaloids that came to be known as the "pyridoacridines".<sup>72</sup> Since then, over forty example of this type of polycyclic heteroaromatic marine alkaloid have been published, including two close analogs of amphimedine. Neoamphimedine (**5.2**) was isolated from two different *Xestospongia* species together with amphimedine (**5.1**) by Guzman, *et al.* at SmithKline Beecham and Scripps Institution of Oceanography in 1999.<sup>73</sup> Deoxyamphimedine (**5.3**) was also reported from a *Xestospongia* sp. in 2001.<sup>74</sup> All three of these compounds were identified as topoisomerase II inhibitors.<sup>75,76</sup>

The name "pyridoacridine" was based on the hypothetical parent structure of 11*H*-pyrido-[4,3,2-*m,n*]-acridine skeleton (**5.4**). However, most reported alkaloids were actually "pyridoacridone" derivatives, with an 8*H*-pyrido-[4,3,2-*m,n*]-acridone iminoquinone structure (**5.5**) (Figure 5-2). The former name, however, prevails in the literature and is accepted as a common name of this type of compound. The partially saturated nitrogen-containing rings in "pyridoacridines" are easily aromatized by air oxidation (self-oxidation) to the more stable "pyridoacridone".<sup>77</sup> Similarly, the imino-

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<sup>72</sup> Schmitz, F. J.; Agarwal, S. K.; Gunasekera, S. P.; Schmidt, P. G.; Shoolery, J. N. Amphimedine, new aromatic alkaloid from a pacific sponge, *Amphimedon* sp. Carbon connectivity determination from natural abundance carbon-13-carbon-13 coupling constants. *J. Am. Chem. Soc.* **1983**, *105*, 4835-4836

<sup>73</sup> Guzman, F. S.; Carte, B.; Troupe, N.; Faulkner, D. J. Neoamphimedine: a new pyridoacridine topoisomerase II inhibitor which catenates DNA. *J. Org. Chem.* **1999**, *64*, 1400-1402

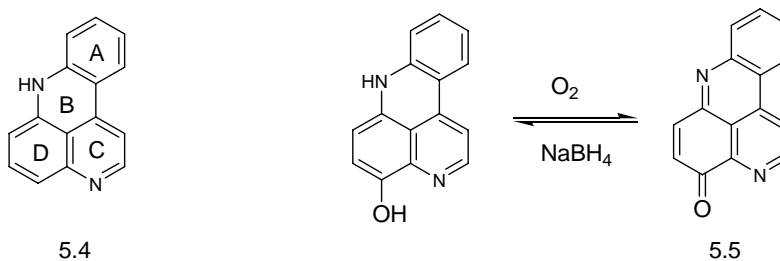
<sup>74</sup> Tasdemir, D.; Marshall, K. M.; Mangalindan, G. C.; Concepcion, G. P.; Deoxyamphimedine, a new pyridoacridine alkaloid from two tropical *Xestospongia* sponges. *J. Org. Chem.* **2001**, *66*, 3246-3248.

<sup>75</sup> Marshall, K. M.; Matsumoto, S.S.; Holden, J. A.; The anti-neoplastic and novel topoisomerase II-mediated cytotoxicity of neoamphimedine, a marine pyridoacridine. *Biochem. Pharm.* **2003**, *66*, 447-458.

<sup>76</sup> Marshall, K. M.; Barrows, L. R. Biological activities of pyridoacridines. *Nat. Prod. Rep.* **2004**, *21*, 731-751

<sup>77</sup> Molinski, T. F. Marine pyridoacridine alkaloids: structure, synthesis, and biological Chemistry. *Chem. Rev.* **1999**, *93*, 1825-1838

quinone substructure of a "pyridoacridone" such as amphimedine and neoamphimedine can be easily reduced by  $\text{NaBH}_4$ .<sup>72,73</sup> Also it should be noted that iminoquinone type pyridoacridine alkaloids can be reduced during ionization stage in a mass spectrometer, which would give an additional dihydro-molecular ion signal in their mass spectrum ( $M + 2$  ion in EIMS,  $MH + 2$  in CI and FABMS), as is typical for quinones.



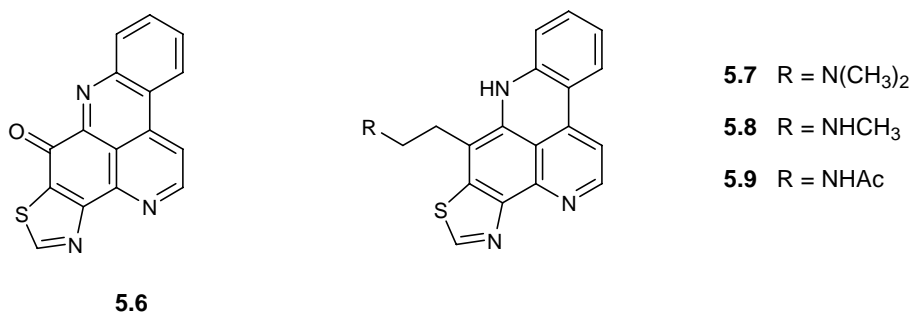
**Figure 5-2** Basic skeleton of Pyridoacridine rings and redox reactions

Pyridoacridine alkaloids are very stable to heat, and most of them have melting points higher than  $300\text{ }^\circ\text{C}$ . Pyridoacridine alkaloids have limited solubility in common organic solvents such as  $\text{CHCl}_3$  or MeOH, DMSO, *etc.* Thus, their NMR spectra were generally recorded in TFA-*d* solution in the protonated salt form. Pyridoacridines are pH indicators and they give a bright yellow solution in  $\text{CHCl}_3$  or MeOH, but a dark red solution in TFA. This was explained by Schmitz *et al.* as being due to a shift of conjugation on the E ring by TFA which altered the compounds' UV-visible absorption band.<sup>72</sup>

Pyridoacridine alkaloids have been found in several marine invertebrates include sponges (Porifera), tunicates (Urochordata), anemones (Cnidaria), and prosobranch (Mollusca). The fact that pyridoacridines have been isolated from different species in more than one major phylum suggests that these alkaloids are actually products of marine

micro-organisms that colonize in these invertebrates. However, no evidence has appeared so far to confirm this hypothesis. It seems just as likely that these compounds are over-expressed secondary metabolites of aromatic amino acid secondary metabolic pathways.<sup>77</sup> Certainly, studies are still needed in alkaloid metabolism in the marine organism world.

Almost all the pyridoacridine alkaloids that have been reported have significant cytotoxic activities. For example, kuanoniamine A, isolated from the lamellarid mollusc *Chelynotus semperi* and its tunicate prey, has shown good activity against the A2780 cell line ( $IC_{50} = 1.0 \mu\text{g/mL}$ ).<sup>78</sup> Its analogs, kuanoniamine B (**5.7**), C (**5.8**) and D (**5.9**) also showed good activities in some other cell lines.<sup>78</sup> In addition, a number of specific biological properties have emerged for different pyridoacridine alkaloids including inhibition of topoisomerase II,<sup>79</sup> anti-HIV activity,<sup>80,81</sup> and DNA intercalation.<sup>82</sup>



**Figure 5-3** Structure of Kuanoniamine alkaloids

<sup>78</sup> Carroll, A. R.; Scheuer, P. J. Kuanoniamines A, B, C, and D: pentacyclic alkaloids from a tunicate and its prosobranch mollusk predator *Chelynotus semperi*. *J. Org. Chem.* **1990**, *55*, 4426-31.

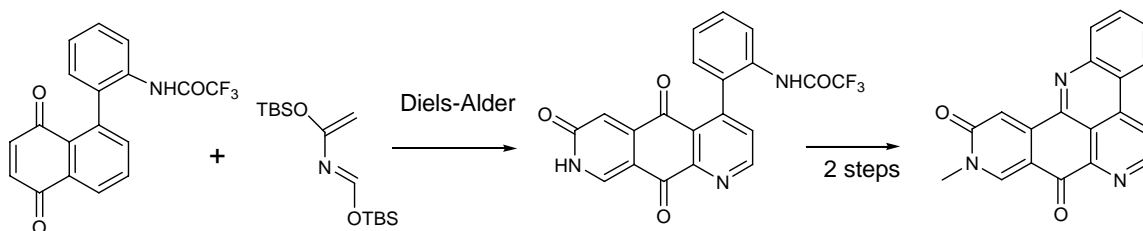
<sup>79</sup> McDonald, L. A.; Eldredge, G. S.; Barrows, L. R.; Ireland, C. M. Inhibition of topoisomerase II catalytic activity by pyridoacridine alkaloids from a *Cystodytes* sp. ascidian: a mechanism for the apparent intercalator-induced inhibition of topoisomerase II. *J. Med. Chem.* **1994**, *37*, 3819-27.

<sup>80</sup> Patil, A. D.; Kumar, N. V.; Kokke, W. C.; Bean, M. F.; Freyer, A. J.; Brosse, C. D.; Mai, S.; Truneh, A.; Carte, B. Novel alkaloids from the sponge *Batzella* sp. Inhibitors of HIV gp120-Human CD4 Binding. *J. Org. Chem.* **1995**, *60*, 1182-96.

<sup>81</sup> Taraporewala, I. B.; Cessac, J. W.; Chanh, T. C.; Delgado, A. V.; Schinazi, R. F. HIV-1 neutralization and tumor cell proliferation inhibition *in vitro* by simplified analogs of pyrido-[4,3,2-mn]-thiazolo-[5,4-b]-acridine marine alkaloids. *J. Med. Chem.* **1992**, *35*, 2744-52.

<sup>82</sup> Gunawardana, G. P.; Koehn, F. E.; Lee, A. Y.; Clardy, J.; He, H. Y.; Faulkner, D. J. Pyridoacridine alkaloids from deep-water marine sponges of the family Pachastrellidae: structure revision of dercitin and related compounds and correlation with the kuanoniamines. *J. Org. Chem.* **1992**, *57*, 1523-32.

Pyridoacridine alkaloids have also drawn attention from synthetic chemists because of their novel heterocyclic skeleton. For example, amphimedines were synthesized by three different groups in 1988 and 1989.<sup>83, 84, 85</sup> All their synthetic schemes utilized Diels-Alder addition of a aza-diene with a substituted quinone to build up the skeleton of amphimedine.



**Figure 5-4** Synthesis of amphimedine

### V.3 Results and Discussion.

The marine organism, *Petrosia*, sp. (Petrosiidae), was unidentified when we began our isolation and structure elucidation. The crude sample collected from the sea near E. New Britain at 15 m depth gave a deep green-blue color in 90% aq. methanol, but when the solution was diluted with water, the color changed to purple. This might be due to the presence of pH indicators such as pyridoacridine type compounds.

The crude extract was found to display weak cytotoxicity toward the A2780 human ovarian cancer cell line ( $IC_{50} = 14 \mu\text{g/mL}$ ). A portion of the crude extract (1 g) was partitioned between 80% aqueous methanol and hexanes (Scheme 5-1). The aqueous methanol fraction was then diluted to 60% methanol with water and extracted with  $\text{CH}_2\text{Cl}_2$ . Each fraction was evaporated and dried under vacuum. The  $\text{CH}_2\text{Cl}_2$  fraction was

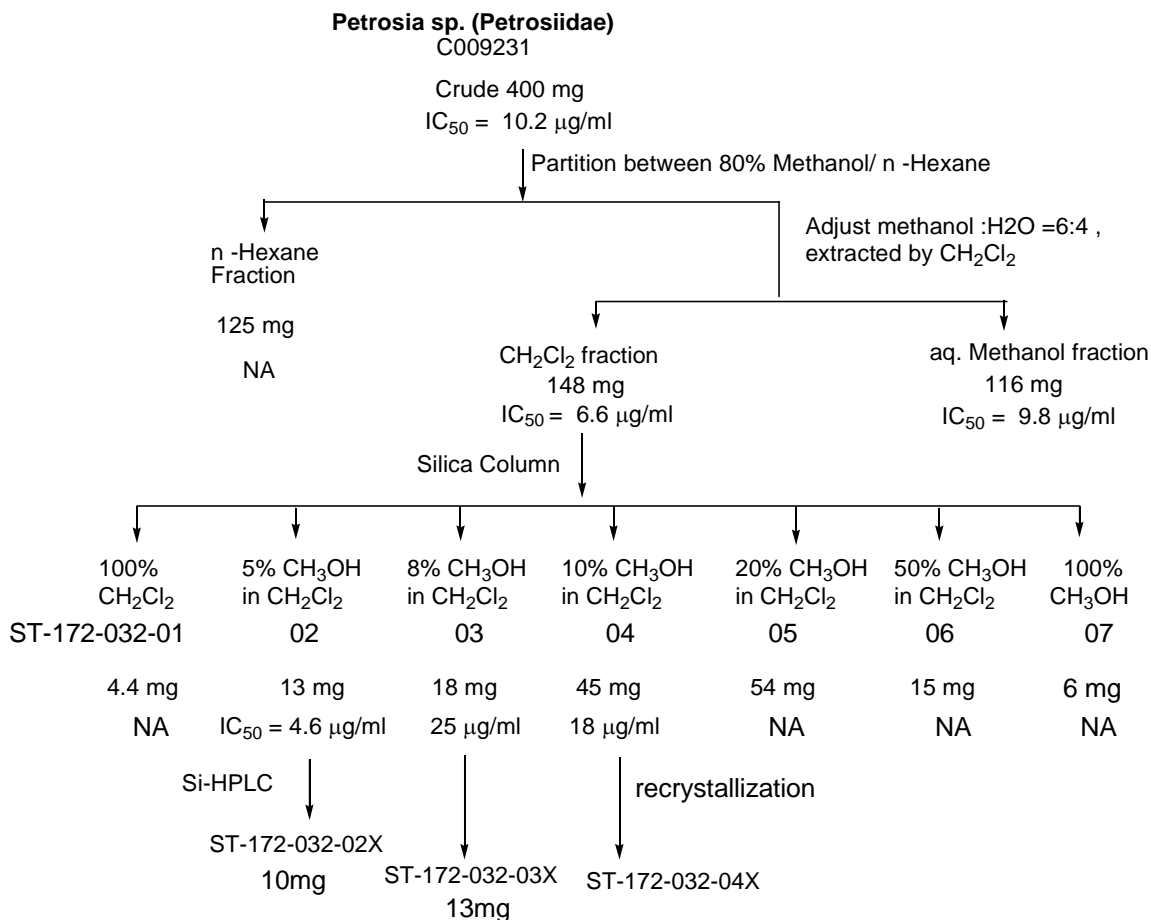
<sup>83</sup> Echavarren, A. M.; Stille, J. K. Total synthesis of amphimedine *J. Am. Chem. Soc.* **1988**, 110, 4051-4056.

<sup>84</sup> Kubo, A.; Nakahara, S. Synthesis of amphimedine, a new fused aromatic alkaloid from a pacific sponge, *Aamphimedon* sp. *Heterocycles* **1988**, 27, 2095-2098.

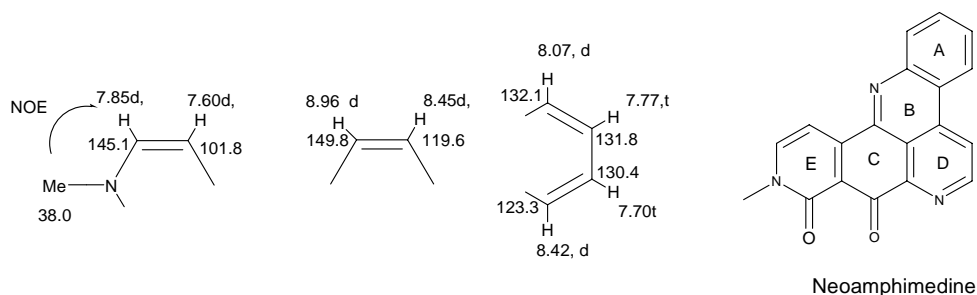
<sup>85</sup> Prager, R. H.; Tsopelas, C. A simple synthesis of amphimedine. *Heterocycles* **1989**, 29, 847-848.

determined as the most active after bioassay. TLC analysis was performed with 8% methanol in  $\text{CHCl}_3$  and visualized by Dragendorff spray, which gave orange-red spots on a yellow background, indicating the presence of alkaloids. Therefore, the  $\text{CH}_2\text{Cl}_2$  fraction was subjected silica column chromatography with a gradient of  $\text{CHCl}_3/\text{MeOH}$  to give three bioactive alkaloid fractions, numbered as ST-172-032-02, 03 and 04. Fractions 032-02, 03 were repurified by HPLC on a silica column to yield pure compound ST-172-032-02X and 03X. Fraction 032-04 was recrystallized from  $\text{CHCl}_3/\text{MeOH}$  to give pure compound ST-172-032-04X.

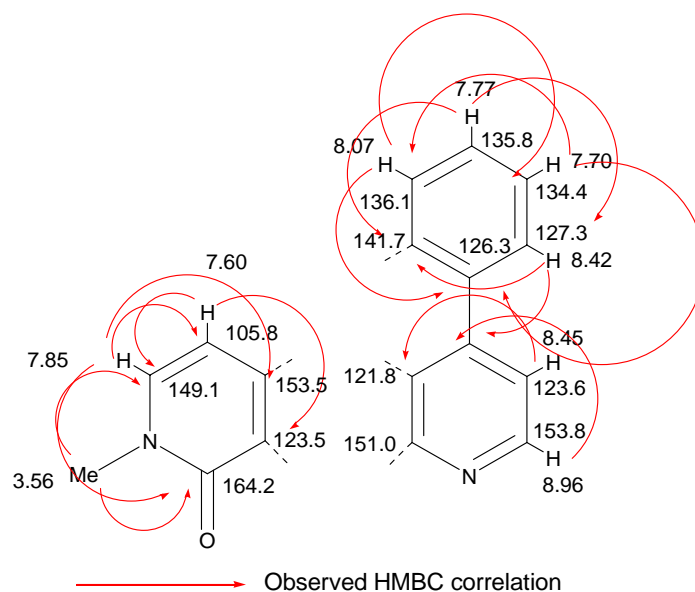
Scheme 5-1. Isolation tree of marine sponge *Petrosia* sp.



Pure compound ST-172-032-03X was obtained as yellow amorphous powder, which was slightly soluble in  $\text{CHCl}_3$ , methanol and pyridine but insoluble in acetone and DMSO. It showed a molecular ion peak at  $m/z$  313 ( $M^+$ ) on EI-MS. High resolution FABMS gave an exact mass consistent with the composition of  $\text{C}_{19}\text{H}_{11}\text{N}_3\text{O}_2$ . Because of the limited solubility in most solvents, the proton NMR experiment was done in  $\text{CDCl}_3/\text{CD}_3\text{OD}$  (2:1) mixed solvent. It showed eight aromatic protons at  $\delta$  8.96 (d,  $J = 5.6$  Hz), 8.45 (d,  $J = 5.6$  Hz), 8.42 (d,  $J = 8.0$  Hz), 8.07 (d,  $J = 8.0$  Hz), 7.85 (d,  $J = 6.8$  Hz), 7.77 (t,  $J = 8.0$  Hz), 7.70 (t,  $J = 8.0$  Hz), 7.60 (d,  $J = 6.8$  Hz) and one N-methyl signal at  $\delta$  3.54 (s). An NOE experiment showed a clear NOE enhancement of this methyl group on the signal of the aromatic proton at  $\delta$  7.85. DQ-COSY and HMQC experiments showed that those aromatic protons were separated into three spin systems with their chemical shifts identical with those of neoamphimedine (**5.2**) (Figure 5-5). An HMBC experiment gave the long range correlations from which the partial structure of three ring systems (A, D and E rings) could be established (Figure 5-6). However, the connectivity of these three rings could not be determined by the HMBC technique because of the presence of so many quaternary carbons. Further comparison of the  $^{13}\text{C}$  and  $^1\text{H}$  NMR data with reference data of neoamphimedine (**5.2**) in both  $\text{CDCl}_3/\text{CD}_3\text{OD}$  (2:1) and  $\text{TFA-}d/\text{CDCl}_3$  (2:1) mixed solvent indicated that this compound was neoamphimedine (Table 5.1).



**Figure 5-5.** COSY HMQC and NOESY correlations of neoamphimedine



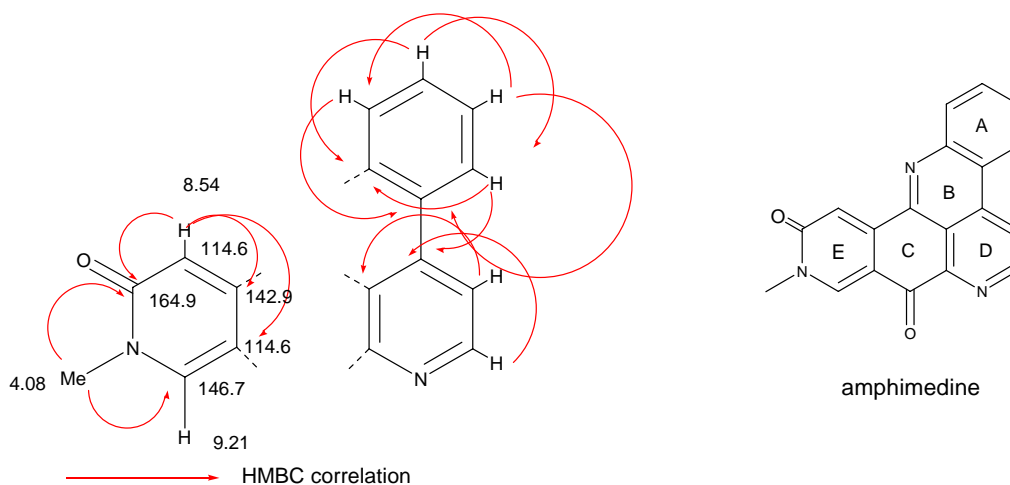
**Figure 5-6.** HMBC correlations of neoamphimedine.

**Table 5-1.** NMR data of ST-172-032-03 and Neoamphimedine (**5.2**)<sup>73</sup>

C #	ST-172-032-03 <sup>a</sup> 2:1 CDCl <sub>3</sub> /CD <sub>3</sub> OD			Neoamphimedine <sup>a</sup> 2:1 CDCl <sub>3</sub> /CD <sub>3</sub> OD		ST-172-032-03 <sup>b</sup> 2:1 TFA/ CDCl <sub>3</sub>		Neoamphimedine <sup>c</sup> 2:1 TFA/ CDCl <sub>3</sub>	
	δ(C)	δ(H)	HMBC	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)
1	132.1	8.09 (d,8.0)	3, 4a	131.9	8.14	133.6	8.69 (d, 8.0)	134.3	8.70
2	131.8	7.77 (t, 8.0)	4, 13a	131.6	7.78	136.9	8.38 (t, 8.0)	137.5	8.38
3	130.4	7.70 (t, 8.0)	1, 4a	130.2	7.69	133.5	8.23 (t, 8.0)	134.3	8.24
4	123.3	8.42 (d,8.0)	2, 4b,12a	123.0	8.48	125.3	8.92 (t, 8.0)	125.7	8.95
4a	122.3			121.9		120.1		120.7	
4b	137.7			137.5		145.0		145.6	
5	119.6	8.45(d, 5.8)	12c,6	119.0	8.53	124.6	9.45(d, 6.6)	125.5	9.48
6	149.8	8.96(d, 5.8)	5, 4b,	149.4	8.98	139.9	9.33(d,6.6)	140.3	9.33
7a	146.0			146.5		137.8		138.7	
8	179.5			179.8		174.2		175.5	
8a	119.5			118.9		116.9		116.7	
9	160.2			159.9		163.2		161.9	
11	145.1	7.85 (d,6.8)	12a, 9, 12, 14	145.2	7.87	145.4	8.43 (d,7.5)	144.2	8.48
12	101.8	7.70(d,6.8)	11,8a	101.7	7.70	106.0	8.47 (d,7.5)	108.1	8.51
12a	149.5			149.5		150.5		151.5	
12b	146.8			146.2		144.8		145.0	
12c	117.8			117.5		117.3		117.9	
13a	145.5			145.2		147.4		148.0	
14Me	38.4	3.56 (s)		38.0	3.50 (s)	39.8	4.08 (s)	40.2	4.06(s)

<sup>a</sup> 500 MHz CDCl<sub>3</sub>/CD<sub>3</sub>OD (2:1), <sup>b</sup> 500 MHz TFA-d / CDCl<sub>3</sub> (2:1), <sup>c</sup> 360 MHz TFA-d / CDCl<sub>3</sub> (2:1)

Fraction ST-172-032-02 was also cytotoxic and was purified by HPLC to yield a yellow amorphous powder ST-172-032-02X. This compound had similar properties to ST-172-032-03X. It also showed a molecular ion peak at  $m/z$  313 ( $M^+$ ) on EI-MS. High resolution FABMS gave the same formula of  $C_{19}H_{11}N_3O_2$  indicating that it was an isomer of neoamphimedine. Since this compound had poor solubility in most solvents including acetic acid, NMR experiments were done in 2:1 TFA- $d$ /CDCl<sub>3</sub>. The <sup>1</sup>H NMR spectrum showed signals for eight aromatic protons at  $\delta$  9.56 (d,  $J = 7.0$  Hz), 9.31 (d,  $J = 7.0$  Hz), 9.21 (s), 8.98 (d,  $J = 8.5$  Hz), 8.68 (d,  $J = 8.5$  Hz), 8.54 (s), 8.39 (t,  $J = 8.5$  Hz), and 8.21 (t,  $J = 8.5$  Hz), and for one N-methyl singlet at  $\delta$  4.08. 2D COSY and HMBC experiments revealed that the partial structure of three ring systems (A, D, E ring) matched perfectly with the skeleton of amphimedine (Figure 5-7). Comparison of the <sup>13</sup>C and <sup>1</sup>H NMR data with Schmitz and Shoolery's data for amphimedine in TFA- $d$ /CDCl<sub>3</sub> (2:1) mixed solvent indicated that this compound was amphimedine.<sup>72</sup> (Table 5-2)



**Figure 5-7.** Major HMBC correlations of amphimedine

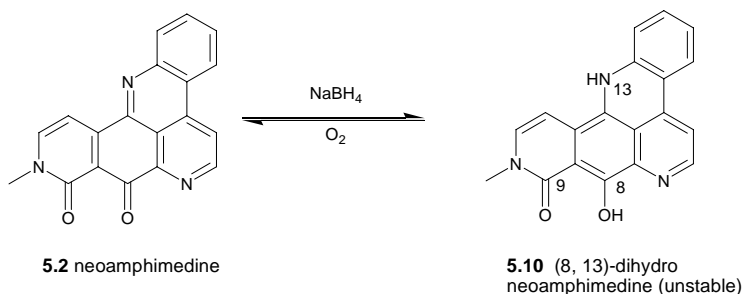
**Table 5-2.** NMR data of ST-172-032-02X and amphimedine (**5.1**).<sup>72</sup>

C #	ST-172-032-02X <sup>a</sup> 2:1 TFA/ CDCl <sub>3</sub> δ(C)	Amphimedine <sup>b</sup> 2:1 TFA/ CDCl <sub>3</sub> δ(C)	ST-172-032-02X <sup>c</sup> 2:1 TFA/ CDCl <sub>3</sub> δ(H)	Amphimedine <sup>d</sup> 2:1 TFA/ CDCl <sub>3</sub> δ(H)
C-1	133.1	133.1	8.68 (d, <i>J</i> = 8.5)	8.68 (d, <i>J</i> = 8.5)
C-2	136.9	137.4	8.39 (t, <i>J</i> = 8.5)	8.39 (t, <i>J</i> = 8.5)
C-3	132.4	132.5	8.21 (t, <i>J</i> = 8.5)	8.22 (t, <i>J</i> = 8.5)
C-4	124.9	125.8	8.98 (d, <i>J</i> = 8.5)	8.97 (d, <i>J</i> = 8.5)
C-4a	119.9	120.5		
C-4b	145.1	146.2		
C-5	124.3	125.2	9.56 (d, <i>J</i> = 7.0)	9.53 (d, <i>J</i> = 7)
C-6	139.3	139.0	9.31 (d, <i>J</i> = 7.0)	9.29 (d, <i>J</i> = 7)
C-7a	139.1	139.8		
C=O 8	173.5	175.0		
C-8a	114.6	113.5		
C-9	146.7	147	9.21 (s)	9.20 (s)
C=O 11	164.9	165.9		
C-12	114.6	115	8.54 (s)	8.52 (s)
C-12a	142.9	143.9		
C-12b	145.0	145.1		
C-12c	118.2	119.0		
C-13a	147.1	147.9		
N-Me	40.8 ppm	40.2 ppm	4.08 (s)	4.10 (s)

<sup>a</sup> 125 MHz TFA-*d* / CDCl<sub>3</sub> (2:1), <sup>b</sup> 75 MHz TFA-*d* / CDCl<sub>3</sub> (2:1), <sup>c</sup> 500 MHz, <sup>d</sup> 300 MHz

Fraction ST-172-032-04 was obtained as a broad yellow band on silica chromatography, immediately following ST-172-032-03. Recrystallization with CHCl<sub>3</sub>/MeOH gave orange yellow hair-like crystals ST-172-032-04X. These crystals gave a strong ion peak at  $m/z=315$  ( $M^+$ ) on EI-MS. High resolution FABMS showed major molecular ion peak at  $m/z = 316.1076$  ( $M+H$ ) which corresponded with a composition of C<sub>19</sub>H<sub>13</sub>N<sub>3</sub>O<sub>2</sub>. As noted earlier, *in situ* reduction of the iminoquinone structure of amphimedine or neoamphimedine (C<sub>19</sub>H<sub>12</sub>N<sub>3</sub>O<sub>2</sub>) can occur in the ion source of a mass spectrometer. Further examination of the <sup>13</sup>C NMR data of ST-172-032-04X in TFA-*d*/CDCl<sub>3</sub> showed that the data were identical with those of neoamphimedine (ST-172-032-03). Since this compound had identical NMR spectra to those of

neoamphimedine but different chromatographic behaviour, it must be a salt of neoamphimedine. Under acidic conditions of the NMR experiment in TFA-d/CDCI<sub>3</sub>, both neoamphimedine and neoamphimedinium salt would be expected to yield identical spectra. The structure of ST-172-032-04X was thus assigned as a neoamphimedinium salt.<sup>86</sup> An alternate dihydro-pyridoacrine structure (**5.10**) was not consistent with the presence of the carbonyl signal at  $\delta$ 179.5. To confirm this, (8,13)-dihydro-neoamphimedine (**5.10**) was prepared by treatment of neoamphimedine (ST-172-032-03X) with 2% NaBH<sub>4</sub> in methanol as described.<sup>1</sup> This reaction gave an unstable purple product, which quickly turned yellow by air oxidation on preparative thin layer silica chromatography. The yellow compound after separation with CHCl<sub>3</sub>/MeOH, was identified as the starting material neoamphimedine (Figure 5-8).



**Figure 5-8** (8,13)-dihydro-neoamphimedine.

All three compounds were tested in the A2780 mammalian cancer cell assay. Amphimedine had an IC<sub>50</sub> = 4.6  $\mu$ g/mL, neoamphimedine IC<sub>50</sub> = 20  $\mu$ g/mL, and neoamphimedinium chloride, IC<sub>50</sub> = 18  $\mu$ g/mL. They were also tested in the assay for

<sup>86</sup> The anion was determined as chloride after AgNO<sub>3</sub> test.

inhibitors of Akt kinase. Amphimedine had an  $IC_{50} = 12-13 \mu\text{g/mL}$ , neoamphimedine  $IC_{50} = 24-25 \mu\text{g/mL}$ , and neoamphimedinium chloride,  $IC_{50} = 16-18 \mu\text{g/mL}$ .

#### **V.4 Experimental Section.**

**General methods.** HPLC was carried on Varian Dynamax Si-HPLC column with MeOH/ $\text{CHCl}_3$  as mobile phase. High resolution FABMS were obtained by the staff of the analytical group of Virginia Polytechnic Institute and State University. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR, HMQC and HMBC experiments were obtained on the JEOL-500 MHz spectrometer. UV spectra were recorded on a Shimadzu 1201 UV-VIS spectrometer. The IMAF™ Akt Assay kit including binding buffer, binding reagent, reaction buffer and fluorescein-labeled Akt substrate was obtained from Molecular Devices. Akt kinase enzyme included in the kit was originally prepared by Upstate Inc.

**Plant Material.** The sponge *Petrosia* sp. (Petrosiidae) was collected by collectors from the Australia Institute of Marine Science (AIMS) under contact with the National Cancer Institute. Collection was made at a depth of 15 m in the sea of E. New Britain, Papua New Guinea. The marine material was extracted by ethanol. The marine organism was assigned voucher number Q66C6150. The extract was assigned the NCI number C009231. A voucher specimen of the organism is deposited at the Queensland Museum, Brisbane, Australia. The sample was identified by Dr. Michele Kelly, National Institute of Water and Atmosphere Research, New Zealand.

**Isolation of bioactive compounds.** The crude ethanol extract (400 mg) was partitioned between 90% aq. MeOH and *n*-hexane, and the aq. MeOH layer was then adjusted to 50% H<sub>2</sub>O and partitioned with CH<sub>2</sub>Cl<sub>2</sub>. The CH<sub>2</sub>Cl<sub>2</sub> fraction (158 mg) was subjected to chromatography on Si gel and eluted with a gradient of CHCl<sub>3</sub>/MeOH. Elution started with 100% CHCl<sub>3</sub> and continued with 5% MeOH to 8% MeOH in CHCl<sub>3</sub> to yield fraction ST-172-032-02 (13 mg) and ST-172-032-03 (18 mg). Elution with 8% to 10% MeOH in CHCl<sub>3</sub> gave a long yellow band of ST-172-032-04 (45 mg). Fractions 032-02 and 032-03 were purified by HPLC on silica column with elution with 5% MeOH in CHCl<sub>3</sub> to give pure amphimedine (11 mg) and neoamphimedine (13 mg). Fraction 032-04 was recrystallized from CHCl<sub>3</sub>/MeOH (2:1) to yield orange yellow crystals (21 mg). After determination of its spectra in TFA-*d*/CDCl<sub>3</sub>, the compound was recovered by treatment with 10% Na<sub>2</sub>CO<sub>3</sub> solution and extraction with CH<sub>2</sub>Cl<sub>2</sub> to yield a yellow powder (15 mg) after evaporation of solvent. The <sup>1</sup>H and <sup>13</sup>C NMR spectra of the recovered material in CDCl<sub>3</sub>/MeOH (2:1) were identical to those of the isolated sample ST-172-032-03X (neoamphimedine).

**Reduction of neoamphimedine by NaBH<sub>4</sub>.** Neoamphimedine (20 mg) was added to anhydrous MeOH (10 mL) at 0 °C under nitrogen, then a 2% NaBH<sub>4</sub> solution in MeOH (4 mL) was added drop by drop. The reaction mixture turned a purple color immediately and was stirred for 10 minutes after analytical TLC showed the depletion of starting material. The reaction was quenched with saturated NaHCO<sub>3</sub> (10 mL) and extracted with CHCl<sub>3</sub> (3 × 10 mL). The CHCl<sub>3</sub> layer was evaporated and separated on preparative TLC.

During this procedure, the purple product quickly turned yellow. The isolated yellow compound (11 mg) was identified as neoamphimedine starting material.

**Akt-kinase bioassay.** The assay procedure utilized was performed as per the manufacturer's instructions. A 1 mg/mL stock solution of each compound was prepared in 50% DMSO/H<sub>2</sub>O and serially diluted to the desired range of concentrations in IMAP reaction buffer containing 1 mM DTT. These solutions, along with a staurosporine positive control, were incubated with Akt enzyme for 30 minutes at room temperature in a 96-well high-efficiency microtiter plate. ATP and Akt substrate were added to each well and the whole plate was incubated for a further 60 minutes at room temperature. Binding buffer, containing nano-particle binding beads, was subsequently added. After a 30 minute incubation at room temperature, the plate was read using an Analyst AD instrument in fluorescence polarization mode (excitation  $\lambda = 485$  nm, emission  $\lambda = 530$  nm). Raw data was converted into % inhibition with reference to the average FP values from wells containing enzyme with no inhibitor (100% level) and wells containing no enzyme (0% level). IC<sub>50</sub> values were calculated using a linear extrapolation method.

**Amphimedine (5.1):** Yellow amorphous powder. UV:  $\lambda_{\text{max}}$  (MeOH) nm (log  $\epsilon$ ): 210 (4.29), 233 (4.59), 281 (3.96), 341 (3.78). NMR data: (in Table 5.2). EI-MS:  $m/z = 313$ , HRFABMS:  $m/z = 314.0927$  (M+H)<sup>+</sup>; calculated for C<sub>19</sub>H<sub>12</sub>N<sub>3</sub>O<sub>2</sub>,  $m/z = 314.0930$  ( $\Delta = -1.2$ ppm).

**Neoamphimedine (5.2):** Yellow amorphous powder. UV:  $\lambda_{\max}$  (MeOH) nm (log  $\epsilon$ ): 221 (4.27), 285 (4.54), 371 (3.74). NMR data (in Table 5.1). EI-MS:  $m/z = 313$ , HRFABMS:  $m/z = 314.0937$  (M+H)<sup>+</sup>; calculated for C<sub>19</sub>H<sub>12</sub>N<sub>3</sub>O<sub>2</sub>,  $m/z = 314.0930$ , ( $\Delta = +2.2$  ppm).

**Neoamphimedinium chloride:** Orange-yellow semi-crystals. UV:  $\lambda_{\max}$  (MeOH) nm (log  $\epsilon$ ): 235 (4.11), 385 (3.96). <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  8.96 (1H, d,  $J = 5.6$ ), 8.45 (1H, d,  $J = 5.6$ ), 8.42 (1H d,  $J = 8.0$ ), 8.07 (1H d,  $J = 8.0$ ), 7.85 (1H d,  $J = 6.8$ ), 7.77 (1H, t,  $J = 8.0$ ), 7.70 (1H, t,  $J = 8.0$ ), 7.60 (1H d,  $J = 6.8$ ), 3.54 (3H, s). <sup>13</sup>C NMR (in TFA-*d*/CDCl<sub>3</sub>): same as neoamphimedine. EI-MS:  $m/z = 315$ , HRFABMS:  $m/z = 316.1076$  (M+H<sub>2</sub>+H)<sup>+</sup>; calculated for C<sub>19</sub>H<sub>14</sub>N<sub>3</sub>O<sub>2</sub>,  $m/z = 316.1086$  ( $\Delta = -3.2$  ppm).

## Chapter VI. Isolation of Bromotyrosine Alkaloids from the Sponge *Porphyria flintae*

### VI.1 Introduction.

Marine sponges have been a rich source of halogenated metabolites, perhaps because of their unique metabolic system that can utilize the high concentration of chloride (0.5 mmol/L) or bromide ion (1  $\mu$ mol/L) from sea water. Since the 1970's, large numbers of bromopyrrole alkaloids and bromotyrosine alkaloids have been reported from different sponge species.<sup>87</sup> Bromopyrrole alkaloids, mainly reported from the Agelasidae, Hymeniacidonidae and Axinellida families, have shown a lot of interesting biological activities, including anti-bacterial, anti-viral and anti-inflammatory activities, as well as the inhibition of different cyclin-dependent-kinases such as cdk-4.<sup>88</sup> Bromo-tyrosine alkaloids, mainly reported from the *Verongia* and *Aplysina* species, have also shown good cytotoxicity against KB-cell lines as well as anti-viral and anti-microbial activity.<sup>89,90,91</sup>

Basically, all bromotyrosine alkaloids can be considered as derivatives of a putative 3,5-dibromo-spiro-cyclohexadienyl-1,2-*trans*-dihydroisoxazole carboxylic acid, (**6.1a** or **6.1b**). These secondary metabolites are derived from D or L-tyrosine, and contain a unique spiro-isoxazole moiety (Figure 6-1). Some simple derivatives of **6.1**, such as purealidine (**6.2**), aeroplysinin (**6.3**) and dibromoverongiaquinol (**6.4**) have also

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<sup>87</sup> Kobayashi, J. and Ishibashi, M. In *The Alkaloids: Chemistry and Pharmacology*, Vol 41, Ed.: Brossi, A., Academic Press: New York, **1992**; p 41.

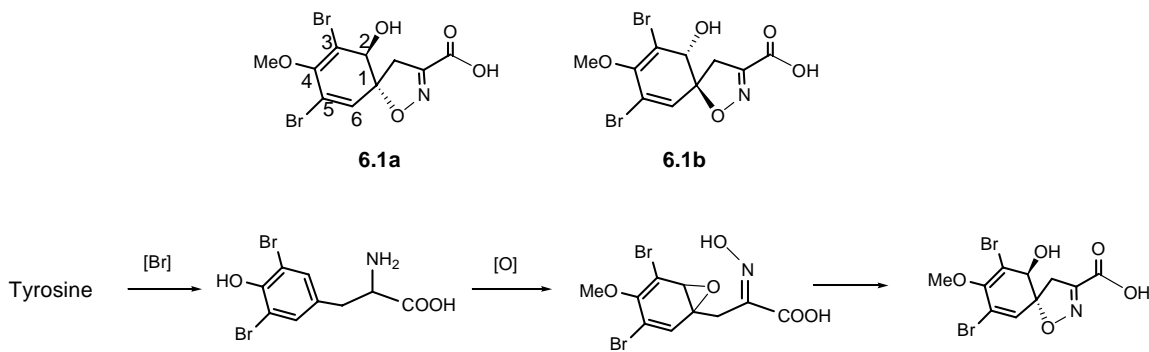
<sup>88</sup> For reviews of bromo-pyrrole alkaloids, see: Faulkner, D. J. Marine natural products, *Nat. Prod. Rep.* **2002**, *19*, 1.; Faulkner, D. J. Marine natural products, *Nat. Prod. Rep.* **1999**, *16*, 155-165.

<sup>89</sup> Mancini, I.; Guella, G.; Laboute, P.; Debitus, C. and Pietra, F. Hemifistularin 3: a degraded peptide or biogenetic precursor? Isolation from a Sponge of the order Verongida from the coral Sea or generation from base treatment of 11-oxofistularin 3. *J. Chem. Soc. Perkin Trans.1.* **1993**, 3121-24.

<sup>90</sup> Gao, H. F.; Kelly, M.; Hamann, M. T. Bromotyrosine-derived metabolites from the sponge *Aiolochoiria crassa*. *Tetrahedron* **1999**, *55*, 9717-21.

<sup>91</sup> Ciminiello, P.; Dell'Aversano, C.; Fattorusso, E.; Magno, S.; Pansini, M. Chemistry of Verongida sponges. 10. Secondary metabolite composition of the Caribbean sponge *Verongula gigantea*, *J. Nat. Prod.* **2000**, *63*, 263-267.

been reported from Verongida sponges (Figure 6-2).<sup>92</sup> Most bromo-tyrosine alkaloids usually comprise two or three dibromo-tyrosine units, such as purealin (**6.5**) from *Psammaplysilla porea*,<sup>93</sup> aerothionin (**6.6**) and homoaerothionin (**6.7**) from *Verongia* sp.,<sup>94</sup> and fistularin 3 (**6.8**) from *Aplysina archeri*<sup>95</sup> and *Aplysina fistularis*.<sup>96</sup> None of these compounds are very stable to acid or base, and they were found to undergo rearrangement of the isoxazole-ring to give the aromatic product (Figure 6-3). Thus, in most references only the relative configuration of the spiro-cyclohexadienyl-(1,2-*trans*)-dihydroisoxazole moiety was given, although in some cases, the absolute configuration of the moiety was clearly elucidated.<sup>97</sup>



**Figure 6-1** Biosynthesis of bromotyrosine derivatives

<sup>92</sup> Ciminiello, P.; Fattorusso, E.; Magno, S.; Pansini, M. Chemistry of Verongida sponges. III. Constituents of a Caribbean *Verongula* sp. *J. Nat. Prod.* **1994**, *57*, 1564-68.

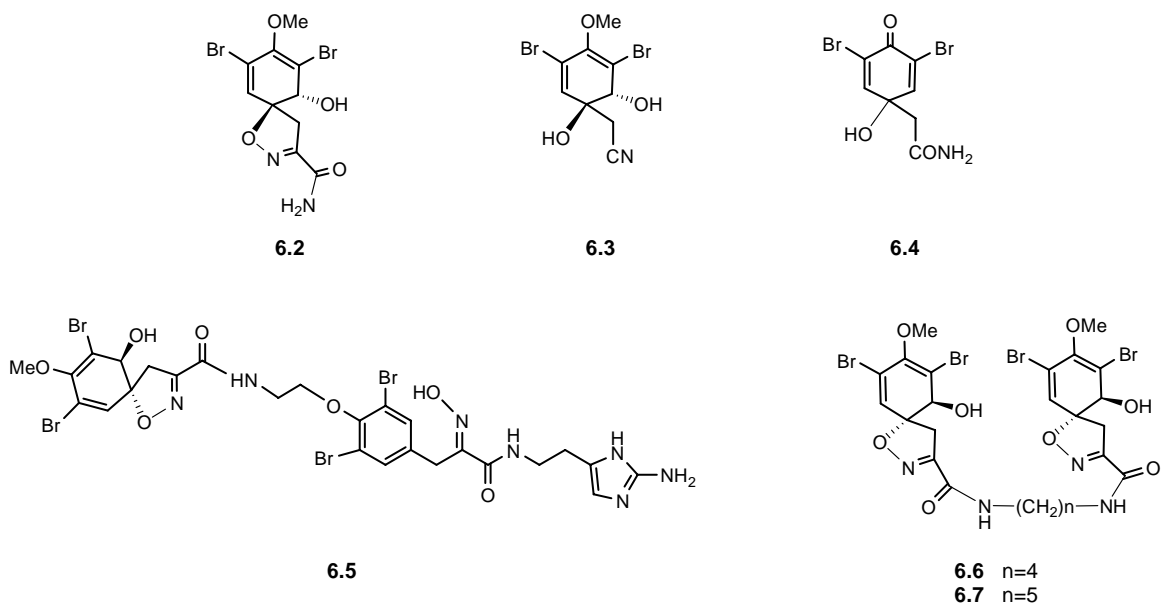
<sup>93</sup> Nakamura, H.; Wu, H.; Kobayashi, J.; Nakamura, Y.; Ohizumi, Y.; Hirata, Y. Physiologically active marine natural products from *Porifera*. IX. Purealin, a novel enzyme activator from the Okinawan marine sponge *Psammaplysilla porea*. *Tetrahedron Lett.* **1985**, *26*, 4517-22.

<sup>94</sup> Moody, K.; Thomson, R. H.; Fattorusso, E.; Minale, L.; Sodano, G. Aerothionin and homoaerothionin: two tetrabromospirocyclohexadienylisoxazoles from *Verongia* sponges. *J. Chem. Soc., Perkin Trans.1.* **1972**, 18-24.

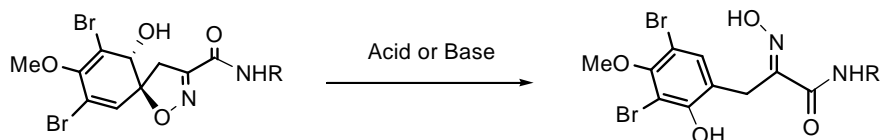
<sup>95</sup> Gunasekera S P; Cross S S. Fistularin 3 and 11-ketofistularin 3. Feline leukemia virus active bromotyrosine metabolites from the marine sponge *Aplysina archeri*, *J. Nat. Prod.* **1992**, *55*, 509-511.

<sup>96</sup> Gopichand, Y.; Schmitz, F. J., Marine natural products: fistularin-1, -2 and -3 from the sponge *Aplysina fistularis* forma fulva, *Tetrahedron Lett.* **1979**, *41*, 3921-28.

<sup>97</sup> Ciminiello, P.; Fattorusso, E.; Forino, M. Magno, S.; Chemistry of Verongida sponges, VIII. Bromocompounds from the Mediterranean Sponges *Aplysina aerophoba* and *Aplysina cavernicola* *Tetrahedron*, **1997**, *53*, 6565-72.



**Figure 6-2** Secondary bromo-tyrosine metabolites



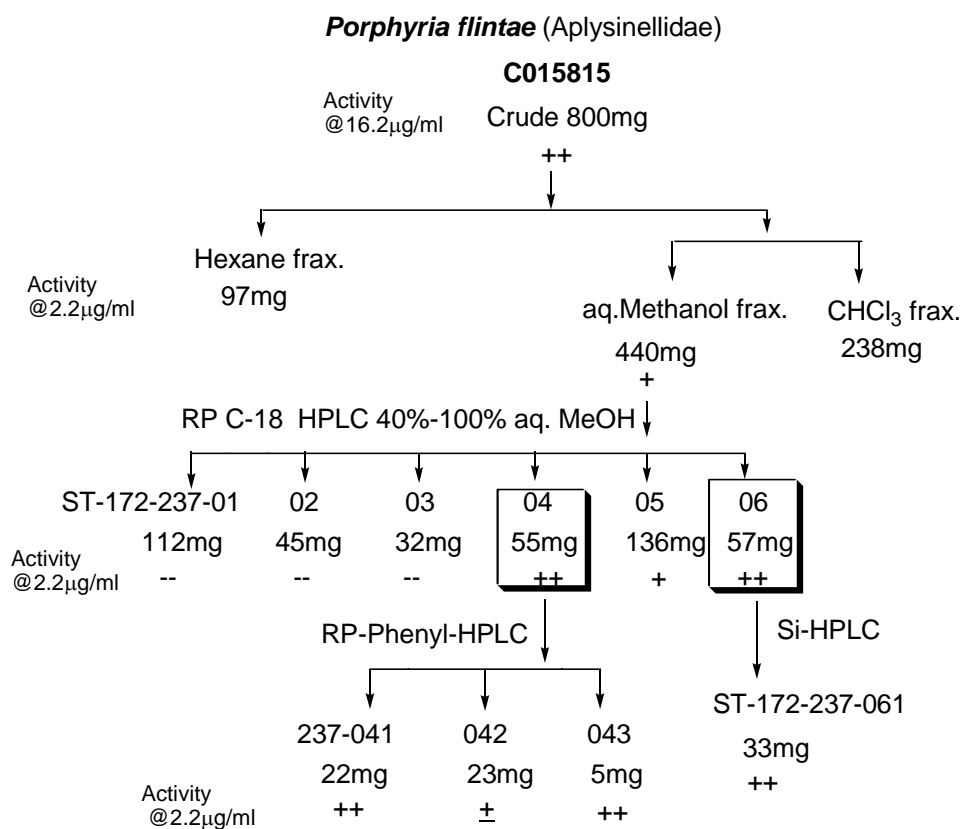
**Figure 6-3** Rearrangement of the spiro-isoxazole rings of bromo-tyrosine alkaloids,

## VI.2 Results and Discussion.

In our search for bioactive natural products as inhibitors of the enzyme DNA polymerase  $\beta$  (pol- $\beta$ ), the marine sponge *Porphyria flintae* (Aplysinellidae) showed mild activity in an assay for inhibitors of pol- $\beta$ . A portion of the sample (0.8 g) was partitioned between 80% aqueous methanol and hexanes (Scheme 6-1). The aqueous methanol fraction was then diluted to 60% methanol with water and extracted with

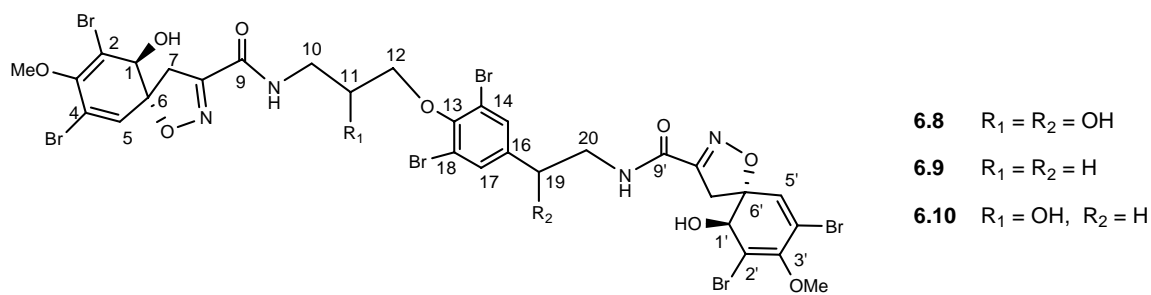
dichloromethane. Each fraction was evaporated and dried under vacuum. The methanol fraction was determined as the most active after bioassay, and this was then subjected to preparative RP-C18 HPLC column with a gradient of aqueous methanol (from 40% to 100% MeOH) to give two active fractions. These fractions were further separated by HPLC to yield three pure compounds, ST-172-237-041, 237-043 and 237-061.

**Scheme 6-1.** Isolation tree of the marine sponge *Porphyria* sp.



Compound ST-172-237-061 was isolated as a yellow powder. LR-FABMS gave a molecular ion signal that consisted of seven isotope peaks with each separated by 2 amu from the neighbor, and the central peak at  $m/z = 1082$  (relative intensity 1:6:15:20:15:6:1). This isotope pattern indicated the presence of 6 bromine atoms in this molecule. High

resolution FABMS gave a formula of  $C_{31}H_{30}Br_6N_4O_9$ , which was identical to the molecular formula of 11,19-di-deoxy-fistularin-3 (**6.9**). The  $^1H$  NMR spectrum of the compound 237-061 in  $CD_3OD$  indicated a symmetrical structure, with overlapped proton signals at  $\delta$  7.42 (2H, s), 6.38 (2H, s), 3.70 (6H, s). The presence of two spiro-isoxazole rings was also indicated by the two pairs of characteristic germinal methylene proton signals. The first pair of signals was at  $\delta$  3.83 (d,  $J = 18$  Hz), 3.16 (d,  $J = 18$  Hz), and the second pair was at  $\delta$  3.81 (d,  $J = 18$  Hz), 3.13 (d,  $J = 18$  Hz). In addition, the  $NH-CH_2-CH_2-CH_2-O$  and  $Ar-CH_2-CH_2-NH$  spin systems were elucidated by a COSY experiment. The  $^{13}C$  NMR data in  $CDCl_3$  closely matched the literature data for 11,19-di-deoxy-fistularin-3 (Table 6-1).<sup>98</sup> Compound ST-172-237-061 was thus determined as 11,19-di-deoxy-fistularin-3 (Figure 6-4).



**Figure 6-4** Structure of fistularin analogs.

<sup>98</sup> Kernan, M. R.; Cambie, R. C. and Bergquist, P. R. Chemistry of sponges, VIII. Anomoian A, a bromotyrosine derivative from *Anomoianthella popeae*. *J. Nat. Prod.* **1990**, 53, 615-618.

**Table 6-1.** Comparison of  $^{13}\text{C}$  NMR data for 237-061 with literature data for **6.9**

	11,19-di-deoxy-fistularin-3 <sup>a</sup> (Lit) <sup>97</sup>	ST-172-26-01 <sup>b</sup>
1, 1'	73.9, 73.8	74.1, 74.0
2, 2'	121.4	121.5
3, 3'	147.9	148.3
4, 4'	112.7	112.8
5, 5'	130.9, 130.7	131.2, 131.1
6, 6'	91.8, 91.6	92.0, 91.9
7, 7'	38.9, 38.8	39.1, 38.9
8, 8'	154.1, 154.0	154.4, 154.3
9, 9'	159.3	159.4
10	37.2	37.4
11	29.2	29.5
12	71.1	71.3
13	151.3	151.7
14, 18	118.1	118.4
15, 17	132.9	133.2
16	137.4	137.5
19	34.2	34.5
20	40.4	40.5
OMe	60.1	60.4

<sup>a</sup> CDCl<sub>3</sub>, 75 MHz. <sup>b</sup> CDCl<sub>3</sub>, 100 MHz

Compound 237-041 was isolated as a yellow solid, and its high resolution FABMS spectrum showed a molecular ion at  $m/z = 1114.7086$  (M+H), consistent with a formula of C<sub>31</sub>H<sub>30</sub>Br<sub>6</sub>N<sub>4</sub>O<sub>11</sub>. Its  $^{13}\text{C}$  spectrum (Table 6-2) matched closely with that of fistularin-3 (**6.8**).<sup>94,95</sup> A COSY experiment also supported the partial structure of a NH-CH<sub>2</sub>-CH(OH)-CH<sub>2</sub>-O and a Ar-CH(OH)-CH<sub>2</sub>-NH moiety. Therefore, compound ST-172-237-041 was determined to be the known compound, fistularin-3 (**6.8**).

**Table 6-2.** Comparison of  $^{13}\text{C}$  NMR data of ST-172-237-041 with literature for **6.8**.

	fistularin-3 <sup>a</sup> (Lit) <sup>95</sup>	ST-172-237-04 <sup>a</sup>
1, 1'	74.7, 74.6	74.3, 74.3
2, 2'	121.8	121.7
3, 3'	147.9	148.1
4, 4'	112.7	113.0
5, 5'	130.9, 130.7	131.1, 131.0
6, 6'	91.9, 91.8	91.4, 91.3
7, 7'	40.3,	40.0, 39.9
8, 8'	155.2, 155.1	154.1, 154.0
9, 9'	160.5	160.6, 160.5
10	43.9	43.6
11	69.4	68.9
12	76.1	75.8
13	152.3	151.7
14, 18	118.4	118.4
15, 17	131.1	131.4
16	143.5	143.9
19	69.5	69.6
20	48.2	47.8
OMe	59.8	59.2

<sup>a</sup> Pyridine-*d*<sub>5</sub> 100 MHz.

Compound 237-043 was isolated as a yellow solid. Its high resolution FABMS spectrum gave a formula of  $\text{C}_{31}\text{H}_{30}\text{Br}_6\text{N}_4\text{O}_{10}$ , which suggested a mono-oxidized analog of 237-061. Its  $^1\text{H}$  NMR spectrum was very similar to that of 237-061. The major difference was that 237-043 had one more oxygenated methine proton at  $\delta$  4.25 (1H, m) but no methylene protons at  $\delta$  2.28. Two spin systems of one  $\text{NH-CH}_2\text{-CH(OH)-CH}_2\text{-O}$  and one  $\text{Ar-CH}_2\text{-CH}_2\text{-NH}$  were indicated by a COSY experiment. Further comparison of its  $^{13}\text{C}$  NMR data (Table 6-3) together with those of 237-061 (11,19-dideoxyfistularin-3) and 237-041 (fistularin-3) in  $\text{CD}_3\text{OD}$  indicated that 237-043 was also a fistularin analog with

the hydroxyl group on C-19 removed. Therefore, this compound was determined as the known compound, 19-deoxy-fistularin-3 (**6.10**).

**Table 6-3.** Comparison of  $^{13}\text{C}$  NMR data of 237-043 with 237-041 and 237-061.

	ST-172-237-043 19-deoxy-fistularin-3 <sup>a</sup>	ST-172-237-041 fistularin-3 <sup>a</sup>	ST-172-237-061 11,19-di-deoxy-fistularin-3 <sup>a</sup>
1, 1'	74.2	74.3, 74.2	74.1
2, 2'	121.4	121.7, 121.6	121.5
3, 3'	148.0	148.1	147.9
4, 4'	112.8	113.0	112.8
5, 5'	130.9	131.1, 131.0	130.9, 130.8
6, 6'	91.2, 91.1	91.4, 91.3	91.5, 91.3
7, 7'	38.8	38.9	38.6, 38.5
8, 8'	153.9	154.0, 153.9	154.1, 154.0
9, 9'	160.5, 160.3	160.6, 160.5	160.0
10	42.4	42.6	37.2
11	68.7	68.9	29.2
12	74.5	74.7	70.9
13	151.3	152.0	151.3
14, 18	117.5	117.7	118.1
15, 17	133.2	131.1	132.9
16	138.5	142.5	138.5
19	33.7	70.4	34.2
20	40.2	48.5	40.4
OMe	59.0	59.2	58.9

<sup>a</sup> CD<sub>3</sub>OD 100 MHz.

All the three isolated fistularin analogs **6.8**, **6.9** and **6.10** showed moderate inhibitory activities to the enzyme pol- $\beta$ . A literature search indicated they also showed good cytotoxicities in several cell lines.<sup>94,96,97</sup> Their ability to inhibit the enzyme pol- $\beta$  made them possible potentiators of DNA damaging agents.

### VI.3 Experimental Section.

**General procedures.**  $^1\text{H}$  and DQCOSY HMQC, HMBC NMR spectra were recorded on a Varian Inova 400 MHz spectrometer,  $^{13}\text{C}$  NMR were obtained on a Varian Unity 400 MHz spectrometer. Preparative HPLC was carried on a Varian Dynamax RPC-18 HPLC column with MeOH and water as mobile phase. UV spectra were recorded on a Shimadzu 1201 UV-VIS spectrometer.

**Bioassay for the inhibitors of Pol- $\beta$ -enzyme.** Bioassay was carried out by Dr. Gao Zhijie at the University of Virginia via previously reported method.<sup>99</sup>

**Isolation of bioactive compounds.** The crude ethanol extract (C015815) (800 mg) was partitioned between 90% aq. MeOH and *n*-hexane, the aqueous MeOH layer was adjusted to 50% with water and then partitioned with  $\text{CH}_2\text{Cl}_2$ . The aqueous MeOH fraction (440 mg) was subjected to chromatography on preparative RP-C18 HPLC starting from 40% aq. MeOH to 95% aq. MeOH to yield 6 fractions. The active fraction ST-172-237-04 was separated by HPLC on a RP-phenyl column with elution by 75% aq. MeOH to give two active pure compounds: ST-172-237-041 (fistularin-3, 23 mg) and ST-172-237-043 (17-deoxy-fistularin-3, 5 mg). The active fraction ST-172-237-06 was purified by HPLC on a silica column with elution by 5% MeOH in  $\text{CHCl}_3$  to give pure ST-172-237-061 (11, 19-dideoxy-fistularin-3), (33 mg).

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<sup>99</sup> Chaturvedula, V. S. P.; Gao, Z.; Hecht, S. M.; Jones, S. H.; Kingston, D. G. I., A new acylated oleanane triterpenoid from *Couepia polyandra* that inhibits the lyase activity of DNA polymerase- $\beta$ , *J. Nat. Prod.* **2003**, *66*, 1463-1465.

**Fistularin-3 (6.8):** Yellow amorphous powder.  $[\alpha]_D = +116^\circ$  (MeOH,  $c = 0.24$ ). UV (MeOH)  $\lambda_{max}$  nm (log  $\epsilon$ ): 225 (4.41), 257 (4.20), 284 (4.02).  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ ):  $\delta$  7.58 (2H, s), 6.39 (1H, s), 6.38 (1H, s), 4.75 (1H, m), 4.18(1H, m), 4.08 (2H, s), 3.98 (2H, m, overlapped). 3.76 (1H, d,  $J = 18.4$ ), 3.73 (1H, d,  $J = 18.4$ ), 3.70 (6H, s, 2OMe), 3.53 (2H, m), 3.27 (2H, m), 3.07 (1H, d,  $J = 18.4$ ), 3.03 (1H, d,  $J = 18.4$ );  $^{13}\text{C}$  NMR: see Table 6-2; HRFABMS:  $m/z = 1114.6989$  (M+H), calculated for  $\text{C}_{31}\text{H}_{30}\text{Br}_6\text{N}_4\text{O}_{11}$   $m/z = 1114.7033$  ( $\Delta = -4.4$  ppm). This compound partially decomposed on standing for one week at room temperature.

**11, 19-di-deoxy-fistularin-3 (6.9):** Yellow powder.  $[\alpha]_D = +86^\circ$  (MeOH,  $c = 0.18$ ). UV (MeOH)  $\lambda_{max}$  nm (log  $\epsilon$ ): 225 (4.41), 257 (4.20), 284 (4.02).  $^1\text{H}$  NMR: ( $\text{CDCl}_3$ )  $\delta$  7.50(2H, s), 6.51 (2H, d,  $J = 1.2$ ), 4.15 (1H, d,  $J = 6.4$ ), 4.07 (2H, t,  $J = 6.4$ ), 3.83 (1H, d,  $J = 18$ ), 3.81(1H, d,  $J = 18$ ), 3.71(6H, s, 2OMe), 3.60 (2H, td,  $J = 7.2$  and 6.4), 3.53 (2H, m), 3.16 (1H, d,  $J = 18$ ), 3.13 (1H, d,  $J = 18$ ), 2.86 (2H, t,  $J = 7.2$ ), 2.11 (2H, m);  $^{13}\text{C}$  NMR: see Table 6-1; HRFABMS:  $m/z = 1082.7091$  (M+H), calculated for  $\text{C}_{31}\text{H}_{30}\text{Br}_6\text{N}_4\text{O}_9$   $m/z = 1082.7135$  ( $\Delta = -3.6$  ppm). This compound partially decomposed on standing for one week at room temperature.

**19-deoxy-fistularin-3 (6.10):** Yellow powder.  $[\alpha]_D = +102^\circ$  (MeOH,  $c = 0.10$ ). UV (MeOH)  $\lambda_{max}$  nm (log  $\epsilon$ ): 220 (4.42), 252 (4.18), 286 (4.04).  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ ):  $\delta$  7.53 (2H, s), 6.56 (2H, s), 4.25 (1H, m), 4.23 (1H, d,  $J = 0.8$ ), 4.18 (1H, d,  $J = 0.8$ ), 4.03 (2H, m), 3.86 (1H, d,  $J = 18$ ), 3.84 (1H, d,  $J = 18$ ), 3.75 (6H, s), 3.72 (1H, m), 3.58 (2H, t,  $J = 7.2$ ), 3.50 (1H, m), 3.12 (1H, d,  $J = 18$ ), 3.10 (1H, d,  $J = 18$ ), 2.88 (2H, t,  $J = 7.2$ );  $^{13}\text{C}$

NMR (CD<sub>3</sub>OD):  $\delta$  160.5, 160.3, 153.9, 153.8, 151.3, 148.0, 138.5, 133.2, 130.9, 121.4, 117.5, 112.8, 91.2, 91.1, 78.1, 74.5, 74.2, 74.1, 68.7, 59.1, 42.4, 40.1, 38.8, 33.7.  
HRFABMS:  $m/z$  = 1098.7100 (M+H), calculated for C<sub>31</sub>H<sub>30</sub>Br<sub>6</sub>N<sub>4</sub>O<sub>9</sub>  $m/z$  = 1098.7084 ( $\Delta$  = 1.6 ppm). This compound partially decomposed on standing for one week at room temperature.

## Chapter VII. Summary of Dropped Extracts

This chapter summarizes several extracts which were dropped in the ICBG or NCDDG projects (listed in Table 7-1). They were dropped either because the bioactivities were too weak, or the active compounds were anticipated to have no value for anticancer purposes, such as polyphenolic compounds (tannins), simple phenolic compounds (ellagic acids), fatty acids, etc.

**Table 7-1.** Index of dropped extracts.

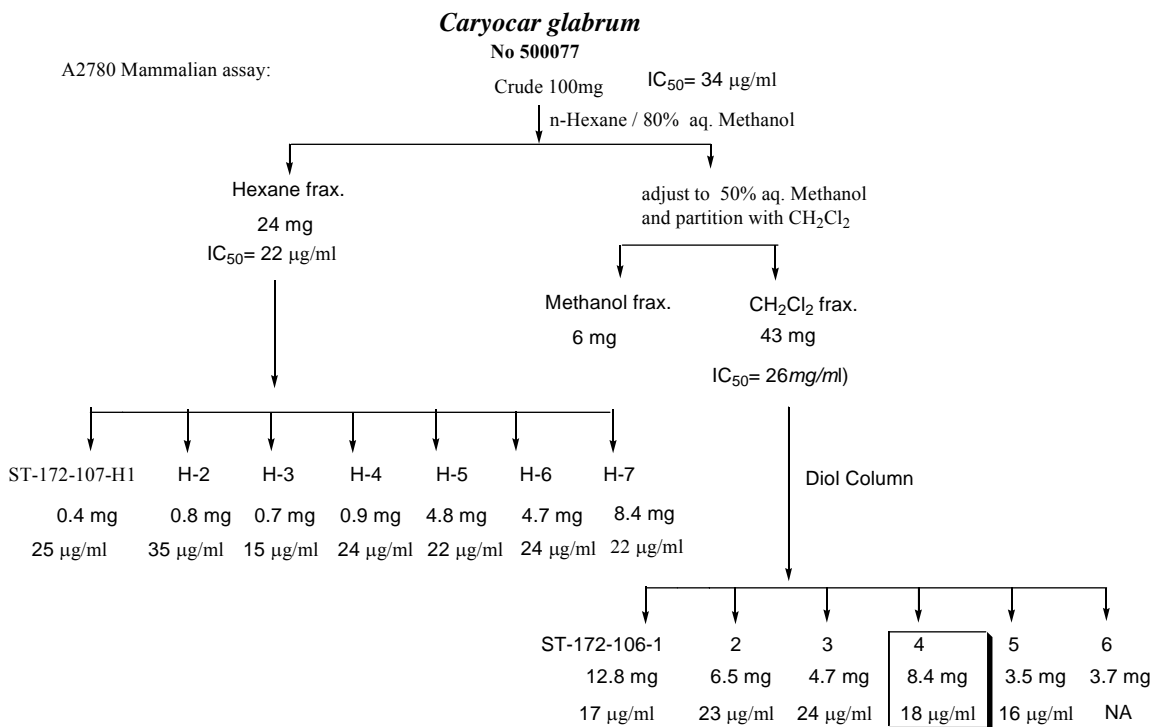
Plant name	Index #	Project	Drop reason
<i>Caryocar glabrium</i>	N500077	ICBG	Bioactivities too weak
<i>Tapura guianensis</i>	N400008	ICBG	Bioactivities too weak
<i>Parkia sumatrana</i>	N011629	NCDDG	Activities mainly from tannins
<i>Tetracoccus halii</i>	B855166	NCDDG	Activities mainly from fatty acids
<i>Brachychiton chillagoensis</i>	B855507	NCDDG	Activities from fatty acids
<i>Pedilanthus tithymaloides</i>	PC-10-114	NCDDG	Activities from fatty acids.
<i>Polyides rotundus</i>	UM 2916	NCDDG	Activities from polyphenols and fatty acids.

## VII.1 Fractionation of the Suriname Plant *Caryocar glabrum*

The higher plant *Caryocar glabrum* is widely distributed in humid forests of northern South America. It is a tree that grows up to 30 m high, with large ellipsoid fruit and oily edible almond.

The crude extract showed weak activity against the A2780 cell line ( $IC_{50} = 34 \mu\text{g/mL}$ ). Both the dichloromethane and the hexane fractions were active after solvent partition (Scheme 7-1). The dichloromethane fraction was subjected to chromatography on a diol column to yield 6 fractions, but none of these 6 fractions had significantly important activity. Chromatography of the hexane fraction also failed to yield any highly active fractions.

Scheme 7-1. Fractionation of *Caryocar glabrum*



Fraction 106-4 gave a positive results in the ferric chloride test for phenols, and examination by TLC showed that it was a pure compound. Recrystallization gave compound 106-04X. Its  $^1\text{H}$  NMR spectrum (in  $\text{DMSO-}d_6/\text{CDCl}_3 = 1:1$ ) was a simple one, with 5 aromatic protons at  $\delta$  7.68 (1H, d,  $J = 7.8$  Hz), 7.25 (1H, br, s), 6.90 (1H, dd,  $J = 7.8$  and 1.2 Hz), 6.40 (1H, s), 6.32 (1H, s) and two methoxyl groups at 3.90 (3H, s), 3.79 (3H, s). Its  $^{13}\text{C}$  NMR spectrum showed 7 oxygenated aromatic carbon signals around  $\delta$  160-150. Its UV spectrum (in EtOH) showed two absorption bands at 251 nm and 350 nm with approximately equal intensities. These data were similar to those of a coumarin type natural product, aureol (also called phytoalexin) (**7.1**).<sup>100</sup> Further comparison of the  $^{13}\text{C}$  NMR data with the aureol analog isotrifoliol (**7.2**)<sup>101</sup> showed good matches of carbon signals except that 106-04X had two methoxyl groups instead of one (Table 7-2). HRFABMS indicated a composition of  $\text{C}_{17}\text{H}_{12}\text{O}_6$ , consistent with the structure of a dimethoxy derivative of aureol. Further 2-D experiments (HMBC and HSQC) determined the position of the two methoxyl groups. The methoxy signal at  $\delta_{\text{H}}$  3.79 (s) was placed at C-9 because it showed HMBC correlation to the carbon signal of C-9 at  $\delta_{\text{C}}$  156.4, which was correlated with both the H-8 and H-10 signals at  $\delta$  6.90 (dd,  $J = 7.8$  and 1.2 Hz) and 7.25 (br, s) (Figure 7-1). The methoxy signal at  $\delta_{\text{H}}$  3.90 (s) was placed at C-1 because it gave an HMBC correlation with the carbon signal at  $\delta_{\text{C}}$  155.1 (C-1). The latter was also correlated with the signal of H-2 at  $\delta$  6.40 (br, s) but not with the signal of H-4 at  $\delta$  6.32 (br, s). Therefore, the structure of this compound was determined as that of a new analog of auroel, 1,9-*O*-dimethyl-aureol (**7.3**).

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<sup>100</sup> Melanie, O. J.; Adesanya, S. A. and Margaret, R. F. Isosojagol, a coumestane from *Phaseolus coccineus*, *Phytochemistry* **1984**, *23*, 2704-08.

<sup>101</sup> Hatano, T.; Aga, Y.; Shintani, Y.; Ito, H.; Okuda, T. and Yoshida, T. Minor flavonoids from licorice, *Phytochemistry* **2000**, *55*, 959-963.



## Experimental Section.

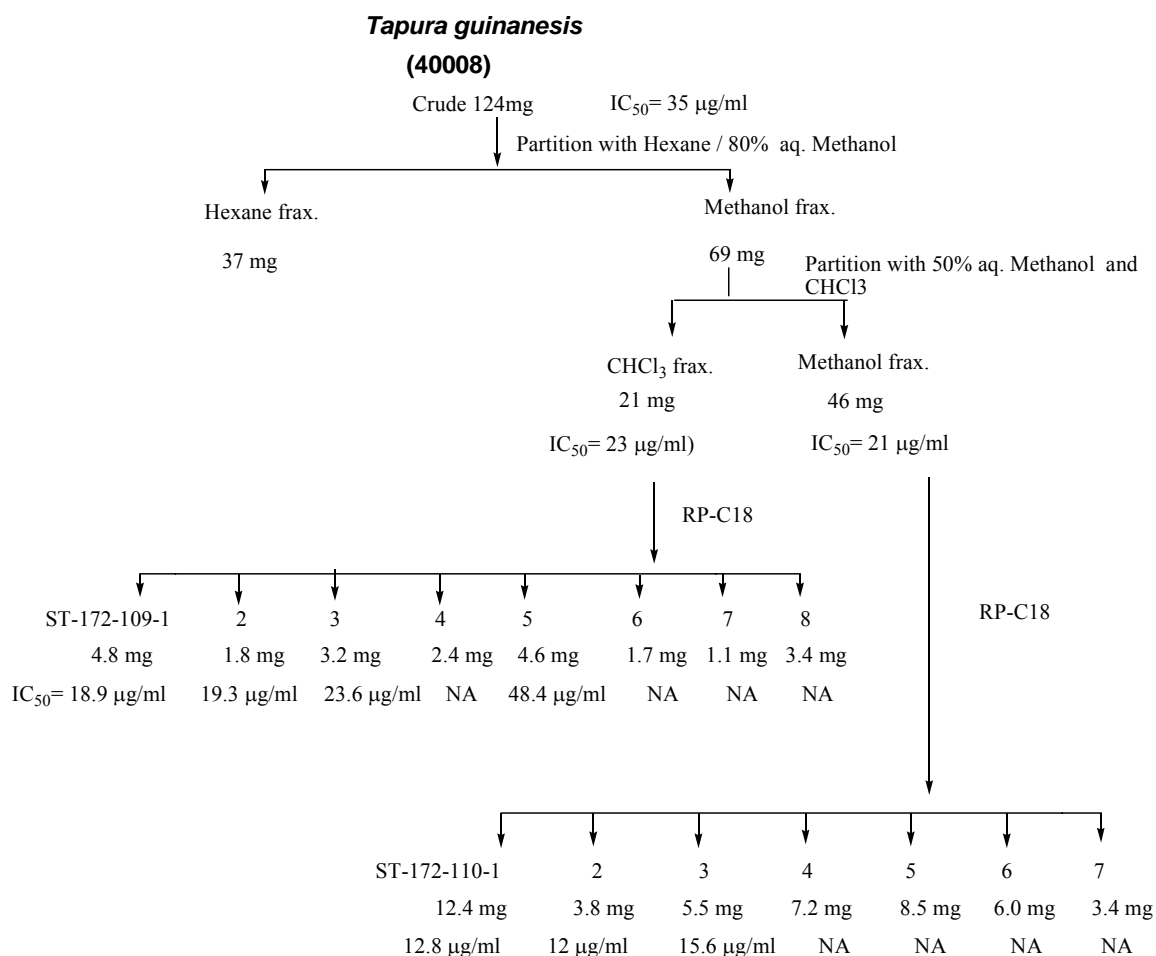
**Isolation of Compound 7.3.** The crude extract of *Caryocar glabrium* (100 mg) was partitioned between 80% aqueous methanol (100 mL) and hexanes (50 mL). The aqueous methanol fraction was then diluted to 60% with water and extracted with CH<sub>2</sub>Cl<sub>2</sub> (40 mL). The CH<sub>2</sub>Cl<sub>2</sub> fraction (43 mg) was subjected to diol column chromatography with a gradient of MeOH/CHCl<sub>3</sub> (0% to 50%) to yield 6 fractions. ST-172-106-4 (8.5 mg) was found to be almost pure after TLC analysis. Recrystallization from CHCl<sub>3</sub>/EtOH gave 4.5 mg of pure ST-172-106-4X (**7.3**).

**1,9-*O*-dimethyl-aureol (ST-172-106-4X, 7.3):** Yellowish powder. UV (EtOH)  $\lambda_{max}$  nm (log  $\epsilon$ ): 208 (4.48), 251 (4.23), 350 (4.24). <sup>1</sup>H NMR (DMSO-*d*<sub>4</sub>/CDCl<sub>3</sub> = 1:1):  $\delta$  7.68 (1H, d, *J* = 7.8), 7.25 (1H, br, s), 6.90 (1H, dd, *J* = 7.8 and 1.2), 6.40 (1H, s), 6.32 (1H, s), 3.90 (3H, s), 3.79 (3H, s). <sup>13</sup>C NMR: (see Table 7-1). HRFABMS: *m/z* = 313.0684 (M+H) calculated for C<sub>17</sub>H<sub>12</sub>O<sub>6</sub>, 313.0712.

## VII.2 Fractionation of the Madagascar Plant *Tapura guianensis*

The crude extract of the Madagascar plant *Tapura guianensis* showed weak activity in the A2780 cell line ( $IC_{50} = 35 \mu\text{g/mL}$ ). Both the dichloromethane and the methanol fractions were active after solvent partition (Scheme 7-2). Both fractions were subjected to RP-C18 flash column chromatography and gave several fractions. However, none of the fractions had significantly improved activity ( $IC_{50} < 10 \mu\text{g/mL}$ ), and the extract was dropped.

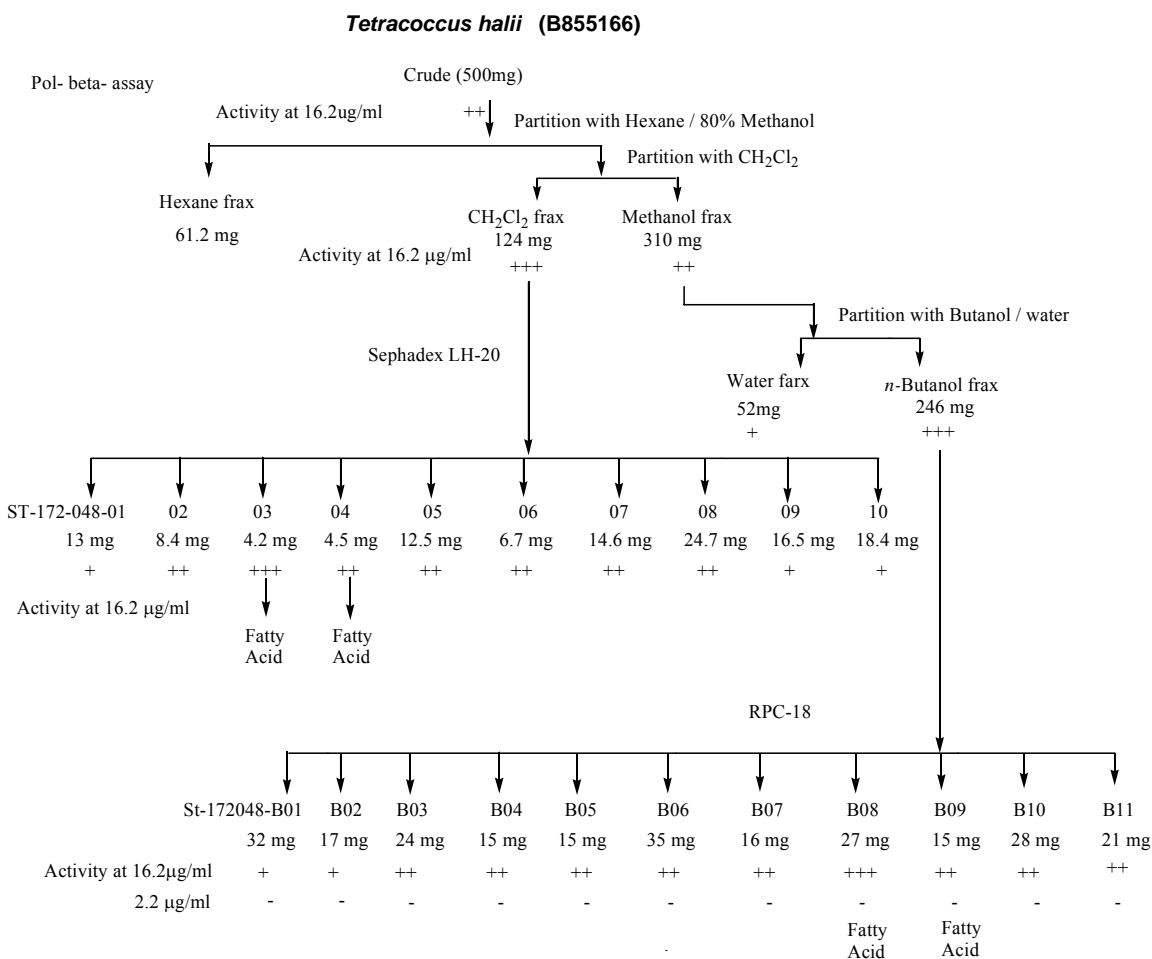
Scheme 7-2. Fractionation of *Tapura guianensis*



### VII.3 Fractionation of the Plant *Tetracoccus halii*.

The crude extract of *Tetracoccus halii* showed weak activity in the pol- $\beta$  bioassay. The dichloromethane and the butanol fraction were the most active fractions after solvent partition (Scheme 7-3). Further isolation and purification revealed that the major active fractions were fatty acids, and this extract was dropped.

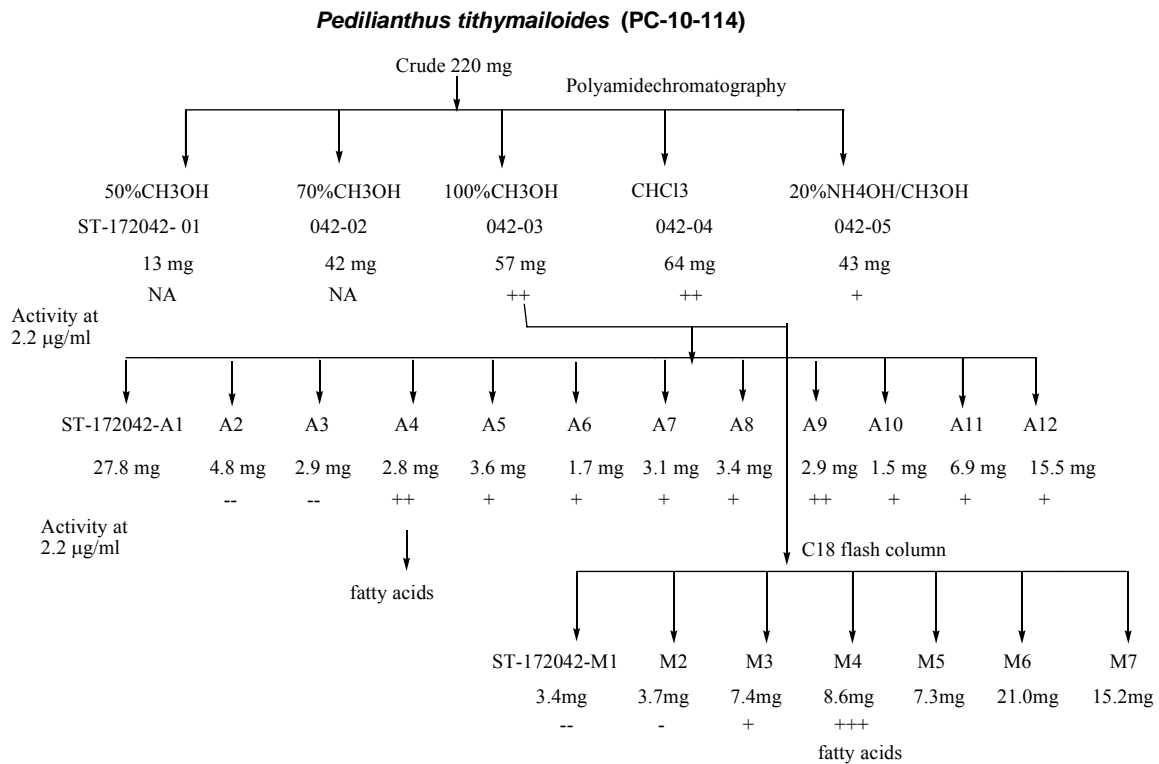
Scheme 7-3. Fractionation of *Tetracoccus halii*



#### VII.4 Fractionation of *Pedilanthus tithymailoides*.

The crude extracts of *Pedilanthus tithymailoides* showed weak activity in the pol- $\beta$  bioassay. It was detanninized by chromatography on a polyamide column and gave two major active fractions. Further isolation and purification revealed that the major active fractions were fatty acids (Scheme 7-4), and this extract was dropped.

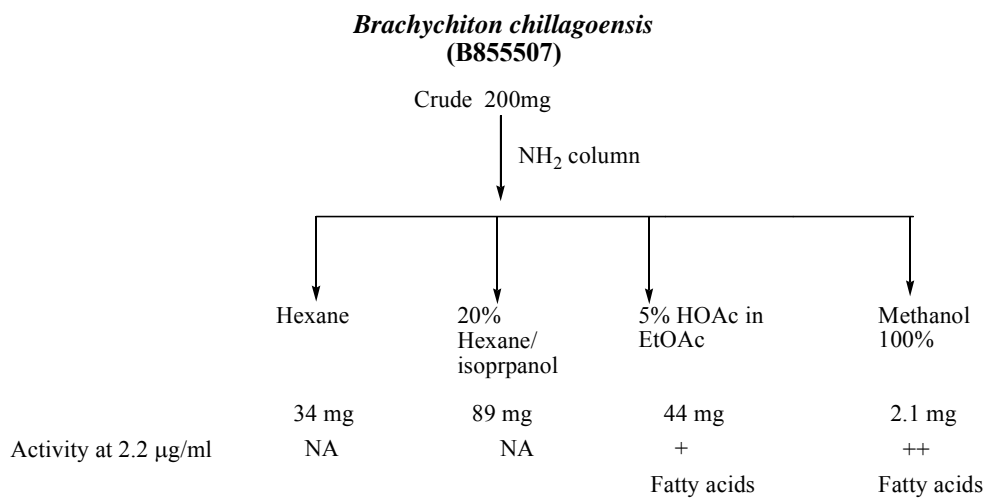
Scheme 7-4. Fractionation of *Pedilanthus tithymailoides*



## VII.5 Fractionation of the Plant *Brachychiton chillagoensis*.

The crude extracts of *Brachychiton chillagoensis* showed weak activity in the pol- $\beta$  bioassay. It was subjected to column chromatography on an amino column and gave two acidic fractions as major active fractions (Scheme 7-5).  $^1\text{H}$  NMR spectra of these fractions indicated that their major compounds were fatty acids, and this extract was dropped.

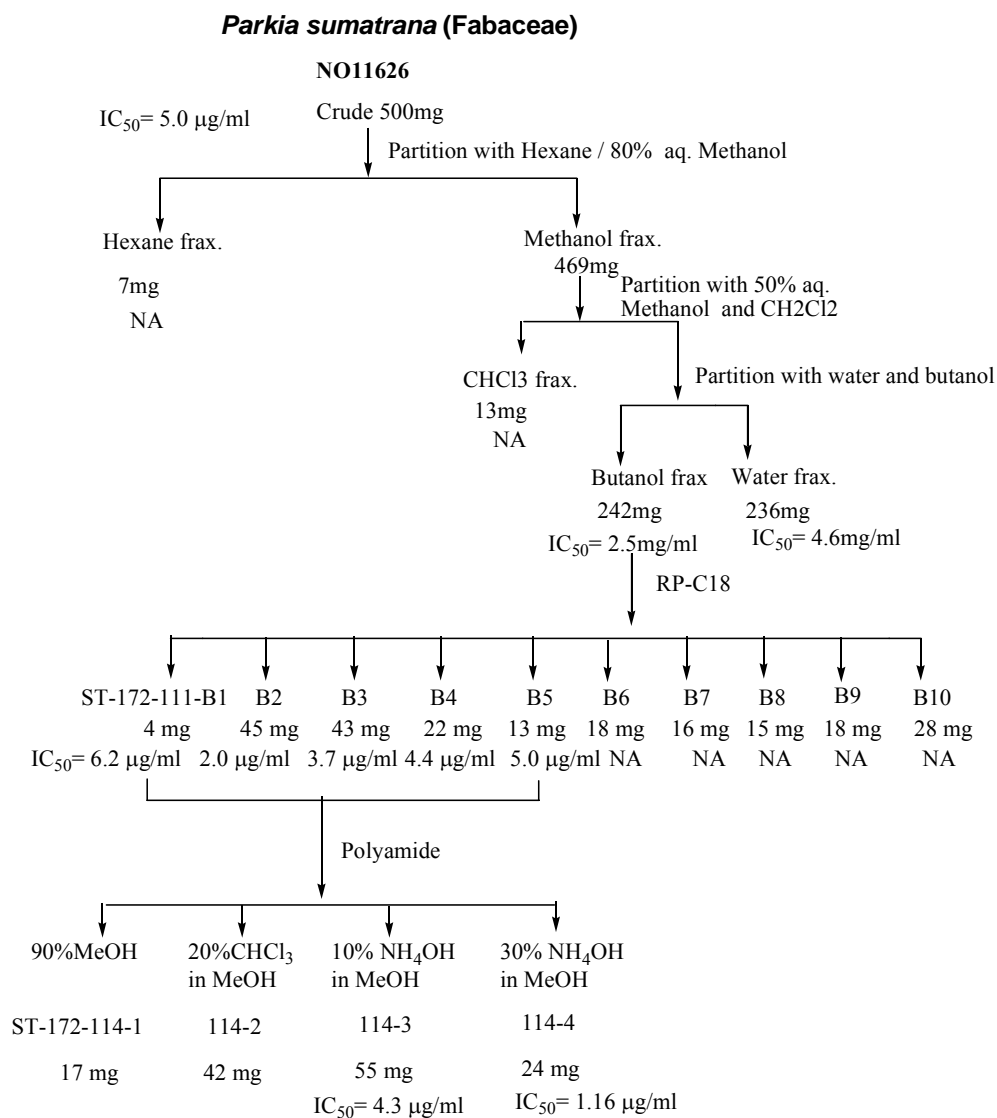
Scheme 7-5. Fractionation of *Brachychiton chillagoensis*



## VII.6 Fractionation of the Plant *Parkia sumatrana*.

The crude extract of *Parkia sumatrana* showed mild activity in an assay for inhibitors of the enzyme Cdc25B. The butanol fraction was the most active fraction after solvent partition (Scheme 7-6). Further isolation and purification yielded several highly polar fractions which were found to be tannins because of their high affinity on a polyamide column. Thus this extract was dropped.

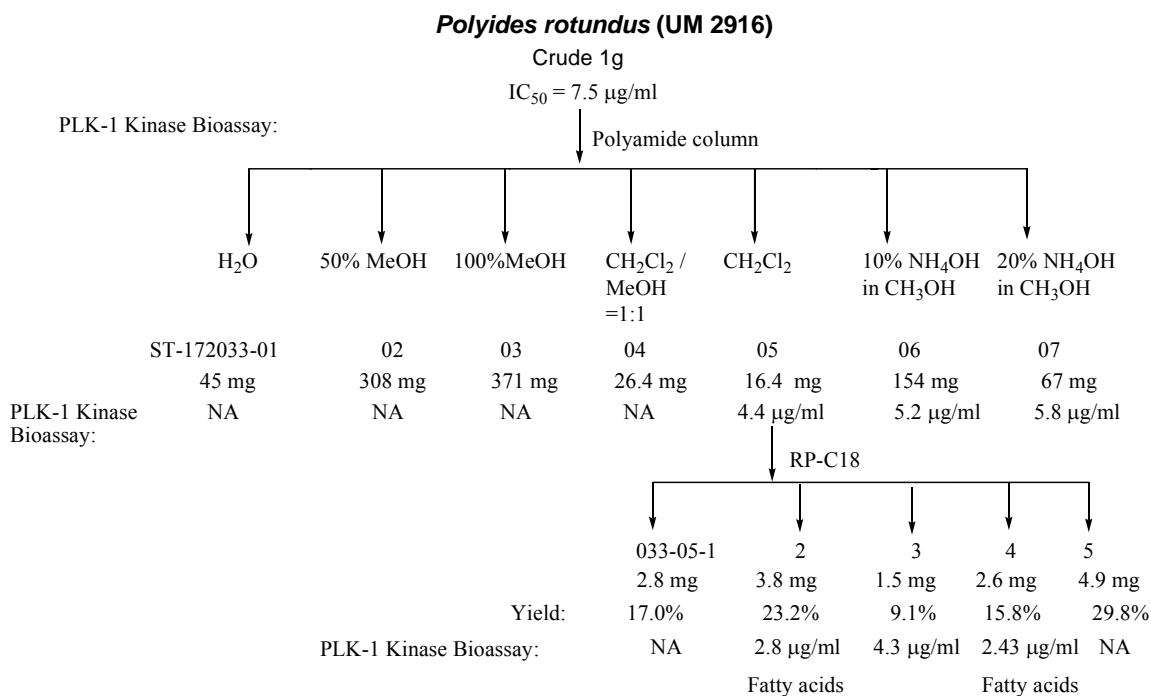
Scheme 7-6. Fractionation of *Parkia sumatrana*.



## VII.7 Fractionation of the Algal Species *Polyides rotundus*.

The crude extract of *Polyides rotundus* was active against the enzyme PLK-1 kinase. It was detanninized by chromatography on a polyamide column and gave one major active fraction as well as two moderately active polyphenolic fractions. Further isolation and purification revealed that the major components of the active fractions were fatty acids (Scheme 7-7). Thus this extract was dropped.

Scheme 7-7. Fractionation of *Polyides rotundus*



## Chapter VIII. Isolation and Synthesis of 6'-Aminoglycolipids

### VIII.1 Introduction to Glycolipids.

Glycolipids are found in all living organisms and occur either as glycosphingolipids or glycoacylglycerolipids (acylated glycoacylglycerols). They are present in cell membranes and their concentration depends on their biological function. Glycosyl-diacyl-glycerides are the major components in chloroplasts and comprise about 50-80% of the total lipids in the cell membrane. Besides their functions as membrane constituent and energy storage materials, these compounds also serve as pheromones, precursors of pheromones or carriers of pheromones.<sup>102</sup> Glycolipids also play a vital role in cellular metabolism. Located mainly at the external surfaces of cell membranes, glycolipids help to regulate cell growth and serve as receptors for toxins, hormones, viruses and other substance.<sup>103</sup> Despite the fact that these compounds could not pass through the cell membrane, glycolipids could serve to modulate the immune response, mainly as antigens which act on the protein receptors of the cell membrane.<sup>104,105</sup> For example, the Lew-X antigen glycosphingolipids (Figure 8-1) found in human liver cancer cells, serve as key signal compounds in cancer cell proliferation.<sup>106</sup> Immunological studies on these kinds of glycolipids have led to the development of a wide range of antitumor vaccines.

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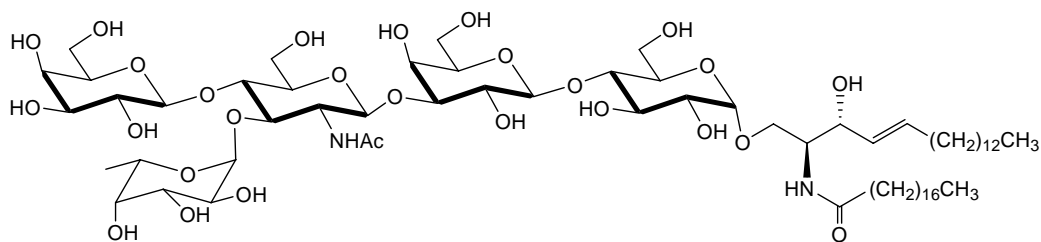
<sup>102</sup> Critchley, D. R. and Vicker, M. G. Glycolipids as membrane receptors important in growth regulation and cell-cell interactions, *Cell. Surf. Rev.* **1977**, *3*, 307-370.

<sup>103</sup> Breimer, M. E.; Hansson, G. C.; Karlson, K. A. and Leffler, H. Separation and characterization of hematosides with different sialic acids and ceramides from rat small intestine. Different composition of epithelial cells versus non-epithelial tissue and of duodenum versus jejunum-ileum. *J. Biochem.*, **1981**, *90*, 589-593.

<sup>104</sup> Perez, E.; Constant, P.; Lemassu, A.; Laval, F.; Daffe, M.; Guilhot, C. Characterization of three glycosyl-transferases involved in the biosynthesis of the phenolic glycolipid antigens from the *Mycobacterium tuberculosis* complex. *J. Bio. Chem.* **2004**, *279*, 42574-42587.

<sup>105</sup> Wu, D.; Xing, G. W.; Poles, M. A.; Horowitz, A.; Kinjo, Y.; Sullivan, B.; Bodmer, V.; Plettenburg, O.; Kronenberg, M.; Tsuji, M.; Ho, D. and Wong, C. H. Bacterial glycolipids and analogs as antigens for CD1d-restricted NKT cells. *Proc. Nat. Aca. Sci.* **2005**, *102*, 1351-1363.

<sup>106</sup> Monteiro, M A.; Zheng, P.; Ho, B.; Yokota, S.; Amano, K.; Pan, Z.; Berg, D. E.; Chan, K. H.; MacLean, L. L.; Perry, M. B. Expression of histo-blood group antigens by lipopolysaccharides of *Helicobacter pylori* strains from asian hosts: the propensity to express type 1 blood-group antigens. *Glycobiology* **2000**, *10*, 701-713.



**Figure 8-1.** Lewis antigen determinant glycosphingolipid

Glycoglycerolipids are carbohydrate derivatives of a 1,2-diacyl-*sn*-glycerol with commonly a mono-saccharide or di-saccharide linked to the third hydroxyl group. The glycoglycerolipid family is a large one, with more than 200 known lipid compounds with different glycosides and fatty acid units. Since the 1960's there have been many phosphorylated or sulfonated lipid derivatives reported with interesting activities. For example, 6'-desoxy-6'-sulfo-glucosyl-diacylglycerolipids have shown strong inhibitory activities against eukaryotic DNA polymerase  $\alpha$  and  $\beta$ ,<sup>107,108</sup> as well as antitumor, anti-HIV,<sup>5,109</sup> and P-selectin receptor inhibition bioactivities.<sup>110</sup> Also synthetic approaches have been developed in the 1980's to make these compounds.

In our search for anticancer agents from natural sources, two new 6'-amino-6'-desoxy-glycolipids (**8.1**) and (**8.2**) were isolated by Dr. Zhou of our group from a marine algal species (UM2972M). These glycolipids showed significant inhibitory activities in

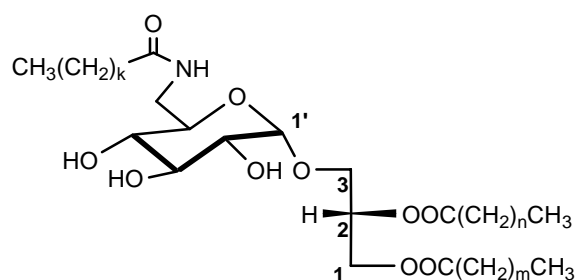
<sup>107</sup> Mizushina, Y.; Watanabe, I.; Ohta, K.; Takemura, M.; Sahara, H.; Takahashi, N.; Gasa, S.; Sugawara, F; Matsukage A; Yoshida, S. and Sakaguchi, K. Studies on inhibitors of mammalian DNA polymerase alpha and beta: sulfolipids from a pteridophyte, *Athyrium niponicum*. *Biochem. Pharmacol.* **1998**, *55*, 537-542.

<sup>108</sup> Ohta, K.; Mizushina, Y.; Hirata N; Takemura, M; Sugawara, F; Matsukage, A; Yoshida, S; Sakaguchi, K. Action of a new mammalian DNA polymerase inhibitor, sulfoquinovosyldiacylglycerol. *Chem. Pharm. Bull.* **1999**, *46*, 684-691.

<sup>109</sup> Loya, S.; Reshef, V.; Mizrahi, E.; Silberstein, C. and Rachamin, Y. The inhibition of the reverse transcriptase of HIV-1 by the natural sulfoglycolipids from cyanobacteria: contribution of different moieties to their high potency. *J. Nat. Prod.* **1998**, *61*, 891-899.

<sup>110</sup> Golik, J.; Dickey, J. K.; Todderund, G.; Lee, D.; Alford, J.; Huang, S.; Klohr, S.; Akino, T. and Kikuchi, K. Isolation and structure determination of sulfonoquinovosyl dipalmitoyl glyceride, a P-selectin receptor inhibitor from the alga *Dictyochloris fragrans*. *J. Nat. Prod.* **1997**, *60*, 387-394.

an assay for inhibitors of the enzyme Myt-1 kinase.<sup>111</sup> Myt1 kinase belongs to a unique class of dual-specificity kinases (DSKs). Myt1 kinase phosphorylates adjacent Thr and Tyr residues in Cdk/Cyclin complexes. Inhibitory phosphorylation of cdc2 by Myt-1 kinase is important for the timing of the cell to enter mitosis. Inhibition of Myt-1 kinase would cause the premature activation of cdc2, which would lead to mitotic catastrophe and cell death. Thus, inhibition of Myt-1 kinase might be a new way of cancer treatment.



Lipid **8.1**  $n = m = k = 14$   
 Lipid **8.2**  $n = 12, m = 14, k = 16$

*Myt-1 Kinase* assay:  $IC_{50} = 0.20 \mu\text{g/ml}$   
 $IC_{50} = 0.12 \mu\text{g/ml}$

**Figure 8-2** 6'-amino-6'-desoxy-glycolipids isolated an algae species

The excellent activity of lipids **8.1** and **8.2** made them attractive synthetic targets. A flexible synthetic route would allow access not only to the natural products but also to various analogs of the natural products. The synthesis of the natural products would not only confirm their structures but would also assure that the observed biological activities were due to the lipids and not to some trace amount of a highly active impurity.

In order to compare the synthetic products with the natural products, it was first necessary to re-isolate the natural products, since the limited supply had become

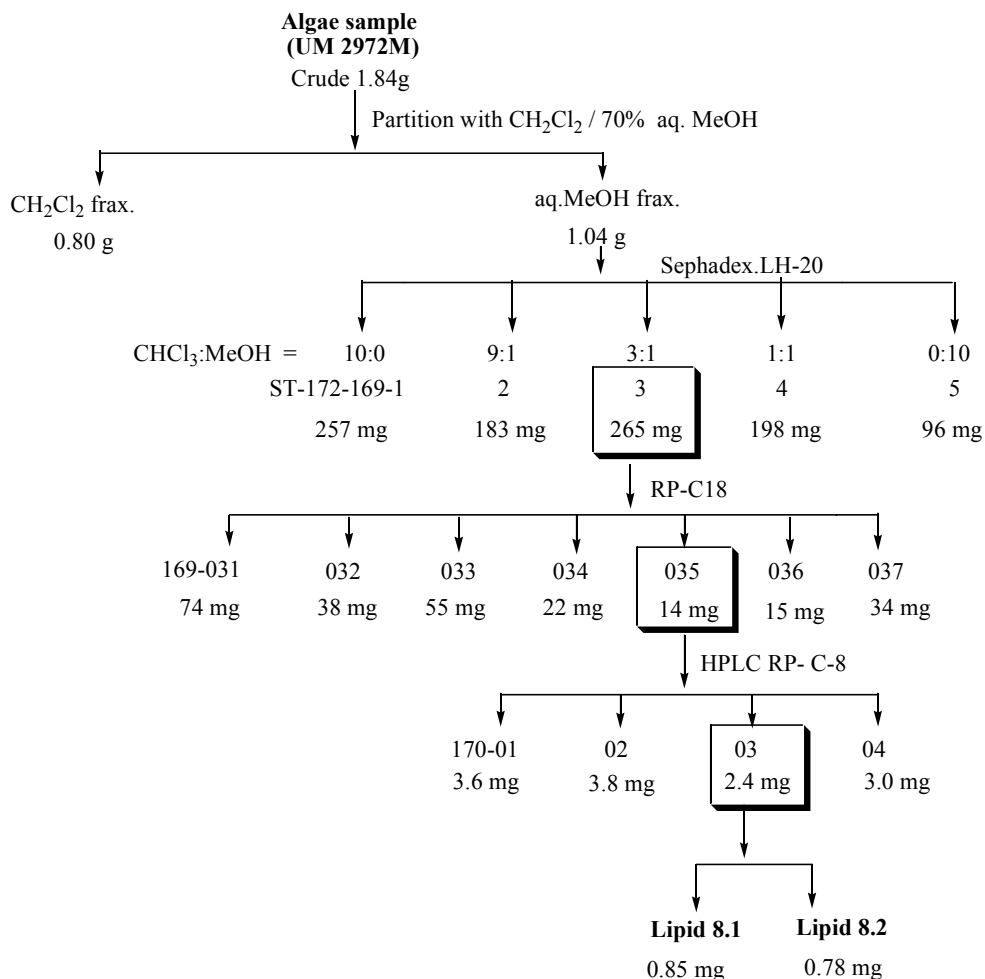
<sup>111</sup> Zhou, B. N.; Tang, S.; Johnson, R. K.; Mattern, M.P.; Harich, K. and Kingston, D.G.I. New glycolipid inhibitors of Myt1 kinase. *Tetrahedron* **2005**, *61*, 883-887

exhausted by testing carried out by our former collaborator Glaxo Smithkline (GSK). The withdrawal of GSK from our collaboration in 2002 was followed by the addition of Dr. John Lazo and his group at the University of Pittsburg, and it was important that both the natural and the synthetic products be tested in the same assay in Dr. Lazo's group.

### **VIII.2 Isolation of Natural 6'-Amino-6'-desoxy-glycolipids.**

Glycolipids **8.1** and **8.2** were reisolated from the crude algae extract by a modification of the scheme Dr. Zhou developed.<sup>111</sup> Briefly, the crude extract was partitioned between CH<sub>2</sub>Cl<sub>2</sub> and 70% aqueous MeOH, and the aqueous MeOH fraction was subjected to chromatography on Sephadex LH-20 with elution by a step gradient of CHCl<sub>3</sub>/MeOH. The lipid containing fraction was purified by reverse-phase chromatography over a C-18 column, followed by RP-HPLC over another C-18 column. The lipid components were detected by a light scattering detector. A total of 1.84 g of crude algae extract yielded 0.78 mg of lipid **8.1** and 0.85 mg of lipid **8.2**. The overall process is summarized in Scheme 8-1.

### Scheme 8-1. Isolation of 6'-amino-6'-desoxy-glycolipids.



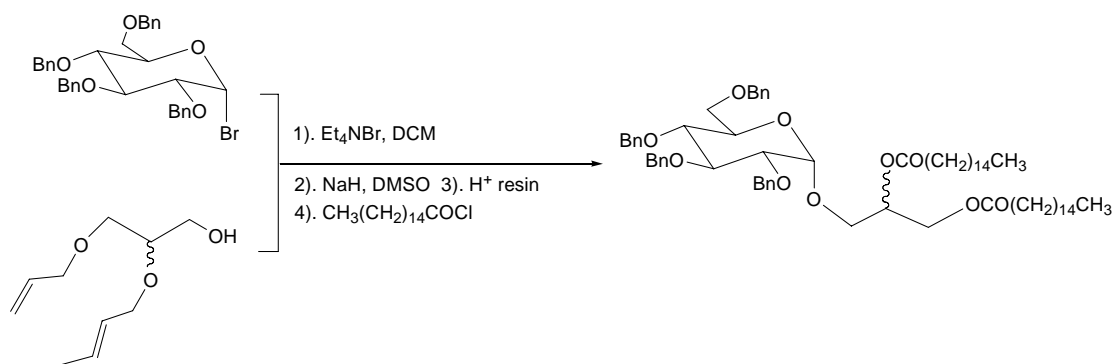
### VIII.3 Previously Reported Syntheses of Glycolipid Derivatives.

Before describing our synthesis, it is useful to give a brief summary of previous reported lipid syntheses. Most of the previously reported work focused on the synthesis of 6'-sulfoquinovosyl-diacylglycolipids, which had attracted some interest from chemists in the saccharide synthesis area because of their novel activities.

In the past few decades, a lot of creative work in saccharide synthesis has been published by many research groups. New sugar coupling techniques and new protective

groups have been continuously developed, some of which have been applied to glycolipid synthesis. The Koenig-Knorr coupling method, the first widely used sugar halide coupling technique, was applied in the synthesis of some  $\alpha$ - glucosyl or galactosyl lipids by Boeckel, et al. in the 1980's (Scheme 8-2).<sup>112</sup> This technique is still used today for the synthesis of some simple saccharides. However, the relative instability of sugar halides narrowed its application.

**Scheme 8-2:** Synthesis of glycolipids via Koenig-Knorr coupling.<sup>112</sup>



The glycosyl trichloroacetimidate coupling method, developed by Schmitz, *et al.*,<sup>113,114</sup> is currently the most commonly used technique in saccharide synthesis. This method was used by Hanashima, *et al.* in the synthesis of both D-type (C-2*S*) and L-type (C-2*R*) 6'-sulfoquinovosylglycolipids (Scheme 8-3).<sup>115</sup> This method requires a non-participating protective group on C-2 of the  $\beta$ -glycosyl donor (generally a benzyl ether

<sup>112</sup> Boekel, C. A. A. and Boom, J. H. Synthesis of phosphatidyl-glucosyl glycerol containing a dioleoyl phosphatidyl moiety, application of the tetraisopropylidisiloxane-1,3-diyl protecting group in sugar chemistry. *Tetrahedron* **1985**, *41*, 4545-4554.

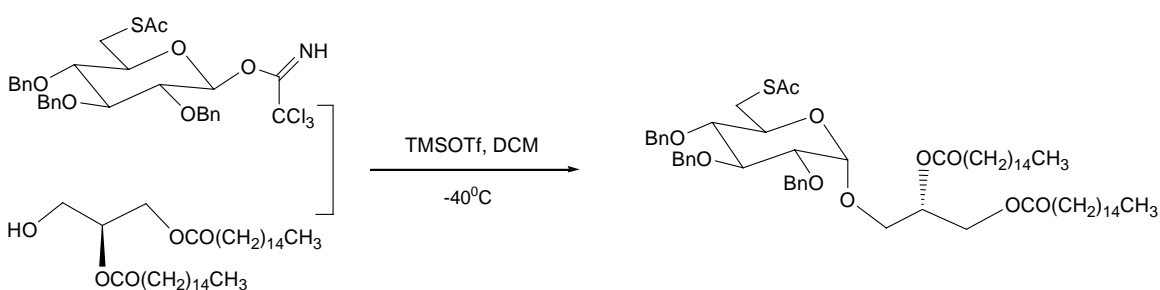
<sup>113</sup> Schmidt, R. R.; Stumpp, M. *Liebigs. Ann. Chem.* **1983**, 1249-1256

<sup>114</sup> Rademann, J.; Geyer, A. and Schmidt, R.R. Solid-phase supported synthesis of the branched pentasaccharide moiety that occurs in most complex type N-glycan chains. *Angew. Chem. Int. Ed. Engl.* **1998**, *37*, 1241-1248

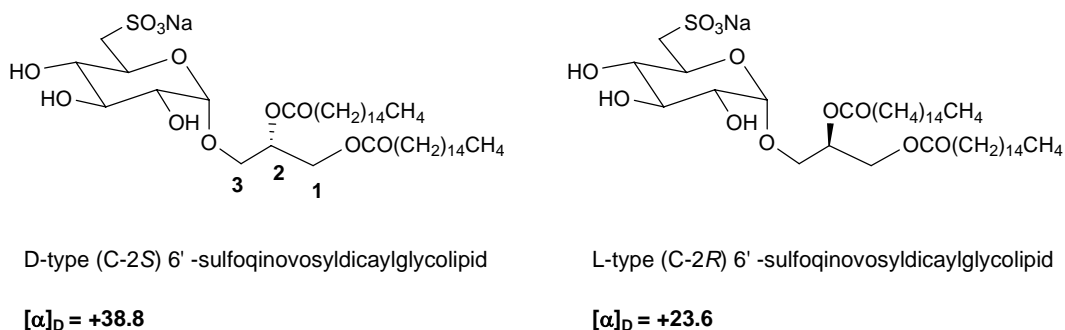
<sup>115</sup> Hanashima, S.; Mizushia, Y.; Yamazaki, T. Ohta, K.; Takahashi, S.; Koshino, H.; Sahara, H. and Sakaguchi, K. Structure-activity relationship of a novel group of mammalian DNA polymerase inhibitors, synthetic sulfoquinovosylacylglycerols. *Tetrahedron Lett.* **2000**, *41*, 4403-4407.

type protection group) for  $\alpha$ -glycosidation. However, the strong Lewis acid catalyzed coupling conditions ( $\text{BF}_3$  or TMSOTf) prevent the usage of some acid-sensitive protective groups on both the glycosyl donor and acceptor. Acyl-migration on the 1,2-*O*-diacyl-glycerol (sugar acceptor) could also occur under these conditions, which could account for the moderate yield of product in this example (62%).

**Scheme 8-3:** Synthesis of glycolipid via trichloroacetimidate coupling.<sup>114</sup>



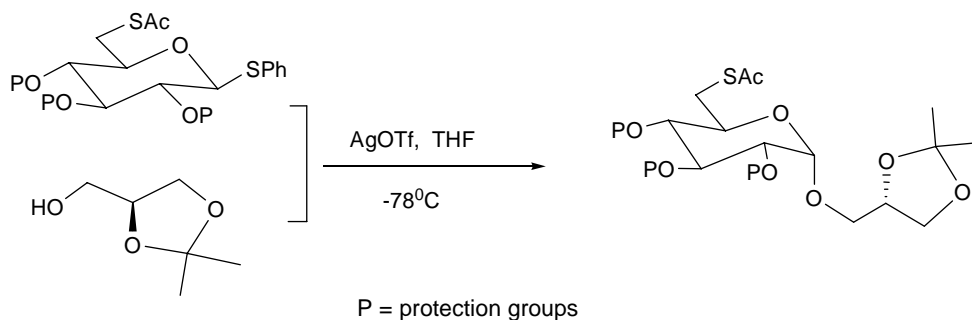
The  $^1\text{H}$  NMR data of the natural D-type (C-2*S*) and unnatural L-type (C-2*R*) 6'-sulfoquinovosyldiacylglycerolipids were quite similar except for the gem-protons on C-1 of glycerol (Figure 8-3). The signals for the two geminal protons on C-1 of the natural D-type 6'-sulfoquinovosyl-diacylglycerolipid appeared more separated with signals at  $\delta$  4.33 (dd,  $J = 12.1, 2.5$  Hz,  $H_{sn-1a}$ ) and  $\delta$  4.10 (dd,  $J = 12.1, 7.6$  Hz,  $H_{sn-1b}$ ) ( $\Delta\delta = 0.23$ ), while these signals appeared at  $\delta$  4.30 (dd,  $J = 11.8, 2.4$  Hz,  $H_{sn-1a}$ ) and  $\delta$  4.22 (dd,  $J = 11.8, 8.6$  Hz,  $H_{sn-1b}$ ) ( $\Delta\delta = 0.08$ ) for the unnatural L-type glycolipid. This difference could be an indirect method of determining the relative configuration of the glycerol aglycone, in addition to the optical rotation data. Both lipids had very similar bioactivities, which suggested that the chirality of C-2 was not an important determinant of activity.<sup>114,117</sup>



**Figure 8-3.** Natural and unnatural 6'-sulfoquinovosyldicacylglycerolipids

Thio-glycosides have also been very commonly used as glycosyl donors in saccharide synthesis. This coupling method was also used by Gordon *et al.* in the synthesis of 6'-sulfoquinovosyl-diacylglycolipids,<sup>116</sup> which used isopropylidene-glycerol as alcohol acceptor under mild conditions (NIS, AgOTf, molecular sieves) (Scheme 8-4). These conditions were compatible with some acid-sensitive protecting groups, including acetonides.

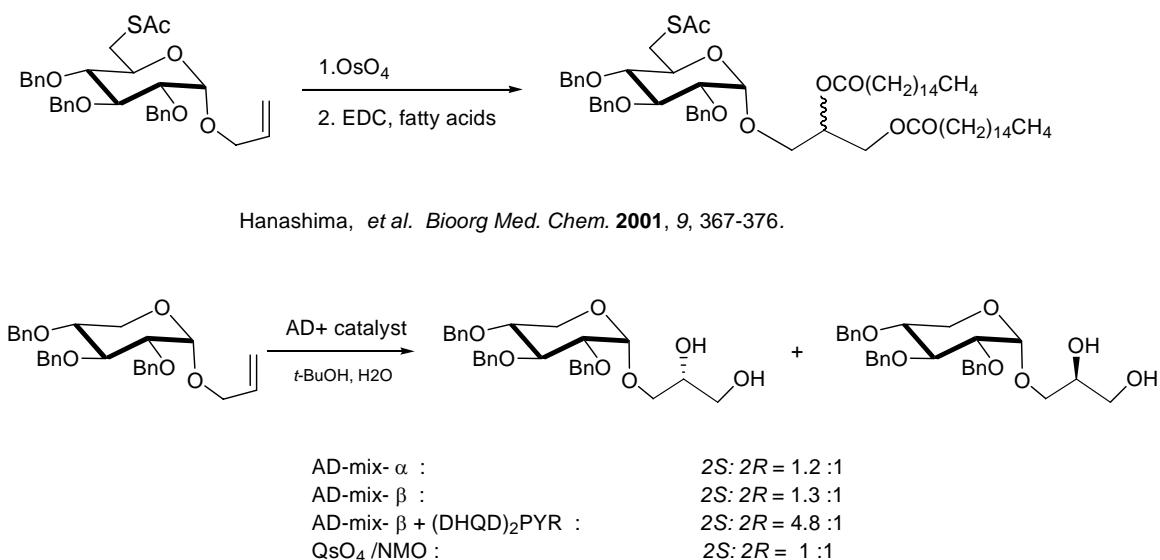
**Scheme 8-4:** Synthesis of glycolipids via thio-glycoside coupling<sup>115</sup>



<sup>116</sup> Gordon, G. M. and Danishefsky, S. J. Synthesis of a cyanobacterial sulfolipid: confirmation of its structure, stereochemistry and anti-HIV-1 activity. *J. Am. Chem. Soc.* **1992**, *114*, 659-662.

Direct dihydroxylation of the  $\alpha$ -allyl group of a 1-*O*-allylglucopyranose derivative could be a simpler synthetic approach to a glycolipid derivatives. This method was reported by Hanashima *et al.* in the synthesis of a C-2 diastereomeric mixture of 6'-sulfoquinovosyl-glycolipids.<sup>117</sup> The major advantage was that this method eliminated the concern about the anomeric selectivity of glycosylation as well as about the synthesis of the glycol-acceptor. However, Sharpless dihydroxylation conditions could not achieve good chiral selectivity on C-2 of the glycerol aglycone. The previous studies by Nicolas, *et al.* on a xylose substrate indicated that both AD-mix- $\alpha$  and AD-mix- $\beta$  preferably produced L-type (C-2*S*)-glycerol-diol, which gave the unnatural L-type (2*R*)-1,2-diacyl-3-glycosylglycerol lipid after acylation (Scheme 8-5).<sup>118</sup> Also, separation of the diastereo-metric C-2 *R, S* isomers by HPLC has turned out to be very difficult.<sup>116</sup>

**Scheme 8-5:** Synthesis of glycolipids via Sharpless dihydroxylation



Nicolas, M.; Françoise, C. and Chapleur, Y. *Tetrahedron Asymmetry* **1997**, 8(17), 2889

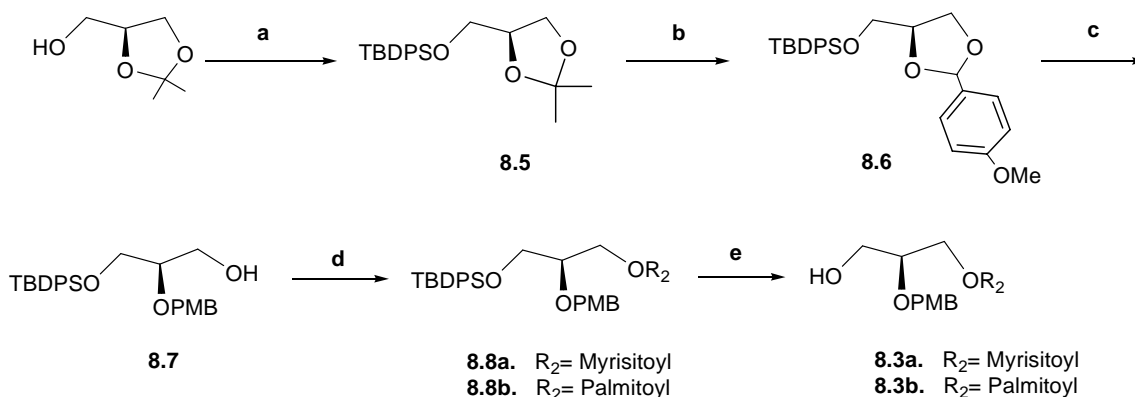
<sup>117</sup> Hanashima, S.; Mizushima, Y.; Yamazaki, T. Ohta, K.; Takahashi, S.; Koshino, H.; Sahara, H. and Sakaguchi, K. Synthesis of Sulfoquinovosylacylglycerols, inhibitors of eukaryotic DNA polymerase  $\alpha$  and  $\beta$ . *Bioorg. Med. Chem.* **2001**, 9, 367-376

<sup>118</sup> Nicolas, M.; Françoise, C. and Chapleur, Y. Asymmetric dihydroxylation of D-xylose-derived allyl ethers. *Tetrahedron Asymmetry*. **1997**, 8, 2889-94.

#### VIII.4 Synthesis of 6-Aminoglycoglycerolipids Bearing Saturated Fatty Acids.

The saturated glycoglycerolipids **8.1** and **8.2** were synthesized by glycosylation of a PMB protected glycerol **8.3** (glucosyl acceptor) with a 6-acylamido-glucosyl-trichloroacetimidate **8.4** (glucosyl donor) according to Schmidt's method as previously described. The synthesis of glucosyl acceptor **8.3** was achieved starting from (2*S*)-isopropylidenglycerol as shown in Scheme 8-6. Protection of the free hydroxyl group as its *tert*-butyldiphenylsilyl ether **8.5** was followed by replacement of the isopropylidene ketal protecting group with a 4-methoxybenzylidene acetal to yield **8.6**. Borane reduction of the 4-methoxybenzylidene acetal **8.6** afforded the primary alcohol **8.7** as the major product. This product **8.7** was then acylated with myristic or palmitic acid to give the esters **8.8a** and **8.8b**, and these esters were subsequently deprotected with HF/pyridine to give the glucosyl acceptors **8.3a** and **8.3b**.

**Scheme 8-6:** Synthesis of the glucosyl acceptor

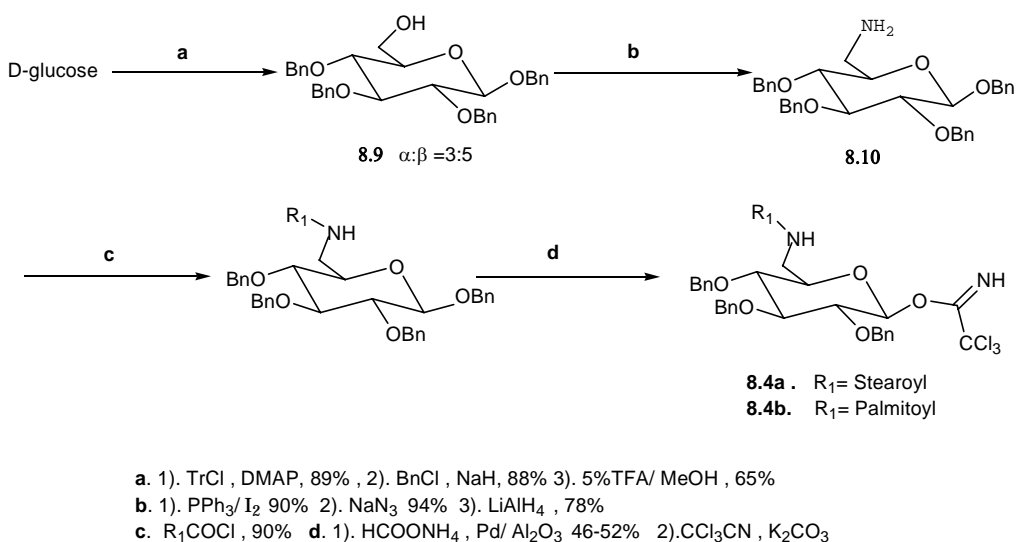


a, 1). TBDPSCl, 96% ; b. 1). HOAc / H<sub>2</sub>O, 100% 2). PPTS / MeOC<sub>6</sub>H<sub>4</sub>CHO, reflux, 90%  
c, BH<sub>3</sub> / THF, 73% d. R<sub>2</sub>COCl, 95% e. HF / Py, 87%

Two schemes were applied to synthesize the glucosyl donor **8.4**. The first approach is shown in Scheme 8-7. In this approach D-glucose was converted to 1,2,3,4-

*O*-tetrabenzyl-glucose **8.9** (the  $\beta$  anomer is the major product), and this was converted to 1,2,3,4-*O*-tetra-benzyl-6-glucosamine **8.10** via an iodide intermediate.<sup>122</sup> However, selective debenzoylation of the anomeric C-1 position by Pd on alumina in MeOH via Bieg's method<sup>119</sup> was found to proceed in low yield (48-54%), and debenzoylation of the C-2, C-3 or C-4 hydroxyl groups of the glucopyranoside was also observed.

**Scheme 8-7:** Synthesis of the glucosyl donor (Part 1)



An alternative synthesis of the glycosyl donors **8.4a** and **8.4b** began with 1-*O*- $\alpha$ -allyl-glucose **8.11** (Scheme 8-8), a common precursor which could be easily prepared from D-glucose.<sup>120</sup> Allyl ether **8.11** was converted to 1-*O*- $\alpha$ -allyl-2,3,4-*O*-tribenzyl-D-glucose (**8.12**) via the reported method.<sup>121, 122</sup> The free C-6 hydroxyl group was replaced

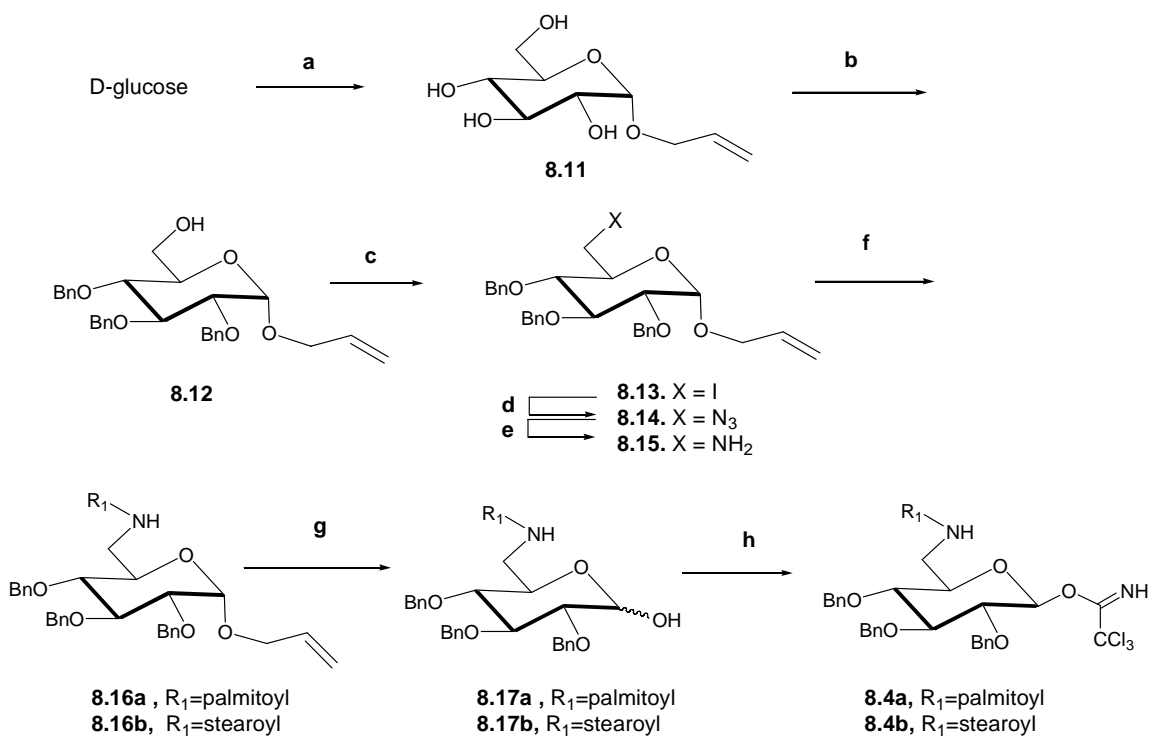
<sup>119</sup> Bieg, T. and Szeja, W. Regioselective hydrogenolysis of benzyl glycosides. *Carbohydr. Res.* **1990**, 205, C10.

<sup>120</sup> Paolo, P. and Richard, W. F. Synthesis of Both Possible Isomers of the Quadrant of Altromycin B. *J. Org. Chem.* **2003**, 68, 8042-8060.

<sup>121</sup> Peer, A. and Vasella, A. Synthesis of an L-fucose-derived cyclic nitron and its conversion to -L-fucosidase inhibitors. *Helv. Chim. Acta.* **1999**, 82, 1044-1063

<sup>122</sup> Ernst, A. and Vasella, A. Oligosaccharide analogs of polysaccharides. Part 8. Orthogonally protected cellobiose-derived dialkynes. A convenient method for the regioselective bromo- and protodegermylation of trimethylgermyl- and trimethylsilyl-protected dialkynes. *Helv. Chim. Acta.* **1996**, 79, 12-19.

**Scheme 8-8:** Synthesis of the glucosyl donor (Part 2)



**a.** 1). Allyl alcohol, CSA, reflux 48% **b.** 1) TrCl, DMAP, 69%, 2). BnCl, NaH, 75%, 3) 5% TFA/ MeOH, 85%;  
**c.** PPh<sub>3</sub>/I<sub>2</sub> 93%; **d.** NaN<sub>3</sub>/DMF 98% **e.** LiAlH<sub>4</sub>, 89%; **f.** Acyl Chloride, TEA, 90-95% **g.** 1). Rh(PPh<sub>3</sub>)<sub>3</sub>Cl, DABCO 2). PTSA, HgCl<sub>2</sub> **h.** CCl<sub>3</sub>CN, K<sub>2</sub>CO<sub>3</sub> 80-85%

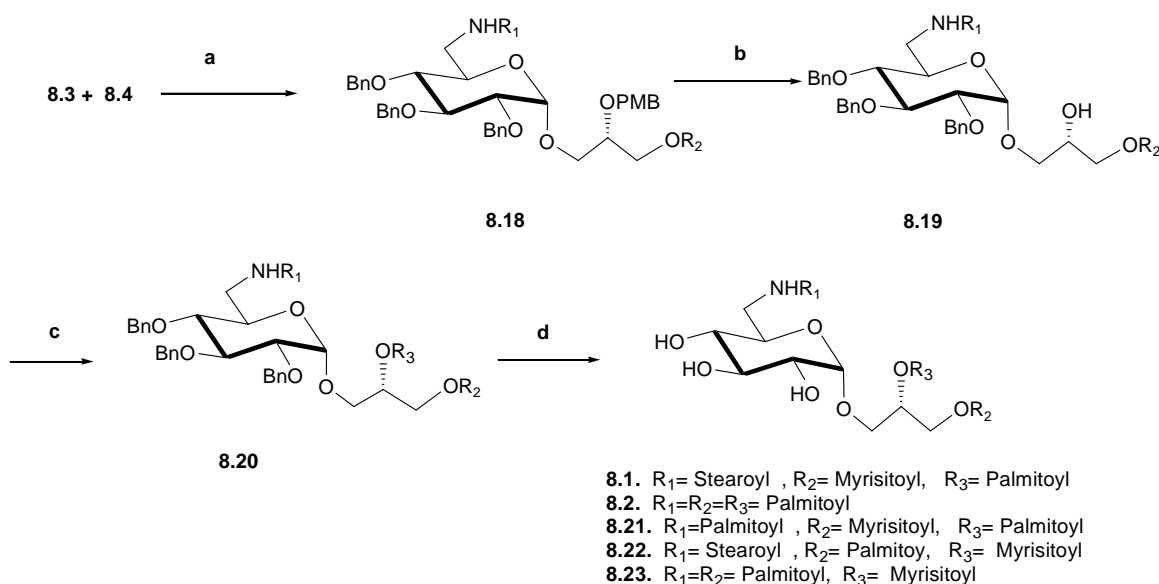
by a free amino group to give **8.15** in 3 high-yielding steps via an iodide intermediate.<sup>123</sup> Amine **8.15** then underwent acylation with a fatty acid chloride followed by deallylation with Rh(PPh<sub>3</sub>)<sub>3</sub>Cl (Wilkinson's catalyst) and HgCl<sub>2</sub> to yield the hemi-acetals **8.17a** and **8.17b**,<sup>124</sup> which were treated with trichloroacetonitrile and K<sub>2</sub>CO<sub>3</sub> in anhydrous CH<sub>2</sub>Cl<sub>2</sub> to achieve β-trichloroacetimidates **8.4a** and **8.4b**, the glucosyl donors. The β-configuration of these glucosyl donors was ascertained by clear anomeric proton signal at δ 5.74 (d, J = 8.0 Hz) in their <sup>1</sup>H NMR spectra.

<sup>123</sup>Hoof, P. A. V.; Marel, G. A.; Boeckel, C. A. A.; Boom, J. H.; Triisobutylaluminium mediated carbocyclisation of sugar derived spiroketals and ketosides. *Tetrahedron Lett.* **2001**, 42; 1769-1778.

<sup>124</sup>Corey, E. J. and Suggs, W. J. Selective cleavage of allyl ethers under mild conditions by transition metal reagents. *J. Org. Chem.* **1973**, 38, 3224-25.

A series of glycosylation reactions between the sugar acceptors (**8.3a**, **8.3b**) and sugar donor (**8.4a**, **8.4b**) were carried out in anhydrous CH<sub>2</sub>Cl<sub>2</sub> with TMSOTf as Lewis acid catalyst (Scheme 8-9). The yields were good, ranging from 72% to 85% with good anomeric selectivity. The products **8.18** were treated with DDQ to remove the PMB protecting group and then esterified by fatty acid with EDCI as the coupling reagent to give the diacyl-glycolipids **8.20**. Hydrogenation of these lipids **8.20** in THF yielded the saturated (2*S*)-1,2-*O*-di-acyl-3-*O*-(6-desoxy-6-acylamido-D-glucosyl)-glycerol products, including lipid **8.1** and lipid **8.2**. This synthetic scheme has the advantage of allowing the introduction of different fatty acids in different steps. However, it is not suitable for the synthesis of the unsaturated glycolipids, since the existence of the double bonds on fatty acid precludes the use of the benzyl protective group.

**Scheme 8-9:** Synthesis of saturated glycosylglycolipids.



a. 1). TMSOTf, 4A Molecular Sieve, 76%-84%  
b. DDQ / H<sub>2</sub>O 75-82%; c. EDC / R<sub>3</sub>COOH 85-90% d. Pd/C , H<sub>2</sub> 90%

The optical rotation values of synthetic lipid **8.1** and lipid **8.2** matched well with the values of the corresponding natural products, which confirmed that the previously reported natural lipids were in the (2*S*)-*D*-glycerol- $\alpha$ -*D*-glucopyranoside form. The C-2*S* configuration was also supported by the <sup>1</sup>H NMR data. Comparative NMR data for the natural and synthetic products **8.1** are listed in Table 8-1.

**Table 8-1.** Comparison of the NMR data of synthetic and natural glycolipids **8.1**.

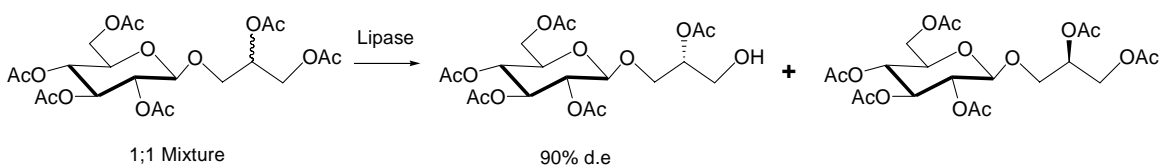
C #	$\delta_{\text{H}}^*$		$\delta_{\text{C}}^\dagger$	
Glycerol-	Synthetic Lipid	Natural- lipid	Synthetic-Lipid	Natural- lipid
1a	4.38(dd, 12.4, 3.6)	4.38 (dd, 12.0, 3.6)	67.1	66.9
1b	4.13(dd, 12.4, 8.4)	4.12 (dd, 12.0, 8.4)		
2	5.24 m	5.23m	70.0	70.1
3a	3.79(dd, 10.4 4.8) ,	3.71(dd, 10.8, 4.4)	62.3	62.4
3b	3.64(dd, 10.4, 6.4)	3.64(dd, 10.8, 6.4)		
6'-acylamino-glucose-				
1'	4.80, (d, 3.6)	4.80 (d, 3.6)	99.6	99.7
2'	3.49, (dd, 9.2 and 3.6)	3.48(dd, 9.2, and 3.6)	71.3	71.2
3'	3.10 (t, 9.2)	3.10(t, 9.2)	73.3	73.4
4'	3.74 (t, 9.2)	3.76(t, 9.2)	72.5	72.6
5'	3.59 ( m )	3.53(m)	70.2	70.6
6'-a	4.04, (ddd, 16.0, 7.6, 1.2),	4.05(m)	39.9	40.0
6'-b	3.03 ( m )	3.03(m)		
1-COO			173.6	173.6
2-COO			175.9	175.8
6'-CONH	6.02(m) N-H	6.05(m) N-H	173.4	173.4

\* CDCl<sub>3</sub>, 400 MHz    † CDCl<sub>3</sub>, 100 MHz

### VIII.5 Synthesis of 6'-Aminoglycoglycerolipids Bearing Linolenic acids.

The unsaturated glycolipids were synthesized by direct dihydroxylation of the allyl group of 1-*O*-allyl-2,3,4-tri-*O*-triethylsilyl- $\alpha$ -glucopyranoside derivatives as Hanashima *et al.* has reported.<sup>17</sup> The problem of diastereometric separation could possibly be solved by enzymatic resolution methods. As Soriente *et al.* has reported, diastereometric mixtures of 1,2-*O*-diacetyl- $\beta$ -glucosyl-glycerol could be enantioselectively purified by enzymatic hydrolysis with *Pseudomonas fluorescens* lipase (Scheme 8-10).<sup>125</sup> Also we<sup>111</sup> and others<sup>126</sup> have reported that lipase selectively hydrolyzes the primary ester on the aglycone of glycolipids in phosphate buffer. It was thus anticipated that the relative hydrolysis rate of the D-type (natural) and L-type (unnatural) glycolipids should be different and that one of the two diastereomers might be purified from the mixture.

**Scheme 8-10.** Enzymatic resolution of glycolipids



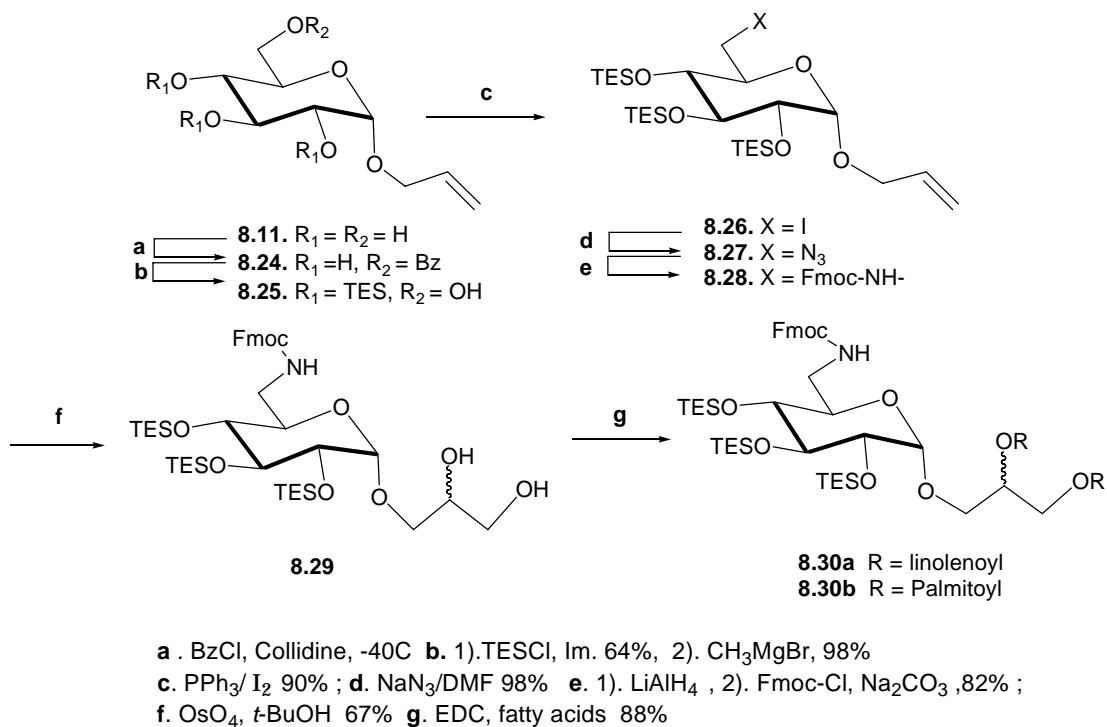
To test the relative hydrolysis rate of acyl esters on glycerol, a mixture of C-2 diastereometric 1,2-di-*O*-palmitoyl-3-*O*-6-(9-fluorenylmethoxycarboamino-6-desoxyglucosyl)-glycosylipids **8.30b** was prepared from the previous intermediate 1-*O*- $\alpha$ -allyl-

<sup>125</sup> Sorient, A.; De Rosa, M.; Trincone, A. and Sodano, G. Enzymatic regio- and diastereoselective hydrolysis of peracetylated glycerol- and erythritol-  $\beta$ -glucosides. *Bioorg Med. Chem. Lett.* **1995**, 5, 2321-2323.

<sup>126</sup> Murakami, N.; Morimoto, T.; Imamura, H. and Nagatsu, A. Enzymatic transformaion of glyceroglycolipids into *sn*-1 and *sn*-2 lysoglyceroglycolipids by use of *Rhizopus arrhizus* Lipase. *Tetrahedron* **1994**, 50, 1993-2002.

glucose (**8.11**) (Scheme 8-11). The primary C-6 hydroxyl group of **8.11** was selectively benzoylated and the C-2, C-3 and C-4 secondary hydroxyl groups were then protected as their triethylsilyl ethers to give **8.24** in 64% yield. The benzoyl ester **8.24** was cleaved by treatment with methyl Grignard reagent to give **8.25**.<sup>127</sup> The C-6 hydroxyl group of **8.25** was converted to an amino compound **8.27** by the same method as previously described, and the free amino group of **8.27** was protected as its 9-fluorenylmethyl-chlorocarbonate **8.28**. Oxidation of **8.28** with OsO<sub>4</sub> gave a diastereometric mixture of diols **8.29**. Diol mixture **8.29** was acylated with linolenic acid or palmitic acid to yield the C-2 diastere-

**Scheme 8-11:** Synthesis of the unsaturated glucosylglycolipid (Part 1).

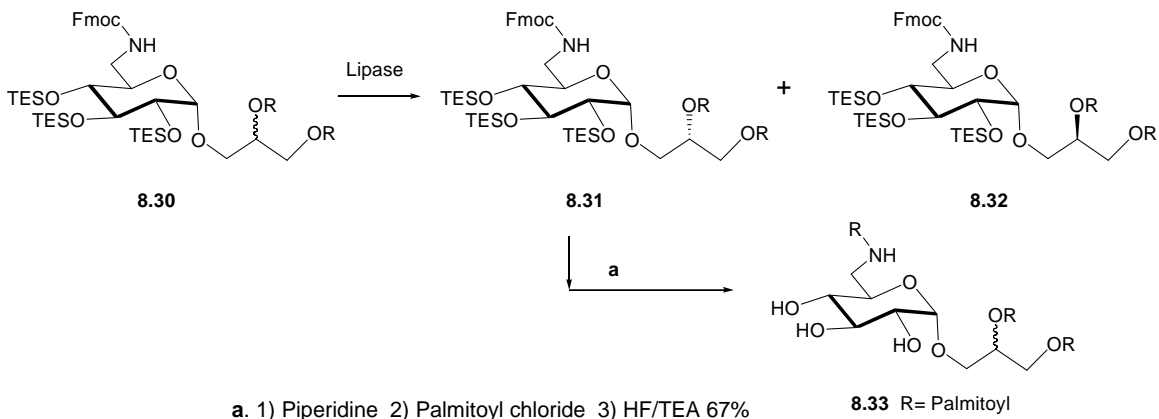


<sup>127</sup> Watanabe, Y.; Fujimoto, T. and Ozaki, S. *J. Chem. Soc. Chem. Commun.* **1992**. 681-684.

-metric mixtures of diacylglycolipids **8.30a** and **8.30b**. The diastereomeric ratio was determined as 1:1 by the equal intensities of proton signals at  $\delta$  4.32 (dd,  $J = 12.0, 3.2$  Hz,  $H_{sn-1a}$  of C-2*S* isomer) and  $\delta$  4.27 (dd,  $J = 11.8, 3.2$  Hz,  $H_{sn-1a}$  of C-2*R* isomer) as well as by the equal intensities of the anomeric carbon signals at  $\delta$  99.6 and 99.5.

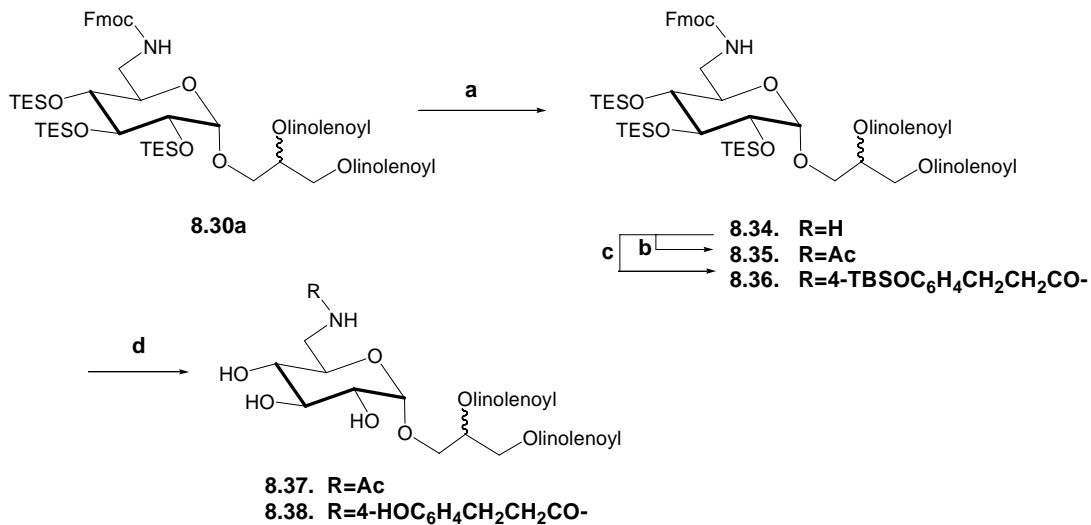
The diastereometric mixture **8.30b** was then treated with lipase (from *Pseudomonas* sp.) in suspension in phosphate buffer for two weeks (**Scheme 8-12**). Usual work-up and chromatographic separation afforded un-hydrolyzed starting material **8.30b** as well as the hydrolyzed monoacyl-glycolipid **8.31**. The unhydrolyzed diacylglycolipid was treated with piperidine to remove the Fmoc protecting group and was then reacylated with palmitic acid in the presence of EDCI, followed with silyl deprotection to give the purified tri-palmitoylglycolipid **8.33**. Comparison of the  $^1\text{H}$  NMR data of **8.33** with the data of the optically pure lipid **8.2** synthesized as described previously in Scheme 8-9 indicated that lipid **8.33** consisted mainly of the C-2*S* form (D-type) diastereomer, since the pair of gem-proton signals on C-1 mainly appeared at  $\delta$  4.38 (dd,  $J = 12.4$  and  $3.6$  Hz,  $H_{sn-1a}$ ) and 4.12 (dd,  $J = 12.4$  and  $8.4$  Hz,  $H_{sn-1b}$ ) which matched well with the pure lipid **8.2** ( $\Delta\delta = 0.26$ ). However, the proton signals of C-1 of the C-2*R* diastereomer (L-type) were also found at  $\delta$  4.27 (dd,  $J = 12.4$  and  $3.8$  Hz,  $H_{sn-1a}$ ) and 4.16 (dd,  $J = 12.4$  and  $8.8$  Hz,  $H_{sn-1b}$ ) ( $\Delta\delta = 0.11$ ), which corresponded to those of the C-2*R* diastereomer previously reported.<sup>114</sup> The C-2 *R/S* ratio was determined to be approximately 1:4 according to the proton integral (80% d.e). This suggested that lipase hydrolyzed the C-2*R* diacyl-lipid diastereomer approximately 4 times faster than the C-2*S* diastereomer in phosphate buffer. Therefore, it is possible to selectively purify the D-type (natural) glycolipids from the diastereometric mixture by enzymatic means.

**Scheme 8-12.** Enzymatic resolution of glucosylglycolipid.



Diastereometric mixtures of unsaturated lipids were synthesized from **8.30a** via similar method (Scheme 8-13). After treatment with piperidine to remove the Fmoc protecting group, the product **8.34** was then reacylated with acetic anhydride or with EDCI/4-*tert*-butyldimethylsiloxy-cinnamic acid to give **8.35** or **8.36**. Final deprotection achieved the diastereomeric mixtures of unsaturated lipids **8.37** and **8.38**.

**Scheme 8-13:** Synthesis of the unsaturated glucosylglycolipid (Part 2).



a. Piperidine 6 b. Ac<sub>2</sub>O/TEA 66% c. EDC, 4-TBSOC<sub>6</sub>H<sub>4</sub>CH<sub>2</sub>CH<sub>2</sub>COOH 75% d. HF/TEA 72%

## VIII.6 Experimental Section.

**General Experiment Methods.** Chemicals were obtained from Aldrich Chemical Co. and were used without further purification. All anhydrous reactions were performed in oven-dried glassware under nitrogen or argon. All solvents were of reagent grade or HPLC grade. Tetrahydrofuran (THF) was distilled over sodium/benzophenone, and CH<sub>2</sub>Cl<sub>2</sub> (DCM) was distilled over calcium hydride. All reactions were monitored by thin layer chromatography (TLC) plates (silica gel 60 GF, aluminum back from the E. Merck.) and spots were detected with 254 nm UV light and/or orcinol/sulfuric acid spray. All <sup>1</sup>H NMR spectral data were obtained in CDCl<sub>3</sub> on Varian Unity 400 or Inova 400 spectrometers (operating at 399.951 MHz for <sup>1</sup>H and 100.578 MHz for <sup>13</sup>C). Chemical shifts are reported as δ-values relative to known solvent residue peaks, and coupling constants are reported in Hertz. HRFABMS spectra were obtained by Mr. William Bebout on a JEOL HX-110 mass spectrometer in the Analytical Services Division in the Department of Chemistry. The known intermediates were prepared by the reported procedures in the literature, and the NMR data of these compounds were identical to literature values.

**Bioassay and discussion.** The bioassay for the inhibitors of Myt-1 Kinase was carried out by our collaborator Ms. Marni Brisson in Dr. John Lazo's group at the University of Pittsburgh via previously reported methods.<sup>111</sup> Unfortunately all the synthetic glycolipids showed very weak activities. The synthetic lipid **8.1** showed weak activity with IC<sub>50</sub> = 4.8 μg/mL and **8.2** was not active, with IC<sub>50</sub> > 20 μg/mL. These IC<sub>50</sub> values were much higher than those of the natural lipids **8.1** and **8.2**, which were previously tested by Glaxo SmithKline. The reason for these weak activities is unknown, although it may be due to

differences in the assay methods between the University of Pittsburgh and Glaxo SmithKline.

**(2R)-3-O-tert-Butyldiphenylsilyl-1,2-O-isopropylidene-glycerol (8.5).** *tert*-Butyldiphenylsilyl chloride (1.4 mL, 5.38 mmol) was added slowly to a solution of 2*S*-isopropylidene glycerol (0.7 g, 5.33 mmol) and imidazole (720 mg, 10.4 mmol) in DMF (15 mL). The reaction mixture was stirred at room temperature overnight, and quenched with saturated NaHCO<sub>3</sub>. The mixture was diluted with EtOAc (100 mL) and washed with distilled water (2×50 mL) and brine (2×50 mL), and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on silica-gel with 5% EtOAc in hexane gave compound **8.5** (1.9 g, 5.24 mmol, 96%) as a colorless oil. <sup>1</sup>H NMR: δ 7.78-7.62 (m, 4H), 7.57-7.38 (m, 6H), 4.12 (m, 1H), 4.04-3.88 (m, overlapped, 4H), 1.48 (s, 3H), 1.22 (s, 3H), 0.98 (s, 9H, 3SiCH<sub>3</sub>); <sup>13</sup>C NMR δ 132.5, 130.2, 126.4, 124.7 (overlapped), 124.6, 106.0, 73.0, 63.7, 61.5, 23.8, 23.7, 22.4, 16.2; HRFABMS *m/z* = 371.2048 [M+H]<sup>+</sup>, calculated for C<sub>22</sub>H<sub>31</sub>O<sub>3</sub>Si 371.2042, Δ = 1.6 ppm.

**(2R)-3-O-tert-Butyldiphenylsilyl-1,2-O-glycerol-4-methoxybenzylidene (8.6).** To a round bottom flask charged with compound **8.5** (1.9 g, 5.24 mmol), 20 mL of 50% aqueous acetic acid was added and the solution was stirred for 4 h at 60 °C until TLC showed the depletion of the starting material. The solvent was removed under reduced pressure and the residue was dried under vacuum and then dissolved in benzene (40 mL). 4-Methoxybenzaldehyde (6.1 mL, 31.6 mmol) and PPTS (165 mg, 0.55 mol) were added and the reaction mixture was refluxed overnight at 85 °C under a Dean-Starck condenser. The reaction was cooled and quenched with saturated aqueous NaHCO<sub>3</sub>. The mixture

was diluted with EtOAc (100 mL) and washed with water (3×100 mL) and brine (2×50 mL), and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on silica gel with 5% EtOAc in hexane yielded compound **8.6** (2.08 g, 4.78 mmol, 90%) as a mixture of two epimers at the acetal carbon.<sup>128</sup> <sup>1</sup>H NMR: δ 7.81-7.90 (overlapped, 4H), 7.42-7.58 (overlapped, 8H), 6.97-7.03 (2H, d, *J* = 8.0), 6.04 (s, 0.8H), 5.92(s, 0.2H), 4.47 (m, 1H), 4.36 (m, 1H), 4.21 (m, 1H), 3.99 (m, 2H), 1.20-1.26 (overlapped, br, 9H, 3CH<sub>3</sub>); <sup>13</sup>C NMR: δ 160.7, 135.9, 133.5, 130.5, 130.2, 128.5, 128.1, 128.0, 114.0, 104.2, 76.6, 67.7, 64.8, 55.4, 27.2, 19.6; HRFABMS: *m/z* = 449.2113 [M+H]<sup>+</sup>, calculated for C<sub>27</sub>H<sub>33</sub>O<sub>4</sub>Si, *m/z* = 449.2148, Δ = -6.6 ppm.

**(2R)-1-O-tert-Butyldiphenylsilyl-2-O-(4-methoxybenzyl)-glycerol (8.7)**. Compound **8.6** (1.95 g, 4.65 mmol) was dissolved in THF (20 mL) and the solution was refluxed under nitrogen while borane/THF (0.5 M, 10 mL) was added slowly. The mixture was refluxed for 2 h and cooled down. The mixture was diluted with EtOAc (20 mL) and washed with saturated NaHCO<sub>3</sub> (3×20 mL), water (2×50 mL) and brine (2×50 mL), and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on silica gel with elution with 13-15% EtOAc in hexane gave compound **8.7** (1.51 g, 3.36 mmol, 73%) as a yellow oil. <sup>1</sup>H NMR: δ 7.78-7.62 (m, 4H), 7.57-7.38 (m, 6H), 7.29(d, 2H, *J* = 8.8), 6.92 (d, 2H, *J* = 8.8), 4.50 (s, 2H, OCH<sub>2</sub>Ph), 3.97 (m, 1H, H-2), 3.82 (s, 3H, OCH<sub>3</sub>), 3.78-3.69 (m, 2H), 3.61-3.54 (m, 2H), 1.18 (s, 9H, 3CH<sub>3</sub>); <sup>13</sup>C NMR δ 159.5, 135.8, 133.5, 130.4, 130.0, 129.6-128.0 (overlapped), 114.1, 73.3, 71.1, 70.9, 65.1, 55.5, 27.1, 19.5; HRFABMS: *m/z* = 451.2284 [M+H]<sup>+</sup>, calculated for C<sub>27</sub>H<sub>35</sub>O<sub>4</sub>Si, *m/z* = 451.2305, Δ = -4.5 ppm

<sup>128</sup> These two epimers were not separated since hydroboration in next step converted them to the same products.

**(2R)-1-O-tert-Butyldiphenylsilyl-2-O-(4-methoxybenzyl)-3-O-palmitoyl-glycerol**

**(8.8a).** Compound **8.7** (0.42 g, 0.93 mmol) was dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (10 mL) with triethylamine (0.35 mL, 2.5 mmol) added, then palmitoyl chloride (0.33 mL, 1.2 mmol) was added and the reaction mixture was stirred for 3 h at RT. The mixture was diluted with EtOAc (100 mL) and washed with water (3×100 mL) and brine (2×50 mL), dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on Silica gel with 15% EtOAc in hexane gave **8.3a** (0.58 g, 0.88 mmol, 95%) <sup>1</sup>H-NMR (CDCl<sub>3</sub>): δ 7.64-7.58 (m, overlapped, 4H), 7.57-7.38 (m, overlapped, 6H), 7.35 (d, 2H, *J* = 8.8), 6.78 (d, 2H, *J* = 8.8), 4.40 (s, 2H, CH<sub>2</sub>OPh), 4.29 (dd, 1H, *J* = 11.6 and 2.3), 4.12 (dd, 1H, *J* = 11.6 and 2.3), 3.73 (s, 3H, OCH<sub>3</sub>), 3.68-3.59 (m, overlapped, 3H), 2.20 (t, 2H, *J* = 7.5, α-CH<sub>2</sub>), 1.50 (m, 2H, β-CH<sub>2</sub>), 1.23-1.15 (overlapped, 20H), 0.80 (t, 3H, *J* = 7.0); <sup>13</sup>C NMR: δ 173.8, 159.5, 135.8, 133.5, 130.4, 130.0, 129.6-128.0 (overlapped), 114.1, 73.3, 71.1, 70.9, 65.1, 55.5, 29.8-29.3 (overlapped), 27.1, 19.5; HRFABMS: *m/z* = 689.4256 [M+H]<sup>+</sup>, calculated for C<sub>43</sub>H<sub>65</sub>O<sub>5</sub>Si, *m/z* = 689.4301, Δ = -6.5 ppm.

**(2R)-1-O-tert-Butyldiphenylsilyl-2-O-(4-methoxybenzyl)-3-O-myristoyl-glycerol**

**(8.8b).** Compound **8.7** (0.53 g, 1.19 mmol) was treated with myristoyl chloride (0.37 mL, 1.38 mmol) and triethylamine (0.39 mL, 2.8 mmol) in THF (10 mL) by the same procedure described above to yield **8.8b** (0.68 g, 1.06 mol, 87%) as a white wax. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.65-7.58 (m, overlapped, 4H), 7.57-7.38 (m, overlapped, 6H), 7.35 (d, 2H, *J* = 8.8), 6.78 (d, 2H, *J* = 8.8), 4.40 (s, 2H, CH<sub>2</sub>OPh), 4.29 (dd, 1H, *J* = 11.6 and 2.3), 4.12 (dd, 1H, *J* = 11.6 and 2.3), 3.73 (s, 3H, OCH<sub>3</sub>), 3.68-3.59 (m, overlapped, 3H), 2.20 (t, 2H, *J* = 7.5, α-CH<sub>2</sub>), 1.50 (m, 2H, β-CH<sub>2</sub>), 1.23-1.15 (overlapped, 16H), 0.81 (t, 3H, *J*

= 7.0);  $^{13}\text{C}$  NMR:  $\delta$  173.8, 159.5, 135.8, 133.5, 130.4, 130.0, 129.6-128.0 (overlapped), 114.1, 73.3, 71.1, 70.9, 65.1, 55.5, 29.7-29.3 (overlapped), 27.1, 19.5; HRFABMS:  $m/z$  = 661.4298  $[\text{M}+\text{H}]^+$ , calculated for  $\text{C}_{41}\text{H}_{61}\text{O}_5\text{Si}$ ,  $m/z$  = 661.4288,  $\Delta$  = 1.5 ppm.

**(2S)-1-O-Palmitoyl-2-O-(4-methoxybenzyl)-glycerol (8.3a).** To a stirred solution of **8.8a** (0.55 g, 0.83 mmol in THF (10 mL) in an ice bath, HF/pyridine (0.5 mL, 70% wt) was added. The reaction mixture was stirred at 0 °C for 2 h and the temperature was then slowly increased to room temperature and the mixture was stirred overnight. The reaction mixture was quenched by slowly adding 10 mL saturated aqueous  $\text{NaHCO}_3$ , and was then diluted with 50 mL of water and extracted with EtOAc (3×50 mL), the combined organic layer was washed with saturated aqueous  $\text{NaHCO}_3$  (3×50 mL) and brine (2×50 mL), and dried over  $\text{Na}_2\text{SO}_4$ . Column chromatography on silica gel with 15% EtOAc in hexane gave compound **8.3a** (0.34 g, 0.8 mmol, 97%).  $[\alpha]_{\text{D}}^{23} = +77^\circ$  ( $c = 0.20$   $\text{CHCl}_3$ ).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.24 (dd, 2H,  $J = 8.0$  and  $2.6$ ), 6.86 (dd, 2H,  $J = 8.0$  and  $2.6$ ), 4.63 (dd, 1H,  $J = 11.4$  and  $2.7$ ), 4.61 (dd, 1H,  $J = 11.4$  and  $2.7$ ), 4.20 (m, 2H), 3.78 (s, 3H,  $\text{OCH}_3$ ), 3.65 (dd, 1H,  $J = 11.2$  and  $2.8$ ), 3.64 (dd, 1H,  $J = 11.2$  and  $2.8$ ), 3.59 (m, 1H), 2.31 (t, 2H,  $J = 7.8$ ,  $\alpha\text{-CH}_2$ ), 1.60 (m, 2H,  $\beta\text{-CH}_2$ ), 1.36-1.20 (overlapped, 28H, 14  $\text{CH}_2$ ), 0.84 (t, 3H,  $J = 7.7$ ).  $^{13}\text{C}$  NMR:  $\delta$  174.0, 159.6, 130.1, 129.7, 114.1, 76.9, 72.0, 62.9, 62.2, 55.4, 34.4, 32.4, 29.9-29.3 (aliphatic, overlapped), 25.1, 22.9, 14.3. HRFABMS:  $m/z$  = 451.3416  $[\text{M}+\text{H}]^+$ , calculated for  $\text{C}_{27}\text{H}_{47}\text{O}_5$ ,  $m/z$  = 451.3423,  $\Delta$  = -1.5 ppm.

**(2S)-1-O-Myristoyl-2-O-(4-methoxybenzyl)-glycerol (8.3b).** Compound **8.8b** (670 mg, 1.01 mmol) was treated with HF/Py (0.7 mL) by the procedure described for **8.3a** to yield

**8.3b** (406 mg, 0.96 mol, 96%) as a white solid.  $[\alpha]_D^{23} = +69^\circ$  ( $c = 0.20$  CHCl<sub>3</sub>). <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  7.24 (dd, 2H,  $J = 8.0$  and  $2.6$ ), 6.86 (dd, 2H,  $J = 8.0$  and  $2.6$ ), 4.63 (dd, 1H,  $J = 11.4$  and  $2.7$ ), 4.61 (dd, 1H,  $J = 11.4$  and  $2.7$ ), 4.20 (m, 2H), 3.78 (s, 3H, OCH<sub>3</sub>), 3.65 (dd, 1H,  $J = 11.2$  and  $2.8$ ), 3.64 (dd, 1H,  $J = 11.2$  and  $2.8$ ), 3.59 (m, 1H), 2.31 (t, 2H,  $J = 7.8$ ,  $\alpha$ -CH<sub>2</sub>), 1.60 (m, 2H,  $\beta$ -CH<sub>2</sub>), 1.36-1.20 (overlapped, 20H, 10 CH<sub>2</sub>), 0.84 (t, 3H,  $J = 7.7$ ); <sup>13</sup>C NMR:  $\delta$  174.0, 159.6, 130.1, 129.7, 114.1, 76.9, 72.0, 62.9, 62.2, 55.4, 34.4, 32.4, 29.9-29.3 (aliphatic, overlapped), 25.1, 22.9, 14.3; HRFABMS  $m/z = 423.3084$   $[M+H]^+$ , calculated for C<sub>25</sub>H<sub>43</sub>O<sub>5</sub> 423.3111,  $\Delta = -6.7$  ppm.

**1-O- $\alpha$ -Allyl-2,3,4-tri-O-benzyl- $\alpha$ -D-glucopyranoside (8.12).** This compound was synthesized from D-glucose via the reported methods.<sup>117,118</sup>  $[\alpha]_D^{23} = +24.3$  ( $c = 0.25$  CHCl<sub>3</sub>). <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  7.35-7.20 (overlapped, 15H, aromatics), 6.02 (m, 1H, CCH=C), 5.42 (d, 1H,  $J = 17.2$  and  $1.4$ , C=CH<sub>a</sub>), 5.30 (dd, 1H,  $J = 11.4$  and  $1.4$ , C=CH<sub>b</sub>), 5.10 (d, 1H,  $J = 11.7$ , CH<sub>a</sub>OPh), 4.99 (d, 1H,  $J = 11.7$ , CH<sub>a</sub>OPh), 4.90 (d, 1H,  $J = 3.5$ , anomeric), 4.86 and 4.54 (d, 2H,  $J = 12.0$ , CH<sub>2</sub>OPh), 4.73 and 4.64 (d, 2H,  $J = 12.0$ , CH<sub>2</sub>OPh), 4.24 (dd, 1H,  $J = 12.8$  and  $5.2$ ), 4.18 (t, 1H,  $J = 9.2$ ), 4.08 (dd, 1H,  $J = 12.8$  and  $6.4$ ), 3.78-3.60 (overlapped, 3H), 3.40-3.23 (overlapped, 2H); <sup>13</sup>C NMR:  $\delta$  139.2, 128.6, 138.5, 134.1, 128.8-127.9 (overlapped), 118.4, 96.0 (C-1), 82.2, 80.4, 77.9, 76.0, 75.3, 73.5, 71.4, 68.6, 61.9 (C-6).

**1-O-Allyl-2,3,4-tri-O-benzyl-6-desoxy-6-iodo- $\alpha$ -D-glucopyranoside (8.13).** To a stirred solution of compound **8.12** (3.6 g, 7.3 mmol) in 20 mL of ether/CH<sub>3</sub>CN, 3:1 (v/v) in an ice bath, triphenylphosphine (3.6 g, 14.7 mmol) and imidazole (1.9 g, 28.9 mmol) were added under nitrogen, then iodine (3.7 g, 14.6 mmol) was slowly added in 3

portions. After stirring for 3 h, the reaction mixture was quenched with saturated NaHCO<sub>3</sub> (20 mL) and extracted with EtOAc (3×50 mL). The combined organic layers were washed with saturated aqueous NaHCO<sub>3</sub> (2×50 mL) and brine (2×50 mL), and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on silica gel with 5% in hexane gave **8.13** (4.1 g, 6.8 mmol, 93%). <sup>1</sup>H NMR: δ 7.44-7.25 (m, 15H, aromatics), 6.05 (m, 1H, CCH=C), 5.46 (d, 1H, C=CH<sub>a</sub> *J* = 17.3 and 1.4), 5.32 (dd, 1H, C=CH<sub>b</sub> *J* = 10.8 and 1.4), 5.18-4.95 (m, 2H), 4.94-4.70 (overlapped, 5H, two CH<sub>2</sub>OPh and anomeric proton), 4.38-4.20 (m, 2H), 4.20-4.08 (m, 2H), 3.68-3.54 (overlapped, 2H), 3.52-3.35 (m, 2H). <sup>13</sup>C NMR: δ 138.8, 138.2, 138.1, 133.7, 128.7-127.8 (overlapped), 118.6, 95.5 (C-1), 81.6, 80.2, 77.5, 75.8, 75.5, 73.3, 69.8, 68.4, 8.0 (C-6). HRFABMS: *m/z* = 624.1547 [M+Na]<sup>+</sup>; calculated for C<sub>30</sub>H<sub>33</sub>IO<sub>5</sub>Na, *m/z* = 624.1531, Δ = -6.7 ppm.

**1-O-α-Allyl-2,3,4-tri-O-benzyl-6-desoxy-6-azido-α-D-glucopyranoside (8.14).** To a solution of **8.13** (4.0 g, 6.8 mmol) in DMF (20 mL) under nitrogen, sodium azide (2.73 g, 42 mmol) was added and the reaction mixture was stirred for 24 h at 50 °C. The mixture was then diluted with EtOAc (100 mL), washed with water (3×100 mL) and brine (2×50 mL), and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on silica gel with 5% EtOAc in hexane gave **8.14** (3.42 g, 6.65 mmol, 98%) as a white wax. <sup>1</sup>H NMR: δ 7.34-7.18 (m, 15H, aromatics), 6.04 (m, 1H, CCH=C), 5.48 (d, 1H, C=CH<sub>a</sub> *J* = 17.3 and 1.4), 5.36 (dd, 1H, C=CH<sub>b</sub> *J* = 10.8 and 1.4), 5.16 (d, 1H, *J* = 11.7, CH<sub>2</sub>OPh), 5.06 (d, 1H, *J* = 11.7, CH<sub>a</sub>OPh), 4.95 (d, 1H, *J* = 3.6, anomeric), 4.92 and 4.68 (d, 2H, *J* = 12.0, CH<sub>2</sub>OPh), 4.78 and 4.70 (d, 2H, *J* = 12.0, CH<sub>2</sub>OPh), 4.32 (dd, 1H, *J* = 12.8 and 5.2), 4.18-4.14 (overlapped, 2H), 3.98 (m, 1H), 3.70 (dd, 1H, *J* = 9.6 and 3.2), 3.58-3.50 (overlapped,

2H), 3.45 (dd, 1H,  $J = 9.6$  and  $3.2$ );  $^{13}\text{C}$  NMR:  $\delta$  138.8, 138.2, 138.1, 133.6, 128.6-127.8 (overlapped), 118.6, 95.5 (C-1), 81.9, 80.1, 78.5, 75.8, 75.3, 73.3, 70.3, 68.4, 51.5 (C-6).

**1-*O*- $\alpha$ -Allyl-2,3,4-tri-*O*-benzyl-6-desoxy-6-amino- $\alpha$ -D-glucopyranoside (8.15).** To a suspension of lithium aluminum hydride (1 g, 27 mmol) in THF (20 mL) at  $-20^\circ\text{C}$ , compound **8.14** (3.3 g, 6.4 mmol) in THF solution (10 mL) was added slowly with stirring and the reaction mixture was stirred at  $0^\circ\text{C}$  for 1 h. The reaction was quenched by adding saturated aqueous  $\text{NH}_4\text{Cl}$  (10 mL) dropwise. Then 10 mL of 2.5 M sodium potassium tartate was added and the solution was stirred for 20 min. The mixture was then extracted with EtOAc (100 mL), washed with water and brine, and dried over  $\text{Na}_2\text{SO}_4$ . Removal of the solvent and chromatography on silica gel with 5% MeOH in  $\text{CHCl}_3$  gave compound **8.15** (2.77 g, 5.7 mmol, 89%) as a white wax-like solid.  $^1\text{H}$ -NMR:  $\delta$  7.38-7.22 (m, 15H, aromatics), 6.06 (m, 1H,  $\text{CCH}=\text{C}$ ), 5.49 (d, 1H,  $\text{C}=\text{CH}_a$ ,  $J = 17.3$  and  $1.4$ ), 5.38 (dd, 1H,  $\text{C}=\text{CH}_b$ ,  $J = 10.8$  and  $1.4$ ), 5.19 (d, 1H,  $J = 11.7$ ,  $\text{CH}_a\text{OPh}$ ), 5.10 (d, 1H,  $J = 11.7$ ,  $\text{CH}_b\text{OPh}$ ), 4.86 (d, 1H,  $J = 3.6$ , anomeric), 4.94 and 4.69 (d, 2H,  $J = 12.0$ ,  $\text{CH}_2\text{OPh}$ ), 4.78 and 4.70 (d, 2H,  $J = 12.0$ ,  $\text{CH}_2\text{OPh}$ ), 4.32 (dd, 1H,  $J = 12.8$  and  $5.2$ ), 4.18-4.14 (overlapped, 2H), 3.98 (m, 1H), 3.70 (dd, 1H,  $J = 9.6$  and  $3.2$ ), 3.58-3.50 (overlapped, 2H), 3.45 (dd, 1H,  $J = 9.6$  and  $3.2$ );  $^{13}\text{C}$  NMR:  $\delta$  138.9, 138.2, 138.1, 133.7, 128.6-127.8 (overlapped, aromatics), 118.6, 95.5 (C-1), 81.9, 80.1, 78.5, 75.8, 75.3, 73.4, 70.3, 68.5, 35.5 (C-6).

**1-*O*-Allyl-2,3,4-tri-*O*-benzyl-6-desoxy-6-stearoylamido- $\alpha$ -D-glucopyranoside (8.16a).**

To a solution of compound **8.15** (0.6 g, 1.23 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (10 mL) in an ice bath, triethylamine (0.45 mL, 3.2 mmol) was added, then stearoyl chloride (0.54 mL,

1.6 mmol) was added dropwise and the reaction mixture was stirred for 3 h at 0 °C and 1 h at room temperature. The mixture was extracted with EtOAc (100 mL) and washed with saturated NaHCO<sub>3</sub> (3×100 mL) and brine (2×50 mL), and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on silica gel with 10% EtOAc in hexane gave compound (0.87 g, 1.12 mmol, 92%) as a white amorphous solid. <sup>1</sup>H NMR: δ 7.44-7.25 (m, 15H, aromatics), 6.05 (m, 1H, CCH=C), 5.78 (m, br, 1H, N-H), 5.38(d, 1H, C=CH<sub>a</sub>, *J* = 17.3 and 1.4), 5.16 (dd, 1H, C=CH<sub>b</sub>, *J* = 10.8 and 1.4), 5.14-4.79 (overlapped, 7H, three CH<sub>2</sub>OPh and anomeric proton), 4.22-4.08 (m, overlapped, 2H), 3.82-3.61(m, overlapped, 2H), 3.50-3.44 (overlapped, 2H), 3.42-3.38 (m, overlapped, 2H), 2.06 (t, 2H, *J* = 7.5, α-CH<sub>2</sub>), 1.60 (m, 2H, β-CH<sub>2</sub>), 1.23-1.15 (overlapped, 28H), 0.80(t, 3H, *J* = 7.0). <sup>13</sup>C NMR: δ 173.5, 138.6, 138.5, 138.0, 133.6, 128.8-128.1(overlapped), 118.4, 96.8 (C-1), 82.4, 80.5, 78.7, 75.9, 75.3, 73.7, 70.3, 68.4, 40.2 (C-6), 37.1, 32.2, 29.9-29.6 (overlapped), 26.1, 22.9, 14.3.

**1-*O*-α-Allyl-2,3,4-tri-*O*-benzyl-6-desoxy-6-palmitoylamido-α-D-glucopyranoside**

**(8.16b).** Compound **8.15b** (0.56 g, 1.14 mmol) was treated with palmitoyl chloride (0.41 mL, 1.5 mmol) and triethylamine (0.40 mL, 2.8 mmol) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (10 mL) as described for compound **8.16a**. Workup and column chromatography gave compound **8.16b** (0.77 g, 1.07 mmol, 94%) as a white amorphous solid. <sup>1</sup>H NMR: δ 7.44-7.25 (m, 15H, aromatics), 6.05 (m, 1H, CCH=C), 5.78 (m, br, 1H, N-H), 5.38(d, 1H, C=CH<sub>a</sub>, *J* = 17.3 and 1.4), 5.16 (dd, 1H, C=CH<sub>b</sub>, *J* = 10.8 and 1.4), 5.14-4.79 (overlapped, 7H, three CH<sub>2</sub>OPh and anomeric proton), 4.22-4.08 (m, overlapped, 2H), 3.82-3.61(m, overlapped, 2H), 3.50-3.44 (overlapped, 2H), 3.42-3.38 (m, overlapped, 2H), 2.06 (t, 2H, *J* = 7.5, α-

CH<sub>2</sub>), 1.60 (m, 2H, β-CH<sub>2</sub>), 1.23-1.15 (overlapped, 28H), 0.80(t, 3H, *J* = 7.0). <sup>13</sup>C NMR: δ 173.5, 138.6, 138.5, 138.0, 133.6, 128.8-128.1(overlapped), 118.4, 96.8 (C-1), 82.4, 80.5, 78.7, 75.9, 75.3, 73.7, 70.3, 68.4, 40.2 (C-6), 37.1, 32.2, 29.9-29.6 (overlapped), 26.1, 22.9, 14.3.

**2,3,4-tri-*O*-Benzyl-6-desoxy-6-stearoylamido-D-glucopyranoside (8.17a).** Compound **8.16a** (0.86 g, 1.1 mmol) was dissolved in 90% aqueous ethanol (40 mL). DABCO (48 mg) and Rh(PPh<sub>3</sub>)<sub>3</sub>Cl (37 mg) was added and the reaction mixture was refluxed at 85°C for 10 h. The reaction mixture was concentrated and partitioned between EtOAc and water. The organic layer was washed with water (3×100 mL) and evaporated. The residue was dissolved again with 95% MeOH at 60°C. Mercury chloride (14 mg) was added together with of *p*-toluenesulfonic acid monohydrate (10 mg). The reaction mixture was refluxed for 4 h and then concentrated in *vacuo*. The mixture was partitioned between EtOAc and water and the organic layer was washed with saturated aqueous NaHCO<sub>3</sub> and brine. Column chromatography on silica gel with 35% EtOAc in hexane gave compound **11a** (0.59 g, 0.82 mmol, 75%) as a mixture of α and β anomers. <sup>1</sup>H-NMR: δ 7.45-7.25 (m, 15H, aromatics), 5.79 (m, N-H, β-isomer), 5.60 (m, N-H, α-isomer), 5.08 (d, *J* = 3.6, α-anomeric proton), 4.88-4.52 (m, overlapped, 5H), 3.93-3.83 (m, overlapped, 2H), 3.61-3.53 (m, overlapped, 2H), 3.42-3.17 (m, overlapped, 2H). 2.23 (t, 2H, *J* = 7.5, α-CH<sub>2</sub>), 1.60 (m, 2H, β-CH<sub>2</sub>), 1.23-1.15 (overlapped, 28H), 0.80 (t, 3H, *J* = 7.0); <sup>13</sup>C NMR: δ 173.8, 173.6, 138.5, 138.2, 138.1, 138.0, 128.9-128.2 (overlapped), 99.5, 96.8, 82.4, 80.6, 80.1, 78.7, 75.9, 75.3, 73.7, 70.3, 68.4, 40.1, 37.1, 32.2, 29.9-29.6 (overlapped), 26.1,

22.9, 14.3; HRFABMS:  $m/z = 716.4854$   $[M+H]^+$ ; calculated for  $C_{45}H_{66}NO_6$ ,  $m/z = 716.4890$ ,  $\Delta = -5.0$  ppm.

**2,3,4-tri-*O*-Benzyl-6-desoxy-6-palmitoylamido-D-glucopyranoside (8.17b).** Reaction of compound **8.16b** (0.76 g, 1.07 mmol) by the same procedure described above gave compound **8.17b** (0.49 g, 0.72 mmol, 67%) a mixture of  $\alpha$  and  $\beta$  anomers.  $^1H$ -NMR:  $\delta$  7.45-7.25 (m, 15H, aromatics), 5.79 (m, N-H), 5.60 (m, N-H), 5.08 (d,  $J = 3.6$ ,  $\alpha$ -anomeric proton), 4.88-4.52 (m, overlapped, 5H), 3.93-3.83 (m, overlapped, 2H), 3.61-3.53 (m, overlapped, 2H), 3.42-3.17 (m, overlapped, 2H). 2.23 (t, 2H,  $J = 7.5$ ,  $\alpha$ -CH<sub>2</sub>), 1.60 (m, 2H,  $\beta$ -CH<sub>2</sub>), 1.22-1.15 (overlapped, 24H), 0.80 (t, 3H,  $J = 7.0$ );  $^{13}C$  NMR:  $\delta$  173.8, 173.6, 138.5, 138.2, 138.1, 138.0, 128.9-128.2 (overlapped), 99.5, 96.8, 82.4, 80.6, 80.1, 78.7, 75.9, 75.3, 73.7, 70.3, 68.4, 40.1, 37.1, 32.2, 29.9-29.6 (overlapped), 26.1, 22.8, 14.4; HRFABMS:  $m/z = 688.4554$   $[M+H]^+$ ; calculated for  $C_{43}H_{62}NO_6$ ,  $m/z = 688.4577$ ,  $\Delta = -3.3$  ppm.

**1-*O*-Trichloroacetimidoyl-2,3,4-tri-*O*-benzyl-6-desoxy-6-stearoylamido- $\beta$ -D-glucopyranoside (8.4a).** Compound **8.17a** (0.55 g, 0.72 mmol) was dissolved in anhydrous  $CH_2Cl_2$  (20 mL) under nitrogen, and  $K_2CO_3$  (0.9 g, 6.6 mmol) and trichloroacetonitrile (1 mL) were added. The mixture was stirred vigorously at RT for 12 h and then filtered through Celite to remove  $K_2CO_3$ . The Celite was washed with 20 mL of additional  $CH_2Cl_2$  and the filtrate was dried with  $MgSO_4$  and dried *in vacuo* to remove trichloroacetonitrile. The residue gave a yellow syrup **8.4a** (0.47 g, 0.55 mmol, 77%) and was directly used for the next step without purification. TLC indicated this compound was about 95% pure with a small amount of unreacted **8.17a**.  $^1H$  NMR:  $\delta$  8.66 (1H, br,

N-H), 7.38-7.22 (m, 15H, aromatics), 5.78 (1H, m, N-H), 5.74 (1H, d,  $J = 8.0$ , anomeric), 4.95-4.63 (m, overlapped, 6H), 3.78-3.64 (m, overlapped, 2H), 3.57-3.43 (m, overlapped, 3H), 2.17 (t, 2H,  $J = 7.5$ ,  $\alpha$ -CH<sub>2</sub>), 1.60 (m, 2H,  $\beta$ -CH<sub>2</sub>), 1.23-1.15 (overlapped, 28H), 0.82 (t, 3H,  $J = 7.0$ ); <sup>13</sup>C NMR:  $\delta$  173.5, 162.1, 138.2, 138.0, 137.9, 128.8-128.1 (overlapped), 98.3 (C-1), 82.4, 81.8, 78.2, 75.9, 75.7, 75.6, 75.3, 40.5, 37.8, 32.3, 30.2-29.9 (overlapped), 26.2, 23.0, 14.3.

**1-O-Trichloroacetimidoyl-2,3,4-tri-O-benzyl-6-desoxy-6-palmitoylamido- $\beta$ -D-glucopyranoside (8.4b).** Compound **8.17b** (0.49 g, 0.72 mmol) was treated with K<sub>2</sub>CO<sub>3</sub> (0.8 g, 5.9 mmol) and trichloroacetonitrile (0.85 mL, large excess) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (20 mL) as described above to give **8.4b** (0.45 g, 0.52 mmol, 71%) as a yellow syrup which was directly used for the next step without purification. TLC indicated that this compound was about 90% pure with a small amount of unreacted **8.17b**. <sup>1</sup>H NMR:  $\delta$  8.66 (1H, br, N-H), 7.38-7.22 (m, 15H, aromatics), 5.78 (1H, m, N-H), 5.74 (1H, d,  $J = 8.0$ , anomeric), 4.95-4.63 (m, overlapped, 6H), 3.78-3.64 (m, overlapped, 2H), 3.57-3.43 (m, overlapped, 3H), 2.17 (t, 2H,  $J = 7.5$ ,  $\alpha$ -CH<sub>2</sub>), 1.60 (m, 2H,  $\beta$ -CH<sub>2</sub>), 1.23-1.15 (overlapped, 28H), 0.82 (t, 3H,  $J = 7.0$ ); <sup>13</sup>C NMR:  $\delta$  173.5, 162.1, 138.2, 138.0, 137.9, 128.8-128.1 (overlapped), 98.3 (C-1), 82.4, 81.8, 78.2, 75.9, 75.7, 75.6, 75.3, 40.5, 37.8, 32.3, 30.2-29.9 (overlapped), 26.2, 23.0, 14.3.

**(2S)-1-O-(2,3,4-tri-O-Benzyl-6-desoxy-6-stearoylamido-glucopyranosyl)-2-O-(4-methoxybenzyl)-3-O-palmitoyl-glycerol (8.18a).** Compounds **8.3a** (0.42 g, 0.48 mmol) and **8.4a** (170 mg, 0.4 mmol) were dissolved together in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (10 mL). Pre-activated 4Å molecular sieves (1.5 g) were added at -20 °C under nitrogen. After stirring

for 10 min tetramethylurea (0.15 mL, 1.3 mmol) was added, followed by trimethylsilyl-trifluoromethanesulfonate (25  $\mu$ l, 0.14 mmol) and the mixture was stirred at -20  $^{\circ}$ C for 8 h and then 4 h at room temperature. The mixture was diluted with EtOAc (100 mL) and filtered through Celite, washed with saturated aqueous NaHCO<sub>3</sub> (3 $\times$ 50 mL) and brine (2 $\times$ 50 mL), dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on silica gel with 10% EtOAc in hexane gave compound **8.18a** (382 mg, 0.33 mmol, 84%) as a white solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  7.34-7.18 (overlapped, 17H, aromatics), 6.82 (d, 2H,  $J$  = 8.0), 5.62 (m, 1H, N-H), 4.98-4.60 (overlapped, 9H, 4CH<sub>2</sub>OPh and anomeric H), 4.30 (dd, 1H,  $J$  = 11.6 and 3.6), 4.18 (dd, 1H,  $J$  = 11.6 and 3.6), 3.97 (t, 1H,  $J$  = 9.6), 3.82-3.77 (m, 2H), 3.75 (s, 3H, OCH<sub>3</sub>), 3.73-3.67 (overlapped, 2H), 3.55-3.45 (overlapped, 2H), 3.40-3.28 (overlapped, 2H), 2.31(t, 2H,  $J$  = 7.8, ester  $\alpha$ -CH<sub>2</sub>), 2.04(t, 2H,  $J$  = 7.8, amide  $\alpha$ -CH<sub>2</sub>), 1.60 (m, 4H, 2 $\beta$ -CH<sub>2</sub>), 1.38-1.20 (overlapped, 52H, 26 CH<sub>2</sub>), 0.84 (t, 6H,  $J$  = 7.7, 2CH<sub>3</sub>); <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  173.8, 173.3, 159.5, 138.8, 138.4, 138.2, 129.6-127.9 (aromatics, overlapped), 114.0, 97.7, 81.8, 80.3, 78.9, 76.0, 75.5, 75.3, 73.2, 72.0, 69.7, 68.3, 63.4, 55.4, 39.7, 37.1, 34.4, 32.2, 29.9-29.3 (aliphatic, overlapped), 14.4; HRFABMS:  $m/z$  = 1170.7927 [M+Na]<sup>+</sup>; calculated for C<sub>72</sub>H<sub>109</sub>NO<sub>10</sub>  $m/z$  = 1170.7949,  $\Delta$  = -1.9 ppm.

**1-O-(2,3,4-tri-O-Benzyl-6-desoxy-6-palmitoylamido-glucopyranosyl)-2-O-(4-methoxybenzyl)-3-O-palmitoyl-glycerol (8.18b).** Treatment of compounds **8.3b** (0.46 g, 0.51 mmol) and **8.4a** (166 mg, 0.39 mmol) as described above yielded **8.18b** (345 mg, 0.31 mmol, 78%) as a white solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  7.34-7.18 (overlapped, 17H, aromatics), 6.82 (d, 2H,  $J$  = 8.0), 5.62 (m, 1H, N-H), 4.98-4.60 (overlapped, 9H, 4CH<sub>2</sub>OPh and anomeric H), 4.30 (dd, 1H,  $J$  = 11.6 and 3.6), 4.18 (dd, 1H,  $J$  = 11.6 and

3.6), 3.97 (t, 1H,  $J = 9.6$ ), 3.82-3.77 (m, 2H), 3.75 (s, 3H, OCH<sub>3</sub>), 3.73-3.67 (overlapped, 2H), 3.55-3.45 (overlapped, 2H), 3.40-3.28 (overlapped, 2H), 2.31 (t, 2H,  $J = 7.8$ , ester  $\alpha$ -CH<sub>2</sub>), 2.04 (t, 2H,  $J = 7.8$ , amide  $\alpha$ -CH<sub>2</sub>), 1.60 (m, 4H, 2 $\beta$ -CH<sub>2</sub>), 1.38-1.20 (overlapped, 48H, 24 CH<sub>2</sub>), 0.84 (t, 6H,  $J = 7.7$ , 2CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  173.8, 173.3, 159.5, 138.8, 138.4, 138.2, 129.6-127.9 (aromatics, overlapped), 114.0, 97.7, 81.8, 80.3, 78.9, 76.0, 75.5, 75.3, 73.2, 72.0, 69.7, 68.3, 63.4, 55.4, 39.7, 37.1, 34.4, 32.2, 29.9-29.3 (aliphatic, overlapped), 14.5, 14.3; HRFABMS:  $m/z = 1142.7629$  [M+Na]<sup>+</sup>; calculated for C<sub>70</sub>H<sub>105</sub>NO<sub>10</sub>  $m/z = 1142.7636$ ,  $\Delta = -0.6$  ppm.

**1-O-(2,3,4-tri-O-Benzyl-6-desoxy-6-stearoylamido-glucopyranosyl)-3-O-palmitoyl-glycerol (8.19a)**. To a stirred solution of compound **8.18a** (380 mg, 0.33 mmol) in 10 mL of CH<sub>2</sub>Cl<sub>2</sub>:water, 9:1 (v/v), DDQ (105 mg, 0.46 mmol) was added and the mixture was stirred for 1.5 h at room temperature. The reaction mixture was then diluted with aqueous NaHCO<sub>3</sub> and extracted with EtOAc (3 $\times$ 25 mL), and the combined organic layers were washed with saturated aqueous NaHCO<sub>3</sub> (3 $\times$ 50 mL) and brine (2 $\times$ 50 mL), and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on silica gel with 15% EtOAc in hexane gave compound **8.19a** (275 mg, 0.27 mmol, 82%). <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  7.34-7.20 (overlapped, 15H, aromatics), 5.64 (m, 1H, N-H), 4.95 and 4.82 (d, 2H,  $J = 11.2$ , CH<sub>2</sub>OPh), 4.84 and 4.62 (d, 2H,  $J = 10.8$ , CH<sub>2</sub>OPh), 4.78 and 4.64 (d, 2H,  $J = 12.0$ , CH<sub>2</sub>OPh), 4.69 (d, 1H,  $J = 3.6$ , anomeric H), 4.16 (dd, 1H,  $J = 11.6$  and 3.6), 4.08 (dd, 1H,  $J = 11.6$  and 3.6), 3.97 (t, 1H,  $J = 9.6$ ), 3.78-3.69 (overlapped, m, 3H), 3.47 (dd, 1H,  $J = 9.6$  and 3.6), 3.41 (dd, 1H,  $J = 10.8$  and 3.6), 3.35 (m, 1H), 3.30 (t, 1H,  $J = 9.2$ ), 2.32 (t, 2H,  $J = 7.8$ , ester  $\alpha$ -CH<sub>2</sub>), 2.04 (t, 2H,  $J = 7.8$ , amide  $\alpha$ -CH<sub>2</sub>), 1.60 (m, 4H, 2 $\beta$ -CH<sub>2</sub>), 1.38-1.20 (overlapped,

52H, 26CH<sub>2</sub>), 0.84 (t, 6H, *J* = 7.7, 2CH<sub>3</sub>); <sup>13</sup>C NMR(CDCl<sub>3</sub>): 174.2, 173.4, 138.6, 138.1, 138.0, 128.8-127.9 (aromatics, overlapped), 98.7, 81.9, 80.2, 79.0, 76.0, 75.5, 73.7, 70.9, 69.8, 69.3, 65.2, 39.8, 37.0, 34.3, 32.1, 31.8, 29.9-29.2 (aliphatic, overlapped), 26.0, 25.1, 22.9, 22.8, 21.2, 14.4; HRFABMS: *m/z* = 972.6953 [M+H]<sup>+</sup>; calculated for C<sub>60</sub>H<sub>94</sub>NO<sub>9</sub>, *m/z* = 972.6928, Δ = 2.5 ppm.

**(2S)-1-O-(2,3,4-tri-O-Benzyl-6-desoxy-6-stearoylamido-glucopyranosyl)-2-O-**

**myristoyl-3-O-palmitoylglycerol (8.20a).** To a solution of myristic acid (145 mg, 0.54 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) was added EDCI (106 mg, 0.55 mmol). After 15 min stirring, DMAP (10 mg, cat.) was added and the solution was stirred for 30 min before compound **8.19a** (133 mg, 0.13 mmol) was added. The reaction mixture was stirred overnight and was then diluted with EtOAc (100 mL), washed with saturated aqueous NaHCO<sub>3</sub> (3×100 mL) and brine (2×50 mL), and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on silica gel with 15% EtOAc in hexane gave compound **8.20a** (148 mg, 0.12 mmol, 92%). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.34-7.20 (overlapped, 15H, aromatics), 5.64 (m, 1H, N-H), 5.21 (m, 1H), 4.96 and 4.82 (d, 2H, *J* = 10.8, CH<sub>2</sub>OPh), 4.84 and 4.63 (d, 2H, *J* = 10.4, CH<sub>2</sub>OPh), 4.75 and 4.62 (d, 2H, *J* = 12.0, CH<sub>2</sub>OPh), 4.68 (d, 1H, *J* = 3.6, anomeric), 4.40 (dd, 1H, *J* = 12.0 and 3.6), 4.18 (dd, 1H, *J* = 10.8 and 3.6), 3.95 (t, 1H, *J* = 9.6), 3.80-3.64 (overlapped, m, 3H), 3.56 (dd, 1H, *J* = 10.8 and 5.6), 3.45 (dd, 1H, *J* = 9.6 and 3.6), 3.34 (m, 1H), 3.27 (t, 1H, *J* = 9.2), 2.32 (t, 4H, *J* = 7.8, ester α-CH<sub>2</sub>), 2.04 (t, 2H, *J* = 7.8, amide α-CH<sub>2</sub>), 1.60 (m, 6H, 3β-CH<sub>2</sub>), 1.38-1.20 (overlapped, 72H, 36 CH<sub>2</sub>), 0.84 (overlapped, 9H, 3 CH<sub>3</sub>); <sup>13</sup>C NMR (CDCl<sub>3</sub>): 173.6, 173.4, 173.3, 138.7, 138.4, 138.2, 128.7-128.0 (aromatics, overlapped), 97.8, 81.7, 80.3, 78.8, 76.0, 75.5, 73.4, 70.1, 69.8, 66.8, 62.6,

39.8, 37.0, 34.5, 34.3, 32.1, 29.9-29.3 (aliphatic, overlapped), 26.0, 25.1, 24.9, 22.9, 22.8, 21.2, 14.4, 14.3 (2CH<sub>3</sub> overlapped); HRFABMS:  $m/z = 1260.9305$  [M+Na]<sup>+</sup>; calculated for C<sub>78</sub>H<sub>127</sub>NO<sub>10</sub>Na,  $m/z = 1260.9420$ ,  $\Delta = 4.9$  ppm.

**(2S)-1-O-(2,3,4-tri-O-Benzyl-6-desoxy-6-palmitoylamido-D-gluco-  
pyranosyl)-2,3-di-O-palmitoylglycerol (8.20b).** Compound **8.19b** (73 mg, 0.072 mmol) was treated with palmitic acid (116 mg, 0.29 mmol) and EDCI (89 mg, 0.45 mmol) by the same procedure as described above to yield **8.20b** (75 mg, 0.06 mmol, 85%). <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  7.34-7.20 (overlapped, 15H, aromatics), 5.64 (m, 1H, N-H), 5.21 (m, 1H), 4.96 and 4.82 (d, 2H,  $J = 10.8$ , CH<sub>2</sub>OPh), 4.84 and 4.63 (d, 2H,  $J = 10.4$ , CH<sub>2</sub>OPh), 4.75 and 4.62 (d, 2H,  $J = 12.0$ , CH<sub>2</sub>OPh), 4.68 (d, 1H,  $J = 3.6$ , anomeric H), 4.40 (dd, 1H,  $J = 12.0$  and 3.6), 4.18 (dd, 1H,  $J = 10.8$  and 3.6), 3.95 (t, 1H,  $J = 9.6$ ), 3.80-3.64 (overlapped, m, 3H), 3.56 (dd, 1H,  $J = 10.8$  and 5.6), 3.45 (dd, 1H,  $J = 9.6$  and 3.6), 3.34 (m, 1H), 3.27 (t, 1H,  $J = 9.2$ ), 2.32 (t, 4H,  $J = 7.8$ , ester  $\alpha$ -CH<sub>2</sub>), 2.04 (t, 2H,  $J = 7.8$ , amide  $\alpha$ -CH<sub>2</sub>), 1.60 (m, 6H, 3 $\beta$ -CH<sub>2</sub>), 1.38-1.20 (overlapped, 72H, 36 CH<sub>2</sub>), 0.84 (overlapped, 9H, 3 CH<sub>3</sub>); <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  173.6, 173.4, 173.3, 138.7, 138.4, 138.2, 128.7-128.0 (aromatics, overlapped), 97.8, 81.7, 80.3, 78.8, 76.0, 75.5, 73.4, 70.1, 69.8, 66.8, 62.6, 39.8, 37.0, 34.5, 34.3, 32.1, 29.9-29.3 (aliphatic, overlapped), 26.0, 25.1, 24.9, 22.9, 22.8, 21.2, 14.4, 14.3 (2CH<sub>3</sub> overlapped); HRFABMS:  $m/z = 1238.9623$  [M+H]<sup>+</sup>; calculated for C<sub>78</sub>H<sub>127</sub>NO<sub>10</sub>  $m/z = 1238.9538$ ,  $\Delta = 6.5$  ppm.

**(2S)-1-O-Palmitoyl-2-O-myristoyl-3-O-(6-desoxy-6-stearoylamido-D-gluco-  
pyranosyl)-glycerol (8.1).** Compound **8.20a** (142 mg, 0.11 mmol) was dissolved in THF (10 mL) and Pd/C (10 wt %, 55 mg) was added. Hydrogenation was carried at 30 psi for

10 h. The mixture was filtered through Celite and the filtrate was concentrated *in vacuo*. The residue was subjected to column chromatography on silica gel with 5% MeOH in CHCl<sub>3</sub> to yield compound **8.1** (148 mg, 11.9 mmol, 90%).  $[\alpha]_D^{23} = +34.6$  ( $c = 0.26$  MeOH); <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 5.98 (m, 1H, N-H), 5.24 (m, 1H, H-2), 4.80 (d, 1H,  $J = 3.6$ , H-1', anomeric H), 4.38 (dd, 1H,  $J = 12.0$  and 3.6, H-<sub>1a</sub>), 4.13 (dd, 1H,  $J = 12.0$  and 3.6, H-<sub>1b</sub>), 4.04 (ddd, 1H,  $J_1 = 16.0$ ,  $J_2 = 7.6$ ,  $J_3 = 1.2$ , H-6'<sub>a</sub>), 3.79 (dd, 1H,  $J = 10.4$  and 4.8, H-<sub>1a</sub>), 3.74 (t, 1H,  $J = 9.6$ , H-4'), 3.64 (dd,  $J = 10.4$  and 6.4, H-<sub>1b</sub>), 3.59 (m, 1H, H-5'), 3.49 (dd, 1H,  $J = 9.6$  and 3.6, H-2'), 3.10 (t, 1H,  $J = 9.6$ , H-3'), 3.03 (m, 1H, H-6'<sub>b</sub>), 2.38-2.33 (overlapped, m, 4H, 2 ester α-CH<sub>2</sub>), 2.26 (t, 2H,  $J = 7.8$ , amide α-CH<sub>2</sub>), 1.68-1.57 (overlapped, 6H, 3β-CH<sub>2</sub>), 1.38-1.20 (overlapped, 72H, 36 CH<sub>2</sub>), 0.86-0.72 (overlapped, 9H, 3 CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 175.9, 173.6, 173.4, 99.6, 73.3, 72.5, 71.3, 70.2, 70.0, 67.1, 62.3, 39.9, 36.6, 34.5, 34.3, 34.0, 32.1, 29.9-29.3 (aliphatic, overlapped), 25.8, 25.1, 25.0, 24.9, 22.9, 22.8, 14.3 (3-CH<sub>3</sub> overlapped). HRFABMS:  $m/z = 968.8178$  [M+H]<sup>+</sup>; calculated for C<sub>57</sub>H<sub>110</sub>NO<sub>10</sub>,  $m/z = 968.8131$ , Δ = 5.0 ppm.

**(2S)-1,2-di-O-Palmitoyl-3-O-(6-desoxy-6-palmitoylamido-D-glucopyranosyl)-**

**glycerol (8.2).** Hydrogenation of compound **8.20b** (75 mg, 0.06 mmol) as described above gave **8.2** (48 mg, 0.051 mmol, 85%) as a white solid.  $[\alpha]_D^{23} = +57.8$  ( $c = 0.18$  MeOH). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 5.98 (m, 1H, N-H), 5.24 (m, 1H, H-2), 4.80 (d, 1H,  $J = 3.6$ , H-1', anomeric H), 4.38 (dd, 1H,  $J = 12.0$  and 3.6, H-<sub>1a</sub>), 4.13 (dd, 1H,  $J = 12.0$  and 3.6, H-<sub>1b</sub>), 4.04 (ddd, 1H,  $J_1 = 16.0$ ,  $J_2 = 7.6$ ,  $J_3 = 1.2$ , H-6'<sub>a</sub>), 3.79 (dd, 1H,  $J = 10.4$  and 4.8, H-<sub>1a</sub>), 3.74 (t, 1H,  $J = 9.6$ , H-4'), 3.64 (dd,  $J = 10.4$  and 6.4, H-<sub>1b</sub>), 3.59 (m, 1H, H-5'), 3.49 (dd, 1H,  $J = 9.6$  and 3.6, H-2'), 3.10 (t, 1H,  $J = 9.6$ , H-3'), 3.03 (m, 1H, H-6'<sub>b</sub>), 2.38-

2.33 (overlapped, m, 4H, 2 ester  $\alpha$ -CH<sub>2</sub>), 2.26 (t, 2H,  $J = 7.8$ , amide  $\alpha$ -CH<sub>2</sub>), 1.68-1.57 (overlapped, 6H, 3 $\beta$ -CH<sub>2</sub>), 1.38-1.20 (overlapped, 72H, 36 CH<sub>2</sub>), 0.86-0.72 (overlapped, 9H, 3 CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  175.9, 173.6, 173.4, 99.6, 73.3, 72.5, 71.3, 70.2, 70.0, 67.1, 62.3, 39.9, 36.6, 34.5, 34.3, 34.0, 32.1, 29.9-29.3 (aliphatic, overlapped), 25.8, 25.1, 25.0, 24.9, 22.9, 22.8, 14.3 (3CH<sub>3</sub> overlapped). HRFABMS:  $m/z = 968.8154$  [M+H]<sup>+</sup>; calculated for C<sub>57</sub>H<sub>110</sub>NO<sub>10</sub>  $m/z = 968.8131$ ,  $\Delta = 2.5$  ppm.

**(2S)-1-O-Myristoyl-2-O-palmitoyl-3-O-(6-desoxy-6-palmitoylamido-D-glucopyranosyl)-glycerol (8.21)**. A similar procedure as described above for **8.1** and **8.2** gave compound **8.21** (17 mg, 0.018 mmol) from **8.18b**.  $[\alpha]_D^{23} = +38.5$  ( $c = 0.14$  MeOH). <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  5.98 (m, 1H, N-H), 5.24 (m, 1H, H-2), 4.80 (d, 1H,  $J = 3.6$ , H-1', anomeric H), 4.38 (dd, 1H,  $J = 12.0$  and  $3.6$ , H-<sub>1a</sub>), 4.13 (dd, 1H,  $J = 12.0$  and  $3.6$ , H-<sub>1b</sub>), 4.04 (ddd, 1H,  $J_1 = 16.0$ ,  $J_2 = 7.6$ ,  $J_3 = 1.2$ , H-6'<sub>a</sub>), 3.79 (dd, 1H,  $J = 10.4$  and  $4.8$ , H-<sub>1a</sub>), 3.74 (t, 1H,  $J = 9.6$ , H-4'), 3.64 (dd,  $J = 10.4$  and  $6.4$ , H-<sub>1b</sub>), 3.59 (m, 1H, H-5'), 3.49 (dd, 1H,  $J = 9.6$  and  $3.6$ , H-2'), 3.10 (t, 1H,  $J = 9.6$ , H-3'), 3.03 (m, 1H, H-6'<sub>b</sub>), 2.38-2.33 (overlapped, m, 4H, 2 ester  $\alpha$ -CH<sub>2</sub>), 2.26 (t, 2H,  $J = 7.8$ , amide  $\alpha$ -CH<sub>2</sub>), 1.68-1.57 (overlapped, 6H, 3 $\beta$ -CH<sub>2</sub>), 1.36-1.20 (overlapped, 68H, 34 CH<sub>2</sub>), 0.86-0.72 (overlapped, 9H, 3 CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  175.9, 173.6, 173.4, 99.6, 73.3, 72.5, 71.3, 70.2, 70.0, 67.1, 62.3, 39.9, 36.6, 34.5, 34.3, 34.0, 32.1, 29.9-29.3 (aliphatic, overlapped), 25.8, 25.1, 25.0, 24.9, 22.9, 22.8, 14.3 (3CH<sub>3</sub> overlapped). HRFABMS:  $m/z = 940.7775$  [M+H]<sup>+</sup>; calculated for C<sub>55</sub>H<sub>106</sub>NO<sub>10</sub>,  $m/z = 940.7817$ ,  $\Delta = -4.5$  ppm.

**(2S)-1-O-Myristoyl-2-O-palmitoyl-3-O-(6-desoxy-6-stearoylamido-D-glucopyranosyl)-glycerol (8.23).** A similar procedure as described above for **8.1** and **8.2** gave compound **8.23** (14 mg, 0.014 mmol) from **8.18a**,  $[\alpha]_D^{23} = +47.5$  ( $c = 0.15$  MeOH).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  5.98 (m, 1H, N-H), 5.24 (m, 1H, H-2), 4.80 (d, 1H,  $J = 3.6$ , H-1', anomeric H), 4.38 (dd, 1H,  $J = 12.0$  and  $3.6$ , H-<sub>1a</sub>), 4.13 (dd, 1H,  $J = 12.0$  and  $3.6$ , H-<sub>1b</sub>), 4.04 (ddd, 1H,  $J_1 = 16.0$ ,  $J_2 = 7.6$ ,  $J_3 = 1.2$ , H-6'<sub>a</sub>), 3.79 (dd, 1H,  $J = 10.4$  and  $4.8$ , H-<sub>1a</sub>), 3.74 (t, 1H,  $J = 9.6$ , H-4'), 3.64 (dd,  $J = 10.4$  and  $6.4$ , H-<sub>1b</sub>), 3.59 (m, 1H, H-5'), 3.49 (dd, 1H,  $J = 9.6$  and  $3.6$ , H-2'), 3.10 (t, 1H,  $J = 9.6$ , H-3'), 3.03 (m, 1H, H-6'<sub>b</sub>), 2.38-2.33 (overlapped, m, 4H, 2 ester  $\alpha\text{-CH}_2$ ), 2.26 (t, 2H,  $J = 7.8$ , amide  $\alpha\text{-CH}_2$ ), 1.68-1.57 (overlapped, 6H,  $3\beta\text{-CH}_2$ ), 1.38-1.20 (overlapped, 72H, 36  $\text{CH}_2$ ), 0.86-0.72 (overlapped, 9H, 3  $\text{CH}_3$ ).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  175.9, 173.6, 173.4, 99.6, 73.3, 72.5, 71.3, 70.2, 70.0, 67.1, 62.3, 39.9, 36.6, 34.5, 34.3, 34.0, 32.1, 29.9-29.3 (aliphatic, overlapped), 25.8, 25.1, 25.0, 24.9, 22.9, 22.8, 14.3 (3 $\text{CH}_3$  overlapped). HRFABMS:  $m/z = 968.8076$   $[\text{M}+\text{H}]^+$ ; calculated for  $\text{C}_{57}\text{H}_{110}\text{NO}_{10}$ ,  $m/z = 968.8131$ ,  $\Delta = -5.5$  ppm.

**1-O-Allyl-2,3,4-tri-O-triethylsilyl-6-benzoyl- $\alpha$ -D-glucopyranoside (8.25).** To a stirred solution of 1-O-allyl- $\alpha$ -D-glucopyranose (2.8 g, 12.7 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (20 mL), collidine (4 mL) was added at  $-40$  °C, then benzoyl chloride (2.2 mL, 15.2 mmol) was added slowly. The reaction mixture was stirred at  $-40$  °C for 3 h and then 1 h at room temperature. Then imidazole (4.9 g, 72 mmol) and chlorotriethylsilane (6.8 mL, 37 mmol) were added and the solution was stirred overnight at room temperature. The reaction mixture was poured into ice water (500 mL) and extracted with EtOAc (4 $\times$ 50 mL), and the combined organic layers were washed with saturated aqueous  $\text{NaHCO}_3$  (3 $\times$ 50 mL)

and brine (2×50 mL), and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on silica gel with 4% EtOAc in hexane gave compound **8.25** (5.5 g, 64.7%) as a colorless syrup. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 8.03 (d, 2H, *J* = 8.0), 7.52 (m, 1H), 7.22-7.18 (m, 2H), 5.95 (m, 1H, CCH=C), 5.30 (dd, 1H, *J* = 17.3 and 1.5, C=CH<sub>2a</sub>), 5.16 (dd, *J* = 10.4 and 1.4, C=CH<sub>2b</sub>), 4.70 (d, 1H, *J* = 3.4, anomeric H), 4.18-4.08 (m, overlapped, 3H), 3.77-3.82 (overlapped, m, 2H), 3.45 (dd, 1H, *J* = 18.6, 7.5), 0.92-0.77 (overlapped, 27H, 9CH<sub>3</sub>), 0.62-0.40 (18H, overlapped, 9SiCH<sub>2</sub>); <sup>13</sup>C NMR: δ 168.9, 134.2, 133.0, 131.2, 130.1, 128.7, 116.8, 98.4, 79.0, 75.8, 73.4, 72.1, 65.3, 7.4-7.1 (overlapped), 5.8-5.3 (overlapped).

**1-*O*-Allyl-2,3,4-tri-*O*-triethylsilyl- $\alpha$ -D-glucopyranoside (8.26).** To a stirred solution of compound **8.25** (5.3 g, 12.2 mmol) in THF (20 mL) at -20 °C, CH<sub>3</sub>MgBr (3M solution in THF) (10 mL, 30 mmol) was added slowly under nitrogen. The reaction mixture was stirred at -20 °C for 2 h and then 1 h at room temperature. The reaction mixture was quenched with water (20 mL) and extracted with EtOAc (50 mL), the organic layer was washed with brine (2×50 mL), and dried over Na<sub>2</sub>SO<sub>4</sub>. After drying *in vacuo* at 0 °C, the residue was used directly for the next step.

**1-*O*-Allyl-2,3,4-tri-*O*-triethylsilyl-6-desoxy-6-iodo- $\alpha$ -glucopyranoside (8.26).** To a stirred solution of **8.25** (1.82 g, 3.2 mmol) in 20 mL of ether/CH<sub>3</sub>CN, 3:1 (v/v) in an ice bath, triphenylphosphine (1.62 g, 6.7 mmol) and imidazole (0.92 g, 13.2 mmol) were added under nitrogen, then iodine (1.63 g, 6.4 mmol) was slowly added. After stirring for 2 h, the reaction mixture was poured into 200 mL of water and extracted with EtOAc (100 mL), the combined organic layers were washed with saturated aqueous NaHCO<sub>3</sub> (3×50 mL) and brine (2×50 mL), and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on

silica gel with 5% EtOAc in hexane gave compound **8.26** (1.94 g, 2.9 mmol, 90%):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  5.95 (m, 1H, CCH=C), 5.34 (dd, 1H,  $J = 17.3$  and  $1.4$  C=CH<sub>2</sub>), 5.21 (dd,  $J = 10.6$ , C=CH<sub>2</sub>), 4.80 (d, 1H,  $J = 3.4$ , anomeric H), 3.96 (dd, 1H,  $J = 13.6$  and  $8.4$ ), 3.77-3.51 (overlapped, m, 4H), 3.28 (dd, 1H,  $J = 9.6$  and  $9.4$ ). 0.91-0.78 (27H, overlapped, 9CH<sub>3</sub>), 0.60-0.35 (18H, overlapped, 9SiCH<sub>2</sub>);  $^{13}\text{C}$  NMR:  $\delta$  134.2, 116.9, 98.4(C-1), 77.5, 76.8, 74.8, 72.0, 68.3, 8.0 (C-6), 7.2-6.9 (overlapped), 5.9-5.7 (overlapped).

**1-O-Allyl-2,3,4-tri-O-triethylsilyl-6-desoxy-6-amino- $\alpha$ -glucopyranosyl)-9-fluorenyl-methyl-carbamate (8.28).** Compound **8.26** (1.6 g, 2.4 mmol) and sodium azide (98 mg, 1.52 mmol) were dissolved in 6 mL of toluene: DMF, 3:1 (v/v), and the mixture was stirred at 50 °C for 12 h, then the solvent was diluted with EtOAc (100 mL) and washed with water (3×100 mL) and brine (2×50 mL), and dried over Na<sub>2</sub>SO<sub>4</sub>. After removal of the solvent, the residue was dried *in vacuo* at 0 °C and redissolved in 20 mL anhydrous THF at 0 °C, and lithium aluminum hydride (80 mg) was added and the suspension was stirred in an ice bath for 1 h. The reaction mixture was quenched with water (20 mL) and extracted with diethylether (2×20 mL). The combined organic layers were evaporated and the syrupy residue was dissolved in dioxane (20 mL) and treated with saturated aqueous NaHCO<sub>3</sub> (5 mL) and 2M aqueous Na<sub>2</sub>CO<sub>3</sub> (5 mL) at 0 °C, followed by 9-fluorenylmethyl-chlorocarbonate (238 mg). The reaction mixture was stirred in an ice bath for 6 h, and was then diluted with water (100 mL) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (3×20 mL). The extract was concentrated and subjected to column chromatography on silica gel with 7% EtOAc in hexane to give compound **8.28** (720 mg, 58%) as a white wax-like

solid.  $[\alpha]_D^{25} = +33.6$  ( $c = 0.4$ ,  $\text{CHCl}_3$ );  $^1\text{H NMR}(\text{CDCl}_3)$ :  $\delta$  7.78 (d, 2H,  $J = 8.5$ ), 7.62 (d, 2H,  $J = 8.5$ ), 7.40 (t, 2H,  $J = 8.5$ ), 7.33 (t, 2H,  $J = 8.5$ ), 5.95 (m, 1H, CCH=C), 5.31 (dd, 1H,  $J = 17.3$  and 1.6 C=CH<sub>2a</sub>), 5.18 (d, 1H, C=CH<sub>2b</sub>,  $J = 10.6$  and 1.6), 5.12 (br, 1H, NH), 4.78 (d, 1H,  $J = 3.6$ , anomeric), 4.44-4.43 (m, 2H), 4.25 (t, 1H,  $J = 6.8$ ), 4.14 (dd,  $J = 12.8$  and 5.2), 3.96 (dd,  $J = 12.8$  and 5.6), 3.90 (t, 1H,  $J = 8.8$ , H-4'), 3.78 (m, 1H, H-6<sub>a</sub>), 3.68 (m, 1H), 3.54 (dd, 1H,  $J = 9.2$  and 3.6, H-2'), 3.32 (t, 1H,  $J = 8.8$ ), 3.11 (m, 1H, H-6<sub>b</sub>), 1.07-0.95 (27H, overlapped, 9CH<sub>3</sub>), 0.80-0.65 (18H, overlapped, 9SiCH<sub>2</sub>);  $^{13}\text{C NMR}$ :  $\delta$  156.5, 144.2, 141.5, 134.4, 127.9, 127.2, 125.3, 120.2, 117.1, 98.7 (C-1), 75.1, 74.8, 74.4, 68.7, 47.5, 43.1, 7.4-7.1 (overlapped), 5.8-5.3 (overlapped); HRFABMS:  $m/z = 783.4366$ ; calculated for  $\text{C}_{42}\text{H}_{69}\text{NO}_7\text{Si}_3$   $m/z = 783.4381$  ( $\Delta = -1.9\text{ppm}$ ).

**1-O-[2,3,4-tri-O-Triethylsilyl-6-desoxy-6-(9-fluorenylmethylcarbamino)-glucopyranosyl]-glycerol (8.29).** OsO<sub>4</sub> (14 mg, 0.05 mmol) was dissolved in 3.5 mL of water and 1.5 mL *tert*-butyl alcohol. This mixture was slowly added to a stirred solution of **8.28** (550 mg, 0.71 mmol) in *tert*-butyl alcohol (4.5 mL), and then *tert*-butylhydroperoxide (0.3 mL, 1.42 mmol) was added. The reaction mixture was stirred vigorously at room temperature for 1 day before quenching with 3M aqueous sodium sulfite (10 mL). The resulting mixture was diluted with 20 mL of water and extracted with EtOAc (3×20 mL), and the combined organic layers were washed with water (3×50 mL) and brine (2×50 mL), and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on Silica gel with 35% EtOAc in hexane yielded compound **8.29** (380 mg, 68%) as a mixture of epimers at C-2.  $^1\text{H NMR}(\text{CDCl}_3)$ :  $\delta$  7.78 (d, 2H,  $J = 8.5$ ), 7.62 (d, 2H,  $J = 8.5$ ), 7.40 (t, 2H,  $J = 8.5$ ), 7.33 (t, 2H,  $J = 8.5$ ), 5.31 (br, 1H, NH), 4.72-4.68 (m, 1H), 4.43-4.36 (overlapped, m, 2H), 4.24-4.20

(m, 1H), 3.88-3.62 (overlapped, 8H), 3.56-3.41 (overlapped, 2H), 3.28 (1H, m), 3.18-3.04 (overlapped, 2H), 1.07-0.95 (27H, overlapped, 9CH<sub>3</sub>), 0.80-0.65 (18H, overlapped, 9SiCH<sub>2</sub>). <sup>13</sup>C NMR: δ 156.5, 144.2, 141.5, 134.4, 127.9, 127.2, 125.2, 120.2, 117.0, 98.7, 98.6, 75.1, 74.9, 74.4, 71.6, 68.7, 66.9, 60.5, 47.5, 43.0, 29.9, 21.1, 14.3, 7.4-7.1 (overlapped), 5.8-5.3 (overlapped). HRFABMS: *m/z* = 818.4486 (M+H)<sup>+</sup>; calculated for C<sub>42</sub>H<sub>69</sub>NO<sub>7</sub>Si<sub>3</sub>, *m/z* = 818.4515, Δ = -3.8 ppm.

**1,2-di-*O*-Palmitoyl-3-*O*-[2,3,4-tri-*O*-triethylsilyl-6-desoxy-6-(9-fluorenylmethyl-carbamino)-glucopyranosyl]-glycerol (8.30a).** To a solution of palmitic acid (67 mg, 0.26 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (2 mL) was added EDCI (49 mg, 0.254 mmol). DMAP (1 mg) was then added and the solution was stirred for 30 min before compound **8.29** (73 mg, 0.09 mmol) was added. The reaction mixture was stirred overnight and was then diluted with EtOAc and washed with saturated aqueous NaHCO<sub>3</sub> and water and brine, and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on silica gel with 15% EtOAc in hexane gave compound **8.30a** (107 mg, 0.084 mmol, 93%) as a mixture of epimers at C-2. <sup>1</sup>H-NMR. δ 7.72 (d, 2H, *J* = 8.4), 7.58 (d, 2H, *J* = 8.4), 7.34 (t, 2H, *J* = 8.4), 7.28 (t, 2H, *J* = 8.4), 5.19 (m, 1H), 4.69 (1H, m), 4.40-4.34 (m, 2H), 4.32 (dd, *J* = 12.0, 3.2), δ 4.27 (dd, *J* = 11.8, 3.2), 4.23 (t, 1H, *J* = 6.8), 4.11 (m, 1H), 3.84-3.76 (m, 2H), 3.67 (m, 1H), 3.58-3.44 (overlapped, m, 4H), 3.28 (t, 1H, *J* = 8.4), 3.16 (m, 1H), 2.26 (t, 4H, *J* = 8.0), 1.58 (m, 4H, overlapped), 1.36-1.20 (48H, 24CH<sub>2</sub>), 1.02-0.95 (27H, overlapped, 9CH<sub>3</sub>), 0.87 (6H, 2CH<sub>3</sub>), 0.74-0.66 (18H, overlapped, 9SiCH<sub>2</sub>). <sup>13</sup>C NMR: δ 173.5, 173.4, 173.2, 173.1, 156.4, 144.1, 141.4, 127.7, 127.1, 125.2, 120.0, 99.6, 99.5, 74.5, 74.4, 73.9, 71.8, 69.8, 66.9, 65.5, 62.5, 47.3, 42.7, 32.0, 29.8, 29.5-29.2 (overlapped), 24.9, 22.8, 14.2, 7.4-7.1

(overlapped), 5.8-5.3 (overlapped). HRFABMS: observed  $m/z = 1316.8574$   $[M+Na]^+$ ; calculated for  $C_{74}H_{131}NO_{11}Si_3$   $m/z = 1316.8627$ ,  $\Delta = -4.0$  ppm.

**1,2-di-*O*-Linolenoyl-3-*O*-[2,3,4-tri-*O*-triethylsilyl-6-desoxy-6-(9-fluorenylmethyl-carbamino)-glucopyranosyl]-glycerol (8.30b).** To a solution of linolenic acid (214 mg, 0.7 mmol) in 5 mL  $CH_2Cl_2$  was added EDCI (148 mg, 0.77 mmol). After 15 min stirring, DMAP (2 mg, cat.) was added and keep stirring for 30 min before compound **8.29** (210 mg, 0.256 mmol) was added. The reaction mixture was stirred overnight workup. Column chromatography on silica gel with 15% EtOAc in hexane gave compound (310 mg, 0.23 mmol, 92%) as a mixture of epimers at C-2.  $^1H$  NMR  $\delta$  7.72 (d, 2H,  $J = 8.4$ ), 7.58 (d, 2H,  $J = 8.4$ ), 7.34 (t, 2H,  $J = 8.4$ ), 7.28 (t, 2H,  $J = 8.4$ ), 5.37-5.28 (m, 12H, overlapped, olefine protons), 5.20 (m, 1H), 4.67 (d, 1H,  $J = 3.2$ ), 4.38-4.31 (m, 3H, overlapped), 4.20 (t, 1H,  $J = 6.8$ ), 4.10(m, 1H), 3.80-3.72 (m, 2H) , 3.60 (m, 1H), 3.56-3.44 (overlapped, m, 4H), 3.26 (t, 1H,  $J = 8.4$ ), 3.14 (m, 1H), 2.78-2.70 (overlapped, 8H), 2.26-2.20 (m ,4H), 2.10-1.94 (overlapped, 12H), 1.56 (m, 4H), 1.31-1.20 (overlapped, 20H), 1.00-0.92 (overlapped, 27H, 9 $CH_3$ ), 0.70-0.61 (overlapped, 18H, 9 $SiCH_2$ );  $^{13}C$  NMR:  $\delta$  170.6, 170.5, 170.3, 170.2, 156.5, 144.0, 141.4, 132.2, 130.5, 130.4, 128.5, 128.3, 128.0, 127.9, 127.3, 99.6, 99.5, 73.4, 72.4, 71.3, 70.2, 70.1, 66.9, 62.6, 40.3, 34.4, 34.3, 32.1, 29.9-29.3 (aliphatic, overlapped), 27.4, 26.4, 25.8, 25.7, 25.1, 25.0, 23.1, 22.9, 20.8, 14.5, 14.3, 7.4-7.1 (overlapped), 5.8-5.3 (overlapped); HRFABMS:  $m/z = 1360.8568$   $(M+Na)^+$ ; calculated for  $C_{78}H_{127}NO_{11}Si_3Na$ ,  $m/z = 1360.8615$  ( $\Delta = -3.4$  ppm).

**Enzymatic resolution of compound 8.30a.** Compound **8.30a** (84 mg, 0.065 mmol) was dissolved in acetonitrile (1 mL) and added to a suspension of lipase (from *Pseudomonas*

sp. dispersed on porous silicate) (100 mg) in 2.5 mL of phosphate buffer (0.5M NaH<sub>2</sub>PO<sub>4</sub>: Na<sub>2</sub>HPO<sub>4</sub> = 1:1, pH = 7.2). The colloidal mixture was stirred at room temperature and the reaction was monitored by TLC. After two weeks the reaction mixture was filtered, extracted with EtOAc and purified by preparative TLC on silica gel. Development with 15% EtOAc in hexane gave compound **8.31** (41 mg, 0.032 mmol).  $[\alpha]_D^{23} = +44.5$  ( $c = 0.2$  CHCl<sub>3</sub>). <sup>1</sup>H-NMR.  $\delta$  7.72 (d, 2H,  $J = 8.4$ ), 7.58 (d, 2H,  $J = 8.4$ ), 7.34 (t, 2H,  $J = 8.4$ ), 7.28 (t, 2H,  $J = 8.4$ ), 5.19 (m, 1H), 4.69 (1H, m), 4.40-4.34 (m, 2H), 4.23 (t, 1H,  $J = 6.8$ ), 4.11 (m, 1H), 3.84-3.76 (m, 2H), 3.67 (m, 1H), 3.58-3.44 (overlapped, m, 4H), 3.28 (t, 1H,  $J = 8.4$ ), 3.16 (m, 1H), 2.26 (t, 4H,  $J = 8.0$ ), 1.58 (m, 4H, overlapped), 1.36-1.20 (48H, 24CH<sub>2</sub>), 1.02-0.95 (27H, overlapped, 9CH<sub>3</sub>), 0.87 (6H, 2CH<sub>3</sub>), 0.74-0.66 (18H, overlapped, 9SiCH<sub>2</sub>). <sup>13</sup>C NMR:  $\delta$  173.4, 173.1, 156.4, 144.1, 141.4, 127.7, 127.1, 125.2, 120.0, 99.1, 74.5, 74.4, 73.9, 71.8, 69.8, 66.9, 65.5, 62.5, 47.3, 42.7, 32.0, 29.8, 29.5-29.2 (overlapped), 24.9, 22.8, 14.2, 7.4-7.1 (overlapped), 5.8-5.3 (overlapped).

**1,2-di-O-Palmitoyl-3-O-[2,3,4-tri-O-triethylsilyl-6-desoxy-6-palmitoylamido-glucopyranosyl]-glycerol (8.33)**. To a solution of compound **8.31** (44 mg, 0.032 mmol) in CHCl<sub>3</sub> (1 mL), piperidine (80  $\mu$ L) was added and the mixture was stirred for 1 h. The solvents were then dried under dry nitrogen and the residue was redissolved in CH<sub>2</sub>Cl<sub>2</sub> (2 mL), then triethylamine (60  $\mu$ L, 0.23 mmol) and palmitoyl chloride (33  $\mu$ L, 0.11 mmol) were added and the mixture stirred for 2 h. The mixture was then diluted with EtOAc and washed with saturated aqueous NaHCO<sub>3</sub>, water and brine, and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on silica gel with 10% EtOAc in hexane gave compound **8.33** (24 mg, 0.018 mmol, 56%) as a white solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>): 5.73 (m, 1H, N-H), 5.15

(m, 1H, H-2), 4.68 (d, 1H,  $J = 3.6$ , H-1', anomeric), 4.34 (dd, 1H,  $J = 12.0$  and  $3.6$ , H-1<sub>a</sub>), 4.18 (dd, 1H,  $J = 12.0$  and  $3.6$ , H-1<sub>b</sub>), 3.79-3.70 (overlapped, m, 3H), 3.58-3.47 (overlapped, m, 3H), 3.24 (t, 1H,  $J = 9.6$ , H-3'), 3.14 (m, 1H, H-6'<sub>b</sub>), 2.32-2.28 (overlapped, m, 4H, 2 ester  $\alpha$ -CH<sub>2</sub>), 2.20 (t, 2H,  $J = 7.8$ , amide  $\alpha$ -CH<sub>2</sub>), 1.64-1.52 (overlapped, 6H, 3 $\beta$ -CH<sub>2</sub>), 1.38-1.20 (overlapped, 72H, 36CH<sub>2</sub>), 0.98-0.97 (overlapped, 27H, 9CH<sub>3</sub>), 0.86-0.78 (overlapped, 9H, 3 CH<sub>3</sub>), 0.70-0.62 (overlapped, 18H, 9CH<sub>2</sub>Si); <sup>13</sup>C NMR (CDCl<sub>3</sub>): 173.5, 173.4, 173.2, 99.3, 74.7, 74.5, 73.9, 72.0, 70.5, 70.3, 69.9, 39.9, 36.6, 34.5, 34.3, 34.0, 32.1, 29.9-29.3 (aliphatic, overlapped), 25.8, 25.1, 25.0, 24.9, 22.9, 22.8, 14.3 (3-CH<sub>3</sub> overlapped). 7.4-7.1 (overlapped), 5.8-5.3 (overlapped); HRFABMS:  $m/z = 1317.0762$  [M+Li]<sup>+</sup>; calculated for C<sub>75</sub>H<sub>151</sub>O<sub>10</sub>NSi<sub>3</sub>Li  $m/z = 1317.0807$  ( $\Delta = -3.4$  ppm)

**1,2-di-*O*-Linolenoyl-3-*O*-[2,3,4-tri-*O*-triethylsilyl-6-desoxy-6-acetylamido-glucopyranosyl]-glycerol (8.32).** To a solution of compound **8.30b** (124 mg, 0.1 mmol) in CHCl<sub>3</sub> (5 mL), piperidine (200  $\mu$ l) was added and the mixture was stirred for 1 h. The solvent was then dried under a flow of dry nitrogen and the residue was dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (3 mL). Triethylamine (45  $\mu$ l, 0.33 mmol) and acetic anhydride (20  $\mu$ l, 0.21 mol) were added and the mixture was stirred at room temperature for 2 h. The mixture was then diluted with EtOAc and washed with saturated aqueous NaHCO<sub>3</sub>, water and brine, and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on Silica gel with 10% EtOAc in hexane gave compound **8.32** (77 mg, 66%, 2 steps) as a white solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>): 5.41-5.28 (m, 12H, overlapped, olefine protons), 5.21 (m, 1H), 4.81 (m, 1H), 4.34-4.18 (overlapped, m, 3H), 3.82-3.70 (overlapped, m, 3H), 3.64-3.47 (overlapped, m,

3H), 3.18 (m, 1H), 2.78-2.70 (overlapped, 8H), 2.26-2.20 (m, 4H), 2.10-1.94 (overlapped, 12H), 1.56 (m, 4H), 1.31-1.20 (overlapped, 20H), 1.00-0.92 (overlapped, 27H, 9CH<sub>3</sub>), 0.70-0.61 (overlapped, 18H, 9SiCH<sub>2</sub>); <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 173.7, 173.6, 172.8, 132.2, 130.5, 130.4, 128.5, 128.3, 128.0, 127.9, 127.3, 99.5, 73.4, 72.4, 71.3, 70.2, 70.1, 66.9, 62.6, 40.3, 34.4, 34.3, 32.1, 29.9-29.3 (aliphatic, overlapped), 27.4, 26.4, 25.8, 25.7, 25.1, 25.0, 23.1, 22.9, 20.8, 14.5, 14.3, 7.4-7.1 (overlapped), 5.8-5.3 (overlapped); HRFABMS: *m/z* = 1180.8076 [M+Na]<sup>+</sup>; calculated for C<sub>65</sub>H<sub>119</sub>O<sub>10</sub>NSi<sub>3</sub>Na, *m/z* = 1180.8039 (Δ = 3.1 ppm).

**1,2-di-*O*-Linolenoyl-3-*O*-[2,3,4-tri-*O*-triethylsilyl-6-desoxy-6-{3-(4-*tert*-butyl-dimethylsiloxy)phenylpropionoylamido}-glucopyranosyl]-glycerol (8.36).** To a solution of compound **8.30b** (110 mg, 0.086 mmol) in CHCl<sub>3</sub> (3 mL), piperidine (120 μl) was added and the mixture was stirred for 1 h. The solvent was then dried under a dry nitrogen flow and the residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (1.5 mL). This solution was added to a mixture of 3-(4-*tert*-butyldimethylsiloxyphenyl)propionic acid (56 mg, 0.20 mmol) and EDCI (49 mg, 0.25 mmol) with DMAP (1 mg) in CH<sub>2</sub>Cl<sub>2</sub> (1 mL). After stirring for 5 h, the mixture was diluted with EtOAc and washed with saturated aqueous NaHCO<sub>3</sub>, water and brine, and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on silica gel with 10% EtOAc in hexane gave compound **8.36** (83 mg, 0.067 mmol, 75%, 2 steps) as a white solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 5.73 (m, 1H, N-H), 5.40-5.27 (m, 12H, overlapped, olefinic protons), 5.15 (m, 1H, H-2), 4.49 (m, 1H), 4.18 (m, 1H), 4.02-3.86 (overlapped, m, 2H), 3.64-3.51 (overlapped, m, 3H), 3.38-3.27 (overlapped, m, 3H), 3.08 (t, 1H, *J* = 9.6), 3.14 (m, 1H), 2.30 (m, 2H), 2.16-2.08 (overlapped, m, 4H), 1.96-1.84 (overlapped, 10H), 1.44-1.36 (overlapped, 4H), 1.38-1.20 (overlapped, 72H, 36 CH<sub>2</sub>), 0.88-0.76 (overlapped,

48H), 0.57-0.48 (overlapped, 18H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 173.5, 173.4, 173.2, 132.2, 130.5, 130.4, 128.5, 128.3, 128.0, 127.9, 127.3, 99.3, 74.7, 74.5, 73.9, 72.0, 70.5, 70.3, 69.9, 39.9, 36.6, 34.5, 34.3, 34.0, 32.1, 29.9-29.3 (aliphatic, overlapped), 25.8, 25.1, 25.0, 24.9, 22.9, 22.8, 14.4, 14.3. 7.4-7.1 (overlapped), 5.8-5.3 (overlapped). HRFABMS:  $m/z = 1378.9431$   $[\text{M}+\text{H}]^+$ ; calculated for  $\text{C}_{78}\text{H}_{139}\text{NO}_{11}\text{Si}_4$   $m/z = 1378.9504$  ( $\Delta = -5.2$  ppm).

**1,2-di-*O*-Linolenoyl-3-*O*-[6-desoxy-6-acetylamido-glucoopyranosyl]-glycerol (8.37).**

To a solution of compound **8.35** (76 mg, 0.048 mmol) in THF (8 mL) was added triethylamine-trihydrofluoride (0.15 mL, large excess) at 0 °C and the solution was allowed to warm to room temperature over 1 h and then stirred overnight. The reaction mixture was diluted with EtOAc and washed with saturated aqueous  $\text{NaHCO}_3$ . The organic layer was washed with water and brine, dried over  $\text{Na}_2\text{SO}_4$ , and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with 10% MeOH in  $\text{CHCl}_3$  to give compound **8.37** (48 mg, 0.057 mmol, 72%, 2 steps) as a white solid.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 6.32 (m, 1H, N-H), 5.37-5.24 (m, 12H, overlapped, olefinic protons), 5.17 (m, 1H, H-2), 4.76 (m, 1H), 4.31 (m, 1H), 4.12 (m, 1H), 3.74-3.42 (overlapped, m, 7H), 3.38-3.27 (overlapped, m, 3H), 3.14 (m, 1H), 3.08 (t, 1H,  $J = 9.6$ ), 2.27 (m, 4H), 2.16-2.08 (overlapped, m, 4H), 2.06-1.97 (overlapped, 10H), 1.54 (m, 4H), 1.38-1.20 (overlapped, 72H, 36  $\text{CH}_2$ ), 0.98 (6H, t,  $J = 7.8$ , 2 $\text{CH}_3$ ).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 173.7, 173.6, 172.8, 99.5, 132.1, 130.5, 130.4, 128.5, 128.4, 128.0, 127.9, 127.3, 99.5, 73.4, 72.4, 71.3, 70.2, 66.9, 62.6, 40.3, 34.5, 34.3, 34.0, 32.1, 29.9-29.3 (aliphatic, overlapped), 25.8, 25.1, 25.0, 24.9, 22.9, 23.1, 14.4, 14.3. HRFABMS  $m/z = 838.5439$   $[\text{M}+\text{Na}]^+$ ; calculated for  $\text{C}_{47}\text{H}_{77}\text{NO}_{10}$ ,  $m/z = 838.5445$  ( $\Delta = -0.8$  ppm).

**1,2-di-*O*-Linolenoyl-3-*O*-[6-desoxy-6-(3-[4-hydroxyphenyl]-propionoylamido-glucopyranosyl)]-glycerol (8.38).** To a solution of **8.36** (81 mg, 0.063 mmol) in THF (10 mL) of was added triethylamine-trihydrofluoride (0.23 mL, large excess) and the solution was allowed to warm to room temperature in 1 h and stirred overnight. The reaction mixture was diluted with EtOAc (40 mL) and washed with saturated aqueous NaHCO<sub>3</sub>. The organic layer was washed with water and brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with 8% MeOH in CHCl<sub>3</sub> to give compound **8.38** (39 mg, 0.042 mmol, 66%, 2 steps) as a white solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>): 5.73 (m, 1H, N-H), 5.15 (m, 1H, H-2), 4.49 (m, 1H), 4.18 (m, 1H), 4.02-3.86 (overlapped, m, 2H), 3.64-3.51 (overlapped, m, 3H), 3.38-3.27 (overlapped, m, 3H), 3.08 (t, 1H, *J* = 9.6), 3.14 (m, 1H), 2.30 (m, 2H), 2.16-2.08 (overlapped, m, 4H), 1.96-1.84 (overlapped, 10H), 1.44-1.36 (overlapped, 4H), 1.38-1.20 (overlapped, 72H, 36 CH<sub>2</sub>), 0.98 (6H, t, 2CH<sub>3</sub>); <sup>13</sup>C NMR (CDCl<sub>3</sub>): 173.5, 173.4, 173.2, 132.2, 130.5, 130.4, 128.5, 128.3, 128.0, 127.9, 127.3, 99.3, 74.7, 74.5, 73.9, 72.0, 70.5, 70.3, 69.9, 39.9, 36.6, 34.5, 34.3, 34.0, 32.1, 29.9-29.3 (aliphatic, overlapped), 25.8, 25.1, 25.0, 24.9, 22.9, 22.8, 14.4, 14.3. HRFABMS *m/z* = 944.5838 [M+Na]<sup>+</sup>; calculated for C<sub>54</sub>H<sub>83</sub>NO<sub>10</sub> 944.5864 ( $\Delta$  = -2.7ppm).

## Chapter IX. Synthesis of Isotopically Labeled Paclitaxel Analogs for REDOR NMR Studies.

### IX.1 Introduction.

#### IX.1.1 The History of Paclitaxel.

Paclitaxel (**9.1**) is one of the most important natural anticancer agents that has been introduced over the last twenty-five years. It has been in clinical use for about thirteen years, and has shown great promise in treating breast cancer and ovarian cancer (Figure 9-1). Paclitaxel was first discovered in 1963 from the bark of a Pacific Yew (*Taxus brevifolia*; Taxaceae) which showed significant cytotoxic and antileukemic activity. The complete structure of paclitaxel (which was named taxol at that time) was announced by Wall and Wani in 1971.<sup>129</sup> Paclitaxel showed strong activity against various cancers in mice, including the B16 melanoma, P1534 leukemia, and the MX-1 human mammary tumor xenograft. These excellent activities convinced the NCI to sponsor full-scale pre-clinical development of paclitaxel as an anticancer agent in 1977. Paclitaxel entered Phase I clinical trials in 1983,<sup>130</sup> and Phase II clinical trials in 1985.<sup>131</sup> Clinical reports indicated good activity against ovarian and breast cancer in 1989 and 1991 respectively.

Because of its effectiveness and the relatively high response rate shown in the clinical trials, paclitaxel received Food and Drug Administration (FDA) approval in 1992 for the treatment of advanced ovarian cancer, and for metastatic breast cancer in 1994.

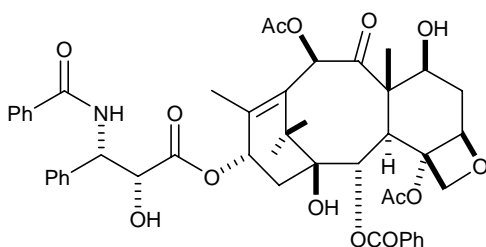
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<sup>129</sup> Wani, M.C.; Taylor, H.L.; Wall, M.E.; Coggon, P.; McPhail, A.T., Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*, *J. Am. Chem. Soc.* **1971**, *93*, 2325–2327.

<sup>130</sup> McGuire, W. P.; Rowinsky, E. K.; Rosenshein, N. B.; Grumbine, F. C.; Ettinger, D. S.; Armstrong, D. K.; Donehower, R. C., Taxol: a unique antineoplastic agent with significant activity in advanced ovarian epithelial neoplasms, *Ann. Intern. Med.*, **1989**, *111*, 273-279.

<sup>131</sup> Holmes, F. A.; Walters, R. S.; Theriault, R. L.; Forman, A. D.; Newton, L. K.; Raber, M. N.; Buzdar, A. U.; Frye, D. K.; Hortobagyi, G. N. Phase II trial of taxol, an active drug in the treatment of metastatic breast cancer. *J. Natl. Cancer Inst.*, **1991**, *83*, 1797-1805.

Currently paclitaxel is being tested against a series of different cancers. Paclitaxel is among the best-selling anticancer drugs in drug history with an estimated sale of one billion US dollars in 2000.



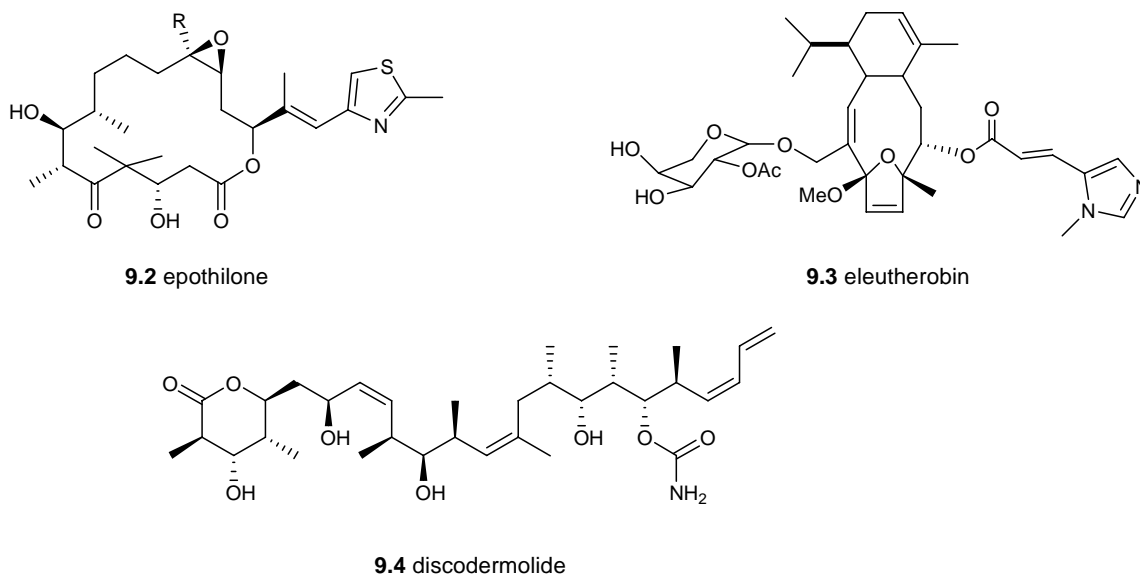
**Figure 9-1** Structure of Paclitaxel.

### IX.1.2 The Tubulin Stabilization Mechanism of Paclitaxel.

In 1979 Horwitz and co-workers discovered the unique mechanism of paclitaxel's activity as a tubulin polymerization stabilizer.<sup>132</sup> They found that paclitaxel promotes polymerization of  $\alpha/\beta$  tubulin subunits to microtubules and stabilizes them irreversibly, thus leading to cell cycle arrest. This new and unique mechanism of action initiated further research in the field of microtubule stabilizing natural products, which has led to the discovery of several new potential anticancer agents with similar mechanisms of action such as epothilone **9.2**, eleutherobin **9.4** and discodermolide **9.5** (Figure 9-2).

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<sup>132</sup> Schiff, P. B.; Fant, J.; Horwitz, S. B., Promotion of microtubule assembly *in vitro* by taxol, *Nature* **1979**, 277, 665–667.



**Figure 9-2** Microtubule stabilizing agents from natural products.

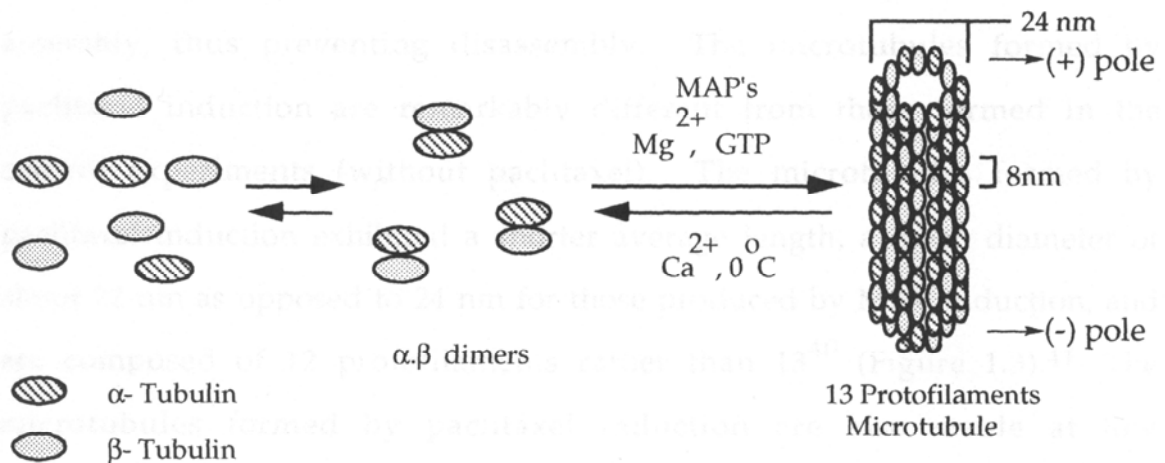
In the mitosis stage of a normal cell, microtubules are key components for the formation of the mitotic spindle, which is essential for separation of the duplicated chromosomes. Microtubules are formed by polymerization of two structurally similar proteins,  $\alpha$ -tubulin and  $\beta$ -tubulin, with 440 and 437 amino acid residues respectively. The molecular weights of these proteins are about 50,000,<sup>133</sup> and they have about 35-40% similarity.<sup>134</sup> When tubulins start to polymerize, one molecule of  $\alpha$ -tubulin and one molecule of  $\beta$ -tubulin form a heterodimer first. The heterodimer dissociation constant to  $\alpha$  and  $\beta$ -tubulin monomers is about  $10^{-6}$  mol/L.<sup>135</sup> Then two energy-rich molecules of guanosine 5'-triphosphate (GTP) bind to the heterodimer with one GTP on the  $\alpha$ -tubulin part and the other one on the  $\beta$ -tubulin part. Then tubulin heterodimers associate with each other to form head-to-tail stacked repeating polymers (8 nm interval) which result

<sup>133</sup> Sullivan, K. F. Structure and utilization of tubulin isotypes. *Annu. Rev. Cell Biol.*, **1988**, *4*, 687-716.

<sup>134</sup> Little, M.; Seehaus, T., Comparative analysis of tubulin sequences. *Comp. Biochem. Physiol. B*, **1988**, *90*, 655-670.

<sup>135</sup> Bershadsky, A. D.; Vasiliev, J. M. In *The Cytoskeleton*, Plenum, New York, **1989**, pp.100-107.

in longitudinal protofilaments. A normal microtubule with a diameter of about 24 nm is formed by 13 protofilaments. The filament structure is surrounded by heterogeneous microtubule-associated proteins (MAPs) to produce biologically active microtubules.<sup>136</sup> At the stable equilibrium stage, both ends of the microtubule are at equilibrium with the same loss and gain rate of tubulin subunits. When microtubules start to grow or decompose, the relative rates of gain or loss of tubulins at these two ends are quite different, which gives the microtubules a growing polarity. The fast growing end is marked as the (+) pole and the relatively slower growing end is thus the (-) pole (Figure 9-3).<sup>137</sup>



**Figure 9-3** The equilibrium of tubulins and microtubules<sup>137</sup>

Antimitotic agents such as the vinca alkaloids and epipodophyllotoxins serve as anticancer drugs by depolymerization of tubulin, because they inhibit microtubule formation when they bind to tubulins. Paclitaxel, on the contrary, was found to be a

<sup>136</sup> Olmsted, J. B. Non-motor microtubule-associated proteins. *Curr. Opin. Cell Biol.*, **1991**, 3, 52-58.

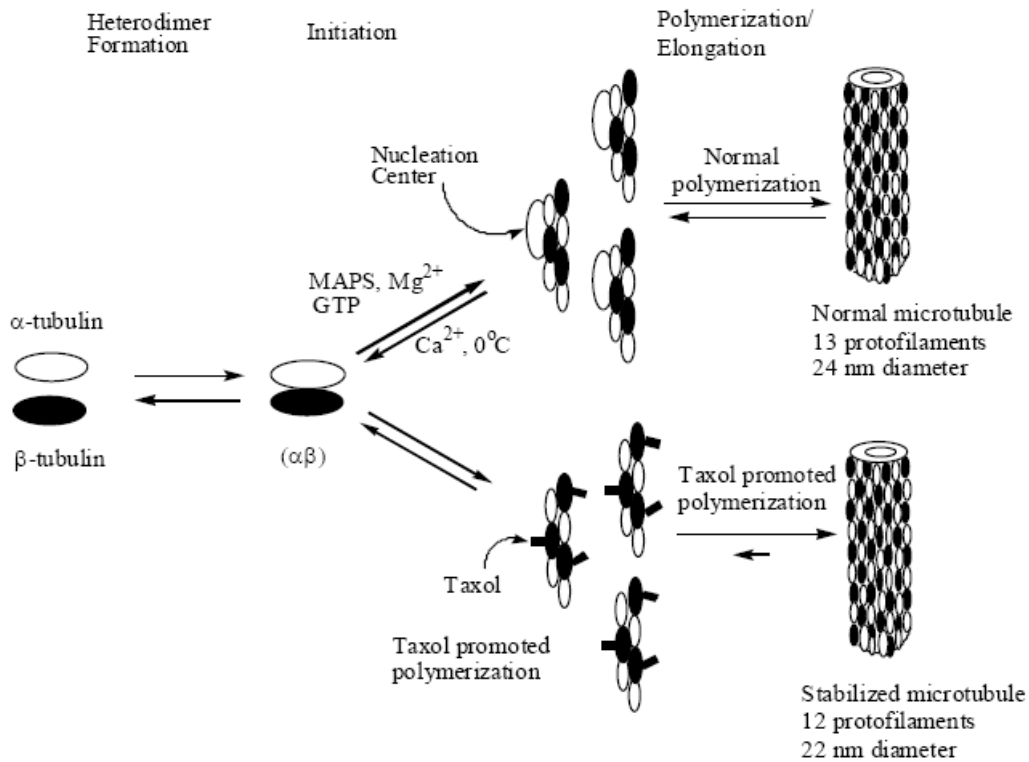
<sup>137</sup> Haimo, L. T. Dynein decoration of microtubules--determination of polarity. *Methods Cell Biol.*, **1982**, 24, 189-206.

promoter of microtubule polymerization.<sup>131</sup> It binds to tubulins and converts them into microtubules irreversibly. Paclitaxel decreases both the critical concentration of tubulin necessary for polymerization and also the induction time for polymerization, and it does this either in the presence or absence of GTP, MAPs, and magnesium ions. The microtubules formed by paclitaxel induction are thinner than normal microtubules with a mean diameter of 22 nm rather than 24 nm, and they are composed of 12 protofilaments instead of the usual 13 protofilaments.<sup>138 139</sup> (Figure 9-4) Furthermore, paclitaxel-induced microtubules are much more stable than normal microtubules and do not depolymerize under normal conditions. Thus, paclitaxel can kill fast proliferating cells by preventing mitosis and ultimately sending the cells into apoptosis.

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<sup>138</sup> Andreu, J. M.; Bordas, J. F.; de Ancos, J. G.; Gil, R.; Medrano, F. J.; Nogales, E.; Pantos, E.; Towns-Andrews, E., Low resolution structure of microtubules in solution. Synchrotron X-ray scattering and electron microscopy of taxol-induced microtubules assembled from purified tubulin in comparison with glycerol and MAP-induced microtubules. *J. Mol. Biol.*, **1992**, 226, 169-184.

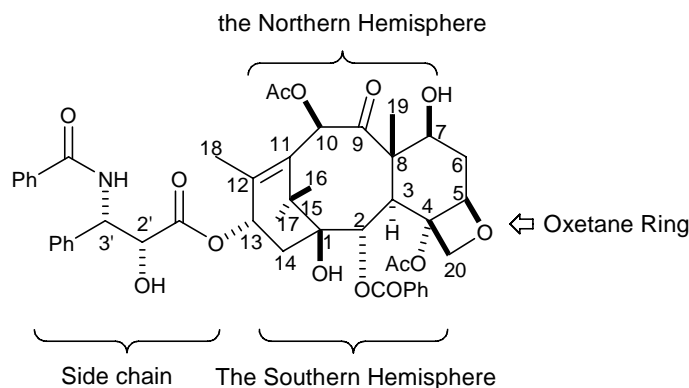
<sup>139</sup> Nicolaou, K. C.; Dai, W. M.; Guy, R. K., Chemistry and Biology of Taxol, *Angew. Chem. Int. Ed. Engl.*, **1994**, 33, 15-44



**Figure 9-4** Paclitaxel affected equilibrium between tubulin and microtubules<sup>139</sup>

### IX.1.3 Structure Activity Relationships (SAR) of Paclitaxel Analogs

In the past 30 years, extensive studies have been carried out on the SAR of paclitaxel and large numbers of analogs have been synthesized. Conventionally the molecular structure of paclitaxel is divided into three regions, the side chain, the northern hemisphere and the southern hemisphere (Figure 9-5).



**Figure 9-5** Conventions used for paclitaxel

The side chain of paclitaxel at the C-13 position is essential for its activity. A free hydroxyl group at C-2' or a hydrolyzable ester linkage is required.<sup>140-142</sup> The C-3' phenyl group is also important. Substitution of the 3'- phenyl group by small alkyl groups such as the methyl group causes a significant loss in bioactivity,<sup>143</sup> but some other substituted phenyl compounds were found to have a similar but slightly decreased activity.<sup>144</sup> Some larger alkyl groups such as isobutyl or isobutenyl give analogs with improved activity.<sup>145</sup> The *N*-acyl group is also required for activity.

The southern hemisphere of paclitaxel's structure turns out to be a very important region for its tubulin binding activities. This part contains C-1 hydroxy, C-2 benzoate,

<sup>140</sup> Denis, J. N.; Greene, A. E.; Serra, A. A.; Luche, M. J., An efficient, enantioselective synthesis of the taxol side chain, *J. Org. Chem.*, **1986**, *51*, 46-50.

<sup>141</sup> Kant, J.; Huang, S.; Wong, H.; Fairchild, C.; Vyas, D.; Farina, V., Studies toward structure-activity relationships of Taxol: synthesis and cytotoxicity of Taxol analogs with c-2' modified phenylisoserine side chains. *Bioorg. Med. Chem. Lett.*, **1993**, *3*, 2471-2474.

<sup>142</sup> Magri, N. F.; Kingston, D. G. I. Modified taxols. 2. Oxidation products of taxol. *J. Nat. Prod.*, **1988**, *51*, 298-306.

<sup>143</sup> Gueritte-Voegelein, F.; Guenard, D.; Lavelle, F.; Le Goff, M. T.; Mangatal, L.; Potier, P., Relationships between the structure of taxol analogs and their antimetabolic activity, *J. Med. Chem.*, **1991**, *34*, 992-998.

<sup>144</sup> (a) Georg, G. I.; Cheruvallath, Z. S.; Himes, R. H.; Mejillano, M. R., Novel biologically active taxol analogs: baccatin III 13-[N-(p-chlorobenzoyl)-(2*R*,3'*S*)-3' phenylisoserinate] and baccatin III 13-(N-benzoyl)-(2*R*,2'*S*)-3'-(p-chlorophenyl)isoserinate, *Bioorg. Med. Chem. Lett.*, **1992**, *2*, 295-298; (b) Georg, G. I.; Cheruvallath, Z. S.; Himes, R. H.; Mejillano, M. R., Semisynthesis and biological activity of taxol analogs, *Bioorg. Med. Chem. Lett.*, **1992**, *2*, 1751-1754; (c) Georg, G. I.; Cheruvallath, Z. S.; Himes, R. H.; Mejilano, M. R.; Burke, C. T., Synthesis of biologically active taxol analogs with modified phenylisoserine side chains *J. Med. Chem.*, **1992**, *35*, 4239-4237.

<sup>145</sup> Kingston, D. G. I.; *History and Chemistry*, In McGuire, W. P., Rowinsky, E. K. (Eds), Taxol in cancer treatment, **1995**, New York: Marcel Dekker, pp. 21-27.

C-4 acetate groups and the oxetane ring. The acyl groups at the C-2 and C-4 positions play an important role in paclitaxel's interaction with tubulin. The benzoyloxy group at the C-2 position is essential to activity.<sup>146</sup> Modifications on the benzoyl ring have achieved a series of highly active analogs.<sup>147</sup> At the C-4 position, the acetyl group is essential to paclitaxel's activity, removal of this group results in significantly reduced activity compared with paclitaxel.<sup>148</sup> Substitution of the acetyl group with some other acyl groups generally causes reduced activities,<sup>149-151</sup> but some C-4 modified analogs have been prepared with enhanced activity compared with paclitaxel.<sup>152</sup> The oxetane ring is a very important structure to maintain the activity of paclitaxel. Ring opened compounds were dramatically less active in both cytotoxicity and microtubule assembly assays.<sup>153</sup>

The northern hemisphere of paclitaxel is less important in comparison with the southern hemisphere, probably because this part does not interact with tubulin directly. Modifications on the C-7 hydroxyl position such as acylation or dehydroxylation have

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<sup>146</sup> Chen, S. H.; Wei, J. M.; Farina, V., Taxol structure-activity relationships, synthesis and biological evaluation of 2-deoxytaxol. *Tetrahedron Lett.*, **1993**, *34*, 3205-3206.

<sup>147</sup> Chaudhary, A. G.; Gharpure, M. M.; Rimoldi, J. M.; Chordia, M. D.; Gunatilaka, A. A. L.; Kingston, D. G. I.; Grover, S.; Lin, C. M.; Hamel, E. Unexpectedly facile hydrolysis of the 2-benzoate group of taxol and synthesis of analogs with increased activities, *J. Am. Chem. Soc.*, **1994**, *116*, 4097-4098.

<sup>148</sup> Datta, A.; Jayasinghe, L.; Georg, G. I., 4-Deacetyltaxol and 10-acetyl-4-deacetyltaxotere: synthesis and biological evaluation, *J. Med. Chem.*, **1994**, *37*, 4258-4260.

<sup>149</sup> Chen, S. H.; Kadow, J. F.; Farina, V.; Fairchild, C.; Johnston, K. First Syntheses of Novel Paclitaxel (Taxol) Analogs Modified at the C4-Position, *J. Org. Chem.*, **1994**, *59*, 6156-6158.

<sup>150</sup> Georg, G. I.; Ali, S. M.; Boge, T. C.; Datta, A.; Falborg, L.; Himes, R. H., Selective C-2 and C-4 deacylation and acylation of taxol: the first synthesis of a C-4 substituted taxol analog, *Tetrahedron Lett.*, **1994**, *35*, 8931-8934.

<sup>151</sup> Chen, S. H.; Fairchild, C.; Long, B. H. Synthesis and biological evaluation of novel C-4 aziridine-bearing paclitaxel (taxol) analogs. *J. Med. Chem.*, **1995**, *38*, 2263-2267.

<sup>152</sup> Chen, S. H.; Wei, J. M.; Long, B. H.; Fairchild, C. A.; Carboni, J.; Mamber, S. W.; Rose, W. C.; Johnston, K.; Casazza, A. M.; Kadow, J. F.; Farina, V.; Vyas, D. M.; Doyle, T. W. Structure-activity relationships of taxol: synthesis and biological evaluation of C2 taxol analogs. *Bioorg. Med. Chem. Lett.*, **1995**, *5*, 2741-2746.

<sup>153</sup> Samaranyake, G.; Magri, N. F.; Jitrangsri, C.; Kingston, D. G. I. Modified taxols. Reaction of taxol with electrophilic reagents and preparation of a rearranged taxol derivative with tubulin assembly activity. *J. Org. Chem.*, **1991**, *56*, 5114-5119.

given analogs with similar activity to paclitaxel.<sup>154, 159</sup> The C-9 keto group can be reduced to a hydroxyl group without loss of activity.<sup>160-161</sup> Deacetylation on C-10 will slightly affect the analog's activity.<sup>162</sup> A number of analogs with some other ester groups on C-10 have also been made with bioactivities at the same level as paclitaxel.

#### IX.1.4 Semisynthesis of Paclitaxel by the Holton-Coupling Method

Isolation of paclitaxel from the bark of the Pacific Yew is difficult, the isolation yield is low and the extraction process is costly. Most of all, yew bark is not a readily renewable resource. The total synthesis of paclitaxel is also impractical for commercialization because of the long synthetic routes.<sup>163-164</sup>

Isolation of the relatively abundant 10-deacetylbaccatin-III (10-DAB) (**9.5**) and baccatin III (**9.6**) from the needles of the English yew has provided an alternative way to obtain paclitaxel. Semisynthesis of paclitaxel from 10-deacetylbaccatin was achieved by

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<sup>154</sup> Lataste, H.; Senilh, V.; Wright, M.; Guenard, D.; Potier, P., Relationships between the structures of taxol and baccatin III derivatives and their in vitro action on the disassembly of mammalian brain and Physarum amebal microtubules, *Proc. Natl. Acad. Sci.*, **1984**, *81*, 4090-4094.

<sup>155</sup> Mathew, A. E.; Mejillano, M. R.; Nath, J. P.; Himes, R. H.; Stella, V. Synthesis and evaluation of some water-soluble prodrugs and derivatives of taxol with antitumor activity *J. Med. Chem.*, **1992**, *35*, 145-151.

<sup>156</sup> Kingston, D. G. I.; Samaranayake, G.; Ivey, C. A., The chemistry of Taxol, a clinical useful anticancer agent, *J. Nat. Prod.*, **1990**, *53*, 1-12.

<sup>157</sup> Senilh, V.; Belchert, S.; Colin, M.; Guenard, D.; Picot, F.; Potier, P.; Varenne, P., Mise en evidence de nouveaux analogues du Taxol extraits de *Taxus baccata*. *J. Nat. Prod.*, **1984**, *47*, 131-137.

<sup>158</sup> Chaudhary, A. G.; Rimoldi, J. M.; Kingston, D. G. I., Modified taxols. 10. Preparation of 7-deoxytaxol, a highly bioactive taxol derivative, and interconversion of taxol and 7-*epi*-taxol. *J. Org. Chem.*, **1993**, *58*, 3798-3799.

<sup>159</sup> Chen, S. H.; Huang, S.; Kant, J.; Fairchild, C.; Wei, J.; Farina, V. Synthesis of 7-deoxy and 7,10-dideoxytaxol via radical intermediates. *J. Org. Chem.*, **1993**, *58*, 5028-5029.

<sup>160</sup> Klein, L. L. Synthesis of 9-dihydrotaxol: a novel bioactive taxane, *Tetrahedron Lett.* **1993**, *34*, 2047-2050.

<sup>161</sup> Georg, G. I.; Cheruvallath, Z. S.; Vander Velde, D. G., *Tetrahedron Lett.*, **1995**, *36*, 1783-1786.

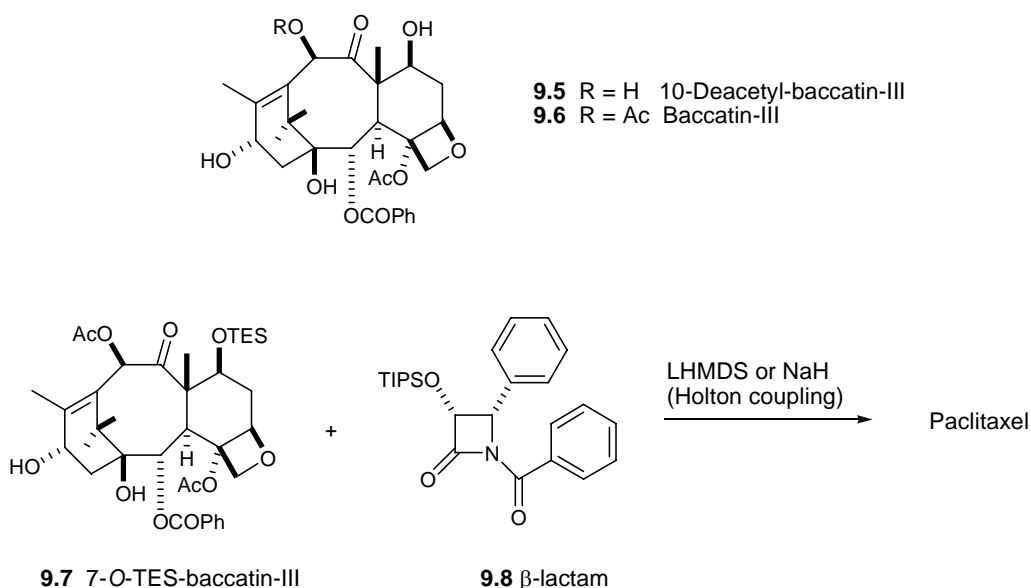
<sup>162</sup> Kant, J.; O'Keeffe, W. S.; Chen, S. H.; Farina, V.; Fairchild, C.; Johnston, K.; Kadow, J. F.; Long, B. H.; Vyas, D. *Tetrahedron Lett.*, **1994**, *35*, 5543-5546.

<sup>163</sup> Nicolaou, K. C.; Zang, Z.; Liu, J. J.; Ueno, H.; Nantermet, P. G.; Guy, R. K.; Claiborne, C. F.; Renaud, J.; Couladouros, E. A.; Paulvannan, K.; Sorensen, E. J. Total synthesis of taxol, *Nature*, **1994**, *367*, 630-634.

<sup>164</sup> Masters, J. J.; Link, J. T.; Snyder, L. B.; Young, W. B.; Danishefsky, S. J. Total synthesis of baccatin-III and taxol, *Angew. Chem. Int. Engl.*, **1995**, *34*, 1723-1726.

<sup>165</sup> Mukaiyama, T.; Shiina, I.; Iwadare, H.; Sakoh, H.; Tani, Y.; Hasegawa, M.; Saitoh, K. P. Asymmetric total synthesis of taxol. *Chem. Eur. J.* **1999**, *5*, 21-28.

Holton's efficient coupling method, which couples a 7-*O*-silyl protected baccatin III core (9.7) with a  $\beta$ -lactam (9.8) to install the paclitaxel side chain on the baccatin core.<sup>163</sup> This method is currently being used in the pharmaceutical industry for the production of paclitaxel, and it is also commonly used in our group for laboratory development of paclitaxel analogs (Figure 9-6).



**Figure 9-6** Holton's paclitaxel semisynthesis scheme.

### IX.1.5 Biological Conformations of Tubulin-Bound Paclitaxel

Despite the tremendous successful development in the chemical and biological studies of paclitaxel, the biological conformation of paclitaxel bound to tubulin has not yet clearly determined. The characterization of the conformation of paclitaxel bound to tubulin is important for the insight it gives into the binding of paclitaxel to tubulin and also as a possible lead in the design of more active analogs of paclitaxel. However,

<sup>166</sup> Holton, R. A. Pat. Appl. EP400, 971, 1990; *Chem. Abstr.* 1990, 114, 164568q.

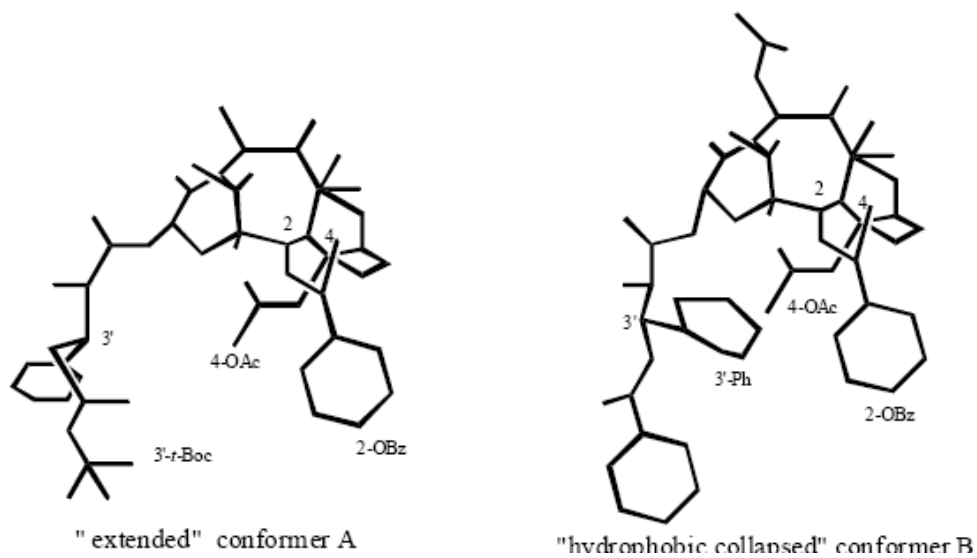
determination of the conformation of microtubule-bound paclitaxel is difficult because of the numerous conformational possibilities of the substitution groups on paclitaxel. Two major conformations have been proposed (Figure 9-7). One is the “extended” conformation, which was found in the crystal structure of the paclitaxel analog as well as in aprotic solvents.<sup>167</sup> In this conformation, the 3'-*t*-BOC-NH on the side chain is close to the 2-benzoate and 4-acetoxy groups. The other conformation, termed “hydrophobic collapsed”, has the 3'-phenyl, 4-acetate and the 2-benzoate groups clustered together. This conformation has been observed from the crystal structure of paclitaxel and from paclitaxel in protic solvents.<sup>168</sup> Snyder and co-workers have also proposed a third conformation of tubulin-bound paclitaxel as a “T-Taxol” or butterfly conformation on the basis of NMR studies and computational chemistry (Figure 9-8). This “T-Taxol” conformation is believed to provide structural rationalization for a major portion of the SAR data and of acquired mutations that led to drug resistance.<sup>169</sup>

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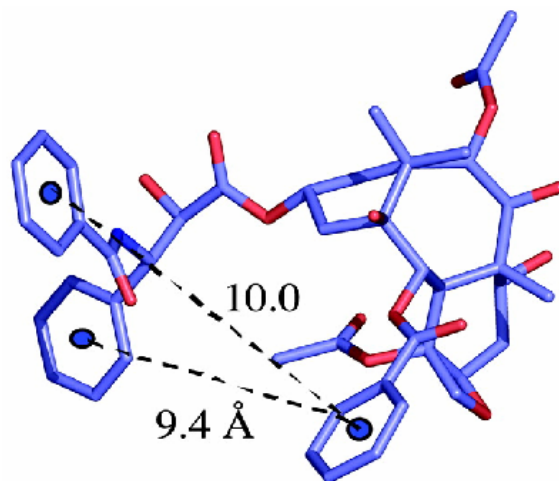
<sup>167</sup> Paloma, L. G.; Guy, R. K.; Wrasidlo, W.; Nicolaou, K. C. Conformation of a water-soluble derivative of taxol in water by 2D NMR spectroscopy, *Chem. Biol.* **1994**, *1*, 107–112.

<sup>168</sup> a). Vander Velde, D. G.; Georg, G. I.; Grunewald, G. L.; Gunn, C. W.; Mitscher, L. A., “hydrophobic collapse” of taxol and taxotere solution conformation in mixture of water and organic solvent. *J. Am. Chem. Soc.* **1993**, *115*, 11650—11651. b). Ojima, I.; Kuduk, S. D.; Chakravarty, S.; Ourevitch, M.; Begue, J. A novel approach to the study of solution structures and dynamic behavior of paclitaxel and docetaxel using flurione-containing analogs as probes, *J. Am. Chem. Soc.* **1997**, *119*, 5519–5527.

<sup>169</sup> Snyder, J. P.; Nettles, J. H.; Cornett, B.; Downing, K. H.; Nogales, E., The binding conformation of taxol in  $\beta$  tubulin, a model based on the electron crystallography, *Proc. Natl. Acad. Sci.* **2001**, *98*, 5312–5316.



**Figure 9.7** Two conformations of paclitaxel



**Figure 9.8** "T-Taxol" conformation

## IX.2 Design of Isotopically Labeled Paclitaxel Analogs for REDOR NMR.

### IX.2.1 Study of the Conformation of Tubulin-bound Paclitaxel by REDOR NMR.

As we have introduced the three proposed paclitaxel conformations, there are several techniques that can be used to examine the biological relevance of these three conformations. One of them is the REDOR NMR technique. REDOR (Rotational–Echo Double Resonance) NMR is a solid–state NMR spectroscopic tool that can be used to obtain accurate internuclear distance data on ligands bound to macromolecules in the solid state. It can thus be used to refine the proposed configurations of a target molecule. REDOR NMR experiments require stable isotope labeled ( $^{19}\text{F}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^2\text{D}$ ) target compounds so as to distinguish the signals of the ligand from the macro-molecule. By subsequent measurement of the heteronuclear dipolar coupling between isolated pairs of labeled nuclei, REDOR NMR gives accurate distances between two labeled nuclei after long acquisition times.<sup>170</sup> For example, the quadruply labeled paclitaxel analog **9.9** was previously synthesized in our group with  $^{13}\text{C}$  labeled on both the C-3' methine carbon and the  $^{15}\text{N}$ -benzamide carbonyl carbon, and  $^{19}\text{F}$  atom labeled on the *para* position of the C-2 benzene ring (Figure 9.9). REDOR experiments gave the distance between the C-3' methine carbon-13 and the fluorine atom as  $9.8 \pm 0.5 \text{ \AA}$  and the distance between the carbonyl carbon-13 on 3'-Ph $^{13}\text{CONH}$  and the fluorine atom as  $10.3 \pm 0.5 \text{ \AA}$ .<sup>171</sup> These data closely match the “extended” paclitaxel conformation with calculated data  $8.64 \text{ \AA}$  and  $10.39 \text{ \AA}$  respectively. However, the protic (hydrophobic) ( $9.60$  and  $10.43 \text{ \AA}$ ) conformation couldn't be ruled out (Figure 9-9), and the T-taxol conformation also fits

<sup>170</sup> Gullion, T.; Schaefer J. *Magn. Reson.* **1989**, *81*, 196.; b) Schaefer, J. In *REDOR NMR of biological solids from protein binding sites to bacterial cell walls; Recent trends in molecular recognition*; Diedrich, F.; Künzer, H, (Eds.); Ernst Schering Research Foundation: Workshop 26, 25–51.

<sup>171</sup> Li, Y.; Poliks, B.; Cegelski, L.; Poliks, M.; Gryczynski, Z.; Piszczek, G.; Jagtap, P.G.; Studelska, D. R.; Kingston, D. G. I.; Schaefer, J.; Bane, S., Conformation of microtubule-bound paclitaxel determined by fluorescence spectroscopy and REDOR NMR. *Biochemistry* **2000**, *39*, 281–291.

these data. Certainly, more labeled paclitaxel analogs are required for further REDOR NMR studies.

### IX.2.2 Deuterium and Fluorine Labeled Paclitaxel Analogs.

Three Deuterium and Fluorine Labeled Paclitaxel analogs were designed as the target compounds for REDOR NMR studies. The first analog, **9.10**, was previously synthesized by Dr. Belhu with the deuterium atoms on the *para* position of the C-2 benzene ring and the C-4 acetyl group, and with the fluorine atom on the *para* position of the C-2 benzene ring. This compound could provide more information of the distance between the C-2 benzoyl group and the C-4 acetyl group, which are crucial parts of paclitaxel.

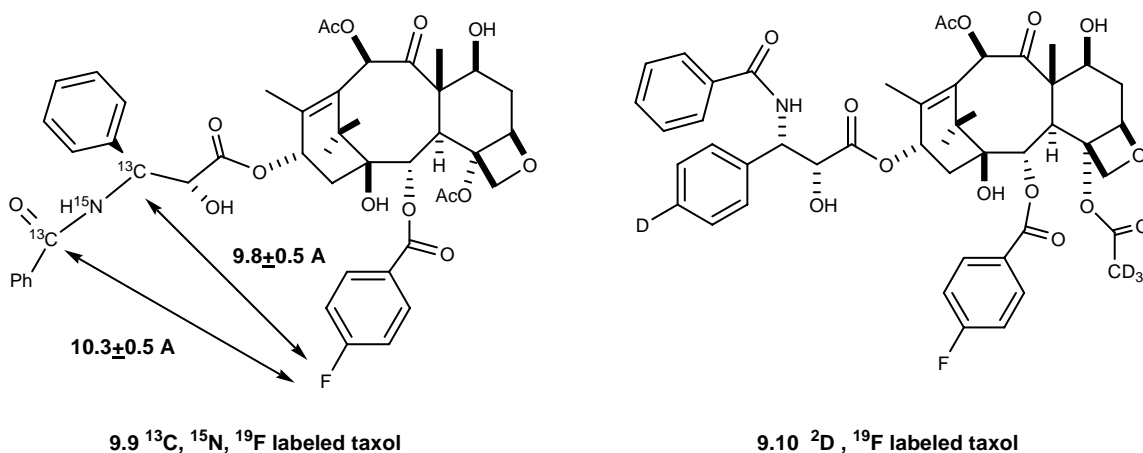
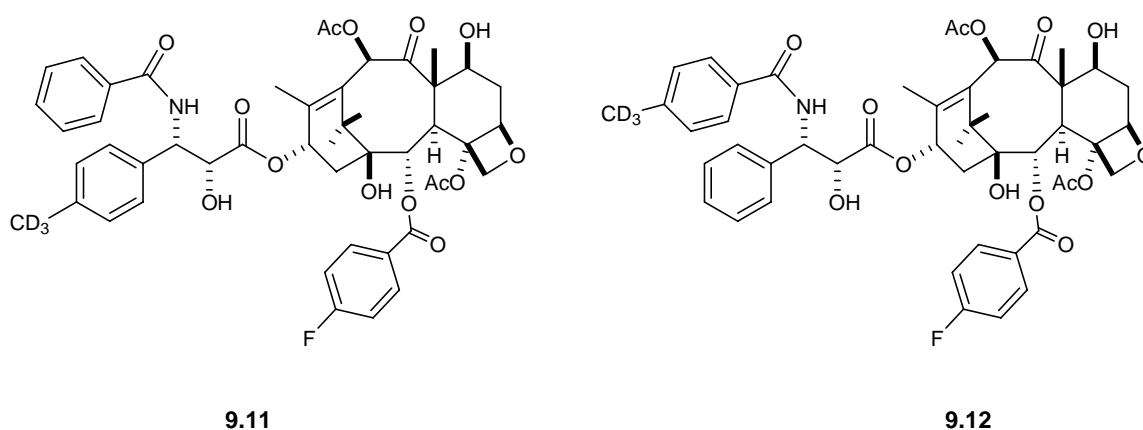


Figure 9-9 Previously prepared labeled paclitaxel by our group

In addition to **9.10**, two more paclitaxel analogs, **9.11** and **9.12** with two different labeling patterns were also anticipated to provide more distance information

from REDOR NMR. In these two analogs, the trideuteromethyl group was placed on the paclitaxel side-chain and the  $^{19}\text{F}$  on the C-2 benzoate of the southern regions of the baccatin skeleton. These analogs could allow us to determine distances between the deuterium atoms on the side chain and the fluorine atom on the C-2 benzoyl group, and the distance data obtained from REDOR NMR experiments were anticipated to provide more information of the tubulin-binding conformation of these two paclitaxel analogs.

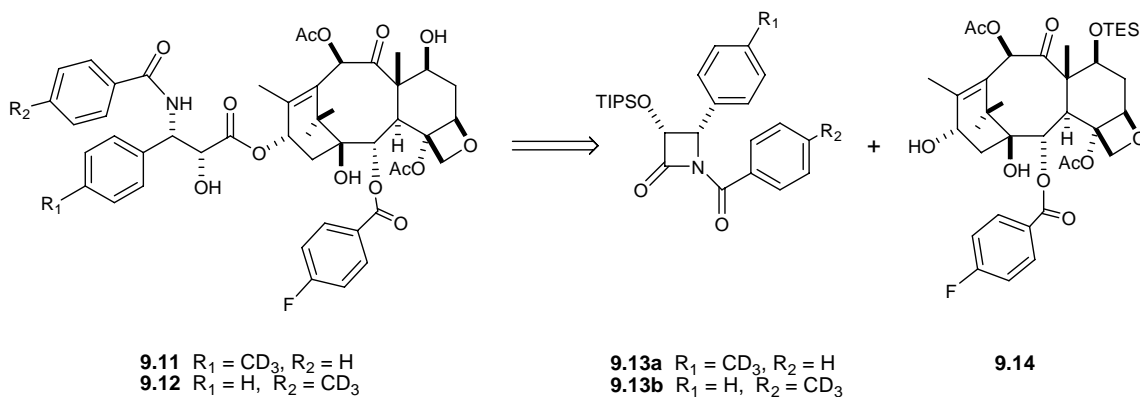


**Figure 9-10** New isotopically labeled paclitaxel **9.11** and **9.12**

### IX.3 Synthesis of the Labeled Paclitaxel Analogs 9.1 and 9.2.

The retrosynthetic analysis of compounds **9.11** and **9.12** is shown in Scheme 9-1. The target compound **9.11** was divided into two parts; the baccatin side chain (3*R*,4*S*)-1-*N*-benzoyl-3-TIPSO-4-(*p*-tri-deuteriomethylphenyl)azetid-2-one (**9.13a**) and 2-debenzoyl-(2-*p*-fluorobenzoyl)-7-triethylsilylbaccatin III (**9.14**). The coupling of **9.1** and **9.14** was accomplished by Holton's coupling method with LHMDS as catalyst.

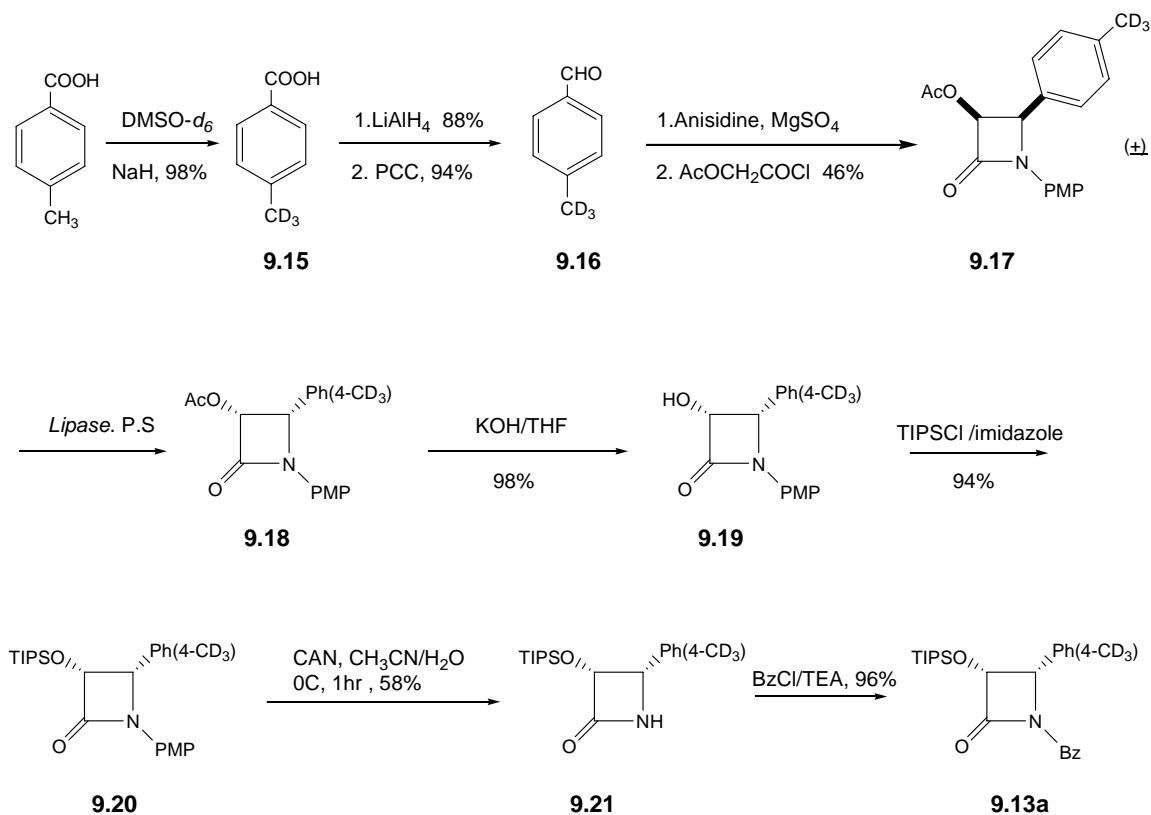
**Scheme 9-1:** Retrosynthesis of labeled paclitaxel.



The synthesis of the  $^2\text{D}$  labeled  $\beta$ -lactam **9.13a** started from commercially available *p*-toluic acid (Scheme 9-2). The methyl group of toluic acid was first deuterated by isotope exchange with  $\text{DMSO-}d_6$  in the presence of sodium hydride as a base.<sup>172</sup> The percentage of deuterium exchange rate was monitored by  $^1\text{H}$  NMR spectra in  $\text{D}_2\text{O}$  until the residue of methyl signal peak at  $\delta$  2.23 diminished to a negligible size (integral < 0.08H). Then the product was acidified to give 4-trideuteromethylbenzoic acid **9.15**.

<sup>172</sup> Atkinson, J. G.; Csakvary, J. J.; Herbert, G. T.; Stuart, R. S., Exchange reactions of carboxylic acid salts. Facile preparation of deuteriocarboxylic acid, *J. Am. Chem. Soc.* **1968**, *90*, 498-9.

**Scheme 9-2:** Synthesis of Labeled  $\beta$ -lactam.



The deuterium-labeled toluic acid **9.15** was converted to the corresponding aldehyde by lithium aluminum hydride reduction followed with PCC oxidation. Then the aldehyde **9.16** reacted with *p*-anisidine to form the corresponding imine. Staudinger [2 + 2] cyclocondensation of the imine with ketene generated from acetoxyacetyl chloride /TEA gave the racemic  $\beta$ -lactam **9.17** in 46% yield. The low yield was due to the substantial amount of unreacted imine and acetoxyacetyl chloride.

Enzyme-controlled hydrolysis with Lipase (PS-Amano) (Kinetic resolution) gave two compounds. The less polar one was the unhydrolyzed (3*R*,4*S*)-3-acetoxy-4-(*p*-tri-deuteriomethylphenyl)-  $\beta$ -lactam **9.18** which had the desired configuration; the polar one was the hydrolyzed (3*S*,4*R*)-3-hydroxyl-4-(*p*-tri-deuteriomethylphenyl)- $\beta$ -lactam which

was not desired. The completion of the reaction was monitored by TLC. After chromatographic separation of the desired compound **9.18**, deacetylation was carried out by potassium hydroxide in aqueous MeOH to give the secondary alcohol **9.19**. Protection of the 3*R* hydroxyl group with triisopropylsilyl ether yielded compound **9.20**. Then Ce(IV)-mediated deprotection of the PMP group gave **9.21** in 58% yield. Benzoylation of the lactam N-H group finally gave the desired labeled sidechain **9.13a** in 96% yield.

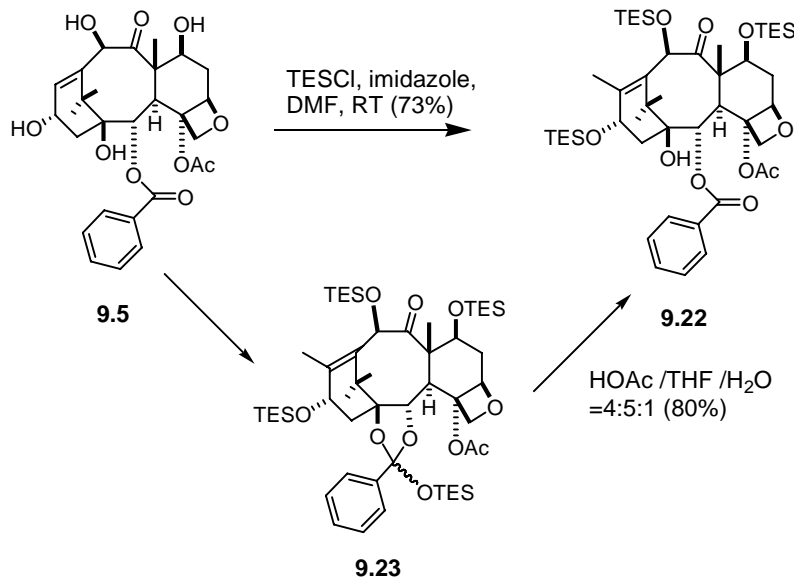
Synthesis of the baccatin part **9.14** started from the natural product 10-deacetyl-baccatin (10-DAB, **9.5**) as shown in Scheme 9-3. Triethylsilyl protection of the hydroxyl groups on C-7, 10 and 13 of 10-DAB was carried out with 4-5 equivalents of triethylsilyl chloride in DMF to give the 7,10,13-tri-*O*-triethylsilyl-baccatin **9.22**. Interestingly, the byproduct **9.23** was also isolated as a mixture of two diastereomers. Compound **9.23** had a higher  $R_f$  value on TLC than **9.22**. NMR experiments showed it was a mixture of two isomers on the orthoester carbon. The  $^1\text{H}$  NMR spectrum indicated the presence of an additional TES group at 0.6-0.8 ppm, and the  $^{13}\text{C}$  NMR spectrum showed that the benzoyl carbonyl signal at 167 ppm had disappeared. This suggested the structure of **9.23** was an overprotected baccatin. Also, when **9.23** was treated with acetic acid in THF/HOAc/ water = 5:4:1, Compound **9.22** was regenerated quickly. This confirmed that **9.17** was a mixture of two orthoesters. A literature search indicated they were known compounds, named as (*R,S*)-7,10,13,1'-*O*-tetra-(triethylsilyl)-2-debenzoyl-10-deacetyl-baccatin III-1,2-semi orthobenzoates.<sup>173,174</sup>

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<sup>173</sup> Appendino, G.; Belloro, E.; Jakupovic, S.; Danieli, B.; Jakupovic J. and Bombardelli, E., Synthesis of paclitaxel (docetaxel) / 2-deacetoxytaxinine J dimmers, *Tetrahedron* **1999**, *55*, 6567-6576.

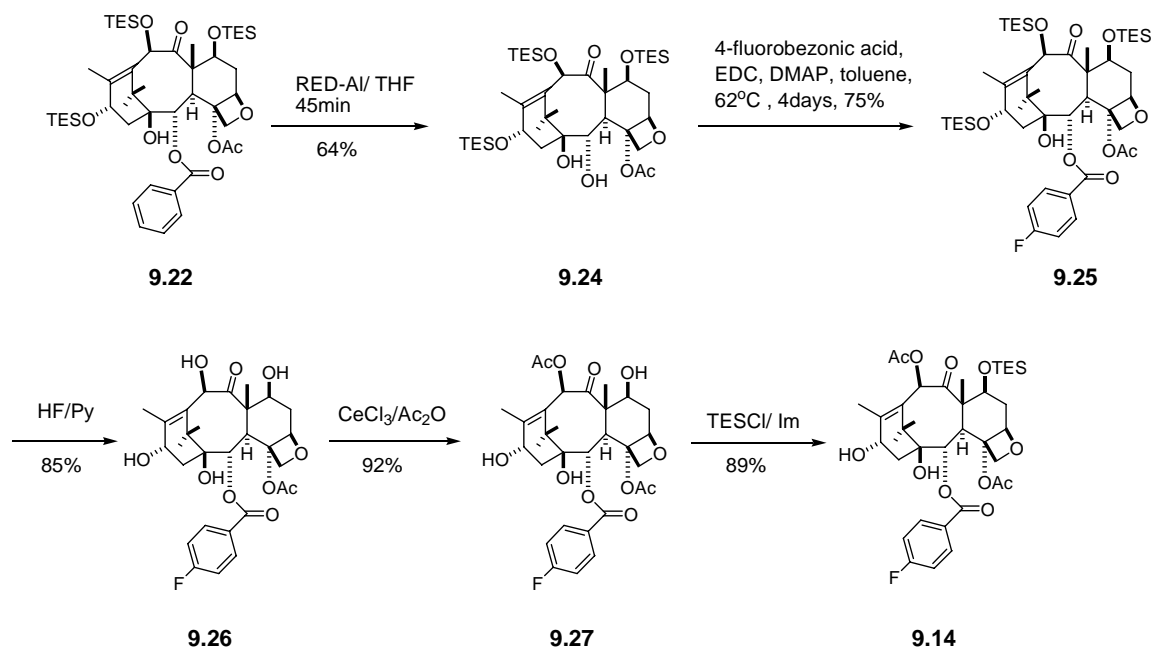
<sup>174</sup> Since these two orthoesters were very less polar and not stable on silica gel over long period, separation was very difficult. See chapter 10 for further study.

**Scheme 9-3:** Synthesis of the 7,10,13- tri-*O*-triethylsilyl baccatin III.



The 7,10,13- tri-*O*-triethylsilyl protected baccatin core **9.22** was treated with Red-Al<sup>®</sup> in THF to remove the C-2 benzoyl ester group in 64% yield to give the diol **9.24**. The relatively low yield was common in this step because the 4-acetyl group was also removed in part under these conditions. Re-esterification of the C-2 hydroxy group with 4-fluorobenzoic acid was achieved with EDCI/DMAP as coupling reagent in dry toluene for 4 days to give the 4-fluorobenzoate **9.25** in 65% yield. The complete deprotection of all the silyl groups to give **9.26** was followed by selective acetylation of the 10-OH mediated by CeCl<sub>3</sub> to give compound **9.27**. Selective reprotection of the 7-OH group completed the synthesis of the labeled baccatin core **9.14**.

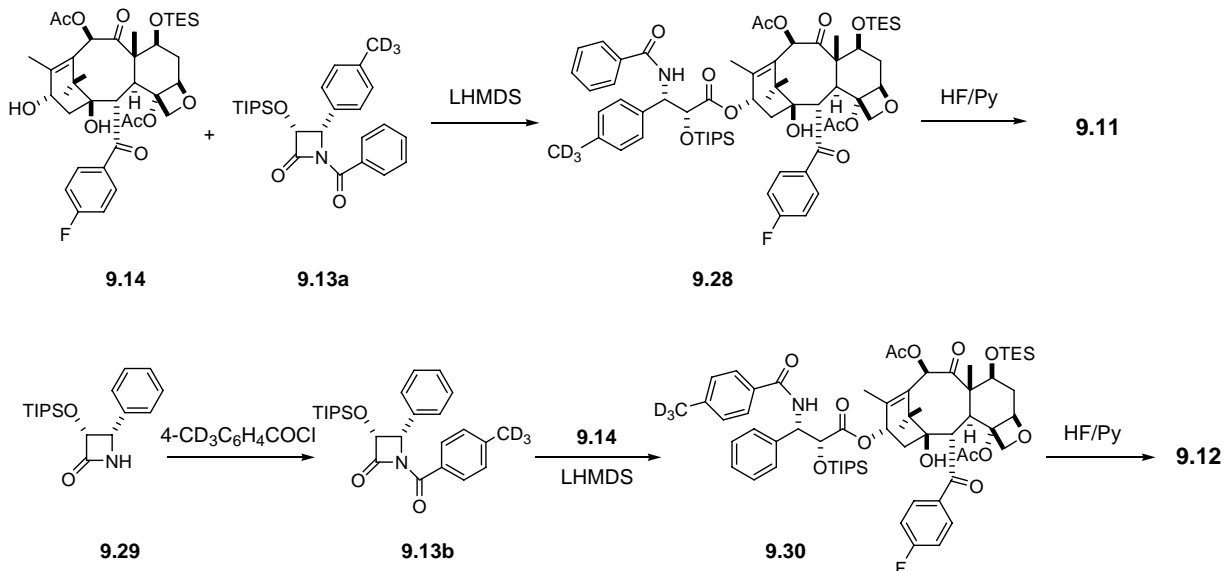
**Scheme 9-4:** Synthesis of fluorine labeled baccatin III.



The synthesis of the target labeled paclitaxel analog **9.11** was completed by Holton's coupling reaction between the baccatin core **9.14** and  $\beta$ -lactam **9.13a** to give the protected compound **9.28**. Deprotection of the silyl groups gave **9.11** (Scheme 9-5).

The  $\beta$ -lactam side chain **9.13b** for paclitaxel analog **9.12** was synthesized from lactam **9.29** which was previously prepared by Dr. Changhui Liu by the same method as in Scheme 9-1. Compound **9.29** was treated with *p*-trideuteriomethylbenzoyl chloride to give **9.13b** in 93% yield. Holton's coupling between the baccatin core **9.14** and **9.13b** gave the product **9.30**, and deprotection gave the final product **9.12**.

### Scheme 9-5: Synthesis of isotopically labeled paclitaxel analogs.



## IX.4 Experimental Results.

**IX.4.1. General Experimental Methods.** The key starting material 10-DAB was obtained from DABUR Chemicals, India. All other reagents and materials were from Aldrich Chemical Company. Anhydrous tetrahydrofuran (THF) was distilled from sodium/benzophenone under nitrogen. Anhydrous  $CH_2Cl_2$  (DCM) was distilled from calcium hydride. Analytical thin layer chromatography (TLC) plates (silica gel 60 GF, with aluminum support) from E. Merck were used for monitoring progress of a reaction and visualized with 254 nm UV light, with vanillin/sulfuric acid spray, or with phosphomolybdic acid/ethanol spray. Silica gel for column chromatography was purchased from E. Merck (230–400 mesh). Preparative thin layer chromatography (PTLC) plates (silica gel 60 GF) were purchased from Analtech. Unless specified, all the  $^1H$  and  $^{13}C$  NMR spectra were obtained from Varian Unity or Inova 400 spectrometers in  $CDCl_3$  at 399.951 MHz frequency. Chemical shifts are reported as  $\delta$ -values relative to

tetramethylsilane (TMS) as internal reference. All  $J$  values are reported in Hertz. High Resolution Fast Atom Bombardment mass spectra (HRFABMS) were obtained by Analytical Services in the Department of Chemistry at Virginia Tech.

#### **IX.4.2 Experimental Procedures for the Preparation of $\beta$ -Lactam:**

***p*-Trideuteromethyl-benzoic acid (9.15).**<sup>171</sup> To a round bottom flask charged with 40 mL of DMSO- $d_6$  (dried over 4 Å molecular sieves), toluic acid (3.4 g, 25 mmol) was added. The solution was stirred at -20° C and NaH (60 wt% in mineral oil) (1.28 g, 28.8 mmol, 1.1 eq) was added slowly under nitrogen. The reaction mixture was stirred until hydrogen evolution ceased. Then the mixture was heated to 110 °C and stirring continues. The reaction was monitored by taking out a small amount of liquid every 12 hours and examining by  $^1\text{H}$  NMR spectroscopy. After 36 hours the  $^1\text{H}$  NMR spectrum in  $\text{D}_2\text{O}$  indicated that the residual methyl signal peak at 2.23 ppm had almost completely diminished (integral < 0.08 H). A few drops of water were then added and the DMSO was removed under vacuum. The residue was acidified with 10% hydrochloric acid and the precipitate was collected and air-dried. Column chromatography on silica gel with 5% MeOH in  $\text{CHCl}_3$  yielded compound **9.15** (3.08 g, 22.1 mmol, 88%) as a white powder.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.84 (2H, d,  $J = 8.4$ ) 7.34 (2H, d,  $J = 8.4$ ), 2.23 (m, <0.08H).

***p*-Trideuteromethyl-benzaldehyde (9.16).** To a stirred solution of **9.15** (3.08 g, 22 mmol) in anhydrous THF (20 mL) at -20 °C, lithium aluminum hydride (3.15 g, 84.8 mmol) was added in small portions and the reaction was stirred at -20 °C for 4 h followed by 1 h at room temperature. Then the reaction was quenched with saturated aqueous

NH<sub>4</sub>Cl (40 mL) and extracted with EtOAc (50 mL × 2). The combined organic layer was washed with water and brine and then dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After the solvent was evaporated, the residue was dried under vacuum and dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (20 mL) at 0 °C, then pyridinium chlorochromate (12.4 g, 69.3 mmol) was added. The reaction mixture was stirred for 40 min in an ice bath and then diluted with 50 mL of saturated aqueous NaHCO<sub>3</sub>, and extracted with EtOAc (50 mL × 3). The combined organic layers were washed with water and brine and then dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on Silica-gel with 5% EtOAc in hexane yielded compound **9.16** (2.38 g, 19.3 mmol, 87% two steps) as a colorless oil. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 9.83 (1H, s), 7.84 (2H, d, *J* = 8.0), 7.21 (2H, d, *J* = 8.0), 2.14 (m, <0.08).

**cis-(±)-1-(*p*-Methoxyphenyl)-3-acetoxyl-4-(*p*-trideuteriomethylphenyl)azetidin-2-one (9.17).** To a solution of the aldehyde **9.16** in CH<sub>2</sub>Cl<sub>2</sub> was added 1.2 equiv of *p*-anisidine (2.89 g, 23 mmol) and a large excess of anhydrous MgSO<sub>4</sub> (pre-activated at 100 °C for 2 hours) and the mixture was stirred at room temperature for 12 h. The yellowish slurry was filtered and concentrated under reduced pressure, and the CH<sub>2</sub>Cl<sub>2</sub> solution of the crude imine was taken to the next step without purification. The CH<sub>2</sub>Cl<sub>2</sub> solution was treated with triethylamine (13 mL) and cooled to -78 °C. Acetoxyacetyl chloride (3.0 mL, 27.3 mmol) was added dropwise to this solution and the thick reaction mixture was allowed slowly to warm up to room temperature and stirred for 12 h. The dark crude reaction mixture was concentrated and purified twice by silica gel column chromatography with EtOAc:hexane, 3:7, to give (3*R*,4*S*) and (3*S*,4*R*) racemic β-lactam mixture **9.17** (3.7 g, 10.8 mmol) as colorless needles. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.28 (2H, d, *J*

= 8.0), 7.06 (2H, d,  $J = 8.4$ ), 6.88-6.82 (overlapped, 4H), 6.10 (1H, d,  $J = 4.0$ ), 5.15 (1H, d,  $J = 4.0$ ), 3.62 (3H, s), 2.03 (3H, s);  $^{13}\text{C}$  NMR:  $\delta$  171.2, 161.9, 156.6, 136.8, 130.2, 128.6, 127.8, 125.2, 117.8, 114.1, 80.4, 62.5, 55.4, 20.8; HRFABMS:  $m/z = 329.1578$  ( $\text{M}+\text{H}$ ) $^+$ , calculated for  $\text{C}_{19}\text{H}_{16}\text{D}_3\text{NO}_4$ :  $m/z = 329.1581$ ,  $\Delta = -1.0$  ppm.

**(3*R*,4*S*)-1-(*p*-Methoxyphenyl)-3-acetoxy-4-(*p*-trideuteriomethylphenyl)azetidin-2-one (9.18).** The racemic  $\beta$ -lactam **9.17** (3.0 g) was then dissolved in 30 mL acetonitrile, and to this solution a phosphate buffer at pH 7.2 (45 mL) was mixed and stirred vigorously. Immobilized Lipase PS Amano enzyme (3.4 g) was added and stirred for 7 days. Reaction progress was monitored by TLC, and after completion of the reaction, the lipase was filtered off and the solution was diluted with 100 mL of water and extracted with EtOAc (50 mL $\times$ 2). The combined organic layers were washed with water and brine and then dried over anhydrous  $\text{Na}_2\text{SO}_4$ . Purification by column chromatography (EtOAc:hexanes, 3:7) gave enantiomerically pure (3*R*,4*S*)-1-(*p*-methoxyphenyl)-3-acetoxyl-4-(*p*-trideuteriomethylphenyl)-azetidin-2-one ( $\beta$ -lactam) **9.18** (1.5 g, 5.4 mmol, 50% yield) as colorless crystals.  $[\alpha]_{\text{D}}^{25} = +16.8^\circ$  ( $\text{CHCl}_3$ ,  $c = 0.32$ ). NMR data was identical to those of **9.17** above.

**(3*R*,4*S*)-1-(*p*-Methoxyphenyl)-3-triisopropylsiloxy-4-(*p*-trideuteriomethylphenyl)azetidin-2-one (9.20).** The solution of **9.18** (1.5 g) in THF (50 mL) was added slowly to 50 mL 1 M aqueous KOH solution at 0  $^\circ\text{C}$ . The solution was stirred for 45 min. After the reaction was completed, the reaction mixture was extracted with EtOAc (100 mL $\times$ 2) and the organic part was washed with water and brine, and then dried over anhydrous  $\text{Na}_2\text{SO}_4$ .

After removal of solvent and drying under vacuum, the product of this reaction **9.19** (1.38 g, 96%) was used directly for the next step without purification. To the solution of **9.19** in 10 mL of DMF was added imidazole (1.64 g, 24 mmol) and triisopropyl chloride (2.38 mL, 2.18 g, 12 mmol) and the mixture stirred at room temperature for 3 h. The reaction mixture was diluted with EtOAc and the combined organic layer was washed with saturated aqueous NaHCO<sub>3</sub> and brine, and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography (EtOAc:hexane, 2:8) on silica gel gave silyl protected β-lactam **9.20** (1.83 g, 4.19 mmol, 84.5%) as a white solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.27 (2H, d, *J* = 8.0), 7.18 (2H, d, *J* = 8.4), 7.05 (2H, d, *J* = 8.4), 6.84 (2H, d, *J* = 8.0), 5.42 (1H, d, *J* = 4.0), 5.15 (1H, d, *J* = 4.0), 3.58 (3H, s), 0.94-0.87 (overlapped, 21H); <sup>13</sup>C-NMR: δ 165.6, 156.5, 137.6, 130.2, 128.6, 127.8, 125.2, 117.1, 115.2, 79.7, 61.4, 55.4, 17.5, 11.8; HRFABMS: *m/z* = 443.2829 (M+H)<sup>+</sup>, calculated for C<sub>26</sub>H<sub>35</sub>D<sub>3</sub>NO<sub>3</sub>Si: *m/z* = 443.2809, Δ = +4.4 ppm.

**(3*R*,4*S*)-3-Triisopropylsilyloxy-4-(*p*-trideuteriomethylphenyl)azetid-2-one (9.21).**

To a solution of **9.20** (0.52 g, 1.2 mmol) in CH<sub>3</sub>CN (25 mL) at -5 °C in an ice bath, (NH<sub>4</sub>)<sub>2</sub>Ce(NO<sub>3</sub>)<sub>6</sub>·2H<sub>2</sub>O (1.36 g, 3.6 mmol) in 15 mL water was added dropwise. The reaction mixture was stirred for 45 min until TLC indicated the consumption of the starting material. Then the mixture was diluted with EtOAc (100 mL) and washed with saturated aqueous NaHCO<sub>3</sub> (2×50 mL), water (2×50 mL), saturated sodium metabisulfite (2×25 mL) and brine, and then the organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>. The crude product was chromatographed on silica gel with 40% EtOAc in hexanes to give the deprotected lactam **9.21** (213 mg, 0.63 mmol, 55% yield). <sup>1</sup>H NMR δ 7.19 (2H, d, *J* = 8.0), 7.11 (2H, d, *J* = 8.0), 5.08 (m, 1H), 4.72 (d, 1H, *J* = 5.5), 0.94-0.86 (overlapped,

21H);  $^{13}\text{C}$  NMR  $\delta$  170.6, 137.6, 133.4, 128.6, 128.2, 79.7, 59.7, 17.5, 11.8; HRFABMS:  $m/z = 337.2384$  (M+H) $^+$ , calculated for  $\text{C}_{19}\text{H}_{29}\text{D}_3\text{NO}_2\text{Si}$ :  $m/z = 337.2391$ ,  $\Delta = -2.1$  ppm.

**(3R,4S)-1-Benzoyl-3-TIPSO-4-(*p*-trideuteriomethylphenyl)azetidin-2-one (9.13a).**

To a solution of **9.21** (182 mg, 0.54 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (1 mL) at 0 °C, triethylamine (190  $\mu\text{l}$ , 1.08 mmol) and benzoyl chloride (92  $\mu\text{l}$ , 0.6 mmol) were added. The mixture was then stirred at room temperature for 3 h, diluted with EtOAc (10 mL), washed with saturated aqueous  $\text{NaHCO}_3$  and brine, and dried over  $\text{Na}_2\text{SO}_4$ . The crude product was purified by chromatography (15% EtOAc in hexane) to give the  $\beta$ -lactam **9.13a** (234 mg, 0.53 mmol, 98% yield).  $[\alpha]_{\text{D}}^{25} = +86.4^\circ$  ( $\text{CHCl}_3$ ,  $c = 0.17$ );  $^1\text{H}$  NMR  $\delta$  8.05 (dd, 2H,  $J = 8.0$  and 1.5), 7.59 (m, 1H), 7.48 (2H, t,  $J = 8.0$ ), 7.31 (2H, dd,  $J = 8.5$  and 1.0), 7.17 (2H, dd,  $J = 8.5$  and 1.0), 5.41 (1H, d,  $J = 6.0$ ), 5.23 (1H, d,  $J = 6.0$ ), 0.98-0.88 (overlapped, 21H);  $^{13}\text{C}$  NMR  $\delta$  166.3, 165.7, 138.0, 133.3, 132.4, 131.0, 129.9, 128.9, 128.3, 128.2, 76.6, 61.2, 17.5, 17.4, 11.8 ppm; HRFABMS  $m/z = 441.2664$  (M+H) $^+$ , calculated for  $\text{C}_{26}\text{H}_{33}\text{D}_3\text{NO}_3\text{Si}$ ,  $m/z = 441.2653$ ,  $\Delta = 2.3$  ppm.

**IX.4.3 Synthesis of The Baccatin Core.**

**7,10,13-Tris(triethylsilyl)-10-deacetylbaccatin (9.22).** To the solution of 10-DAB **9.5** (800 mg, 1.49 mmol) in 5 mL of DMF was added imidazole (1.15 g, 19.8 mmol) and triethylsilyl chloride (1.5 mL, 9.9 mmol) and the mixture stirred at room temperature for 3 h. The reaction mixture was diluted with EtOAc (50 mL) and the combined organic layer was washed with saturated aqueous  $\text{NaHCO}_3$  and brine, and dried over  $\text{Na}_2\text{SO}_4$ . Column chromatography (EtOAc:hexane, 2:8) on silica gel gave compound **9.22** (989

mg, 1.19 mmol, 76%) as a white solid.  $^1\text{H}$  NMR:  $\delta$  8.07 (2H, dd,  $J = 8.0$  and  $1.5$ ), 7.56 (1H, t,  $J = 8.0$ ), 7.45 (2H, t,  $J = 8.0$ ), 5.60 (1H, d,  $J = 6.0$ ), 5.17 (1H, s), 4.93 (1H, dd,  $J = 9.0$  and  $2.5$ ), 4.42 (1H, dd,  $J = 7.5$  and  $1.5$ ), 4.26 (1H, d,  $J = 8.5$ ), 4.11 (1H, dd,  $J = 9.0$  and  $2.0$ ), 4.08 (1H, dd,  $J = 9.5$  and  $2.5$ ), 3.83 (1H, d,  $J = 7.0$ ), 2.48 (1H, m), 2.26 (3H, s), 2.01 (3H, s), 1.62 (3H, s), 1.17 (3H, s), 1.10 (3H, s), 0.98-0.96 (overlapped, 27H), 0.62-0.60 (overlapped, 18H);  $^{13}\text{C}$  NMR:  $\delta$  209.0, 170.0, 167.2, 139.5, 133.5, 130.1, 128.6, 84.1, 80.9, 79.6, 76.8, 76.7, 75.8, 75.6, 72.7, 68.4, 58.3, 47.0, 43.1, 39.9, 37.4, 26.4, 22.4, 20.7, 14.6, 14.2, 10.5, 7.0-6.9 (overlapped), 5.75, 5.44, 5.12; HRFABMS:  $m/z = 887.5047$  (M+H) $^+$ , calculated for  $\text{C}_{47}\text{H}_{79}\text{O}_{10}\text{Si}_3$   $m/z = 887.4981$ ,  $\Delta = +7.4$  ppm.

**7,10,13,1'-O-Tetra(triethylsilyl)-2-debenzoyl-10-deacetyl-baccatin III 1,2-semiortho-benzoate (9.23).**<sup>172,173</sup> To a solution of 10-DAB (**9.5**) (500 mg, 0.93 mmol) in DMF (3.5 mL) was added imidazole (1.26 g, 18.6 mmol) and triethylsilyl chloride (1.35 mL, 8.9 mmol) and the mixture was stirred at room temperature overnight. Then the reaction mixture was quenched with saturated aqueous  $\text{NaHCO}_3$  (2 mL), and diluted with EtOAc (50 mL) and the combined organic layer was washed with saturated aqueous  $\text{NaHCO}_3$ , water and brine, and dried over  $\text{Na}_2\text{SO}_4$ . Column chromatography (EtOAc:hexane, 1:9 to 1:4) on silica gel gave the overprotected product **9.23** (223 mg, 0.022 mmol, 24%) as colorless gum and **9.22** (482 mg, 0.54 mmol, 58%). Compound **9.23**, C-1' *R, S* mixture, HRFABMS  $m/z = 1001.5753$  [M+H] $^+$  calculated for  $\text{C}_{53}\text{H}_{93}\text{O}_{10}\text{Si}_4$   $m/z = 1001.5846$  ( $\Delta = -9.3$  ppm).

**2-Debenzoyl-7,10,13-tris(triethylsilyl)-10-deacetylbaecatin (9.24).** To a solution of **9.22** (750 mg, 0.85 mmol) in anhydrous THF (15 mL) at -20 °C, Red-Al (4M in THF, 1.1 mL) was added dropwise under nitrogen. The reaction was stirred for 45 min until TLC showed the exhaustion of starting material. After quenching with a few drops of water, the reaction mixture was added to 50 mL of 1M sodium potassium tartrate and extracted with EtOAc. The organic part was washed with water and brine, and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on silica gel (EtOAc:hexane, 3:7) gave compound **9.24** (484 mg, 0.42 mmol, 72%). <sup>1</sup>H NMR δ 5.14 (1H, s), 4.72 (1H, d, *J* = 7.0), 4.63 (1H, dd, *J* = 9.5 and 4.0), 4.42 (1H, dd, *J* = 7.5 and 1.5), 4.56 (1H, d, *J* = 9.0), 4.11 (1H, m), 3.98 (1H, dd, *J* = 10.5 and 6.0), 3.74(1H, dd, *J*=10.5 and 5.5), 3.45 (1H, d, *J* = 10.5), 3.23 (1H, d, *J* = 6.0), 2.45-2.37 (3H, overlapped, m), 2.08 (3H, s), 1.98 (3H,s), 1.78 (3H, s), 1.04 (3H, s), 1.01 (3H, s), 0.97-0.94 (overlapped, 27H), 0.63-0.60 (overlapped, 18H); <sup>13</sup>C NMR δ 206.3, 169.7, 139.0, 136.0, 83.7, 82.0, 78.7, 78.0, 76.8, 75.8, 74.7, 72.7, 68.4, 58.2, 46.8, 42.5, 40.4, 37.4, 26.0, 22.4, 20.6, 14.5, 10.6, 6.9-6.8 (overlapped), 5.21, 5.13, 4.82.

**2-Debenzoyl-2-(*p*-fluorobenzoyl)-7,10,13-tris(triethylsilyl)-10-deacetylbaecatin (9.25).** To a solution of of *p*-fluorobenzoic acid (752 mg, 5.40 mmol) in dry toluene (10 mL) was added EDCI (1.03 g, 5.40 mmol) and DMAP (6 mg). The heterogenous solution was stirred at room temperature for 30 min, and then compound **9.23** (280 mg, 0.35 mmol) in 5 mL of toluene was added dropwise and the mixture was stirred for 10 min at room temperature and then warmed up to 55 °C and stirred for 2 days. The reaction mixture was diluted with EtOAc and washed with water and aqueous NaHCO<sub>3</sub>. The combined organic phase was washed with water and brine, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and

concentrated under reduced pressure. Column chromatography (EtOAc:hexane, 1:4) gave **9.23** (223 mg, 0.24 mmol, 73%) as a white solid.  $^1\text{H}$  NMR  $\delta$  8.08 (2H, dd,  $J = 8.5$  and 5.5), 7.07 (2H, dd,  $J = 8.5$  and 8.0), 5.65 (1H, d,  $J = 5.6$ ), 5.08 (1H, s), 4.96 (dd,  $J = 9.0$  and 2.5), 4.42 (1H, dd,  $J = 7.5$  and 1.5), 3.82 (1H, d,  $J = 7.0$ ), 2.83 (overlapped, m, 2H), 2.18 (m, 2H), 2.14 (3H, s), 1.90 (3H, s), 1.63 (m, 1H), 1.53 (3H, s), 1.32 (3H, s), 1.20 (3H, s), 1.09 (3H, s), 0.98-0.92 (overlapped, 27H), 0.61-0.59 (overlapped, 18H);  $^{13}\text{C}$  NMR  $\delta$  206.8, 171.3, 164.4, 159.6, 139.5, 137.9, 132.4, 132.3, 116.2, 116.0, 90.2, 86.6, 77.1, 73.2, 2.6, 70.9, 68.8, 55.8, 43.2, 41.0, 38.1, 25.4, 22.5, 21.1, 14.5, 10.8, 7.0, 5.8, 5.7, 5.4; HRFABMS:  $m/z = 903.4713$  ( $\text{M}+\text{H}$ ) $^+$ , calculated for  $\text{C}_{47}\text{H}_{78}\text{FO}_{10}\text{Si}_3$   $m/z = 903.4730$ ,  $\Delta = -1.9$  ppm.

**2-Debenzoyl-2-(*p*-fluorobenzoyl)-10-deacetylbaecatin (9.26).** To a solution of **9.25** (220 mg, 0.24 mmol) in 2.5 mL of THF was added HF/pyridine (70 wt%, 1.0 mL, large excess) and the solution was stirred at room temperature for 10 h. The reaction mixture was diluted with EtOAc and washed with aqueous  $\text{NaHCO}_3$  solution. The organic layer was washed with water and brine, dried over anhydrous  $\text{Na}_2\text{SO}_4$ , and evaporated under reduced pressure. The residue was purified by chromatography on silica gel (EtOAc:hexane, 2:3) to yield **9.26** (128 mg, 0.22 mmol, 94%) as colorless crystals.  $^1\text{H}$  NMR:  $\delta$  8.08 (2H, dd,  $J = 8.5$  and 5.5), 7.14 (2H, dd,  $J = 8.5$  and 8.0), 5.58 (1H, d,  $J = 5.6$ ), 4.95 (dd,  $J = 9.0$  and 2.5), 4.80 (1H, t,  $J = 7.5$ ), 4.42 (1H, dd,  $J = 7.5$  and 1.5), 3.82 (1H, d,  $J = 7.0$ ), 2.83 (overlapped, m, 2H), 2.18 (m, 2H), 2.14 (3H, s), 1.90 (3H, s), 1.63 (m, 2H), 1.32 (3H, s), 1.20 (3H, s), 1.09 (3H, s);  $^{13}\text{C}$  NMR:  $\delta$  208.4, 170.6, 164.4, 158.7, 139.5, 137.9, 132.4, 132.3, 116.2, 116.0, 90.2, 86.6, 77.1, 73.2, 2.6, 70.9, 68.8, 55.8, 43.2,

41.0, 38.1, 25.4, 22.5, 21.1, 14.5, 10.5., 6.9, 5.8; HRFABMS  $m/z$  = 563.2285, calculated for  $C_{29}H_{36}FO_{10}$   $m/z$  = 563.2293,  $\Delta$  = -1.4 ppm.

**2-Debenzoyl-2-(*p*-fluorobenzoyl)-baccatin (9.27).** To a solution of **9.26** (120 mg, 0.21 mmol) in 1 mL of anhydrous THF was added 5 mg of  $CeCl_3$  at room temperature. The mixture was stirred for 5 min and then acetic anhydride (0.18 mL, 1.8 mmol) was added and stirring continued at room temperature for 1 h. The reaction mixture was then diluted with EtOAc. The organic layer was washed with saturated aqueous  $NaHCO_3$ , water and brine, and dried with  $Na_2SO_4$ . The residue was purified on silica gel chromatography (EtOAc:hexane, 3:7) to yield **9.26** (113 mg, 0.19 mmol, 90%).  $^1H$  NMR  $\delta$  8.09 (2H, dd,  $J$  = 8.5 and 5.5), 7.12 (2H, ddd,  $J$  = 8.5 and 2.0), 6.30 (1H, s), 5.56 (1H, d,  $J$  = 7.0), 4.96 (dd,  $J$  = 9.5 and 2.0), 4.85 (1H, t,  $J$  = 8.0), 4.45 (1H, dd,  $J$  = 7.5 and 1.5), 4.24 (1H, d,  $J$  = 8.5), 4.12 (1H, d,  $J$  = 8.5), 3.84 (1H, d,  $J$  = 7.0), 3.75 (1H, br, s), 2.60-2.53 (overlapped, m, 3H), 2.24 (3H, s), 2.21 (3H, s), 2.02 (3H, s), 1.84 (m, 1H), 1.63 (3H, s), 1.08 (3H, s), 1.06 (3H, s);  $^{13}C$  NMR  $\delta$  204.2, 171.4, 171.3, 170.6, 166.1, 165.2, 146.8, 132.8, 132.7, 131.7, 125.8, 125.7, 116.0, 115.8, 84.5, 80.8, 79.1, 76.4, 76.3, 75.2, 72.3, 67.8, 60.4, 58.7, 46.2, 42.7, 38.6, 35.6, 26.9, 22.6, 21.1, 21.0, 20.9, 15.6, 14.2, 9.47; HRFABMS:  $m/z$  = 605.2384 (M+H) $^+$ , calculated for  $C_{31}H_{38}FO_{11}$   $m/z$  = 605.2398,  $\Delta$  = -2.4 ppm.

**2-Debenzoyl-2-(*p*-fluorobenzoyl)-7-*O*-triethylsilyl-baccatin (9.14).** To a solution of **9.27** (80 mg, 0.13 mmol) in DMF (4 mL) at 0 °C was added imidazole (27 mg, 0.4 mmol) and chlorotriethylsilane (40  $\mu$ L, 0.37 mmol). The progress of the reaction was carefully monitored to avoid the side reaction on the C-13 hydroxyl group. After 2 h the reaction

was completed the mixture was diluted with 20 mL of EtOAc and quenched with saturated aqueous NaHCO<sub>3</sub>. The organic layer was washed with saturated aqueous NaHCO<sub>3</sub>, water and brine, and dried over Na<sub>2</sub>SO<sub>4</sub>. The crude product was purified by preparative silica gel TLC with EtOAc:hexane, 1:4 to give **9.14** (82 mg, 0.11 mmol, 80%) as a glassy solid. <sup>1</sup>H NMR: δ 8.11 (2H, dd, *J* = 8.5 and 5.5), 7.14 (2H, dd, *J* = 8.5 and 8.0), 6.47 (1H, s), 5.60 (1H, d, *J* = 7.0), 4.95 (dd, *J* = 9.0 and 2.5), 4.82 (1H, t, *J* = 7.5), 4.46 (1H, dd, *J* = 7.5 and 1.5), 4.27 (1H, d, *J* = 8.0), 4.10 (1H, d, *J* = 8.0), 3.87 (1H, d, *J* = 7.0), 3.75 (1H, br, s), 2.53 (overlapped, m, 3H), 2.26 (3H, s), 2.18 (3H, s), 2.17 (3H, s), 1.84 (m, 1H), 1.66 (3H, s), 1.18 (3H, s), 1.03 (3H, s), 0.92-0.89 (9H, overlapped), 0.58-0.55 (6H, overlapped); <sup>13</sup>C NMR δ 204.2, 171.4, 171.3, 170.6, 166.1, 165.2, 146.8, 132.8, 132.7, 131.7, 125.8, 125.7, 116.0, 115.8, 84.5, 80.8, 79.1, 76.4, 76.3, 75.2, 72.3, 67.8, 60.4, 58.7, 46.2, 42.7, 38.6, 35.6, 26.9, 22.6, 21.1, 21.0, 20.9, 15.6, 14.2, 9.5, 7.0, 5.56; HRFABMS : *m/z* = 719.3266 (M+H)<sup>+</sup>, calculated for C<sub>37</sub>H<sub>52</sub>FO<sub>11</sub>Si, *m/z* = 719.3263, Δ = 0.5 ppm.

**2'-O-(Triisopropyl)-3'-(*p*-trideuteromethylphenyl)-7-O-triethylsilyl-2-debenzoyl-2-(*p*-fluorobenzoyl)-paclitaxel (9.28).** To a solution of **9.14** (19 mg, 0.026 mmol) in THF (1 mL) at -20 °C was added LHMDS (2.5 M in THF, 40 μl) and the mixture was stirred for 10 min. A THF solution of β-lactam **9.12a** (0.5 mL, 13 mg, 0.031 mmol) was then added slowly. The reaction mixture was stirred for 4 h till TLC showed the complete reaction of the starting material. Then 1 mL of saturated aqueous NH<sub>4</sub>Cl was added and the mixture was extracted with EtOAc. The organic layer was washed with water and brine and then dried under reduced pressure. The crude reaction product was

purified on preparative TLC (developed with EtOAc:hexane, 4:6) to give the protected labeled paclitaxel **9.28** (14.5 mg, 0.13 mmol) in 54% yield. <sup>1</sup>H NMR δ 8.15 (2H, dd, *J* = 8.5 and 5.0), 7.72 (2H, dd, *J* = 8.0 and 1.5), 7.37 (m, 2H), 7.21-7.16 (7H, overlapped), 6.44 (1H, s), 6.21 (1H, t, *J* = 8.0), 5.68 (1H, d, *J* = 7.0), 5.65 (1H, d, *J* = 7.0), 4.92 (2H, m), 4.48 (1H, dd, *J* = 10.5, *J* = 7.0), 4.27 (1H, d, *J* = 8.5), 4.19 (1H, d, *J* = 8.5), 3.83 (1H, d, *J* = 7.0), 2.53 (1H, m), 2.24 (3H, s), 2.18 (2H, m), 2.05 (3H, s), 1.92 (1H, m), 1.68 (3H, s), 1.21 (3H, s), 1.02 (3H, s), 0.92-0.89 (30H, overlapped), 0.62-0.60 (6H, overlapped); <sup>13</sup>C NMR δ 204.2, 171.4, 171.3, 170.6, 166.1, 165.2, 146.8, 132.8, 132.7, 131.7, 125.8, 125.7, 116.0, 115.8, 84.5, 80.8, 79.1, 76.4, 76.3, 75.2, 72.3, 67.8, 60.4, 58.7, 46.2, 42.7, 38.6, 35.6, 26.9, 22.6, 21.1, 21.0, 20.9, 15.6, 14.2, 11.7, 9.47, 6.9, 5.5 ppm; HRFABMS *m/z* = 1143.5867 (M+H)<sup>+</sup>, calculated for C<sub>63</sub>H<sub>84</sub>D<sub>3</sub>FNO<sub>14</sub>Si<sub>2</sub> *m/z* = 1143.5888, Δ = -1.9 ppm.

**3'-(*p*-Trideuteromethylphenyl)-2-debenzoyl-2-(*p*-fluorobenzoyl)-paclitaxel (**9.11**).**

To a solution **9.26** (11 mg, 0.013 mmol) in THF (1.0 mL) was added HF/pyridine (70 wt%, 1.5 mL, large excess) and the solution was stirred at room temperature for 3 h. The reaction mixture was diluted with EtOAc and washed with aqueous NaHCO<sub>3</sub> solution. The organic layer was washed with water and brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated under reduced pressure. The residue was purified by preparative TLC (EtOAc:hexane, 1:4) to afford the desired product (**9.11**, 8.5 mg, 87%). <sup>1</sup>H NMR δ 8.16 (2H, dd, *J* = 8.5 and 5.0), 7.70 (2H, dd, *J* = 8.0 and 1.5), 7.47 (m, 1H), 7.38 (4H, m), 7.22-7.16 (4H, overlapped), 6.88 (1H, d, *J* = 8.5), 6.26 (1H, s), 6.24 (1H, t, *J* = 8.0), 5.76 (1H, dd, *J* = 7.0 and 2.5), 5.64 (1H, d, *J* = 7.0), 4.94 (1H, dd, *J* = 9.0 and 2.0), 4.78 (1H, s),

4.41 (1H, m), 4.28 (1H, d,  $J = 8.5$ ), 4.18 (1H, d,  $J = 8.5$ ), 3.80 (1H, d,  $J = 7.0$ ), 3.51 (1H, m, br), 2.55 (1H, m), 2.44-2.40 (2H, m), 2.38 (3H, s), 2.24 (3H, s), 2.18 (2H, m), 1.92 (1H, m), 1.81(3H, s), 1.63(3H, s), 1.13(3H, s), 1.06(3H, s).  $^{13}\text{C}$  NMR  $\delta$  203.7, 173.0, 171.4, 167.3, 166.1, 142.7, 133.8, 133.1, 133.0, 132.9, 132.1, 129.9, 128.8, 127.1, 126.9, 116.1, 116.0, 84.5, 81.2, 79.2, 75.6, 75.2, 73.2, 72.4, 72.2, 68.1, 58.7, 54.8, 45.6, 43.2, 35.8, 35.6, 26.9, 22.7, 20.9, 14.9, 9.65 ppm; HRFABMS  $m/z = 911.3443$  (M+Na) $^+$ , calculated for  $\text{C}_{48}\text{H}_{49}\text{D}_3\text{FNO}_{14}\text{Na}$ ,  $m/z = 911.3459$ ,  $\Delta = -1.8$  ppm.

**(3R,4S)-1-N-(*p*-trideuteromethylbenzoyl)-3-triisopropylsiloxy-4-azetidin-2-one**

**(9.13b).** *p*-trideuteromethylbenzoic acid **9.15** (90 mg, 0.64 mmol) was added to oxalyl chloride (300  $\mu\text{L}$ , large excess) and the mixture was stirred for 2 h and distilled at 40°C. The residue was dissolved in 1 mL of anhydrous  $\text{CH}_2\text{Cl}_2$  and slowly added to a solution of (3R,4S)-3-triisopropylsiloxy-4-azetidin-2-one **9.28** (57 mg, 0.176 mmol) and  $\text{NEt}_3$  (8  $\mu\text{l}$ ) in 1 mL anhydrous  $\text{CH}_2\text{Cl}_2$ . The mixture was stirred at room temperature for 4 h and diluted with EtOAc (20 mL), and the organic layer was washed with saturated aqueous  $\text{NaHCO}_3$  and brine, and dried over  $\text{Na}_2\text{SO}_4$ . The crude product was purified by TLC (15% EtOAc in hexane) to give the  $\beta$ -lactam **9.11** (76 mg, 0.173 mmol, 97% yield from **9.28**).  $[\alpha]_{\text{D}}^{23} = +99.5^\circ$  ( $\text{CHCl}_3$ ,  $c = 0.19$ );  $^1\text{H}$  NMR  $\delta$  7.96 (dd, 2H,  $J = 7.0$  and 2.0), 7.48 (2H, dd,  $J = 8.5$  and 1.5), 7.34 (2H, t,  $J = 7.0$ ), 7.30-7.25 (3H, overlapped), 5.45 (1H, d,  $J = 6.0$ ), 5.26 (1H, d,  $J = 6.0$ ), 0.98-0.88 (overlapped, 21H);  $^{13}\text{C}$  NMR  $\delta$  168.35, 165.7, 138.0, 133.3, 132.4, 131.0, 129.9, 128.9, 128.3, 128.2, 76.6, 61.2, 17.5, 17.4, 11.7; HRFABMS  $m/z = 441.2655$  (M+H) $^+$ , calculated for  $\text{C}_{26}\text{H}_{33}\text{D}_3\text{NO}_3\text{Si}$   $m/z = 441.2653$ ,  $\Delta = 0.4$  ppm.

**2'-O-(triisopropyl)-3'-N-(p-trideuteromethylbenzoyl)-7-O-triethylsilyl-2-debenzoyl-2-(p-fluorobenzoyl)-paclitaxel (9.29).** To a solution of compound **9.13** (9.5 mg, 0.012 mmol) in THF (1 mL) at -20 °C was added LHMDS (2.5 M in THF, 40  $\mu$ l) and stirred for 10 min, then 0.5 mL THF solution of  $\beta$ -lactam **9.1b** (15 mg, 0.033 mmol) was added slowly. The reaction mixture was stirred for 3 h till TLC showed the complete reaction of the starting material **9.14**. Then 1 mL of saturated aqueous  $\text{NH}_4\text{Cl}$  was added and the mixture was extracted with EtOAc. The organic layer was washed with water and brine and then dried under reduced pressure. The crude reaction product was purified by preparative TLC (developed with EtOAc:hexane, 4:6) to give the protected labeled paclitaxel **9.30** (9.0 mg, 0.0082 mmol, 67%).  $^1\text{H}$  NMR  $\delta$  8.18 (2H, dd,  $J = 8.5$  and  $5.0$ ), 7.62 (2H, dd,  $J = 7.0$  and  $1.5$ ), 7.37-7.31 (overlapped, 5H), 7.20-7.06 (4H, overlapped), 7.07 (1H, d,  $J = 8.5$ ), 6.44 (1H, s), 6.22 (1H, t,  $J = 8.0$ ), 5.74 (1H, dd,  $J = 7.0$  and  $2.5$ ), 5.66 (1H, d,  $J = 7.0$ ), 4.94 (2H overlapped), 4.48 (1H, dd,  $J = 6.0$  and  $2.0$ ), 4.28 (1H, d,  $J = 8.5$ ), 4.20 (1H, d,  $J = 8.5$ ), 3.82 (1H, d,  $J = 7.0$ ), 3.55 (1H, d,  $J = 5.0$ ), 2.55 (1H, m), 2.44-2.40 (2H, m), 2.38 (3H, s), 2.21 (3H, s), 2.18 (2H, m), 1.92 (1H, m), 1.80 (3H, s), 1.64 (3H, s), 1.24 (3H, s), 1.12 (3H, s);  $^{13}\text{C}$  NMR  $\delta$  203.8, 173.0, 171.3, 170.6, 166.1, 165.2, 146.8, 132.8, 132.7, 131.7, 125.8, 125.7, 116.0, 115.8, 84.5, 80.8, 79.1, 76.4, 76.3, 75.2, 72.3, 67.8, 60.4, 58.7, 46.2, 42.7, 38.6, 35.6, 26.9, 22.6, 21.1, 21.0, 20.9, 15.6, 14.2, 11.7, 9.47, 7.4, 5.8; HRFABMS  $m/z = 1143.5867$  (M+H) $^+$ , calculated for  $\text{C}_{63}\text{H}_{84}\text{D}_3\text{FNO}_{14}\text{Si}_2$   $m/z = 1143.5888$ ,  $\Delta = -1.9$  ppm.

**3'-N-(p-trideuteromethylbenzoyl)-2-debenzoyl-2-(p-fluorobenzoyl)-paclitaxel (9.12).** Compound **9.30** (8.5 mg) was treated with HF/Py (0.5 mL, 70% wt, large excess)

overnight to yield **9.12** (5.5 mg, 0.0063 mmol, 84%) as described for compound **9.11**.  $^1\text{H}$  NMR  $\delta$  8.15 (2H, dd,  $J = 6.5$  and  $3.0$ ), 7.61 (2H, dd,  $J = 6.5$  and  $1.5$ ), 7.46 (2H, d,  $J = 7.0$ ), 7.42 (2H, t,  $J = 7.0$ ), 7.35 (1H, m), 7.20-7.16 (4H, overlapped), 6.92 (1H, d,  $J = 8.5$ ), 6.26 (1H, s), 6.24 (1H, t,  $J = 7.0$ ), 5.79 (1H, dd,  $J = 8.5$  and  $2.5$ ), 5.65 (1H, d,  $J = 7.0$ ), 4.94 (1H, dd,  $J = 7.5$  and  $2.0$ ), 4.80 (1H, m), 4.40 (1H, m), 4.28 (1H, d,  $J = 8.5$ ), 4.19 (1H, d,  $J = 8.5$ ), 3.78 (1H, d,  $J = 7.0$ ), 3.56 (1H, d,  $J = 5.0$ ), 2.55 (1H, m), 2.44-2.40 (2H, m), 2.38 (3H, s), 2.21 (3H, s), 2.18 (2H, m), 1.92 (1H, m), 1.80 (3H, s), 1.64 (3H, s), 1.24 (3H, s), 1.12 (3H, s);  $^{13}\text{C}$  NMR  $\delta$  203.8, 173.0, 171.4, 170.4, 166.1, 165.3, 142.1, 138.1, 133.2, 133.0, 132.9, 130.8, 129.4, 129.14, 129.12, 128.5, 127.1, 127.0, 125.6, 116.2, 116.0, 84.5, 81.2, 79.5, 79.2, 76.6, 76.5, 75.6, 75.2, 73.2, 72.4, 72.2, 58.7, 54.9, 45.6, 43.2, 35.8, 35.6, 30.0, 26.9, 22.7, 22.0, 20.9, 19.14, 19.10, 16.8, 14.2, 11.6, 9.5 ppm; HRFABMS  $m/z = 889.35785$  (M+H) $^+$ , calculated for  $\text{C}_{48}\text{H}_{49}\text{D}_3\text{FNO}_{14}$   $m/z = 889.3639$ ,  $\Delta = -6.1$  ppm.

#### IX.4.4 Bioassay Results.

Both labeled compounds were tested for cytotoxicity in the A2780 mammalian cell line with normal paclitaxel as a standard. Compound **9.11** was tested with  $\text{IC}_{50} = 1.10$   $\mu\text{g/mL}$  when paclitaxel was  $\text{IC}_{50} = 0.015$   $\mu\text{g/mL}$ . Compound **9.12** was tested with  $\text{IC}_{50} = 0.092$   $\mu\text{g/mL}$  when paclitaxel was  $\text{IC}_{50} = 0.022$   $\mu\text{g/mL}$ . These data indicated that **9.12** was a suitable substrate for REDOR NMR studies, but that compound **9.11** was not active enough for these studies.

## Chapter X. Study of the Chemistry of A-nor-Paclitaxel Analogs

### X.1 Introduction to A-nor-Paclitaxel Analogs.

A-nor-paclitaxel (**10.1**) is an A-ring contracted paclitaxel analog that was first reported in 1991.<sup>174</sup> These studies indicated that paclitaxel (**10.2**) could undergo rearrangement under acidic conditions to give the ring contracted product A-nor-paclitaxel, possibly via a cyclopropane intermediate.<sup>175</sup> Biological studies revealed that A-nor-paclitaxel was much less cytotoxic than paclitaxel towards the KB cell line, but that it still had tubulin assembly ability at a level about one third as great as paclitaxel's.<sup>176</sup> A molecular modeling study showed that the rearranged A-nor-baccatin core of A-nor-paclitaxel has a similar "inverted cup-shape" conformation to that the baccatin core of paclitaxel. A number of A-nor-paclitaxel analogs with modifications on the C-1 isopropenyl moiety were prepared, and some of them showed enhanced tubulin binding activities, in some cases to the same level as that of paclitaxel.<sup>177</sup> However, none of them showed significant cytotoxicity to the KB cell line. Interestingly, unlike paclitaxel, where some modifications on the C-2 benzoyl group could increase tubulin binding activity,<sup>178</sup> the same modifications on the C-2 benzoyl group of A-nor-paclitaxel uniformly decreased tubulin binding activity.

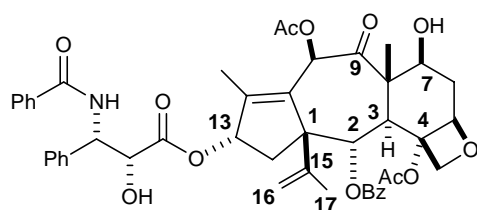
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<sup>175</sup> Samaranyake, G.; Magri, N. F.; Jitrangsi, C. and Kingston, D. G. I., Modified taxols: Reaction of taxol with electrophilic reagents and preparation of a rearranged taxol derivative with tubulin assembly activity., *J. Org. Chem.* **1991**, *56*, 5114-19.

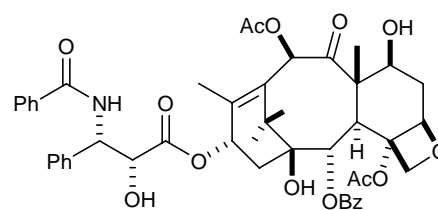
<sup>176</sup> Yuan, H.; Kingston, D. G. I.; Long, B. H.; Fairchild, C. A. and Johnston, K. A. Synthesis and biological evaluation of C-1 and ring modified A-nor-paclitaxels. *Tetrahedron* **1999**, *55*, 9089-9100.

<sup>177</sup> Chordia, M. D.; Kingston, D. G. I.; Hamel, E.; Lin, C. M.; Long, B. H.; Fairchild, C. A.; Johnston, K. A. and Rose, W. C. Synthesis and biological activity of A-nor-paclitaxel analogs. *Bioorg. & Med. Chem.* **1997**, *5*, 941-947.

<sup>178</sup> Kingston, D. G. I.; Chaudhary, A. G.; Chordia, M. D.; Gharpure, M.; Gunatilaka, A. A. L.; Higgs, P. I.; Rimoldi, J. M.; Samala, L.; Jagtap, P. G.; Giannakakou, P.; Jiang, Y. Q.; Lin, C. M.; Hamel, E.; Long, B. H.; Fairchild, C. R.; Johnston, K. A. Synthesis and biological evaluation of 2-acyl analogs of paclitaxel. *J. Med. Chem.* **1998**, *41*, 3715-3726.



10.1 *A-nor*-paclitaxel

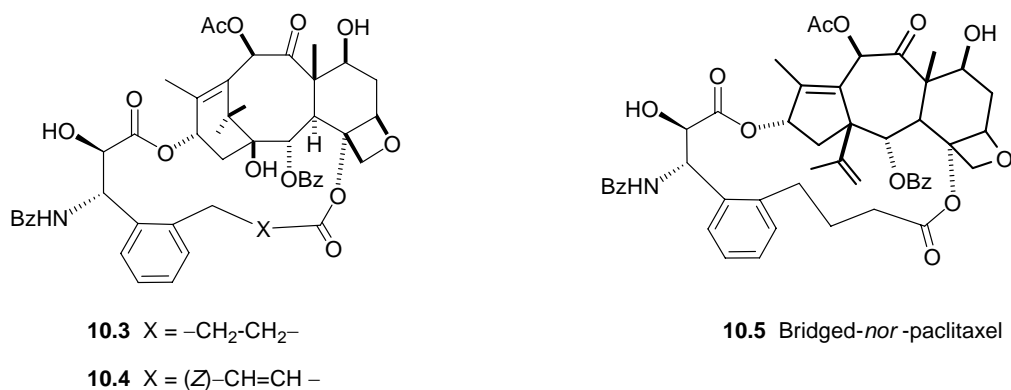


10.2 Paclitaxel

**Figure 10-1** Structure of *A-nor*-paclitaxel and paclitaxel.

Bridged paclitaxel analogs **10.3** and **10.4** were synthesized recently in our group with both enhanced cytotoxicities and tubulin binding activities.<sup>179</sup> A modeling study indicated that the carbon bridge that linked the C-3' phenyl group and the C-4 acetoxyl group helped to lock the conformation of these molecules into the “T-taxol” conformation, which fits well into the taxol-binding site of  $\beta$ -tubulin. Because of the similarity of structure between paclitaxel and *A-nor*-paclitaxel, it was anticipated that an *A-nor*-paclitaxel analog could also be locked into the same “T-taxol” conformation by making a carbon bridge between the C-3' phenyl group *ortho*-position and the C-4 acetoxyl group. Therefore, the bioactivity of *A-nor*-paclitaxel could also be increased as well as its tubulin affinity. For this purpose, a new *A-nor*-paclitaxel analog (**10.5**) was synthesized and its bioactivities were examined.

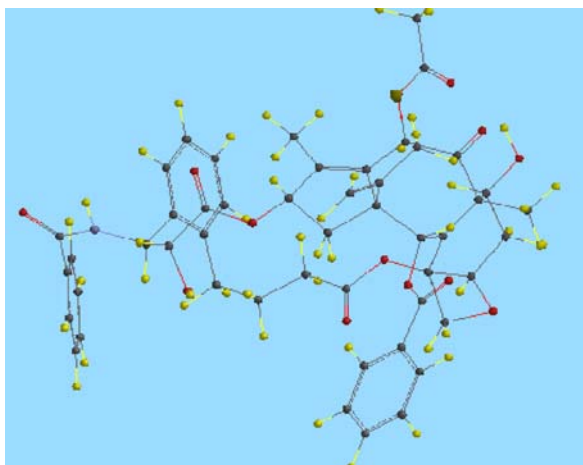
<sup>179</sup> Ganesh, T.; Guza, R. C.; Bane, S.; Ravindra, R.; Shanker, N.; Lakdawala, A. S.; Snyder, J. P.; Kingston, D. G. I. The bioactive taxol conformation on tubulin: Experimental evidence from highly active constrained analogs. *Proc. Nat. Acad. Sci.* **2004**, *101*, 10006-10011



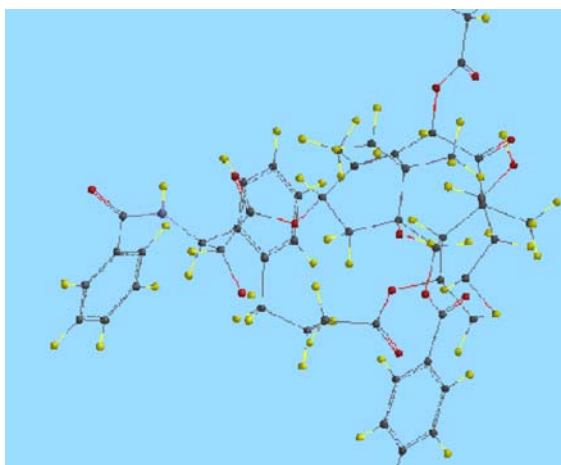
**Figure 10-2.** Structure of bridged-*nor*-paclitaxel analogs

### X.2 Conformation Study of Bridged-*nor*-Paclitaxel.

The conformations of both the bridged paclitaxel **10.3** and the corresponding bridged *A-nor*-paclitaxel **10.5** were compared using the molecular mechanics capability of the Spartan software program. Compound **10.3** was chosen for this comparison because previous work<sup>179</sup> showed it to be the most active of the bridged analogs. The structures were input into the Spartan program, and energy minimization was carried out on each compound separately (Figure 10-3). The structures were then compared by determining several key internuclear distances for each compound (Data listed in Table 10-1).



**10.5** Bridged-*nor*-paclitaxel



**10.3** Bridged-paclitaxel

**Figure 10-3.** Computer model of bridged-*A-nor*-paclitaxel and bridged-paclitaxel.

**Table 10-1.** Comparison of the modeling distance data between **10.3** and **10.5**

Distance between selected atoms ( Å )		bridged- <i>nor</i> -taxol ( <b>10.3</b> )	bridged-taxol ( <b>10.5</b> )
Oxygen on C-13	Oxygen on C-2	4.817	4.639
3'phenyl (C-2'')	C-2 benzoyl (C-1'')	9.004	8.685
3'phenyl (C-1'')	C-2 benzoyl (C-1'')	9.349	8.973
3'phenyl (C-1'')	C-2 benzoyl (C-4'')	10.613	10.194
3'phenyl (C-2'')	C-2 benzoyl (C-4'')	9.236	8.872
Oxygen on C-4	Oxygen on C-5	3.395	3.268
Oxygen on C-7	Oxygen on C-9	3.427	3.194

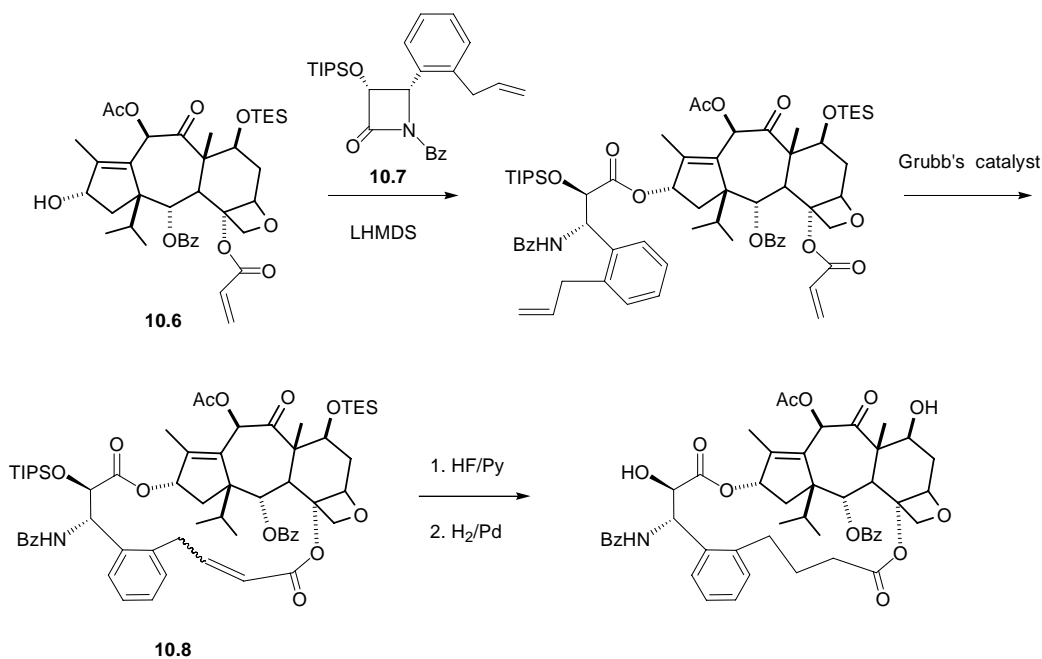
The data listed above suggested that the conformations of these two molecules are very close. Bridged-*A-nor*-taxol (**10.5**) has larger values of the distance between the C-2 benzoyl group and the C-3' phenyl group, but these differences are very slight.

### X.3 Chemical Investigations of Rearranged *A-nor*-Baccatin.

The rearranged diterpenoid core of *A-nor*-paclitaxel is named *A-nor*-baccatin, consistent with its baccatin precursor. It can be easily characterized by its  $^1\text{H}$  NMR spectrum, which reveals two weakly coupled vinyl proton signals at  $\delta$  4.78 (d,  $J = 0.9$ -1.2 Hz) and  $\delta$  4.57 (d,  $J = 0.9$ -1.2 Hz).<sup>180</sup> Also the proton on C-2 is shifted up-field from  $\delta$  5.5 (d,  $J = 8.5$  Hz) to  $\delta$  5.0 (d,  $J = 8.0$  Hz).

Our original synthetic scheme was to synthesize a 4-*O*-deacetyl-4-*O*-acryloyl-*A-nor*-baccatin-III derivative (**10.6**) (Scheme 10-1). Coupling (**10.6**) with a  $\beta$ -lactam (**10.7**) followed by ring-closing metathesis could give the bridged-*nor*-taxol (**10.8**) (Scheme 10-1). It was elected to synthesize the silyl-protected *A-nor*-baccatin precursor (**10.9**) from the starting material 7,10,13-tri-triethylsiloxy-baccatin III (**9.21**) and investigate the chemistry of this molecule.

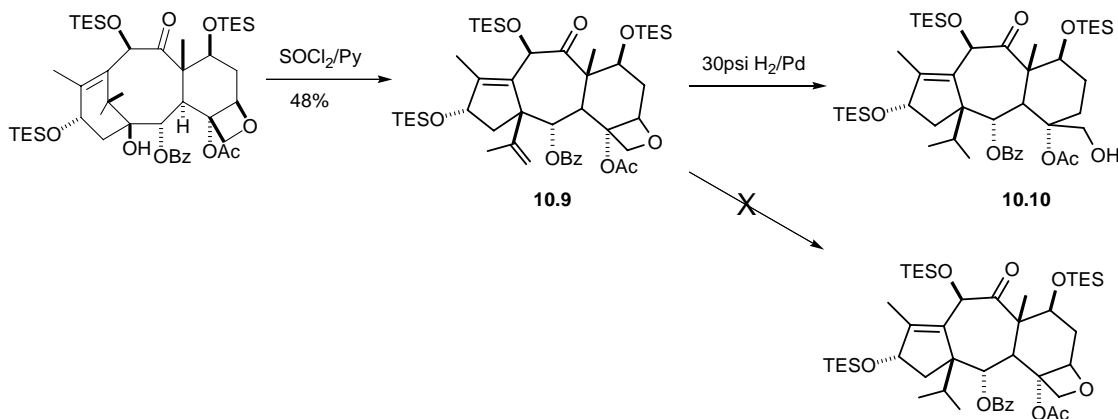
**Scheme 10-1.** Proposed synthesis of bridged-*nor*-taxol



<sup>180</sup> These two signals generally appear as two broad singlets.

Compound **10.9** was synthesized in 48% yield by treatment of 7,10,13-tris(triethylsilyl)-baccatin III (**9.21**) with sulfonyl chloride (Scheme 10-2). Several other Lewis acids have been reported to contract the A-ring, including boron trifluoride, methanesulfonyl chloride, sulfonyl fluoride, etc.<sup>181,182</sup> All these reactions were carried out at low temperature with relatively low yields, ranging from 20% to 50%. Hydrogenation of **10.9** on Pd/C at 30 psi, however, surprisingly gave product **10.10** in which both the isopropenyl group on C-1 and the oxetane ring were hydrogenated. This result indicated that the oxetane-ring of *nor*-baccatin was not as stable as that of baccatin III, since **9.21** was not hydrogenated under same condition.

**Scheme 10-2.** Synthesis of rearranged *nor*-baccatin III



The structure of product **10.10** was determined by  $^1\text{H}$ ,  $^{13}\text{C}$  and 2-D NMR spectroscopy. Its  $^1\text{H}$  NMR spectrum showed the disappearance of the proton signal of H-

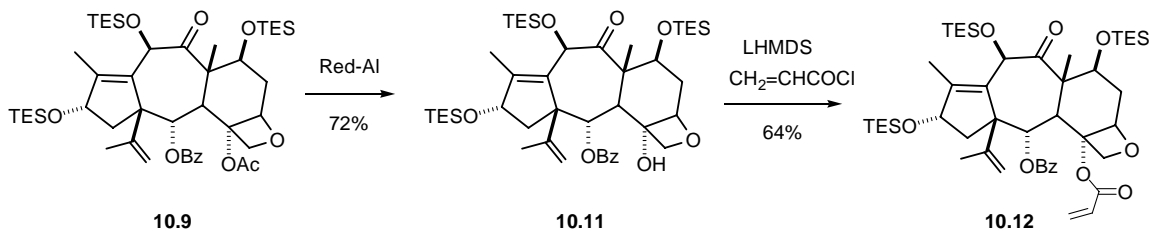
<sup>181</sup> Chen, S. H.; Huang, S.; Wei, J. and Farina, V. Serendipitous synthesis of a cyclopropane-containing taxol analog via anchimeric participation of an unactivated angular methyl group. *J. Org. Chem.* **1993**, 58, 4520-1.

<sup>182</sup> Wahl, A.; Gueritte-Voegelein, F.; Guenard, D. Rearrangement reactions of taxanes: structural modifications of 10-deacetylbaccatin III. *Tetrahedron* **1992**, 48, 6965-74.

5 at  $\delta$  4.88 (dd, 1H,  $J = 9.5, 4.5$  Hz). Also the two signals for the gem-coupled proton ( $H_{22a}, H_{22b}$ ) shifted up-field from  $\delta$  4.30 and  $\delta$  4.12 to  $\delta$  3.96 and 3.84 (d,  $J = 10.4$  Hz), which matched well with the spectra of other oxetane-ring opened taxol analogs.<sup>1,2</sup> HRFABMS gave a molecular ion peak at  $m/z = 873.5115$   $[M+H]^+$  which indicated a formula of  $C_{47}H_{80}O_9Si_3$ , also consistent with a tetra-hydrogenated baccatin structure. The  $\Delta^{11-12}$  double bond was not reduced, as evidenced by the presence of signals for two tertiary  $sp^2$  carbon at  $\delta$  137.4 and 143.2 (C-11, C-12) in the  $^{13}C$  NMR spectrum of **10.10**.

The reactivity of groups on the southern hemisphere of *A-nor*-baccatin is similar to that of baccatin III, as shown by the fact that the C-4 acetyl group of *A-nor*-baccatin **10.9** could be selectively removed by Red-Al and replaced with acryloyl group to yield 4-acryloyl-4-deacetyl-*A-nor*-baccatin III **10.11** (Scheme 10-3).

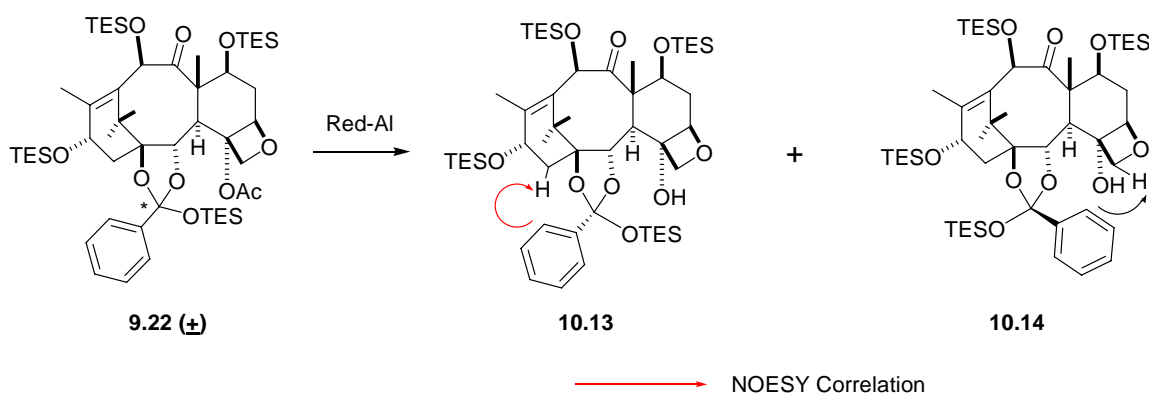
**Scheme 10-3.** Preparation of 4-acryloyl-*A-nor*-baccatin analog (Part 1)



The *ortho*-ester of baccatin III (**9.17**) could also be used to synthesize **10.12** (Scheme 10-4). Compound **9.17** was a diastereomeric mixture which was very hard to separate on silica chromatography. Separation of the two isomers was achieved after deacetylation of C-4 by treatment with Red-Al to give compounds **10.13** and **10.14** in a ratio of about 2:1 and in a combined yield of 72%. Compound **10.13** was assigned as the *R* *ortho*-ester because an NMR experiment showed clear a NOE effect between the *ortho*-

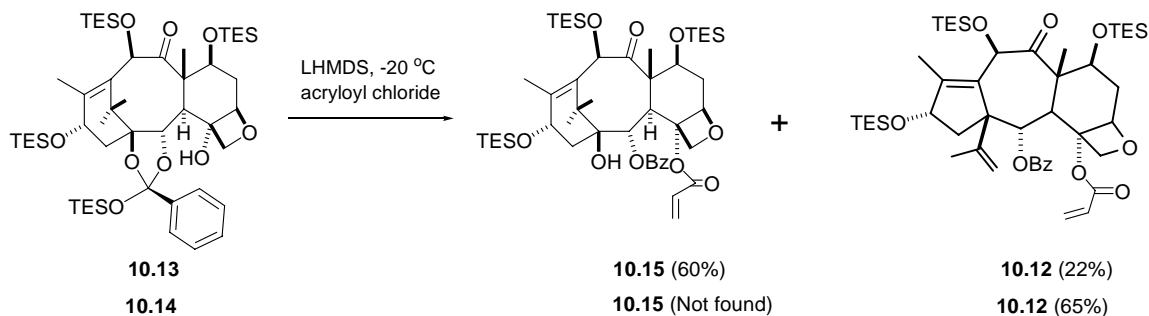
proton of the phenyl ring and the proton of C-14a. Compound **10.14** was assigned as the *S* ortho-ester because an NMR experiment showed a NOE effect between the phenyl *ortho*-proton and the proton of C-20a.

**Scheme 10-4:** Preparation of 4-acryloyl-A-nor-baccatin analog (Part 2)



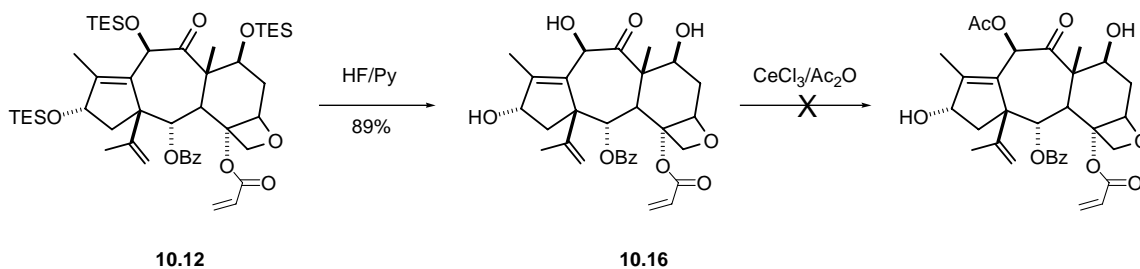
When compound **10.13** was treated with LHMDS and acryloyl chloride, the 4-*O*-deacetyl-4-*O*-acryloyl-baccatin III analog **10.15** was formed as the major product and the *nor*-baccatin analog **10.12** was obtained as a byproduct. Compound **10.14**, however, gave mainly the *nor*-baccatin **10.12** under same conditions (Scheme 10-5).

**Scheme 10-5.** Preparation of 4-acryloyl-A-nor-baccatin analog (Part 3)



The silyl groups of **10.12** were deprotected with hydrogen fluoride to give the product **10.16**. When **10.16** was treated with cerium (III) chloride and acetic anhydride in anhydrous THF,<sup>183</sup> no acetylation reaction was observed over 4 h (Scheme 10-6). A modeling study with Spartan indicated that the distance between the two oxygen atoms at C-7 and C-9 increased from 3.194 to 3.427 Å (Table 10-1). It thus appears that these two oxygen atoms are too far apart to chelate easily with Ce<sup>3+</sup> to activate the C-10 hydroxyl group. Due to the problems of the instability of the oxetane ring of *nor*-baccatin and the difficulty in achieving selective acylation of the C-10 hydroxyl group, the synthesis Scheme 10-1 was abandoned. The new Scheme 10-7 was adopted, in which bridged A-*nor*-paclitaxel **10.5** was prepared directly from a bridged paclitaxel analog.

**Scheme 10-6.** Preparation of 4-acryloyl-A-*nor*-baccatin analog (Part 4)

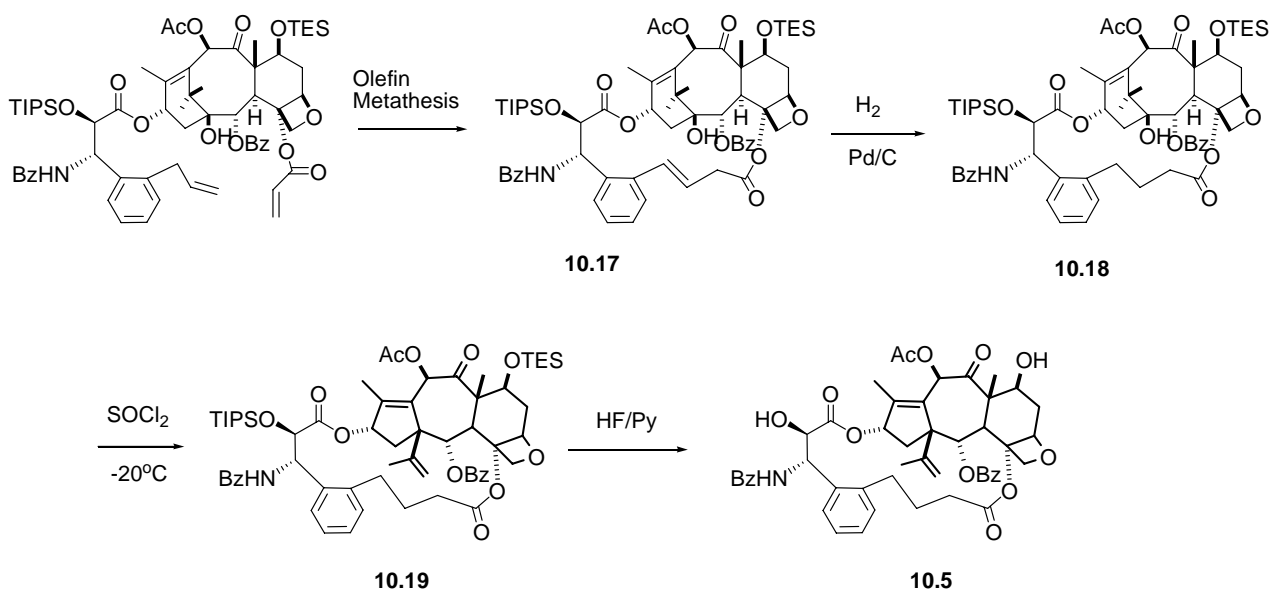


<sup>183</sup> These conditions have been used frequently for acetylation on the C-10 position of 10-deacetyl baccatin III analogs, and usually give a rapid and high-yielding reaction.

#### X.4 Synthesis of The Bridged A-nor-Paclitaxel.

The bridged A-nor-paclitaxel was synthesized directly from the *trans*-alkene-bridged-paclitaxel analog **10.17** (prepared by Mr. Chao Yang in our group).<sup>184</sup> Compound **10.17** was hydrogenated to yield compound **10.18**. Thionyl chloride-mediated ring contraction gave **10.19** in 46% yield. After silyl-deprotection, the bridged A-nor-paclitaxel analog **10.5** was formed.

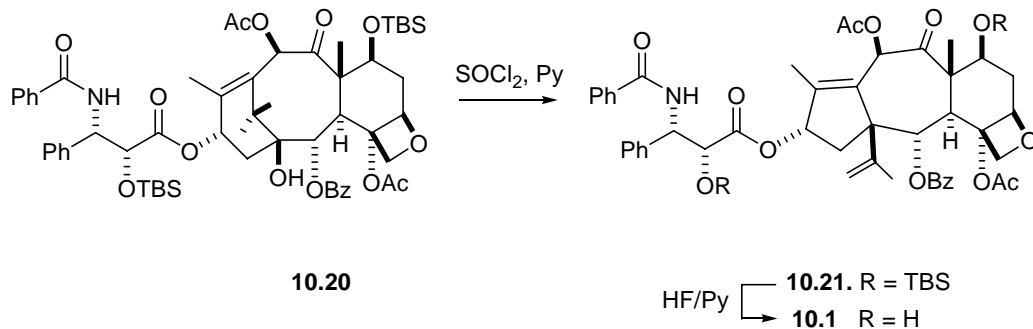
**Scheme 10-7.** Preparation of bridged A-nor-paclitaxel.



A-nor-paclitaxel **10.1** was also prepared for comparison purposes from a paclitaxel analog **10.20** via the reported method (Scheme 10-8).<sup>2</sup> It was also tested in a few cell lines to compare with the activities of compound **10.5**.

<sup>184</sup> The product **10.17** was formed as a mixture of *cis* and *trans*-alkenes. These were hydrogenated together to give **10.18**.

**Scheme 10-8.** Preparation of A-nor-paclitaxel



**X.5 Bioactivities of The Bridged A-nor-Paclitaxel.**

The bridged A-nor-paclitaxel analog **10.5** was tested together with the bridged A-nor-paclitaxel **10.5** as well as the unbridged A-nor-paclitaxel **10.1** in the A2780 cytotoxicity assay and the tubulin-binding assay. Both assays employed paclitaxel **10.2** as standard. The bioactivities were listed in Table 10-2.

**Table 10-2.** Bioassay results of A-nor-paclitaxel and paclitaxel analogs

	A2780 cytotoxicity assay (nM)	Tubulin-binding assay (μM)
Bridged A-nor-paclitaxel <b>10.5</b>	89 ± 9	0.90 ± 0.10
Bridged-paclitaxel <b>10.3</b>	0.56 ± 0.05	0.64 ± 0.08
Paclitaxel <b>10.2</b>	2.4 ± 0.2	1.77 ± 0.20
A-nor-paclitaxel <b>10.1</b>	> 2000 (NA)	5.4 ± 0.6

The bridged *A-nor*-paclitaxel analog **10.5** showed good cytotoxic activity in the A2780 cell line ( $IC_{50} = 89$  nM), which made it about 37 times less active than paclitaxel ( $IC_{50} = 2.3$  nM). In contrast, the unbridged *A-nor*-paclitaxel **10.1** was much less cytotoxic, with  $IC_{50} > 2000$  nM in the A2780 assay. The bridged *A-nor*-paclitaxel **10.5** also showed better tubulin binding activity ( $IC_{50} = 0.90$   $\mu$ M) than both paclitaxel **10.2** ( $IC_{50} = 1.77$   $\mu$ M) and the unbridged *A-nor*-paclitaxel **10.1** ( $IC_{50} = 5.4$   $\mu$ M) in a tubulin assembly assay. It was only slightly less active than the best bridged-paclitaxel analog **10.3** ( $IC_{50} = 0.64$   $\mu$ M). These results indicated that a bridged *A-nor*-paclitaxel which can maintain a “T-taxol” conformation also retains all of paclitaxel’s tubulin-assembly activity and much of its cytotoxicity. This work offers further evidence for the significance of the T-taxol conformation for tubulin binding and tubulin assembly.

## **X.6 Experimental Section.**

**General Experimental Methods.** Unless otherwise specified, all the reagents and materials were from Aldrich Chemical Company. Anhydrous tetrahydrofuran (THF) was distilled from sodium/benzophenone under nitrogen. Anhydrous  $CH_2Cl_2$  (DCM) was distilled from calcium hydride. Analytical thin layer chromatography (TLC) plates (silica gel 60 GF, with aluminum support) from E. Merck were used for monitoring progress of a reaction and visualized with 254 nm UV light with vanillin/sulfuric acid spray or phosphomolybdic acid/ethanol spray. Silica gel for column chromatography was purchased from E. Merck (230–400 mesh). Preparative thin layer chromatography (PTLC) plates (silica gel 60 GF) were purchased from Analtech.  $^1H$  and  $^{13}C$  NMR spectra were obtained on Varian Unity or Inova 400 MHz spectrometer in  $CDCl_3$  at 399.951 MHz

frequency. Chemical shifts are reported as  $\delta$ -values relative to tetramethylsilane (TMS) as internal reference and  $J$  values are reported in Hertz. High Resolution Fast Atom Bombardment mass spectra (HRFABMS) were obtained by Analytical Services in the Department of Chemistry at Virginia Polytechnic and State University.

**Bioassay for the tubulin-binding activities of paclitaxel analogs.** Bioassay was carried out by Dr. Ravindra in Dr. Susan Bane's group at the State University of New York at Binghamton via previously reported methods.<sup>175-176</sup>

**15(16)-Anhydro-10-deacetyl-7,10,13-tris(triethylsiloxy)-11(15→1)-abeo-baccatin III (10.9).** 7,10,13-Tris(triethylsilyl)-10-deacetyl-baccatin III **9.21**<sup>185</sup> (250 mg) and pyridine (0.25 mL) were dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (10 mL). The mixture was cooled to -20°C, and thionyl chloride (145  $\mu$ L) was added with stirring. After half an hour, the reaction was quenched with saturated NaHCO<sub>3</sub> and extracted with EtOAc. The organic phase was dried under vacuum and the residue was separated on silica chromatography with 5% EtOAc in hexane to give compound **10.9** as a white solid (117 mg, 48% yield). <sup>1</sup>H NMR  $\delta$  8.00 (dd, 2H,  $J$  = 8.0 and 1.5), 7.57 (1H, t,  $J$  = 8.0), 7.42 (2H, t,  $J$  = 8.0), 5.57 (1H, d,  $J$  = 7.6), 5.26 (1H, s), 5.01 (1H, d,  $J$  = 8.4), 4.90 (1H, d,  $J$  = 2.0), 4.63 (1H, d,  $J$  = 2.0), 4.55 (1H, t,  $J$  = 7.2), 4.47 (1H, dd,  $J$  = 9.6 and 2.4), 4.24 (1H, d,  $J$  = 8.0), 4.18 (1H, d,  $J$  = 8.0), 3.54 (1H, d,  $J$  = 7.6), 2.58 (1H, ddd,  $J_1$  = 16.0,  $J_2$  = 7.2 and  $J_3$  = 1.6), 2.27 (1H, m), 2.23 (3H, s), 1.94-1.82 (2H, m), 1.79 (3H, s), 1.73 (3H, s), 1.63 (3H, s), 1.00-0.93 (27H, 9CH<sub>3</sub>), 0.72-0.57 (18H, 9CH<sub>2</sub>); <sup>13</sup>C-NMR  $\delta$  207.3, 170.2, 165.4, 146.2, 145.9,

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<sup>185</sup> See chapter 9 for preparation details.

137.8, 133.5, 130.0, 129.9, 128.7, 112.1, 84.8, 79.0, 77.0, 76.1, 74.8, 73.9, 72.6, 71.4, 63.5, 56.7, 44.2, 42.8, 38.5, 22.0, 21.3, 11.6, 9.6, 7.23, 7.18, 7.0, 6.11, 5.69, 4.99; HRFABMS  $m/z = 869.4861$   $[M+H]^+$  calculated for  $C_{47}H_{77}O_9Si_3$  869.4875 ( $\Delta = -1.7$  ppm).

**15(16)-Anhydro-10-deacetyl-7,10,13-tris(triethylsiloxy)-(4,5,15,16)-tetrahydro-11**

**(15→1) abeo-baccatin III (10.10).** Compound **10.9** (50 mg) was dissolved in THF (10 mL) and Pd/C (10% wt, 50 mg) was added. The mixture was then hydrogenated at 30 psi for 12 hrs. After filtration through Celite, the filtrate was evaporated and subjected to PTLC on silica gel developed with 10% EtOAc in hexane to give compound **10.10** (23 mg, 46%) as a white solid.  $^1H$  NMR  $\delta$  7.99 (2H, d,  $J = 7.8$ ), 7.70 (2H, d,  $J = 7.0$ ), 7.54 (1H, t,  $J = 7.2$ ), 5.27 (1H, s), 5.12 (1H, m), 5.03 (1H, d,  $J = 6.8$ ), 4.28 (1H, dd,  $J = 11.2$  and 4.4), 3.63 (1H,  $J = 6.8$ ), 3.49 (1H, d,  $J = 10.4$ ), 3.37 (1H, d,  $J = 10.4$ ), 3.21 (1H, br, s), 2.79 (1H, m), 2.35 (1H, m), 2.24 (1H, m), 2.10 (3H, s), 1.96 (1H, m), 1.91 (3H, s), 1.84-1.73 (2H, overlapped, m), 1.28 (3H, s), 1.00-0.92 (21H, overlapped), 0.79 (3H, d,  $J = 6.4$ ), 0.70-0.53 (18H, overlapped);  $^{13}C$  NMR  $\delta$  208.0, 170.5, 166.5, 142.6, 137.2, 133.1, 130.7, 130.2, 128.5, 77.0, 75.1, 73.6, 72.9, 71.7, 71.2, 66.8, 62.8, 58.3, 44.1, 37.4, 36.0, 33.9, 25.4, 21.3, 19.2, 14.5, 11.9, 7.29, 7.15, 6.63, 5.99, 5.68, 4.14; HRFABMS  $m/z = 873.5115$   $[M+H]^+$  calculated for  $C_{48}H_{85}O_9Si_3$  873.5110 ( $\Delta = 0.5$  ppm).

**15(16)-Anhydro-4,10-di-deacetyl-7,10,13-tris(triethylsiloxy)-11(15→1)-abeo-**

**baccatin III (10.11).** To a solution of **10.9** (70 mg, 0.085 mmol) in anhydrous THF (10 mL) at  $-20^\circ C$ , Red-Al (0.18 mL) was added dropwise under nitrogen. The reaction was stirred for 30 min until TLC showed the exhaustion of starting material. After quenching

with a few drops of water, 1M sodium potassium tartate (10 mL) was added. The mixture was stirred for 0.5h before it was extracted with EtOAc. The combined organic phase was washed with water and brine, and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on silica gel with elution with EtOAc:hexane, 1:4 gave compound **10.11** (43 mg, 61%) as a colorless gum. <sup>1</sup>H NMR δ 8.03 (dd, 2H, *J* = 7.6 and 1.2), 7.57 (1H, t, *J* = 7.6), 7.42 (2H, t, *J* = 7.6), 5.27 (1H, s), 5.13 (1H, m), 5.03 (1H, d, *J* = 6.8), 4.28 (1H, dd, *J* = 11.2 and 4.4), 3.64 (1H, d, *J* = 6.8), 4.18 (1H, d, *J* = 8.0), 3.54 (1H, d, *J* = 7.6), 2.58 (1H, ddd, *J*<sub>1</sub> = 16.0, *J*<sub>2</sub> = 7.2 and *J*<sub>3</sub> = 1.6), 2.27 (1H, m), 2.23 (3H, s), 1.94-1.82 (2H, m), 1.79 (3H, s), 1.73 (3H, s), 1.63 (3H, s), 1.00-0.93 (27H, 9CH<sub>3</sub>), 0.72-0.57 (18H, 6CH<sub>2</sub>Si); <sup>13</sup>C NMR δ 207.0, 165.1, 145.7, 138.3, 133.3, 129.7, 129.6, 128.7, 128.6, 128.5, 127.7, 127.0, 111.7, 87.3, 78.3, 76.7, 73.7, 72.9, 71.7, 63.4, 56.6, 48.7, 42.0, 38.2, 21.0, 11.5, 9.47, 6.97, 6.93, 6.73, 5.72, 5.34, 5.23, 4.70; HRFABMS *m/z* = 827.4736 [M+H]<sup>+</sup> calculated for C<sub>45</sub>H<sub>75</sub>O<sub>8</sub>Si<sub>3</sub> 827.4770 (Δ = -4.2 ppm).

**15(16)-Anhydro-4,10-di-deacetyl-4-acryloyl-7,10,13-tris(triethylsiloxy)-11(15→1)-nor-baccatin III (10.12).** To a solution of **10.11** (41 mg, 0.048 mmol) in THF (2 mL) at -20°C was added LHMDS (40 μL, 2.5 M in THF) and the mixture was stirred for 10 min. Acryloyl chloride (67 μL, 0.1 mmol) was then added. The reaction mixture was stirred for 1 h before being quenched with 2 mL of saturated aqueous NH<sub>4</sub>Cl. The mixture was extracted with EtOAc, and the organic layer was washed with water and brine and then dried with Na<sub>2</sub>SO<sub>4</sub>. The product was purified on preparative TLC (4:6 = EtOAc:hexanes) to give compound **10.12** (25 mg, 0.028 mmol, 63%) as a white solid. <sup>1</sup>H NMR δ 8.04 (dd, 2H, *J* = 8.0 and 1.5), 7.64 (1H, t, *J* = 8.0), 7.51 (2H, t, *J* = 8.0), 6.51 (1H, dd, *J* = 17.2

and 1.0), 6.17 (1H, dd,  $J = 17.2$  and  $10.4$ ), 6.01 (1H, dd, 1H, dd,  $J = 10.4$  and  $1.0$ ), 5.74 (1H, d,  $J = 8.0$ ), 5.02 (1H, d,  $J = 8.4$ ), 4.79 (1H, d,  $J = 1.2$ ), 4.67 (1H, d,  $J = 1.2$ ), 4.54 (1H, t,  $J = 7.2$ ), 4.37 (1H, d,  $J = 8.0$ ), 4.32 (1H, d,  $J = 8.0$ ), 3.53 (1H, d,  $J = 8.4$ ), 2.55 (1H, ddd,  $J_1 = 15.2$ ,  $J_2 = 7.8$  and  $J_3 = 1.6$ ), 2.21 (1H, dd,  $J = 15.2$  and  $8.0$ ), 2.05 (1H, m), 1.98 (3H, s), 1.91 (1H, m), 1.68 (3H, s), 1.11 (3H, s), 1.01-0.94 (27H, 9CH<sub>3</sub>), 0.72-0.58 (18H, 6CH<sub>2</sub>Si); <sup>13</sup>C-NMR  $\delta$  207.3, 165.4, 164.7, 146.9, 145.9, 137.3, 133.5, 131.5, 130.0, 129.9, 129.1, 128.7, 112.1, 84.8, 79.4, 76.9, 75.8, 75.1, 73.9, 72.7, 71.5, 63.4, 56.8, 44.0, 42.9, 38.5, 21.3, 11.7, 9.68, 7.27, 7.22, 7.05, 6.15, 5.69, 5.02. HRFABMS  $m/z = 881.4859$  [M+H]<sup>+</sup>, calculated for C<sub>48</sub>H<sub>77</sub>O<sub>9</sub>Si<sub>3</sub> 881.4875 ( $\Delta = -1.7$  ppm)

**(R)-7,10,13,1'-O-Tetra(triethylsilyl)-2-debenzoyl-4,10-di-deacetyl-baccatin III 1,2-semi-orthobenzoate (10.13)** and **(S)-7,10,13,1'-O-Tetra(triethylsilyl)-2-debenzoyl-4,10-di-deacetyl-baccatin III 1,2-semi-orthobenzoate (10.14)**.<sup>186</sup> To the solution of **9.23** (350 mg, 0.35 mmol) in anhydrous THF (20 mL) at -20°C, RedAl<sup>®</sup> (0.8 mL) was added dropwise under nitrogen. The reaction was stirred for 1.5 h before it was quenched with a few drops of water. Then 50 mL of 1M sodium potassium tartate was added and the mixture was extracted with EtOAc. The organic phase was washed with water and brine, and dried over Na<sub>2</sub>SO<sub>4</sub>. Column chromatography on silica gel with elution with EtOAc:hexane, 1:8 gave compounds **10.13** (162 mg, 0.16 mmol) and **10.14** (79 mg, 0.078 mmol). Compound **10.13**: colorless gum. <sup>1</sup>H NMR:  $\delta$  7.41 (2H, dd,  $J = 8.0$  and  $2.4$ ), 7.29-7.24 (3H, m, overlapped), 5.20 (1H, s), 4.85 (1H, dd,  $J = 9.6$  and  $2.4$ ), 4.75 (1H, d,  $J = 8.0$ ), 4.65 (1H, d,  $J = 8.0$ ), 4.46 (1H, d,  $J = 8.0$ ), 4.17 (2H, m), 3.40 (1H, br, s), 3.07

<sup>186</sup> Appendino, G.; Belloro, E.; Jakupovic, S.; Danieli, B.; Jakupovic J. and Bombardelli, E., Synthesis of paclitaxel (docetaxel) / 2-deacetoxytaxinine J dimmers, *Tetrahedron* **1999**, *55*, 6567-6576.

(1H, d,  $J = 5.2$ ), 2.51 (1H, m), 2.19 (1H, dd,  $J = 16.0$  and  $2.8$ ), 2.04 (3H, s), 1.98 (1H, m), 1.92 (3H, s), 1.25 (3H, s);  $^{13}\text{C}$  NMR:  $\delta$  206.7, 142.6, 138.8, 137.3, 127.7, 127.4, 125.4, 116.7, 87.8, 86.5, 79.3, 76.8, 76.5, 73.9, 72.7, 69.4, 60.2, 49.1, 40.1, 37.9, 37.8, 28.8, 18.2, 17.2, 13.9, 10.4, 6.84, 6.67, 6.50, 6.40, 5.75, 5.12, 4.96, 4.35; HRFABMS  $m/z = 958.5589$   $[\text{M}+\text{H}]^+$  calculated for  $\text{C}_{51}\text{H}_{91}\text{O}_9\text{Si}_4$  958.5662 ( $\Delta = -7.6$ ppm). Compound **10.14**: colorless gum.  $^1\text{H}$  NMR:  $\delta$  7.41 (2H, dd,  $J = 8.0$  and  $2.0$  Hz), 7.38-7.25 (3H, m, overlapped), 5.16 (1H, s), 4.85 (1H, dd,  $J = 9.2$  and  $2.0$ ), 4.75 (1H, m), 4.69 (1H, d,  $J = 8.0$ ), 4.63 (1H, d,  $J = 8.0$ ), 4.14 (2H, m), 3.67 (1H, d,  $J = 5.2$ ), 3.41 (1H, br, s), 2.99 (1H, d,  $J = 5.2$ ), 2.62 (1H, m), 2.51 (1H, m), 1.96 (3H, s), 1.52 (3H, s), 1.01-0.94 (27H,  $9\text{CH}_3$ ), 0.72-0.58 (18H,  $6\text{CH}_2\text{Si}$ );  $^{13}\text{C}$  NMR  $\delta$  207.3, 141.8, 138.8, 137.6, 128.5, 128.2, 127.8, 125.9, 125.2, 117.2, 87.1, 85.5, 80.2, 79.3, 76.9, 74.3, 73.2, 69.6, 60.1, 49.1, 40.9, 39.4, 38.4, 28.4, 19.2, 17.2, 10.4, 7.1, 7.0, 6.86, 6.13, 5.54, 5.46, 5.37, 5.31, 5.10, 4.76; HRFABMS  $m/z = 958.5573$   $[\text{M}+\text{H}]^+$ , calculated for  $\text{C}_{51}\text{H}_{91}\text{O}_9\text{Si}_4$  958.5662 ( $\Delta = -9.2$ ppm).

**7,10,13-Tris(triethylsiloxy)-4-deacetyl-4-acryloyl-baccatin (10.15)**. To a solution of **10.13** (142 mg, 0.15 mmol) in THF (8 mL) at  $-20$  °C was added LHMDS (120  $\mu\text{L}$ , 2.5 M in THF). The mixture was stirred for 5 min, and then acryloyl chloride (130  $\mu\text{L}$ , 0.3 mmol) was added. The reaction mixture was stirred for 1 h before being quenched with 2 mL of saturated  $\text{NH}_4\text{Cl}$ . The mixture was then extracted with EtOAc. The organic layer was washed with water and brine and then dried with  $\text{Na}_2\text{SO}_4$ . The product was purified by preparative TLC on silica gel, developed with EtOAc:hexane, 1:9, to give compound **10.15** (84 mg, 0.091 mmol, 60%) as a white solid together with the by-product compound **10.12** (18 mg, 0.02 mmol, 22%). Compound **10.15**:  $^1\text{H}$  NMR:  $\delta$  8.10 (2H, dd,  $J = 8.0$

and 1.2), 7.59 (1H, t,  $J = 8.0$ ), 7.43 (2H, t,  $J = 8.0$ ), 6.52 (1H, dd, 1H, dd,  $J = 16.0$  and 1.0), 6.21 (1H, dd,  $J = 16.0$  and 10.4), 6.00 (1H, dd,  $J = 10.4$  and 1.0), 5.63 (1H, d,  $J = 8.0$ ), 5.21 (1H, s), 4.94 (1H, dd,  $J = 9.6$  and 2.0), 4.84 (1H, t,  $J = 7.2$ ), 4.47 (1H, dd,  $J = 10.8$  and 6.0), 4.34 (1H, d,  $J = 8.0$ ), 4.21 (1H, d,  $J = 8.0$ ), 3.95 (1H, d,  $J = 8.0$ ), 2.55 (1H, m), 2.20 (1H, dd,  $J = 15.2$  and 8.0), 2.05 (1H, m), 1.98 (3H, s), 1.91 (1H, m), 1.68 (3H, s), 1.20 (3H, s), 1.12 (3H, s), 1.01-0.95 (27H, 9CH<sub>3</sub>), 0.70-0.56 (18H, 9CH<sub>2</sub>Si); <sup>13</sup>C NMR:  $\delta$  206.0, 167.4, 164.7, 140.0, 135.7, 133.7, 130.7, 130.2, 130.1, 129.9, 128.7, 84.2, 81.3, 79.8, 76.0, 75.8, 72.9, 68.4, 58.6, 47.1, 43.2, 40.1, 37.6, 26.6, 20.8, 14.9, 10.7, 7.20, 7.17, 7.11, 6.21, 5.47, 5.11. HRFABMS  $m/z = 899.4964$  [M+H]<sup>+</sup>, calculated for C<sub>48</sub>H<sub>79</sub>O<sub>10</sub>Si<sub>3</sub> 899.4981 ( $\Delta = -1.9$  ppm).

**15(16)-Anhydro-4,10-di-deacetyl-4-acryloyl-11(15→1)-abeo-baccatin III (10.16).** To a solution of **10.12** (42 mg, 4.5  $\mu$ mol) in THF (5 mL) at 0 °C was added pyridine (1 mL) and HF/pyridine (100  $\mu$ L, 70 wt%, large excess). The solution was allowed to warm up to room temperature in 1 h and stirred overnight. The reaction mixture was quenched with saturated aqueous NaHCO<sub>3</sub> and extracted with EtOAc. The organic layer was washed with water and brine, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Purification by TLC on silica gel developed with EtOAc: hexane, 2:5, gave compound **10.16** (24 mg, 3.9  $\mu$ mol, 85%) as a white solid. <sup>1</sup>H NMR:  $\delta$  8.04 (dd, 2H,  $J = 8.0$  and 1.5), 7.64 (1H, t,  $J = 8.0$ ), 7.51 (2H, t,  $J = 8.0$ ), 6.51 (1H, dd,  $J = 17.2$  and 1.0), 6.17 (1H, dd,  $J = 17.2$  and 10.4), 6.01 (1H, dd, 1H, dd,  $J = 10.4$  and 1.0), 5.74 (1H, d,  $J = 8.0$ ), 5.02 (1H, d,  $J = 8.4$ ), 4.79 (1H, d,  $J = 1.2$ ), 4.67 (1H, d,  $J = 1.2$ ), 4.54 (1H, t,  $J = 7.2$ ), 4.37 (1H, d,  $J = 8.0$ ), 4.32 (1H, d,  $J = 8.0$ ), 3.53 (1H, d,  $J = 8.4$ ), 2.55 (1H, ddd,  $J_1 = 15.2$ ,  $J_2 = 7.8$  and  $J_3 = 1.6$ ), 2.21 (1H, dd,  $J$

= 15.2 and 8.0), 2.05 (1H, m), 1.98 (3H, s), 1.91 (1H, m), 1.68 (3H, s), 1.11 (3H, s);  $^{13}\text{C}$ -NMR  $\delta$  207.3, 165.4, 164.7, 146.9, 145.9, 137.3, 133.5, 131.5, 130.0, 129.9, 129.1, 128.7, 112.1, 84.8, 79.4, 76.9, 75.8, 75.1, 73.9, 72.7, 71.5, 63.4, 56.8, 44.0, 42.9, 38.5, 21.3. HRFABMS  $m/z = 539.2297$   $[\text{M}+\text{H}]^+$  calculated for  $\text{C}_{30}\text{H}_{35}\text{O}_9$  539.2281 ( $\Delta = +2.6\text{ppm}$ ).

**2'-Triisopropylsiloxy-7-triethylsiloxy-bridged-paclitaxel (10.18).** To a solution of **10.17** (20 mg, 0.017 mmol) in methanol (10 mL) was added Pd/C (10 wt %, 20 mg). Hydrogenation was carried at 40 psi for 12 h. The mixture was filtered through Celite and the filtrate was concentrated *in vacuo*. The residue was subjected to preparative TLC on silica gel with 15% EtOAc in Hexane to yield compound **10.18** (18.5 mg, 0.016 mmol, 92%) as a white solid.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 8.12 (d,  $J = 7.5$ , 2H), 7.74 (d,  $J = 7.2$ , 2H), 7.59 (t,  $J = 6.5$ , 1H), 7.51 (m, 3H), 7.43 (t,  $J = 6.0$ , 2H), 7.31 (m, 1H), 6.99 (m, 2H), 6.93 (d,  $J = 8.0$ , 1H), 6.27 (bs, 2H), 6.13 (d,  $J = 10.0$ , 1H) 5.74 (d,  $J = 7.2$ , 1H), 5.02 (s, 1H), 4.99 (s, 1H), 4.47 (m, 1H), 4.34 (d,  $J = 8.4$ , 1H), 4.31 (m, 1H), 4.24 (d,  $J = 8.4$ , 1H), 4.10 (m, 1H), 3.75 (d,  $J = 7.6$ , 1H), 3.3 (m, 1H), 3.06 (bs, 1H), 2.90 (m, 1H), 2.60 (m, 1H), 2.48-2.30 (m, 2H), 2.26 (m, 1H), 2.24 (s, 3H), 1.90(m, 1H), 1.86 (s, 3H), 1.70 (s, 3H), 1.30 (s, 3H), 1.25 (s, 3H), 0.96-0.88 (30H, overlapped), 0.64-0.61 (6H, overlapped).  $^{13}\text{C}$  NMR : 203.8, 174.3, 172.8, 171.6, 167.2, 155.0, 142.5, 134.1, 133.8, 133.3, 132.1, 130.3, 129.4, 129.0, 127.2, 127.0, 126.0, 121.3, 111.1, 84.8, 81.4, 79.4, 76.69, 76.61, 75.6, 75.5, 73.1, 72.2, 71.4, 65.5, 58.5, 45.6, 43.6, 36.1, 35.6, 31.8, 29.9, 27.3, 24.7, 23.1, 22.5, 21.1, 21.0, 14.5, 11.8, 10.0, 9.5, 6.9, 5.5. HRFABMS:  $m/z = 1150.5665$   $[\text{M}+\text{H}]^+$ , calculated for  $\text{C}_{64}\text{H}_{87}\text{NO}_{14}\text{Si}_2$ :  $m/z = 1150.5743$  ( $\Delta = -7.0\text{ppm}$ ).

**2'-Triisopropylsiloxy-7-triethylsiloxy-bridged-11(15→1)-abeo-paclitaxel (10.19).** To a solution of **10.18** (18 mg) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (5 mL) was added pyridine (0.15 mL). The mixture was cooled to -20 °C and thionyl chloride (53 μL) was added. After stirring for 0.5 h, the reaction was quenched with saturated aqueous NaHCO<sub>3</sub> and extracted with EtOAc. The organic phase was dried under vacuum and the residue was separated by PTLC on silica gel, developed with 8% EtOAc in hexane, to give compound **10.19** (8.5 mg, 49%) as a white solid. <sup>1</sup>H NMR δ 7.99 (2H, d, *J* = 7.8), 7.70 (2H, d, *J* = 7.0), 7.54 (1H, t, *J* = 7.2), 6.30 (1H, s), 5.88 (1H, t, *J* = 8.0), 5.64 (1H, d, *J* = 8.0), 5.50 (1H, d, *J* = 7.2), 4.94 (1H, d, *J* = 8.4), 4.79 (1H, br, s), 4.65 (1H, d, *J* = 2.0), 4.59 (1H, br, s), 4.43 (1H, t, *J* = 8.4), 4.24 (1H, d, *J* = 8.0), 4.13 (1H, d, *J* = 8.0), 3.47 (1H, d, 7.2), 3.21 (1H, m), 2.82 (1H, m), 2.74 (1H, m), 2.64 (1H, m), 2.60- 2.52 (3H, overlapped), 2.08 (3H, s), 1.96 (1H, m), 1.83 (3H, s), 1.61 (3H, s), 1.58 (3H, s), 1.08-0.82 (30H, overlapped), 0.75-0.64 (6H, overlapped); <sup>13</sup>C NMR: δ 201.8, 172.5, 171.3, 169.2, 166.8, 165.7, 145.9, 144.2, 139.5, 138.5, 137.2, 134.3, 133.8, 131.9, 130.5, 130.1, 129.3, 129.0, 128.9, 128.4, 127.2, 127.2, 126.8, 113.5, 84.9, 79.4, 77.9, 75.4, 75.3, 72.9, 71.0, 70.7, 63.5, 57.5, 52.1, 43.7, 38.8, 38.6, 35.4, 33.6, 26.1, 21.0, 20.7, 18.3, 18.1, 17.9, 13.2, 11.6, 9.48, 7.10, 5.5; HRFABMS *m/z* = 1132.5347 [M+H]<sup>+</sup>, calculated for C<sub>64</sub>H<sub>85</sub>NO<sub>13</sub>Si<sub>2</sub> 1132.5438 (Δ = - 7.9ppm).

**Bridged-11(15→1)-abeo-paclitaxel (10.5).** To a solution of **10.19** (8.5 mg, 7.5 μmol) in THF (2.5 mL) at 0 °C was added HF/pyridine (0.10 mL, 70 wt%, large excess) and the solution was allowed to warm to room temperature over 1 hour and then stirred overnight. The reaction mixture was quenched with aqueous NaHCO<sub>3</sub> and extracted with EtOAc.

The organic layer was washed with water and brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and evaporated under reduced pressure. The residue was purified by PTLC on silica gel, developed with EtOAc:hexane, 1:3, to yield **10.5** (5.8 mg, 6.7 μmol, 89%) as a white solid. <sup>1</sup>H NMR δ 8.03 (2H, d, *J* = 8.0), 7.70 (2H, d, *J* = 8.0), 7.53 (1H, t, *J* = 7.6), 7.34 (1H, t, *J* = 7.6), 6.30 (1H, s), 5.88 (1H, t, *J* = 8.0), 5.64 (1H, d, *J* = 7.8), 5.50 (1H, d, *J* = 7.8), 4.94 (1H, d, *J* = 8.0), 4.79 (1H, br, s), 4.65 (1H, d, *J* = 1.0), 4.59 (1H, br, s), 4.43 (1H, t, *J* = 8.4), 4.23 (1H, d, *J* = 8.0), 4.14 (1H, d, *J* = 8.0), 3.48 (1H, d, 7.2), 3.24 (1H, m), 2.82 (1H, m), 2.75 (1H, m), 2.64-2.52 (4H, m), 2.08 (3H, s), 1.97-1.91 (2H, m), 1.83 (3H, s), 1.62 (3H, s), 1.58 (3H, s). <sup>13</sup>C NMR δ 203.5, 173.5, 172.5, 171.6, 166.9, 165.6, 146.2, 144.9, 140.2, 139.1, 135.8, 134.0, 133.8, 132.1, 130.4, 130.3, 129.4, 129.1, 128.8, 128.7, 127.7, 127.4, 113.7, 85.1, 79.1, 78.8, 75.1, 73.2, 72.7, 71.9, 71.0, 64.1, 57.4, 49.7, 44.1, 39.2, 36.4, 35.4, 33.6, 29.9, 26.8, 20.9, 20.7, 11.9, 8.4. HRFABMS *m/z* = 862.3470 [M+H]<sup>+</sup>, calculated for C<sub>49</sub>H<sub>52</sub>NO<sub>13</sub> 862.3434 (Δ = 3.1 ppm).

**2',7-di-*tert*-Butyldimethylsiloxy-paclitaxel (10.20)**. Obtained as a byproduct from the preparation of 2'-*O-tert*-butyldimethylsilyl-paclitaxel by treatment of paclitaxel (150 mg) in DMF at 70 °C with 4 equivalents of *tert*-butyldimethylsilyl chloride and imidazole. Yield **10.20** (28 mg, 16%). <sup>1</sup>H NMR δ 8.08 (dd, 2H, *J* = 8.0 and 1.2), 7.77 (2H, d, *J* = 7.0), 7.58 (1H, t, *J* = 7.2), 7.48-7.34 (4H, overlapped, m), 7.32-7.27 (6H, overlapped, m), 7.04 (1H, d, *J* = 8.0), 6.39 (1H, s), 6.23 (1H, t, *J* = 7.6), 5.76 (1H, dd, *J* = 8.8 and 1.2), 5.72 (1H, d, *J* = 7.6), 4.96 (1H, d, *J* = 8.0), 4.64 (1H, br, s), 4.41 (1H, dd, *J* = 10.4 and 6.8), 4.32 (1H, d, *J* = 8.0), 4.21 (1H, d, *J* = 8.0), 3.82 (1H, d, *J* = 8.0), 2.60 (3H, s), 2.56 (1H, m), 2.40 (1H, dd, *J* = 15.2 and 8.0), 2.08 (3H, s), 2.02 (3H, s), 1.91 (1H, m), 1.75

(3H, s), 1.24 (3H, s), 1.12 (3H, s), 0.92-0.88 (18H, 6CH<sub>3</sub>), 0.10 (3H, s), 0.08 (3H, s), -0.01 (3H, s), -0.15 (3H, s); <sup>13</sup>C NMR 201.9, 171.6, 170.4, 169.7, 167.3, 140.8, 138.5, 134.3, 133.8, 133.7, 132.0, 130.5, 129.4, 128.9, 128.2, 127.2, 126.6, 84.4, 81.4, 79.0, 75.5, 75.3, 75.1, 72.4, 71.6, 58.8, 55.9, 46.8, 43.6, 37.8, 35.7, 29.9, 26.8, 25.7, 25.6, 23.3, 22.9, 21.7, 21.1, 18.3, 18.0, 14.4, 14.3, 10.3, -2.55, -4.92, -5.63, -5.71; HRFABMS *m/z* = 1082.5077 [M+H]<sup>+</sup> calculated for C<sub>59</sub>H<sub>80</sub>NO<sub>14</sub>Si<sub>2</sub> *m/z* = 1082.5117 ( $\Delta$  = -3.7 ppm).

**2',7-di-*tert*-Butyldimethylsiloxy-15(16)-anhydro-11(15→1)-abeo-paclitaxel (10.21).**

Compound **10.20** (20 mg, 15.3  $\mu$ mol) was dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (5 mL) with pyridine (0.13 mL) added. The mixture was cooled to -20 °C and thionyl chloride (47  $\mu$ L) was added. After stirring for half an hour, the reaction was quenched with saturated aqueous NaHCO<sub>3</sub> and extracted with EtOAc. The organic phase was evaporated and the residue was separated by PTLC on silica gel with 12% EtOAc in hexane to give compound **10.21** (9.3 mg, 8.1  $\mu$ mol, 53%) as a white solid. <sup>1</sup>H NMR  $\delta$  8.05 (dd, 2H, *J* = 8.0 and 1.2), 7.71 (2H, d, *J* = 7.0), 7.52 (1H, t, *J* = 7.2), 7.48-7.25 (10H, overlapped, m), 6.46 (1H, s), 5.83 (1H, t, *J* = 7.6), 5.67 (1H, d, *J* = 8.8), 5.59 (1H, d, *J* = 8.0), 4.75 (1H, br, s), 4.67 (1H, d, *J* = 2.0), 4.57 (1H, d, *J* = 2.0), 4.47 (1H, t, *J* = 8.4), 4.26 (1H, d, *J* = 8.0), 4.21 (1H, d, *J* = 8.0), 4.05 (1H, t, *J* = 6.8), 3.54 (1H, d, *J* = 8.0), 2.62 (1H, m), 2.45 (3H, s), 2.42 (1H, m), 2.11 (3H, s), 2.06 (3H, s), 1.98-1.85 (2H, m), 1.77 (3H, s), 1.65 (3H, s), 0.81 (9H, 3CH<sub>3</sub>, overlapped), 0.77 (9H, 3CH<sub>3</sub>, overlapped), 0.12 (3H, s), 0.06 (3H, s), -0.12 (3H, s), -0.32 (3H, s); <sup>13</sup>C NMR:  $\delta$  202.6, 171.8, 171.3, 169.6, 167.6, 166.1, 146.0, 144.8, 139.4, 137.8, 135.0, 134.3, 132.5, 131.9, 130.5, 130.8, 129.9, 129.6, 129.5, 129.4, 128.6, 127.8, 127.2, 113.8, 85.4, 79.7, 78.7, 77.5, 76.3, 75.5, 72.9, 71.3, 63.9, 57.8, 56.7,

44.3, 39.5, 39.3, 35.4, 26.5, 26.3, 23.1, 21.5, 21.1, 19.0, 18.8, 12.2, 10.2, -2.13, -4.72, -4.80, -5.18. HRFABMS  $m/z = 1064.4958$   $[M+H]^+$  calculated for  $C_{59}H_{78}NO_{13}Si_2$   $m/z = 1064.5012$  ( $\Delta = -5.4$  ppm).

**15(16)-Anhydro-11(15→1)-abeo-paclitaxel. (10.1)** To a solution of **10.21** (9.3 mg, 8.1  $\mu$ mol) in THF (3.5 mL) at 0 °C was added HF/pyridine (150  $\mu$ L, 70 wt %, large excess) and the solution allowed to warm to room temperature over 1 hour and then stirred overnight. The reaction mixture was quenched with saturated aqueous  $NaHCO_3$  and extracted with EtOAc. The organic layer was washed with water and brine, dried over anhydrous  $Na_2SO_4$ , and evaporated under reduced pressure. The residue was purified by PTLC on silica gel, developed with EtOAc:hexane, 1:4, to yield **10.1** (6.5 mg, 7.1  $\mu$ mol, 86%) as a white solid.  $^1H$  NMR  $\delta$  8.08 (2H, dd,  $J = 8.0$  and 1.2), 7.71 (2H, d,  $J = 7.0$ ), 7.55 (1H, t,  $J = 7.2$ ), 7.44-7.25 (10H, overlapped, m), 6.98 (1H, d,  $J = 8.4$ ), 6.41 (1H, s), 5.80 (1H, t,  $J = 7.6$ ), 5.75 (1H, d,  $J = 8.8$ ), 5.61 (1H, d,  $J = 8.0$ ), 4.99 (1H, d,  $J = 8.4$ ), 4.79 (1H, br, s), 4.70 (1H, br, s), 4.43 (1H, t,  $J = 8.4$ ), 4.26 (1H, d,  $J = 8.0$ ), 4.21 (1H, d,  $J = 8.0$ ), 3.52 (1H, d,  $J = 8.0$ ), 2.60 (1H, m), 2.49 (1H, m), 2.39 (3H, s), 2.10 (3H, s), 1.99 (1H, m), 1.90 (1H, m), 1.75 (3H, s), 1.60 (3H, s), 1.55 (3H, s);  $^{13}C$  NMR  $\delta$  201.8, 172.5, 171.3, 169.2, 166.8, 165.7, 145.9, 144.2, 139.5, 138.5, 137.2, 134.3, 133.8, 131.9, 130.5, 130.1, 129.3, 129.0, 128.9, 128.4, 127.2, 127.2, 126.8, 113.5, 84.9, 79.4, 77.9, 75.4, 75.3, 72.9, 71.0, 70.7, 63.5, 57.5, 52.1, 43.7, 38.8, 38.6, 35.4, 33.6, 26.1, 21.8, 10.8. HRFABMS  $m/z = 836.3256$   $[M+H]^+$  calculated for  $C_{47}H_{50}NO_{13}$   $m/z = 836.3282$  ( $\Delta = -3.1$  ppm).

## XI. Summary and Conclusion

Extracts from ten plants were studied in a search for anticancer compounds. Four of these extracts were further fractionated to yield three new compounds and ten known compounds. The structures of these compounds were elucidated using 1-D and 2-D NMR and mass spectrometry.

A number of 6'-amino-glycoglycerolipids were synthesized and their bioactivities against Myt1 kinase were determined. The synthetic 6'-amino-glycoglycerolipid did not show any significant bioactivity.

Two isotopically labeled paclitaxel analogs ( $^2\text{D}$ ,  $^{19}\text{F}$ ) were prepared in preparation for studies of the tubulin-binding conformation of paclitaxel by REDOR NMR. They are undergoing further REDOR NMR study by our collaborators.

A new macrocyclic *A-nor*-paclitaxel was synthesized, and was found to have good cytotoxicity and improved tubulin-binding activity as compared with paclitaxel. This compound provided additional evidence for the "T-taxol" conformation of paclitaxel as the tubulin-binding conformation responsible for tubulin assembly activity.