III. The Isolation of Deoxypodophyllotoxin from *Bridelia tulasneana* (Euphorbiaceae)

3.1 Introduction

Through our partnership with Madagascar as a part of the ICBG program an extract of *Bridelia tulasneana* was obtained and found to be active in the A2780 bioassay. The extract was subjected to fractionation, which resulted in the isolation of compound **A**.

3.1.1 Chemical Investigation of *Bridelia*

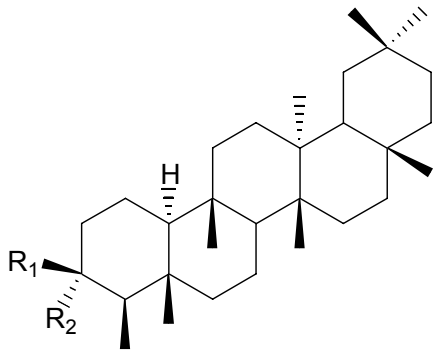
Bridelia tulasneana is a tree belonging to the family Euphorbiaceae. The genus *Bridelia* is made up of about 60 species scattered throughout Asia, Africa, and Australia.[1] They are used medicinally in the treatment of many different ailments. *B. ferruginea* Benth. is used in African traditional medicine as a decoction of the stem bark to treat diarrhea, dysentery, gastro-intestinal disorders, gynecological disorders (including sterility), and rheumatic pains. A decoction of the leaves is used to treat diabetes. It is also used as a purgative and a vermifuge.[2,3] It has even been used as a source of dye by local peoples.[4] In Thailand *B. ovata* Dcne. (known locally as Ma-Ga), *B. tomentosa* Bl. (syn. *B. monoica* Merr.) (known locally as Knon or Khon non), and *B. siamensis* Craib.

---

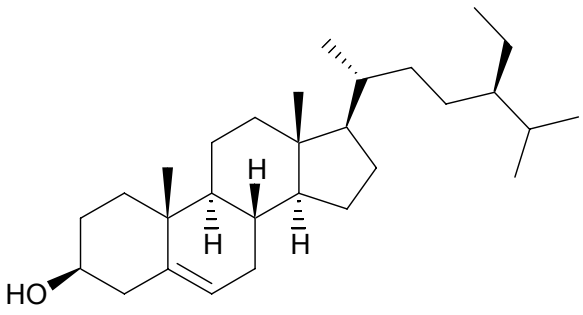
[1] Rashid, M. A.; Gustafson, K. R.; Cardellina, J. H., II; Boyd, M. R. A New Podophyllotoxin Derivative from *Bridelia ferruginea*. *Nat. Prod. Let*. **2000**, *14*, 285-292.
[2] Cimanga, K.; De Bruyne, T.; Apers, S.; Pieters, L.; Totté, J.; Kambu, K.; Tona, L.; Bakana, P.; van Ufford, L. Q.; Beukelman, C.; Labadie, R.; Vlietinck, A. J. Complement-Inhibiting Constituents of *Bridelia ferruginea* Stem Bark. *Planta Med*. **1999**, *65*, 213-217.
[3] De Bruyne, T.; Cimanga, K.; Pieters, L.; Claeys, M.; Dommisse, R.; Vlietinck, A. Gallocatechin-(4′→O→7)-Epigallocatechin, A New Biflavonoid Isolated from *Bridelia ferruginea*. *Nat. Prod. Let.* **1997**, *11*, 47-52.
[4] Addae-Mensah, I.; Achenbach, H. Terpenoids and Flavonoids of *Bridelia ferruginea*. *Phytochemistry*. **1985**, *24*, 1817-1819.

are used as medicinal plants. A decoction of their leaves is used as an expectorant and a laxative, and a decoction of their bark or leaves is used to treat colic. The bark is used medicinally as an astringent. A decoction of their leaves, along with other parts of the plant, is used to treat high fever. Their roots are taken the first three days after childbirth.[5,6,7]

The chemical constituents of *Bridelia* have not been thoroughly investigated. From *B. moonii* Thw., the triterpenes friedelin (**3.1**), friedelan-3α-ol (**3.2**), friedelan-3β-ol (**3.3**), sitosterol (**3.4**), and glochidone (**3.5**) have been isolated.[8] Some additional triterpenes have been isolated from *B. tomentosa* Bl. (*B. monoica* Merr.) including the previously unknown triterpene, 24-methyllanosta-9(11),25-dien-3-one (**3.6**).[7]

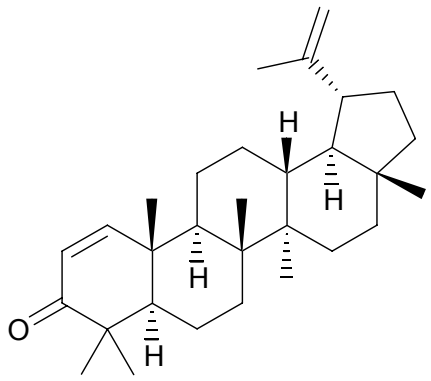


3.1 Friedelin $R_1,R_2 = O$
3.2 Friedelan-3α-ol $R_1 = H, R_2 = OH$
3.3 Friedelan-3β-ol $R_1 = OH, R_2 = H$

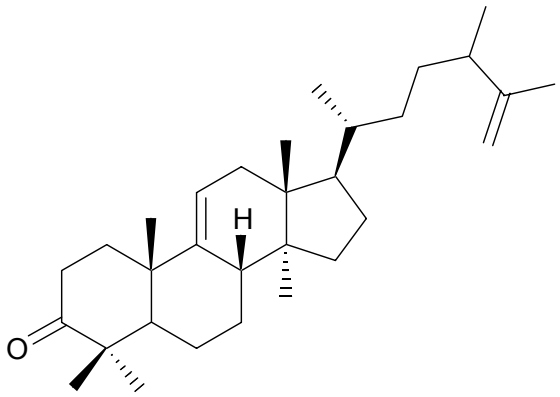


3.4 Sitosterol

[5] Delgado, A.; Clardy, J. Total Synthesis of (−)-Ovatolide. *J. Org. Chem.* **1993**, *58*, 2862-2866.
[6] Boonyaratavej, S.; Tantayanontha, S.; Kitchanachai, P.; Chaichantipyuth, C.; Chittawong, V.; Miles, D. H. Trans-Triacontyl-4-Hydroxy-3-Methoxycinnamate, A New Compound from the Thai Plant *Bridelia ovata*. *J. Nat. Prod*. **1992**, *55*, 1761-1763.
[7] Boonyaratavej, S.; Bates, R. B.; Caldera, S.; Suvannachut, K. A New Triterpenoid from *Bridelia tomentosa*. *J. Nat. Prod*. **1990**, *53*, 209-211.
[8] Carpenter, R. C.; Sotheeswaran, S.; Sultanbawa, M. U. S.; Balasubramaniam, S. Triterpenes of Five Euphorbiaceae Species of Sri Lanka. *Phytochemistry*. **1980**, *19*, 1171-1174.

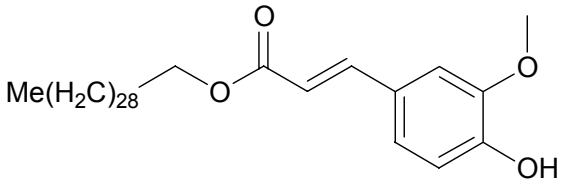


3.5 Glochidone

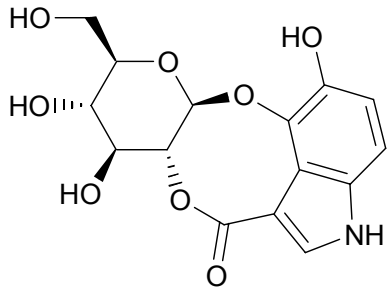


3.6 24-methyllanosta-9(11),25-dien-3-one

Trans-triacontyl-4-hydroxy-3-methoxycinnamate (**3.7**) has been isolated from *B. ovata* Dcne., along with various triterpenes.[6] The indole glycoside, (–)-ovatolide (**3.8**), has been isolated from *B. ovata* Dcne. and *B. siamensis* Craib.[5]



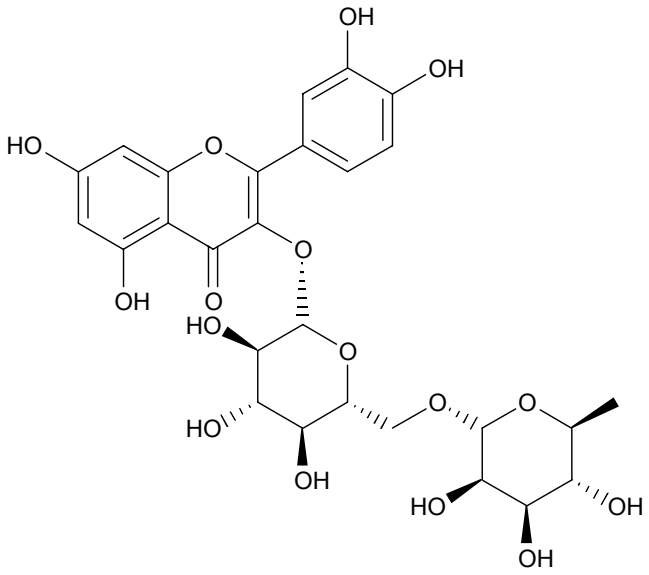
3.7 *Trans*-triacontyl-4-hydroxy-3-methoxycinnamate



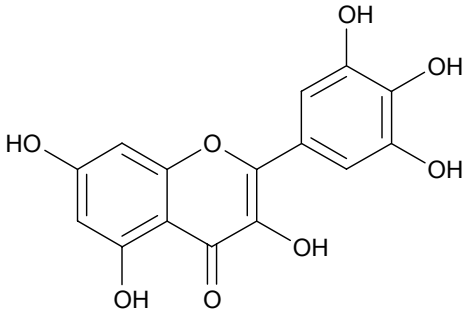
3.8 (-)-Ovatolide

B. ferruginea Benth. has been found to contain various triterpenes, the flavonoids and flavonoid glycosides quercetin derivatives such as rutin (**3.9**), myricetin derivatives (**3.10**) gallocatechin-(4′-O-7)-epigallocatechin (**3.11**); 3,5-dicaffeoylquinic acid (**3.12**) and 1,3,4,5-tetracaffeoylquinic acid (**3.13**); and the lignans deoxypodophyllotoxin (**3.14**),

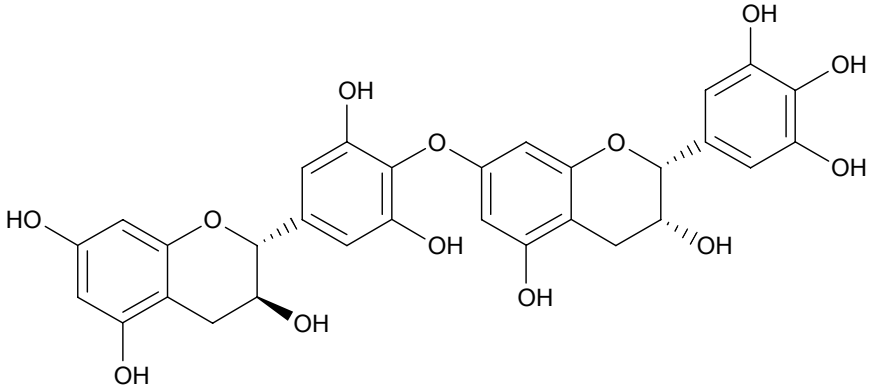
β-peltatin (**3.15**), β-peltatin-5-*O*-β-D-glucopyranoside (**3.16**), and 5′-demethoxy-β-peltatin-5-*O*-β-D-glucopyranoside (**3.17**).[1-4]



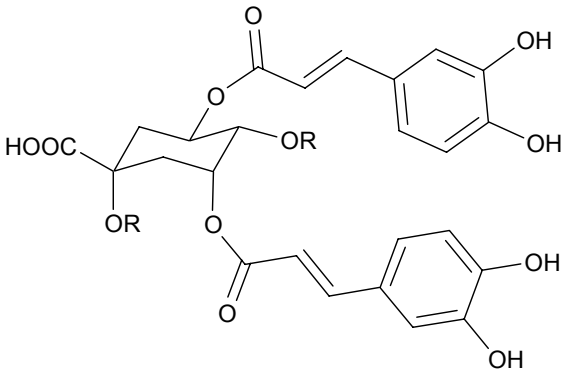
3.9 Rutin



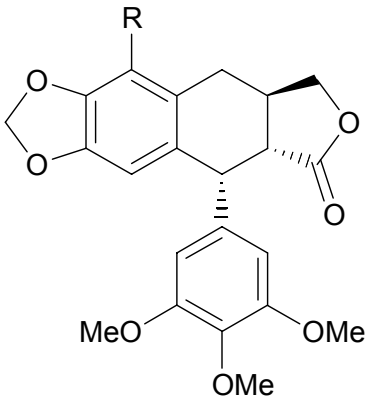
3.10 Myricetin

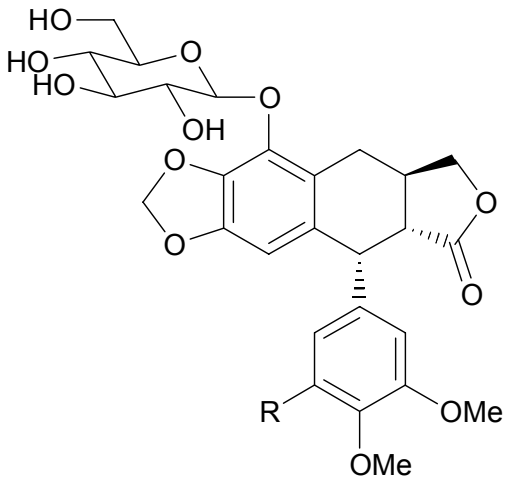


3.11 Gallocatechin-(4'-O-7)-epigallocatechin



3.12 3,5-dicaffeoylquinic acid R = H
3.13 1,3,4,5-tetracaffeoylquinic acid R = caffeoyl



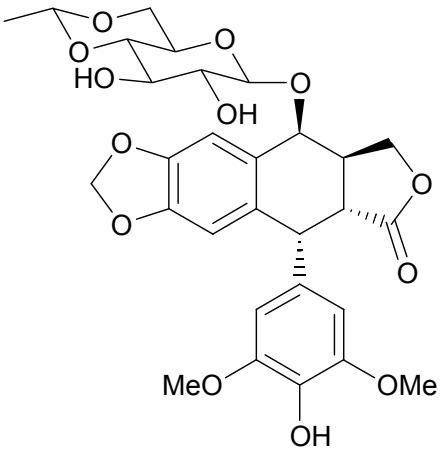


3.14 Deoxypodophyllotoxin R = H
3.15 β-peltatin R = OH

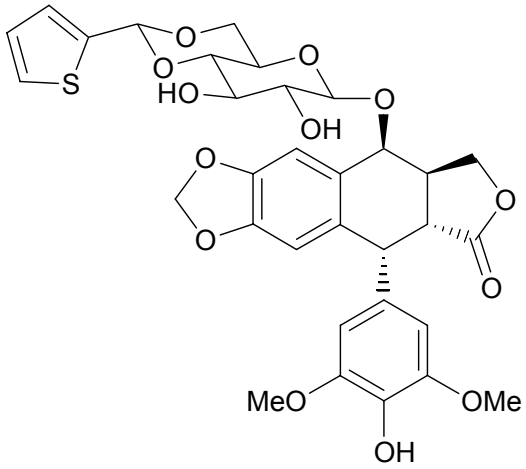
3.16 β-peltatin-5-*O*-β-D-glucopyranoside R = OMe
3.17 5'-demethoxy-β-peltatin-5-*O*-β-D-glucopyranoside R = H

Investigations have shown extracts of *B. ferruginea* Benth. to be antimicrobial, anti-HIV, and antispasmodic.[2,9,10] Rutin (**3.9**) has been shown to exhibit hypoglycemic activity.[11] The biflavanol (**3.11**) and the quinic acid derivatives (**3.12** and **3.13**) have been shown to inhibit the complement system, which is an immune response that plays a role in inflammation and allergic reactions.[2,12] The lignans (**3.14-17**) were found to be cytotoxic and are structurally related to the semi-synthetic podophyllotoxin analogs etoposide (**3.18**) and teniposide (**3.19**).[1] This is the first reported investigation of *B. tulasneana*.

[9] Akinpelu, D. A.; Olorunmola, F. O. Antimicrobial activity of Bridelia ferruginea fruit. *Fitoterapia*. **2000**, *71*, 75-76.
[10] Muanza, D. N.; Euler, K. L.; Williams, L.; Newman, D. J. Screening for antitumor and anti-HIV activities of nine medicinal plants from Zaire. *Int. J. Pharmacogn.* **1995**, *33*, 98-106.
[11] Onunkwo, G. C.; Akah, P. A.; Udeala, O. K. Studies on *Bridelia ferruginea* leaves (1). Stability and hypoglycemic actions of the leaf extract tablets. *Phytother. Res.* **1996**, *10*, 418-420.
[12] Stryer, L. *Biochemistry*. W. H. Freeman and Company, New York, **1995**, 376-377.



3.18 Etoposide

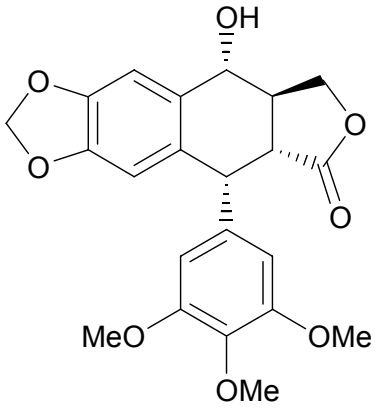


3.19 Teniposide

3.1.2 The Podophyllotoxins

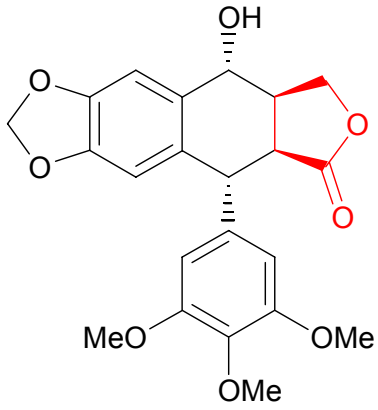
The lignans, including podophyllotoxin and its analogs, are produced via the shikimate pathway from coniferyl alcohol.[13] Two semisynthetic podophyllotoxin analogs, etoposide (**3.18**) and teniposide (**3.19**) are used clinically to treat cancer. It is interesting, as discussed previously in chapter 1, that these epi-analogs of podophyllotoxin exhibit cytotoxicity by a mechanism that is different from podophyllotoxin itself. The epi-analogs act via an interaction with topoisomerase II, whereas most natural podophyllotoxin analogs are microtubule inhibitors. Podophyllotoxin (**3.20**) and deoxypodophyllotoxin (**3.14**) can cause complete inhibition of tubulin polymerization at concentrations as low as 5 μM.[14]

[13] Dewick, P. M. *Medicinal Natural Products: A Biosynthetic Approach*. John Wiley & Sons, Inc., New York, **1997**, 123-124.
[14] Bohlin, L.; Rosén, B. Podophyllotoxin Derivatives: Drug Discovery and Development. *Drug Disc. Today*. **1996**, *1*, 343-351.



3.20 Podophyllotoxin

The activity of the podophyllotoxins is dependent on the presence of the *trans*-fused lactone. When the ring is converted to the *cis*-fused lactone, as in picropodophyllin (**3.21**), the compound loses almost all of its cytotoxic activity (Figure 3.1).[13]



3.21 Picropodophyllin

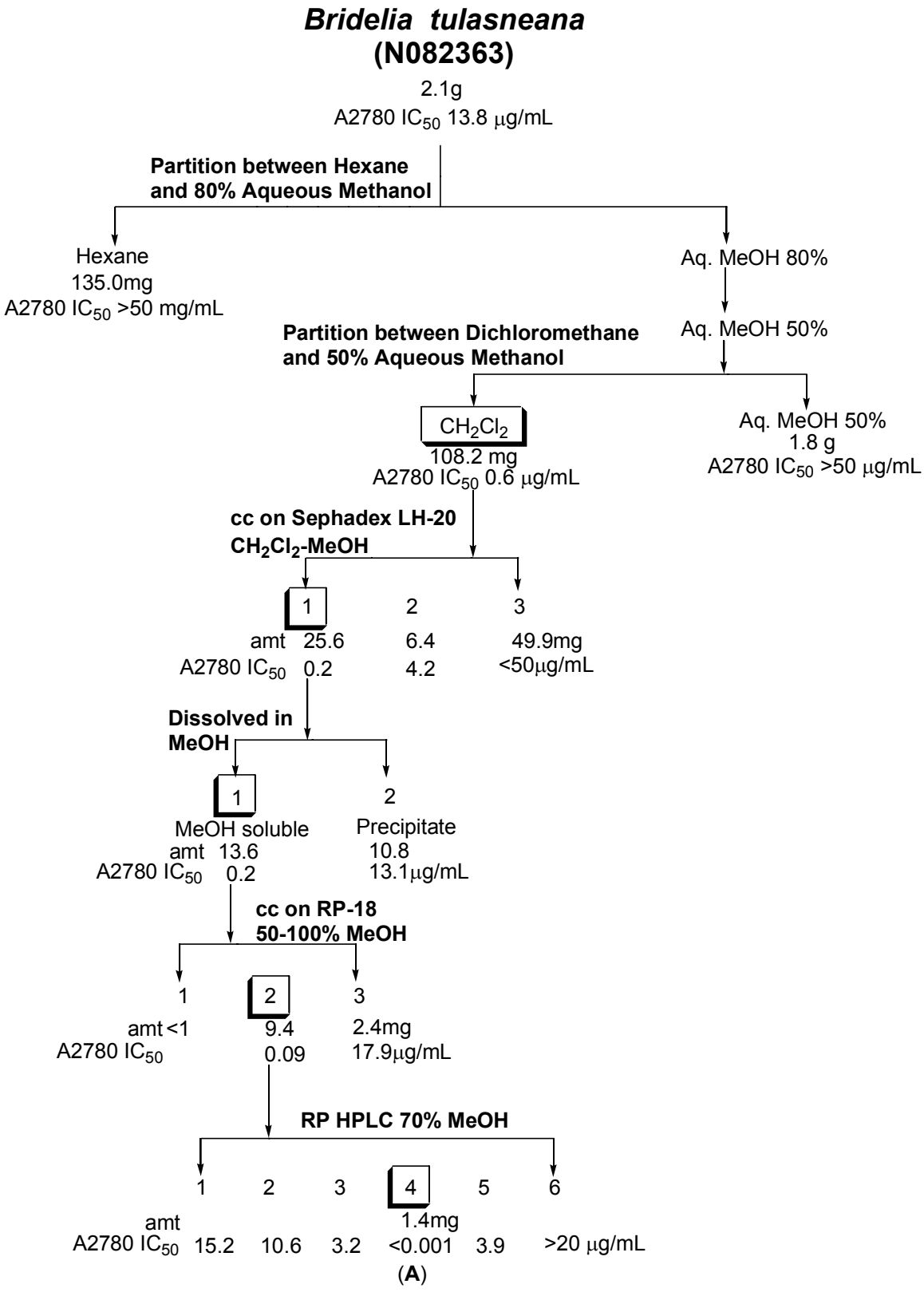
Figure 3.1 The *cis*-fused ring of picropodophyllin.

3.2 Results and Discussion

3.2.1 Isolation of Compound A from *Bridelia tulasneana*

An extract of *Bridelia tulasneana* was obtained from Madagascar as a part of our continuing search for anticancer compounds through the ICBG program. Testing revealed that the crude extract was weakly cytotoxic (IC_{50} = 14 μg/ml) in the A2780 human ovarian cell line and thus was selected for further investigation.

A portion (2.1 g) of the extract was taken for bioassay-guided fractionation (Scheme 3.1). It was partitioned between 80% aqueous methanol and hexane, then the methanol fraction was diluted to 50% aqueous methanol and washed with dichloromethane. The fractions were concentrated via rotary evaporation and tested for cytotoxicity. The dichloromethane fraction was the sole active fraction with a very impressive increase in activity to an IC_{50} of 0.6 μg/ml. The dichloromethane fraction was then subjected to fractionation using Sephadex LH-20 eluted with a dichloromethane/methanol gradient starting from 100% dichloromethane and going to 100% methanol. Fractions were combined based on TLC analysis. The most active fraction (1) was dissolved in methanol, at which point it formed a white precipitate. The precipitate was filtered out, and the two fractions were tested. The methanol soluble portion was found to be active and was fractionated further using a reversed phase C-18 open column eluted with an aqueous methanol gradient starting at 50% and going to 100%. 11 fractions were collected and combined based on TLC analysis to give 3 fractions. Fraction 2, being the most active, was fractionated further using reversed phase HPLC eluted with 70% aqueous methanol. Fraction 4 collected at 16 minutes with a flow rate of 2 ml/min. yielded 1.4 mg of compound **A**.



Scheme 3.1 Fractionation tree for the isolation of compound **A**.

3.2.2 Structure Elucidation of Compound A from *Bridelia tulasneana*

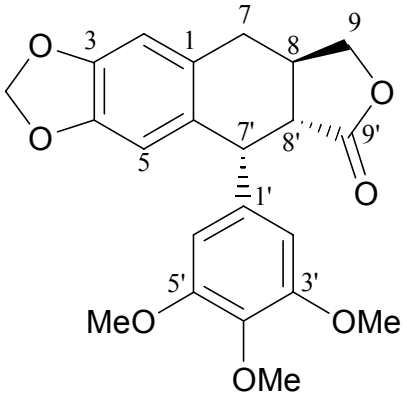
Compound **A** was isolated as a colorless amorphous solid. High-resolution positive ion FABMS indicated a molecular formula of $C_{22}H_{22}O_7$ (*m/z* 399.1431 $[M+1]^+$).

The ^1H NMR spectrum in $CDCl_3$ was relatively simple, with only eleven signals. It indicated the presence of three methoxy groups, two of which were equivalent (δ 3.73(s), 3.73(s), 3.79(s)), two protons that were coupled to each other (δ 5.91(d, *J*=1.5 Hz), 5.93(d, *J*=1.5 Hz)), and four aromatic protons (δ 6.33(s), 6.33(s), 6.50(s), 6.65(s)).

The ^{13}C NMR spectrum, also ran in $CDCl_3$, contained eighteen signals. Eight of the ^{13}C signals appeared to be from saturated carbons, while ten signals appeared to be from unsaturated carbons. One of the saturated carbons (δ 101.2) was exceptionally deshielded, indicating that it was probably bonded to two oxygens. One of the unsaturated carbon signals (δ 174.9) indicated the presence of a carbonyl group, possibly from an ester or an acid.

In most cases the bioassay data is not used in the elucidation of a structure. However, in this case the exceptional activity, combined with the prior precedent for the genus to contain podophyllotoxin analogs, indicated that compound **A** might also be a podophyllotoxin analog. This hypothesis was supported by the presence of the three methoxy groups. The signals at δ 5.91 and δ 5.93 in the ^1H NMR and δ 101.2 in the ^{13}C NMR are also characteristic of podophyllotoxin analogs and indicate a methylene oxide group. A quick comparison of molecular formulas indicated that deoxypodophyllotoxin was the most likely possibility. A comparison of the ^1H and ^{13}C NMR values to literature

values showed a close correlation (Table 3.1 and 3.2).[15] Additionally, it was possible to compare the spectra to that of an authentic sample.[16] The spectra of compound **A** also matched the spectra of the authentic sample. The only discrepancy amongst the spectra was that the ^{13}C NMR of compound **A** and the known sample contained an unsaturated quaternary carbon signal that was weaker than expected. The intensity of the signals is not given in the literature, so it must be assumed that that same signal was also weak in the literature. In the known sample it was intense enough to be clearly distinguished above the noise. However, in compound **A** it was only just distinguishable above the noise. This is in all probability due to the small sample size of compound **A**. Since the chemical shifts match both the literature and the known sample there is little doubt that compound **A**is deoxypodophyllotoxin.



3.14 Deoxypodophyllotoxin

Figure 3.2 Numbering scheme for Deoxypodophyllotoxin.

[15] Ikeda, R.; Nagao, T.; Okabe, H.; Nakano, Y.; Matsunaga, H.; Katano, M.; Mori, M. Antiproliferative Constituents in Umbelliferae Plants. III. Constituents in the Root and Ground Part of *Anthriscus sylvestris* Hoffm. *Chem. Pharm. Bull*. **1998**, *46*, 871-874.
[16] Kingston, D. G. I.; Rao, M. M.; Zucker, W. V. Plant Anticancer Agents. IX. Constituents of *Hyptis tomentosa*. *J. Nat. Prod*. **1979**, *42*, 496-499.

Table 3.1 ^{1}H NMR of Compound **A**.

	Deoxypodophyllotoxin Lit.[15]	Deoxypodophyllotoxin Authentic Sample[16]	Compound **A**
2	6.65 s	6.65 s	6.65 s
5	6.52 s	6.50 s	6.50 s
7	~ 2.73	~2.75	~2.75
	3.07 dd (17.0, 10.0)	3.06 m	3.06 m
8	2.72 m	2.72	2.72
9	3.91 m	3.90 m	3.90 m
	4.44 m	4.44 m	4.44 m
2′	6.35 s	6.33 s	6.33 s
6′	6.35 s	6.33 s	6.33 s
7′	4.59 d (3.5)	4.58 d (3.5)	4.58 d (3.5)
8′	2.72 m	2.72	2.72
3′&5′ OCH_3	3.75 s	3.73 s	3.73 s
4′ OCH_3	3.80 s	3.79 s	3.79 s
OCH_2O	5.92 d (1.0)	5.91 d (1.0)	5.91 d (1.5)
	5.94 d (1.0)	5.93 d (1.0)	5.93 d (1.5)

All spectra recorded in $CDCl_3$.

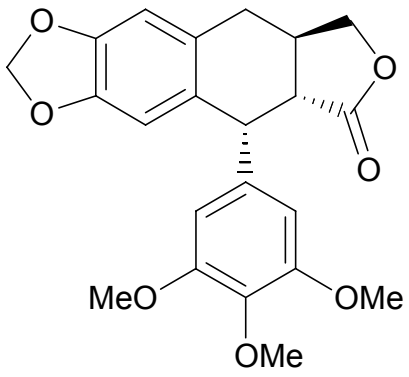
Table 3.2 ^{13}C NMR of Compound **A**.

	Deoxypodophyllotoxin Lit.[15]	Deoxypodophyllotoxin Authentic Sample[16]	Compound **A**
1	128.3	128.3	128.3
2	108.5	108.5	108.5
3	147.1	147.0	147.0
4	146.8	146.7	146.7
5	110.5	110.5	110.5
6	130.7	130.6	130.6
7	33.1	33.1	33.1
8	32.8	32.7	32.7
9	72.0	72.1	72.1
1′	136.2	136.3	136.3
2′	108.5	108.2	108.3
3′	152.5	152.5	152.5
4′	137.3	137.0	137.1
5′	152.5	152.5	152.5
6′	108.5	108.2	108.3
7′	43.8	43.7	43.7
8′	47.5	47.5	47.5
9′	174.8	174.9	174.9
3′ & 5′ OCH_3	56.3	56.2	56.2
4′ OCH_3	60.8	60.8	60.8
OCH_2O	101.2	101.2	101.2

All spectra recorded in $CDCl_3$.

In order to confirm that the stereochemistry of compound **A** was in fact the same as that of deoxypodophyllotoxin, its optical rotation was measured. The optical rotation was determined to be $[\alpha]_D$ = -103.8° (*c* 0.08), whereas the optical rotation found in literature was $[\alpha]_D$ = -103.0 (*c* 0.11).[17] Since compound **A** has the same optical rotation as the literature, and since they have identical NMR spectra they must have the same absolute stereochemistry.

Based on the molecular weight, the NMR data, the extreme cytotoxicity, and the optical rotation compound **A** was determined to be deoxypodophyllotoxin (**3.14**).



3.14 Deoxypodophyllotoxin

3.2.3 Biological Evaluation of Deoxypodophyllotoxin

Deoxypodophyllotoxin (**3.14**) as previously stated is a known compound and has been extensively studied. It, like podophyllotoxin (**3.20**), is a microtubule inhibitor.[14] Due to a lack of material the deoxypodophyllotoxin (**3.14**) that was isolated from *Bridelia tulasneana* could not be assayed for cytotoxicity, and the final bioassay results were

[17] Chang, L. C.; Song, L. L.; Park, J. E.; Luyengi, L.; Lee, K. J.; Farnsworth, N. R.; Pezzuto, J. M.; and Kinghorn, A. D. Bioactive Constituents of *Thuja occidentalis*. *J. Nat. Prod.* **2000**, *63*, 1235-1238.

determined using the known sample. The cytotoxicity in the A2780 human ovarian cancer cell line was found to be greater than could be accurately measured (IC_{50} < 0.001 μg/ml). Deoxypodophyllotoxin (**3.14**) is not used clinically in the treatment of cancer due to toxic side effects.[13]

3.3 Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. UV spectra were obtained using a Shimadzu UV-1201 spectrophotometer. High resolution FABMS were taken on a JEOL HX-110 instrument. 1D NMR data collected on a JEOL Eclipse instrument at 500 MHz for proton and 125 MHz for carbon. Thin layer chromatography was performed on Whatman $MKC_{18}F$ reversed phase TLC plates, and Sigma-Aldirch silica gel TLC plates. Column chromatography was performed using Sigma lipophilic Sephadex LH-20, and Whatman LRP2. HPLC was performed using a Shimadzu 10A vp instrument with a YMC ODS C-18 column (250 × 10 mm) with a flow rate of 2 ml/min.

Cytotoxicity Bioassay. The A2780 human ovarian cell line was employed as an *in vitro* cytotoxicity assay as previously reported.[18]

Plant Material. The twigs of *Bridelia tulasneana* Baill. (Euphorbiaceae) were collected at 18°2′3″ S, 48°31′23″ E, elev. 920 m, in Toamasina, Madagascar, on April 22, 1995, by R. E. Gereau, P. -J. Rakotomalaza, C. Rasolomalana, T. Razafindrabeaza, S. Rapanarivo,

[18] See Chapter 1 Bioassay Section.

and F. Andriatsiferana of the Missouri Botanical Garden. A voucher specimen (GEREAU 5762) can be found at the Missouri Botanical Garden (MO), and was identified by G. McPherson of the Missouri Botanical Garden. The tree was 10 m tall with green fruit and a trunk and buds that were covered with thorns.

Extract Preparation. The twigs of *Bridelia tulasneana* were extracted to give the crude extract N082363.

Bioassay Guided Fractionation and Isolation of Deoxypodophyllotoxin (3.14). 2.1 g of N082363 was taken for fractionation. It was partitioned between hexane and 80% aqueous MeOH. The aqueous methanol fraction was diluted with water to give a 50% aqueous MeOH fraction, which was further partitioned with CH_2Cl_2. The fractions were concentrated via rotary evaporation to give an active CH_2Cl_2 fraction. The CH_2Cl_2 fraction was then chromatographed on Sephadex LH-20 using a 100% CH_2Cl_2 to 100% MeOH gradient. The fractions were pooled to give three factions. Fraction 1 was the most active. Fraction 1 was further partitioned by removal of a non-MeOH soluble precipitate. The more active MeOH soluble portion was further fractionated using RP C-18 eluted with an aqueous MeOH gradient (50-100%). 11 fractions were collected and combined based on TLC to give 3 fractions. Fraction 2, being the most active, was further fractionated using RP-HPLC. Fraction 4, collected at 16 min., gave 1.4 mg of deoxypodophyllotoxin (**3.14**).

Deoxypodophyllotoxin (3.14): colorless amorphous solid; $[\alpha]_D$ −103.8° (*c* 0.08, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 290 (3.38), 216 (4.15); ^1H NMR, see Table 3.1; ^{13}C NMR, see Table 3.2; HRFABMS *m/z* 399.1431 $[M+H]^+$ (calcd for $C_{22}H_{23}O_7$, 339.1444).