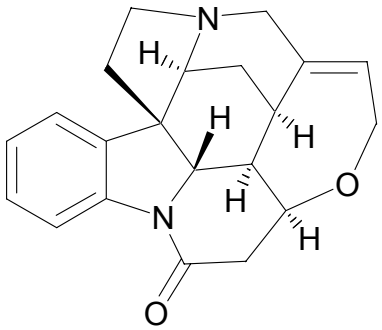
I. Medicinal Natural Products

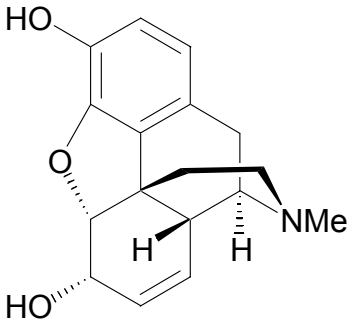
1.1 History and Representative Structures

Written records of the use of plants as medicinal agents date back thousands of years. The oldest records come from Mesopotamia and date from about 2600 BC. Those records are not simply a case of one or two plant based 'drugs' finding their way into popular use, because the documents indicate that there were many drugs in use that contained plants (up to 1,000 in the case of Mesopotamia).

However, it was not until the early 1800's that the active principles from plants were isolated. It was at this point that the effectiveness of medicinal natural products began to be attributed to science and not to magic or witchcraft. Among the first active principles to be isolated were strychnine (**1.1**), morphine (**1.2**), atropine (**1.3**), and colchicine (**1.4**). In 1826, this resulted in E. Merck producing the first commercially pure natural product, morphine (**1.2**).[1]

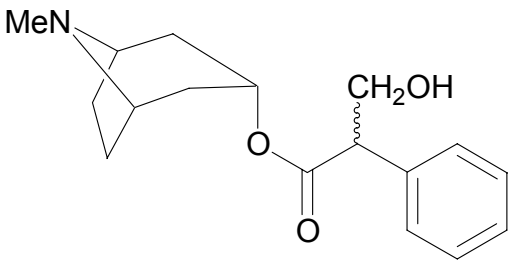


1.1 Strychnine

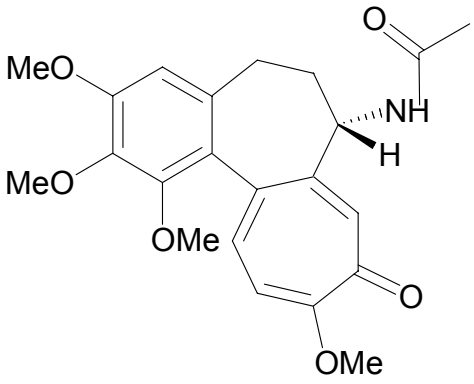


1.2 Morphine

[1] Newman, D. J.; Cragg, G. M.; Snader, K. M. The Influence of Natural Products Upon Drug Discovery. *Nat. Prod. Rep.,* **2000,** *17,* 215-234.



1.3 Atropine



1.4 Colchicine

More recently a study by the World Health Organization (WHO) has shown that about 80% of the world's population still relies on traditional medicine.[2] This is of interest to a natural product chemist for many reasons. There is the possibility that the herb used in the traditional medicine is harmful to the patient, in which case the treatment may do more harm that good. Conversely there is the possibility that the herbs used are not effective at all. That may not be of concern for minor ailments, but in more serious cases an ineffective treatment could result in the death of the patient. Hopefully, however, the herbs used are effective. If that is the case then investigation of that remedy could be of benefit to the remaining 20% of the world's population.

Natural products still play a very important role in the medicine of the remaining 20% of the world's population. Between 1983 and 1994 41% of new approved drugs have natural products as their source (Figure 1.1).

[2] Farnsworth, N. R.; Akerele, O.; Bingel, A. S.; Soejarto, D. D.; Guo, Z. Medicinal Plants in Therapy. *Bull. WHO*. **1985**, *63*, 965-981.

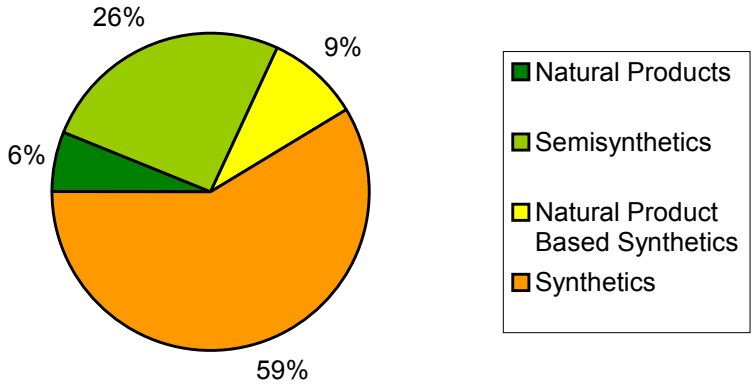


Figure 1.1 The role of natural products in modern medicine.

This percentage becomes even higher when one only examines anti-infective and anticancer compounds. For both classes the percentage of drugs with natural products as their source increases to over 60% (Figure 1.2).[3]

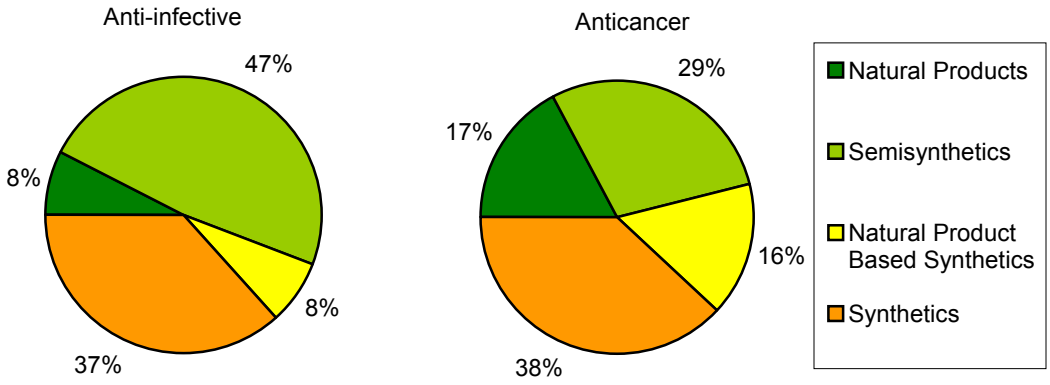


Figure 1.2 The role of natural products in anticancer and anti-infective drugs.

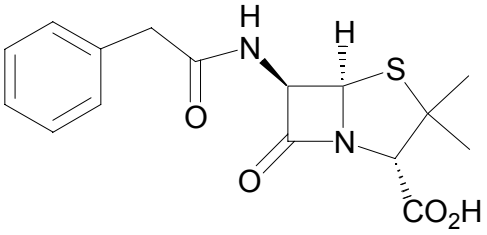
[3] Cragg, G. M.; Newman, D. J.; Snader, K. M. Natural Products in Drug Discovery and Development. *J. Nat. Prod.* **1997**, *60*, 52-60.

1.2 Natural Products In Medicine

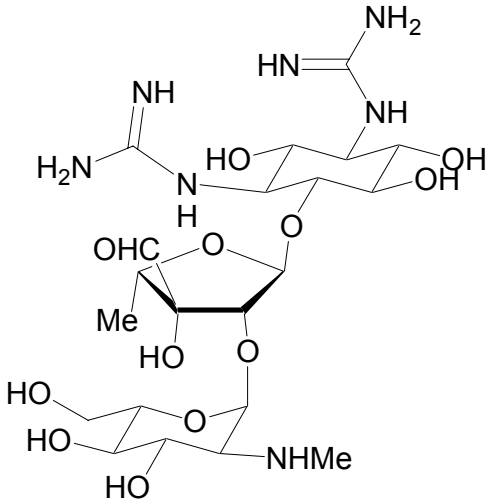
Because natural products are most important in the areas of anti-infective and anticancer agents, some of the important contributions to these drug classes are worth closer inspection. In particular the anticancer drugs will be examined, as that is the area of research that the Kingston group is concerned with.

1.2.1 Natural Products and Anti-infectives

The first real breakthrough in the field of anti-infectives was the discovery of the β-lactam antibiotics such as penicillin G (**1.5**). After its discovery it was suddenly possible to treat diseases that before had been untreatable and sometimes even deadly. The next breakthrough was the discovery of streptomycin (**1.6**). Streptomycin was the first antibiotic treatment for tuberculosis. These discoveries were followed by the discovery of the tetracyclines, chlortetracycline (**1.7**), and the macrolides, which are best represented by erythromycin A (**1.8**).[1,4]

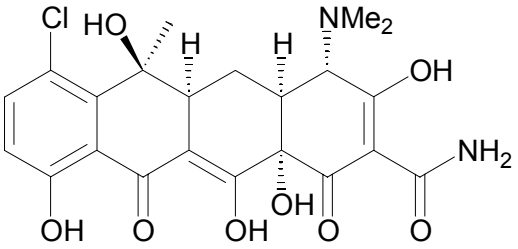


1.5 Penicillin G

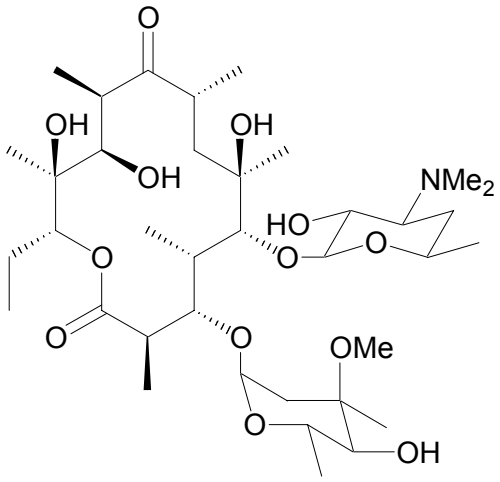


1.6 Streptomycin

[4] Miller, J. B. *The Pharmaceutical Century: Ten Decades of Drug Discovery*, Supplement to ACS Publications, **2000**, 21-63.



1.7 Chlortetracycline

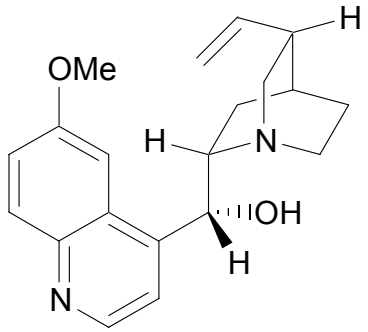


1.8 Erythromycin A

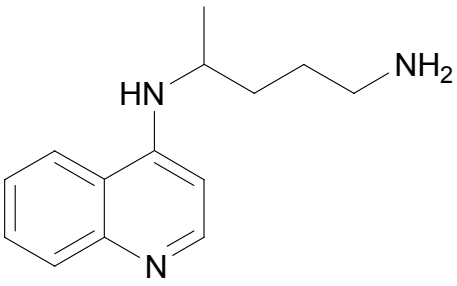
Natural products have also made an important impact in the area of antimalarial drugs. The first antimalarial drug was quinine (**1.9**), which led to the development of other antimalarial drugs such as chloroquine (**1.10**).[4] The newest class of potential antimalarials are peroxy-bridge containing compounds. The first compound of this class to be discovered was artemisinin (**1.11**), which has been used for over 2000 years by the Chinese to treat malaria.[5] The peroxy-bridge compounds, artemisinin in particular, show promise in treating cases of malaria that have become resistant to treatment with chloroquine.[6]

[5] Klayman, D. L.; Lin, A. J.; Acton, N.; Scovill, J. P.; Hoch, J. M.; Milhous, W. K.; Theoharides, A. D.; Dobek, A. S. Isolation of artemisinin (qinghaosu) from Artemisia annua growing in the United States. *J. Nat. Prod*. **1984**, *47*, 715-717.

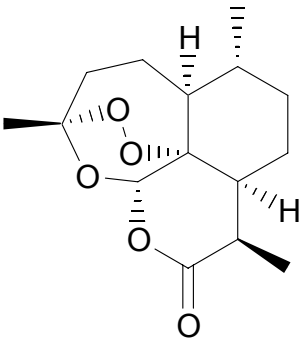
[6] Shu, Y.-Z. Recent Natural Products Based Drug Development: A Pharmaceutical Industry Perspective. *J. Nat. Prod.* **1998**, *61*, 1053-1071.



1.9 Quinine



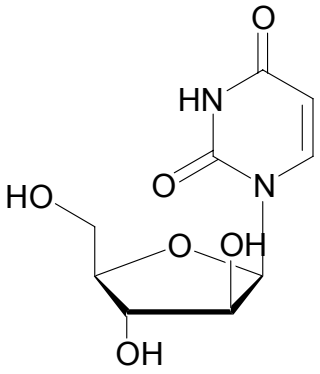
1.10 Chloroquine



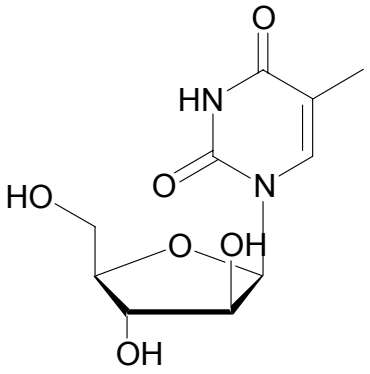
1.11 Artemisinin

Antiviral drugs have also relied heavily on natural products as drugs and/or leads. Spongouridine (**1.12**) and spongothymidine[7] (**1.13**) led to the discovery of the anti-HIV drug AZT (**1.14**). In fact spongouridine (**1.12**) and spongothymidine (**1.13**) can be thought of as the precursors of all nucleoside drugs.[8]

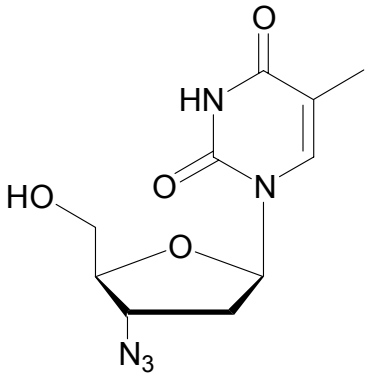
[7] Bergmann, W.; Burke, D. C. Marine products. XXXIX. The nucleosides of sponges. III. Spongothymidine and spongouridine. *J. Org. Chem*. **1955**, *20*, 1501-1507.
[8] Suckling, C. J. Chemical approaches to the Discovery of New Drugs. *Sci. Prog.* **1991**, *75*, 323-359.



1.12 Spongouridine



1.13 Spongothymidine



1.14 AZT

1.2.2 Natural Products and Anticancer Agents

Cancer is the second leading cause of death in the United States; one out of every four deaths is from cancer. During 2002, it is estimated that over 1.28 million people will develop cancer (this figure does not include noninvasive cancers). The death rate for people with cancer is 38%. The National Institutes of Health (NIH) has estimated the costs from cancer to be 156.7 billion dollars.[9] It is also important to note that 77% of all

[9] National Institutes of Health http://www.nih.gov

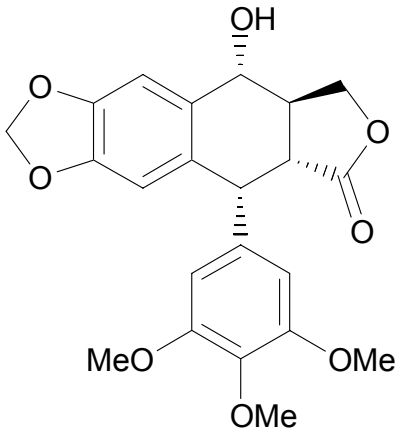
cancers diagnosed are in people 55 years of age or older.[10] With cancer taking such a toll on the population, both in lives and cost, the discovery of anticancer drugs has become very important. When one considers the aging population of the United States, it is clear that these numbers will likely increase in the years to come, and the search for more effective drugs will become even more important.

The goal in the search for new anticancer drugs is to find drugs that act via a specific mode of action. In this manner it is hoped that the cancer cells can be targeted and little or no damage to noncancerous cells. However, the reduction of general cytotoxicity is not a simple matter, because cancerous cells and noncancerous cells are very similar. Due to the deadly nature of cancer, the FDA has allowed drugs that are less than completely specific to be approved. Despite the potential for side effects, these drugs are considered the most successful means by which to treat cancer.[11]

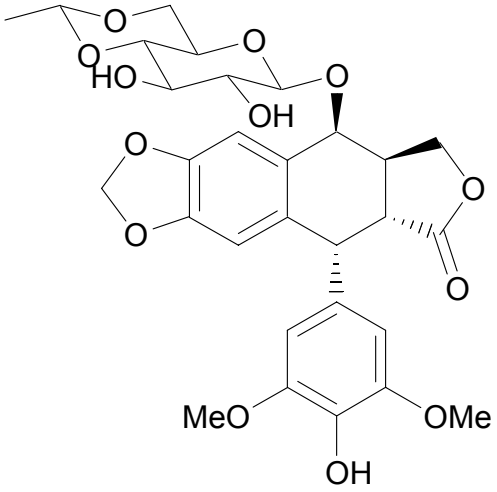
Some of the most effective cancer treatments to date are natural products or compounds derived from natural products. The history of natural products as anticancer compounds began in 1947 with podophyllotoxin (**1.15**) being islolated from *Podophyllum peltatum*.[12,13] Podophyllotoxin (**1.15**), which is too toxic for use as an anticancer agent, is used in the topical treatment of genital warts.[14] Etoposide (**1.16**) and teniposide (**1.17**), which are modifications of an analog, 4′-demethylepipodophyllotoxin (**1.18**), are used

[10] American Cancer Society, *Cancer Facts and Figures 2002*. http://www.cancer.org
[11] Nicolau, K. C.; Hepworth, D.; King, N. P.; Finlay, M. R. V. Chemistry, Biology and Medicine of Selected Tubulin Polymerizing Agents. *Pure Appl. Chem.* **1999**, *71*, 989-997.
[12] Hartwell, J. L.; Shear, M. J. Chemotherapy of cancer. Classes of compounds under investigation and active components or podophyllin. *Cancer Research*. **1947**, *7*, 716-717.
[13] Colegate, S. M.; Molyneux, R. J. *Bioactive natural products: Detection, Isolation, and Structural Determination*. (S. M. Colgate, ed.) CRC Press, Inc., Boca Raton, **1993**, 222.
[14] Bohlin, L.; Rosén, B. Podophyllotoxin Derivatives: Drug Discovery and Development. *Drug Disc. Today*, **1996**, *1*, 343-351.

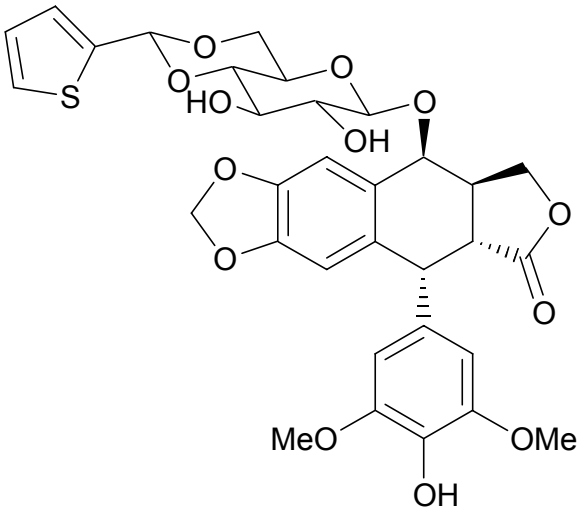
clinically to treat cancer.[15] Podophyllotoxin (**1.15**) acts by preventing the polymerization of tubulin into microtubules. However, the 4′-demethylepipodophyllotoxin analogs do not act via the same mechanism. Instead they inhibit topoisomerase II, preventing the cleavage and resealing of DNA strands.[1,16]



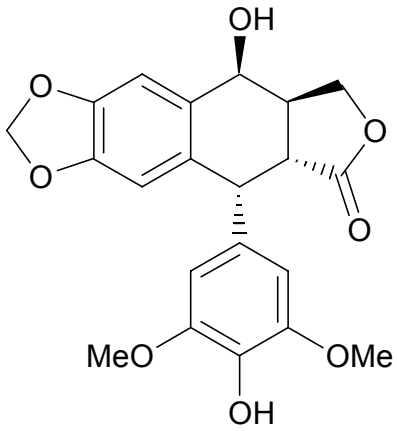
1.15 Podophyllotoxin



1.16 Etoposide



1.17 Teniposide

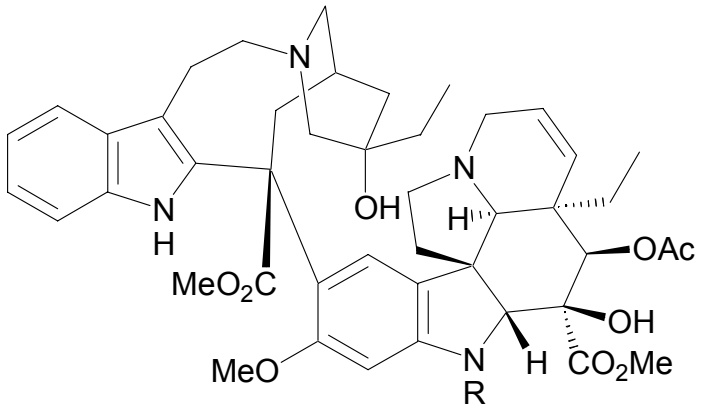


1.18 4'-demethylepipodophyllotoxin

[15] Juslén, C. K.; Kuhn, M.; Wartburg, A. v.; Stähelin, H. Synthesis and Antimitotic Activity of Glycosidic Lignan Derivatives Related to Podophyllotoxin. *J. Med. Chem*. **1971**, *14*, 936-940.
[16] Dewick, P. M. *Medicinal Natural Products: A Biosynthetic Approach*. John Wiley & Sons, Inc., New York, **1997**, 123-124.

The 'vinca alkaloids', vinblastine (**1.19**) and vincristine[17] (**1.20**) from the Madagascan periwinkle, *Catharanthus roseus*, discovered while searching for oral hypoglycemic agents,[1] are used in the treatment of Hodgkin's disease and childhood leukemia, respectively. Both are considered antimitotic drugs because they inhibit cell division. They act by binding to tubulin and preventing it from polymerizing into microtubules. Both treatments are reported to be highly effective. [18]

The success of the podophyllotoxins together with the discovery of the 'vinca alkaloids' led to a much greater priority being placed on investigation of plants. This was achieved in the United States through the National Cancer Institute (NCI), which established the Cancer Chemotherapy National Service Center (CCNSC) in 1955.[19]

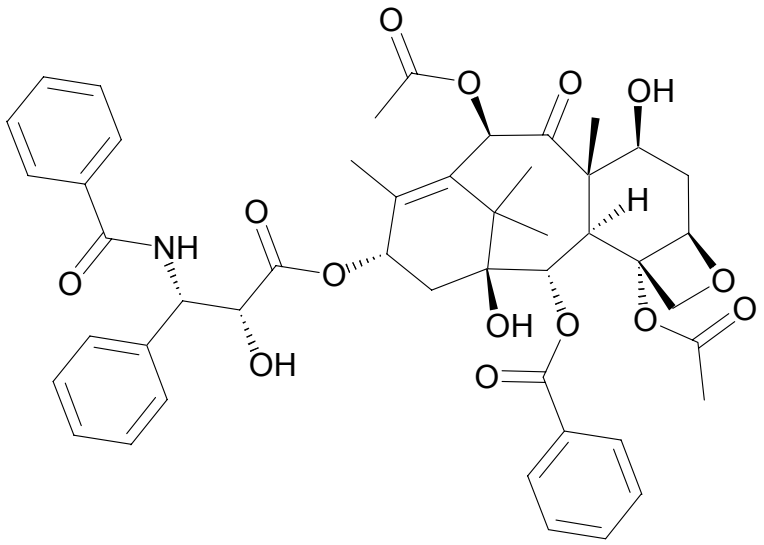


1.19 R = Me, Vinblastine
1.20 R = CHO, Vincristine

[17] Svoboda, G. H. Alkaloids of Vinca rosea. IX. Extraction and characterization of leurosidine and leurocristine. *Lloydia*. **1961**, *24*, 173-178.
[18] Dewick, P. M. *Medicinal Natural Products: A Biosynthetic Approach*. John Wiley & Sons, Inc., New York, **1997**, 329-330.
[19] Cragg, G. M.; Boyd, M. R.; Cardellina, J. H., II; Grever, M. R.; Schepartz, S. A.; Snader, K. M.; Suffness, M. Role of Plants in the National Cancer Institute Drug Discovery and Development Program. *Human Medicinal Agents From Plants*; (A. D. Kinghorn, M. F. Balandrin, eds.), ACS Symposium Series 534; American Chemical Society, Washington, D.C., **1993**, 80-95.

As a result of that increased priority the Pacific yew tree, *Taxus brevifolia*, was investigated. Thus paclitaxel (**1.21**) (Taxol®) was discovered as its active principle.[20] It was later determined that paclitaxel possessed a unique cytotoxic mechanism.

Paclitaxel acts as an antimitotic drug, as do the previously mentioned vinca alkaloids and podophyllotoxin. However, paclitaxel is the first anticancer drug discovered that stabilized microtubules and thus promotes their polymerization. Since its discovery paclitaxel has become a blockbuster drug used in the treatment of lung, ovarian, and breast cancer and Kaposi's sarcoma.[6] Sales of Taxol total over a billion dollars a year and were over 1.6 billion dollars in 2000.[21]

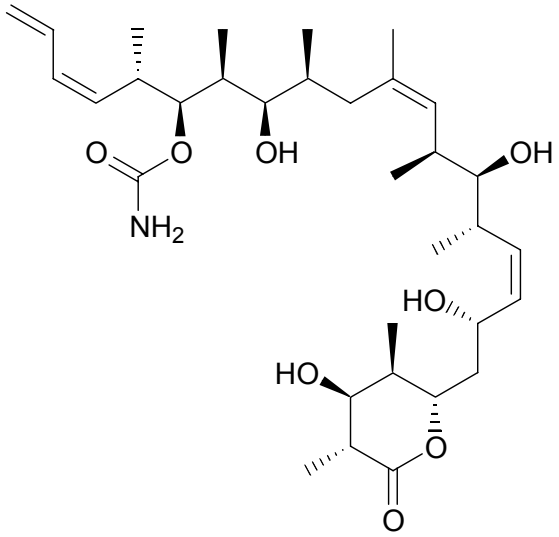


1.21 Paclitaxel (Taxol®)

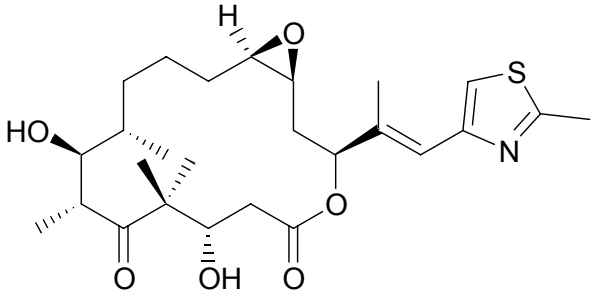
The discovery of paclitaxel has led to the discovery of other compounds that act through the same mechanism.[11] (+)-Discodermolide (**1.22**) and the epothilones such as

[20] Wani, M. C.; Taylor, H. L.; Wall, M. E.; Coggon, P.; McPhail, A. T. The Isolation and Structure of Taxol, a Novel Antileukemic and Antitumor Agent from *Taxus brevifolia*. *J. Am. Chem. Soc*. **1971**, *93*, 2325-2327.
[21] Bristol-Myers Squibb http://www.bms.com

epothilone A (**1.23**) are two such examples.[22,23,24] These compounds show great promise as drug candidates, and even show activity against multidrug-resistant cell lines. It is interesting to note that, despite the structural differences between paclitaxel, discodermolide, and epothilone, they appear to have remarkably similar pharmacophores and binding sites.[25,26] It has even been observed that paclitaxel and discodermolide have a synergistic effect when administered together.[27]



1.22 (+)-Discodermolide



1.23 Epothilone A

[22] Gunasekera, S. P.; Gunasekera, M.; Longley, R. E.; Schulte, G. K. Discodermolide: A New Bioactive Polyhydroxylated Lactone from the Marine Sponge *Discodermia dissoluta*. *J. Org. Chem*. **1990**, *55*, 4912-4915.
[23] Höfle, G.; Bedorf, N.; Gerth, K.; Reichenbach, H.; German patent DE 4138042 A1 1993.
[24] Victory, S. F.; Velde, D. G. V.; Jalluri, R. K.; Grunewald, G. L., Georg, G. I. Relative Stereochemistry and Solution Conformation of the Novel Paclitaxel-like Antimitotic Agent Epothilone A. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 893-898.
[25] Smith, A. B., III; LaMarche, M. J.; Falcone-Hindley, M. Solution Structure of (+)-Discodermolide. *Org. Lett.*, **2001**, *3*, 695-698.
[26] Giannakakou, P.; Gussio, R.; Nogales, E.; Downing, K. H.; Zaharevitz, D.; Bollbuck, B.; Poy, G.; Sackett, D.; Nicolaou, K. C.; Fojo, T. A Common Pharmacophore for Epothilone and Taxanes: Molecular Basis for Drug Resistance Conferred by Tubulin Mutations In Human Cancer Cells. *Proc. Natl. Acad. Sci. USA*, **2000**, *97*, 2904-2909.
[27] Horwitz, S. B.; Martello, L. A.; Yang, C-P. H.; Smith, A. B., III; McDaid, H. M. Discodermolide and Taxol: A Synergistic Drug Combination in Human Carcinoma Cell Lines. *Anticancer Agents*. ACS Symposium Series 796; American Chemical Society, Washington, D.C., **2001**, 81-96.

1.3 Natural Products As a Source of Chemical Diversity

Natural products have the potential to provide medicine with a source of novel structures that are unobtainable from sources such as combinatorial synthesis. Nature is capable of producing complex molecules with multiple chiral centers that are designed to interact with biological systems.[28,29] These compounds are often used by the producing organism as a self defense mechanism.[30]

A prime example of this is paclitaxel (**1.21**). Paclitaxel, as mentioned earlier, is produced by *Taxus brevifolia*. It is a cytotoxic agent that is most likely produced to prevent organisms from feeding on the plant. It has been designed to interact with microtubules and produce a specific response, cell death. Paclitaxel is a very complicated molecule. It contains four fused rings, an amino acid based side chain, and a total of eleven chiral centers. Due to its complex structure it is highly unlikely that it would have ever been produced synthetically prior to its discovery. Another important point to note is that it is not likely that combinatorial chemistry would have ever led to the discovery of paclitaxel. However, the complexity of its structure does make it a good candidate for combinatorial modifications to produce numerous analogs.[31] It has been targeted by many groups, for both semi- and total-synthesis, as a result of its complex structure, unique activity, and low bioavalability.[32] Despite the numerous synthetic

[28] Cordell, G. A. Biodiversity and Drug Discovery: A Symbiotic Relationship. *Phytochemistry*, **2000**, *55*, 463-480.
[29] Young, R. N. Importance of Biodiversity to the Modern Pharmaceutical Industry. *Pure Appl. Chem.*, **1999**, *71*, 1655-1661.
[30] da Rocha, A. B.; Lopes, R. M.; Schwartsmann, G. Natural Products in Anticancer Therapy. *Curr. Opin. Pharmacol.* **2001**, *1*, 364-369.
[31] Ecker, D. J.; Crooke, S. T. Combinatorial Drug Discovery: Which Methods Will Produce the Greatest Value? *Biotechnology*, **1995**, *13*, 351-360.
[32] Lee, K-H. Novel Antitumor Agents from Higher Plants. *Med. Res. Rev*. **1999**, *19*, 569-596.

routes published for paclitaxel Bristol-Myers Squibb (BMS) obtains it via semi-synthesis from its precursor 10-deacetylbaccatin.[33]

At some point the amount of chemical diversity available through Nature must begin to tail off. Perhaps the best manner to determine the amount of chemical diversity remaining is to examine the amount of biodiversity remaining.

1.4 Natural Products and Biodiversity

Because biodiversity is so important to the continued discovery of novel natural products, it is important to know how much biodiversity remains. The greater the amount of remaining biodiversity to be studied, the greater the potential amount of chemical diversity remains to be discovered.

It has been estimated that of the approximately 250,000 plant species only about 5-15% of them have been investigated for bioactive compounds.[34] The biodiversity of the marine ecosystem has barely been scratched.[3] In fact "if you look at the fundamental phyla of life, there are 34; and 17 occur on land, whereas 32 occur in the sea [with some overlap]," says William Fenical of Scripps Institute of Oceanography.[35] Additionally, marine organisms tend to produce a wealth of natural products. Over the past thirty years over 3,000 new compounds have been isolated from marine organisms.[30] However, the area that has perhaps the most potential and definitely the greatest biodiversity is the microbial world. It has been estimated that at best only about 1% of the world's

[33] Holton, R. A.; Biediger, R. J.; Boatman, P. D. Semisynthesis of Taxol and Taxotere. *Taxol® Science and Applications*. (M. Stuffness ed.) CRC Press, Boca Raton, **1995**, 97-121.
[34] Balandrin, M. F.; Kinghorn, A. D.; Farnsworth, N. R. Plant-Derived Natural Products in Drug Discovery and Development: An Overview. *Human Medicinal Agents form Plants*. (A. D. Kinghorn, M. F. Balandrin, eds.) ACS Symposium Series 534; American Chemical Society: Washington, D.C., **1993**; 2-12.
[35] Quote by Willis, R. C. Nature's Pharma Sea. *Mod. Drug Disc*. **2002**, *5*(1) 32-38.

microbes can be cultured with the technology currently being used. As a result the DNA from these microbes is now being isolated and inserted into cultivatable organisms in the hope that the host organism will express the foreign DNA and produce novel compounds that otherwise would be unobtainable.[36,37]

Based on the above information it is obvious that there is still an abundance of species for investigation. However, one should also consider that with the introduction of each new bioassay there is the potential to discover new compounds from old natural product sources.[6] Thus as new bioassays are developed it is important to retest all samples even if they previously showed no activity.[29]

1.5 The ICBG Program

1.5.1 Biodiversity Loss and the ICBG Program

Biodiversity and its conservation has become a very political and popular issue in recent years. Many smaller countries are using their natural resources at an alarming rate. Of particular concern is the consumption of the rainforests. As the rainforests are cleared for timber and farmland plant and animal species are lost forever. This is alarming for several reasons. It is unknown what role many of the species play in the environment, and, from a natural products point of view, it is unknown what chemicals they may contain.[28]

As a result natural products research now has three goals: drug discovery, biodiversity conservation, and economic development. Through involvement in the

[36] Brady, S. F.; Chao, C. J.; Handelsman, J.; Clardy, J. Cloning and Heterologous Expression of a Natural Product Biosynthetic Gene Cluster from eDNA. *Org. Lett.* **2001**, *3*, 1981-1984.
[37] Wang, G-Y-S.; Graziani, E.; Waters, B.; Pan, W.; Li, X.; McDermott, J.; Meurer, G.; Saxena, G.; Anderson, R. J.; Davies, J. Novel Natural Products from Soil DNA Libraries in a Streptomycete Host. *Org. Lett.* **2000**, *2*, 2401-2404.

International Cooperative Biodiversity Grant (ICBG) program, the Kingston group aids in this conservation. As a part of the ICBG program agreements are made with the source country providing them with a percentage of any profits that result from the sale of a successful drug isolated from a plant collected in that country. The Kingston group is currently partnered with Suriname and Madagascar for the extracts that it studies.

Suriname was the original country that was chosen for collaboration by the Kingston group. Suriname was chosen because nearly 90% of its area is undisturbed forest that is estimated to contain over 5,000 species of plant. It also has good working relationships with the group.[38] Madagascar, the second country to partner with the group, has a very high level of biological diversity due to its isolation. It is believed to contain between 10,000 and 12,000 species of flowering plants and 80% of them are believed to be endemic. This amazing level of biodiversity coupled with the rapid loss of natural forest habitat (as much as 80% has already been lost) makes Madagascar a prime area for conservation efforts and has resulted in its classification as a biodiversity hotspot.[39]

The ICBG program at VPI&SU currently consists of six collaborating groups. The lead group is Virginia Polytechnic Institute and State University. Missouri Botanical Garden (MBG) (Dr. James Miller) and Conservation International (CI) (Dr. Russell Mittermeier) are involved in plant collection and identification. The Centre National d'Application et des Recherches Pharmaceutiques (CNARP) (Dr. Rabodo Andriantsiferana) is involved in sample preparation and phytomedicine development.

[38] Kingston, D. G. I.; Abdel-Kader, M.; Zhou, B.-N.; Yang, S.-W.; Berger, J. M.; van der Werff, H.; Miller, J. S.; Evans, R.; Mittermeier, R.; Famolare, L.; Guerin-McManus, M.; Malone, S.; Nelson, R.; Moniz, E.; Wisse, J. H.; Vyas, D. M.; Wright, J. J. K.; Aboikonie, S. The Suriname International Cooperative Biodiversity Group Program: Lessons from the First Five Years. *Pharmaceutical Biol*. **1999**, *37*, 22-34.
[39] Conservation International, Conservation Strategies, Hotspots, Madagascar and Indian Ocean Islands, http://www.conservation.org/xp/CIWEB/strategies/hotspots/madagascar.xml

Bedrijf Geneesmiddelen Voorziening Suriname (BGVS) (Dr. Jan Wisse) is involved in sample preparation and antimicrobial drug discovery. DowAgrosciences (Dr. Cliff Gerwick) is involved in agrochemical discovery.

1.5.2 Plant Collection in the ICBG Program

There are two basic methods of plant collection, the random method and the ethnobotanical method. Under the random method plants are randomly selected based on what is available and what can be identified (in the reproductive stage). The ethnobotanical method relies on the knowledge of local indigenous people. Plants are collected based on their use in the traditional medicine system of the local inhabitants. Both methods have advantages and disadvantages. Under the random method the percentage of hits generated are slightly less than those from the ethnobotanical method.[40] However, under the ethnobotanical method hits may be missed simply because the plant is not used medicinally. In that case the random method has an advantage over the ethnobotanical method.[41] An additional disadvantage to the ethnobotanical method involves the collection method. As previously stated plants are collected based on what the local people use, and they may not be in a growth stage that allows for their identification. It is important that the identity of the plant be known in order to plan an isolation strategy. More importantly, it avoids wasting time on a plant that has already been investigated with the same assay.

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[40] Kingston, D. G. I. Biodiversity Conservation and Drug Discovery in Suriname. Explorations in Nature's Combinatorial Library. *Pure Appl. Chem*. **2001**, *73*, 595-599.

[41] Soejarto, D. D. Logistics and Politics in Plant Drug Discovery: The Other End of the Spectrum. *Human Medicinal Agents from Plants*. (A. D. Kinghorn, M. F. Balandrin, eds.) ACS Symposium Series 534; American Chemical Society: Washington, D.C., **1993**, 96-111.

Under the ICBG program both techniques are used. The collections made by MBG tend to be random collections, and the collections made by CI tend to be ethnobotanical.

1.6 The Use of Bioassays in Natural Products Research

1.6.1 Bioassay Guided Fractionation

Bioassay guided fractionation is essential to natural products chemistry. Without bioassay guided fractionation it would be an overwhelming task to isolate active compounds from a crude extract. In order to have any assurance of isolating the active compound(s) it would be necessary to isolate every component of an extract or at the very least all of the major components. All of this would be time consuming and costly. In the end the only viable way to isolate bioactive compounds is to use bioassay guided fractionation.[42]

There are numerous bioassays available for use in testing for many different activities. In choosing a bioassay there are several important factors to consider; cost, sensitivity, selectivity, simplicity, and throughput. The ideal bioassay would be inexpensive, sensitive to small amounts of active material, selective for a specific type of bioactivity, simple to run and maintain, and be capable of high throughput.[42]

The bioassay used by the Kingston group in conjunction with the ICBG program is a cell-based assay. The A2780 human ovarian cancer cell line is used as a general cytotoxicity assay. Because it is a general cytotoxicity assay it is not possible, using this assay, to determine the mechanism in which the compound works. DNA damaging

[42] Rahman, A.; Choudhary, M. I.; Thomsen, W. J. *Bioassay Techniques for Drug Development*. Hardwood Academic Publishers, Amsterdam, **2001**, 1-3.

agents, antimitotics, etc, are all measured as general cytotoxic agents in this assay. There are some advantages to this type of assay. Though it is not possible to determine the mechanism involved with this assay it is possible to detect a wide range of compounds with different mechanisms of action. It is also important to note that, as this is a cellular based assay, it only detects compounds that are capable of passing through the cell membrane. As a result any compounds that are discovered have a greater chance of becoming drug candidates without requiring any modification. However, this same benefit also means that it is possible to miss compounds that could potentially be developed into drug candidates via synthetic modification to improve membrane permeability. As such there is a trade off between fewer dead ends and a potential loss in chemical diversity.

1.6.2 Operation of the Bioassay

The A2780 human ovarian cell line is employed as an *in vitro* cytotoxicity assay. The assay is a microtiter plate assay using 96 well tissue culture plates. The A2780 cells are seeded in columns 1 through 11 at a cell density of 2.7×10^5 cells/mL in a medium made up of RPMI 1640 medium plus L-glutamine (Gibco) and 10% Fetal Bovine Serum (Gibco) (180μL). Column 12 contains only medium (200μL) as a positive control. The cells are then incubated for 3 hours in a 5% CO_2 atmosphere at 37°C to allow the cells to adhere to the wells. The samples to be tested are dissolved in 50/50 DMSO/water at a concentration of 1000 μg/mL. Then the samples are diluted 1:50 (20 μg/mL). Seven 1:3 dilutions are then carried out to give a range of concentrations between 20 and 0.00122 μg/mL. Columns 1 through 10 then receive 20μL of each sample dilution. The final four

dilutions of a series of eight dilutions of actinomycin D (20μL), at an initial concentration of 2 μg/mL, are added to wells A-D of column 11 as a positive control. The remaining four wells, E-H, in column 11 contain only cells and medium as a negative control. The plates are then incubated for 48 hours under the same conditions as above. At the end of the 48 hours the medium is replaced with fresh medium plus 1% alamarBlue™ (Biosource International). The plates are then allowed to incubate for another 3 hours. At the end of the three hours the plates are read using a cytofluor (PerSeptive Biosystems) at an excitation of 530 nm and an emission of 590 nm with a gain of 45. A dose response scheme is calculated by dividing the fluorescence of the sample by the average fluorescence of the wells that contain only cells and medium. The activity is calculated via linear regression of the dose response scheme in order to determine the concentration (μg/mL) required to cause 50% inhibition of the cells, the IC_{50}.

The assay uses alamarBlue™ as a fluorometric indicator of metabolic activity. AlamarBlue™ contains an oxidation-reduction indicator that fluoresces and changes color when reduced. The reduction of the indicator is believed to require uptake by the cells as they grow. It is possible, based on relative redox potentials, that reduction takes the place of oxygen in the final step of respiration by the reduction of the indicator by acting as an electron acceptor for any oxidoreductase (i.e. the cytochromes). As such it does not shut down the respiratory process. Since cell growth generates a reduced environment and inhibition of cell growth generates an oxidized environment alamarBlue™ can be used to indicate metabolic activity and thus the percentage of inhibited cells.[43] This is possible by using the following equation:

[43] Anonymous. Biosource International, *alamarBlue™ Assay*.

$$\text{Output}_{\lambda} = \kappa_{\lambda} c$$

Where κ is a proportionality constant, c is the concentration, and λ is the wavelength the luminescence is measured at. Since only the cells whose metabolic activity has not been inhibited will reduce alamarBlue™ the percentage of living or non-inhibited cells is proportional to output_{λ}.

According to the above procedure, the cells are incubated with a potentially cytotoxic agent. During this period some or all of the cells are killed or inhibited. Then the alamarBlue™ is added and the cells are incubated again to allow any living cells to metabolize the indicator. At the end of this incubation period the amount of oxidized alamarBlue™ is measured. This is then compared to the fluorescence of the negative control. This makes it possible to solve for κ_{λ} in the equation above. The following equation is then used to solve for the percentage of reduced alamarBlue™ in the sample:

$$c_{\text{sample}}/c_{\text{control}} = \text{Output}_{\lambda\text{sample}}/\text{Output}_{\lambda\text{control}}$$

This percentage is inversely proportional to the percentage of cells that have been killed or inhibited, and it is these values that are used to calculate the IC_{50}.

1.7 Structure Elucidation[44]

Currently the two tools most vital to the structure elucidation of natural products are mass spectrometry and nuclear magnetic resonance spectroscopy (NMR). With these two tools the structures of most natural products can be determined. Although X-ray crystallography is a more powerful tool it requires that the compound in question be capable of producing good quality crystals that are at least 1 mm^2. That requirement is

[44] Crews, P.; Rodríguez, J.; Jaspars, M. *Organic Structure Analysis*. Oxford University Press, New York, **1998**, 1-16.

the source of two problems. Some compounds do not produce crystals and if they do their dimensions may not match those required for X-ray crystallography. The second problem is the sample size required to produce crystals. Natural products are isolated in increasingly small amounts, and as a result crystals are more difficult to produce.

1.7.1 Mass Spectrometry

This is generally the first step in structure elucidation. If a good mass spectrum can be obtained then the molecular formula can be determined. Without mass spectrometry it is difficult to determine the types of heteroatoms present in the compound. Thus until a good mass spectrum is obtained and the molecular formula is determined it is not always possible to propose a structure with any certainty.

1.7.2 Nuclear Magnetic Resonance Spectroscopy[45]

With the production of more powerful instruments and the invention of newer pulse sequences, especially the 2-D pulse sequences, NMR has become increasingly more important in structure elucidation. It has almost completely replaced degradation studies in the determination of novel structures, and in many cases synthesis is no longer necessary as a structural proof.

There has been little recent change in the techniques used in basic 1-D NMR spectra, although there have been increases in field strength and newer probes capable of handling smaller samples have been produced. The result has been an increase in the dispersion of the spectra, and a decrease in the amount of sample needed and the time

[45] Reynolds, W. F.; Enríquez, R. G. Choosing the Best Pulse Sequences, Acquisition Parameters, Postacquisition Processing Strategies, and Probes for Natural Product Structure Elucidation by NMR Spectroscopy. *J. Nat. Prod.* **2002**, *65*, 221-244.

required to collect good spectra. In the case of simple small molecules 1-D NMR coupled with MS may be enough to elucidate a compound's structure. However, most natural products are not small molecules and are certainly not simple. In these cases more powerful techniques are required.

This need has driven the creation of the more sophisticated and more powerful 2-D NMR pulse sequences. Among the most commonly used 2-D pulse sequences are heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond correlation (HMBC), and nuclear Overhauser enhancement spectroscopy (NOESY). An HMQC spectrum gives one-bond H-C correlations. This allows the assignment of protons to their respective carbons, and makes it possible to analyze the HMBC spectrum. HMQC has replaced the heteronuclear correlation experiment (HETCOR) owing to its shorter experiment time. Heteronuclear single quantum correlation (HSQC) in turn is replacing HMQC. HSQC should produce spectra with better ^{13}C resolution and better signal to noise ratio than those produced by HMQC. HMBC spectra give multiple bond H-C correlations, generally two and three bond correlations. This allows one to assign carbon and proton positions in the molecule based on shared correlations. Once the structure is determined NOESY can be used to determine the relative stereochemistry of the molecule. NOESY spectra produce through space H-H correlations. The through space correlations are in contrast to the correlations seen in the above spectral techniques, since HMQC, HSQC, and HMBC all give correlations through bonds. Thus the NOESY spectrum gives the proton correlations relative to the distance through space that separates them, and consequently their relative stereochemistry.

These NMR experiments are commonly used in natural products chemistry. In most cases they are capable of providing molecular structures without the need of further experimentation. However, it is still possible to find examples from the literature where the total synthesis of a compound resulted in the revision of its structure.