NONPOLAR PENTACYCLIC TRITERPENES OF THE MEDICINAL FERN *POLYPODIUM SUBPETIOLATUM*

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ABSTRACT.—The rhizomes of the medicinal Honduran fern *Polypodium leucotomos*, commonly called "Calaguala", are claimed to exhibit antineoplastic activity (1). We were prompted to investigate the nature of the compounds present in the rhizomes of the Guatamalan fern *Polypodium subpetiolatum* Hook because this plant is also used medicinally and is referred to by the same common name "Calaguala". This report describes our isolation, characterization, and identification of the nonpolar fractions of a pentane extract of the rhizomes. The following pentacyclic triterpenes were characterized by chemical and physical methods: 9(11)-fernene (**3**), 13(18)-neohopene (**4**), diploptene (**5**), 7-fernene (**6**), 17(21)-hopene (**7**), serratene (**8**), 3β-methoxy-9(11)-fernene (**9**), 3β-hydroxy-9(11)-fernene (**10**), 3β-acetoxy-9(11)-fernene (**11**) and fernenol palmitate (**12**). Neither **11** nor **12** have been previously isolated from natural sources.

Our interest in the fern *Polypodium subpetiolatum* Hook stems from the fact that this species, called "Calaguala", is used by some inhabitants of Guatamala as a medicinal plant. An infusion, prepared by boiling the ground rhizomes with water, is taken internally and used primarily for treatment of open sores on the skin. A related species of fern used medicinally in Honduras, *Polypodium leucotomos*, is also called "Calaguala". An infusion of its rhizomes is claimed to be active against malignant tumors and leukemias (1, 2). In view of the same common name for the two species and the fact that they are both used medicinally, we undertook experiments to determine the structure of the extractable molecules from *P. subpetiolatum* rhizomes. In this article we report the results of our initial work dealing with an analysis of the nonpolar compounds present in the pentane extract of the rhizomes. Several pentacyclic triterpene hydrocarbons, as well as oxygenated derivatives related to hopane (**1**) and fernane (**2**), were isolated and their structures determined. A pentacyclic hydrocarbon identified as serratene (**8**) was the only triterpene isolated which was not directly related to **1** or **2**.

Dried, ground rhizomes were extracted with pentane in a continuous extractor. After removal of the pentane *in vacuo*, the residue was taken up in ether and separated into acidic, basic, and neutral fractions by appropriate extractions. Fractions A (acidic) and B (basic) were set aside for future analysis. Fraction C (neutrals) was chromatographed on Al_2O_3 and eluted consecutively with pentane (fraction D), 10% ether in pentane (fraction E), 25% ether in pentane (fraction F) and 50% ether in pentane (fraction G). More polar substances remaining on the column were removed with ethyl acetate (fraction H) and set aside.

The fraction (D), analyzed by tlc on 20% silver nitrate impregnated Silica Gel G, was shown to consist of a mixture of eight unsaturated hydrocarbons. The hydrocarbon fraction (D) was rechromatographed on a column of 10% silver nitrate impregnated Al_2O_3 with pentane as eluent. An initial fraction, which crystallized from methanol-chloroform, contained pure 9(11)-fernene (**3**), as established by mp, mmp, rotation, and spectra in comparison with an authentic sample. Intermediate pentane fractions contained an unseparated mixture of five hydrocarbons (Fraction I). The two final fractions contained pure 13(18)-neohopene (**4**, hopene-II) and diploptene (**5**), whose structures were verified by

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comparison of mp, mmp and IR spectra with authentic materials.   In the case of
13(18)-neohopene (**4**), authentic material was obtained by the known conversion
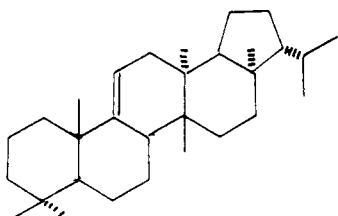of 9(11)-fernene (**3**) to **4** under the action of acid (3, 4).

A small quantity of Fraction I from the silver nitrate chromatography was
subjected to high pressure liquid chromatography (hplc) with 20% silver nitrate
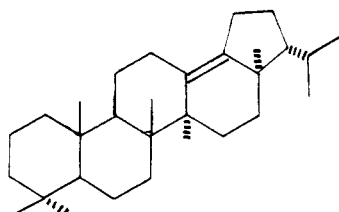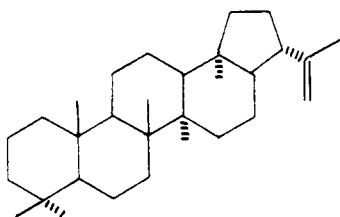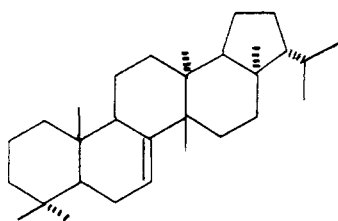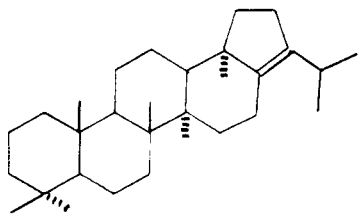
1

2

3

4

5

6

7

8

9   R = CH$_3$

10 R = H

11 R = CH$_3$CO

12 R = n-C$_{15}$H$_{31}$CO
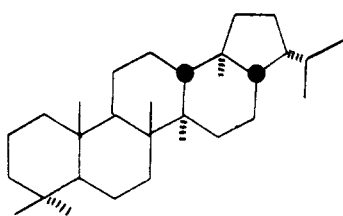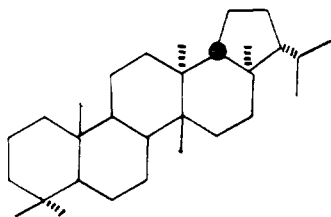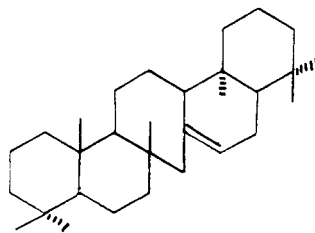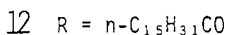
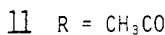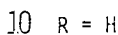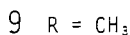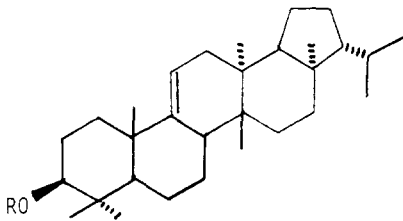impregnated Silica Gel as the absorbant and hexane as the eluent. This mixture was cleanly separated into five fractions which appeared homogeneous on analysis by silver nitrate tlc. Three other pentacyclic hydrocarbons, characterized by comparison of their mps, mmps, ir spectra and rotation to authentic samples, were identified as 7-fernene (6), 17(21)-hopene (7, hopene-I), and serratene (8). The two remaining hydrocarbons could not be characterized due to a lack of material.

Nonpolar oxygenated triterpene derivatives present in the neutral fraction (C) were eluted from the original Al$_2$O$_3$ column with solvent systems more polar than pentane. Fraction E (10% ether in pentane) was rechromatographed on Al$_2$O$_3$ and eluted with 5% ether in pentane to afford a compound which, after crystallization from hexane, was characterized by mp, mmp and superimposable ir spectra as 9, 3β-methoxy-9(11)-fernene (arundoin) as synthesized by methylation of 3β-hydroxy-9(11)-fernene (10, fernenol).

Fraction F, eluted by 25% ether in pentane from the initial large scale Al$_2$O$_3$ separation, was rechromatographed on Silica Gel with 5% ethyl acetate in pentane. Two fractions of interest were obtained. The least polar of the two fractions (fraction J), after further purification, was identified as 3β-acetoxy-9(11)-fernene (11). The more polar fraction, K, would not crystallize; however, on standing for approximately one week in acetone solution, a white amorphous solid separated. The substance possessed a broad melting point range, but appeared as a single spot on Silica Gel G and 10% silver nitrate impregnated Silica Gel G plates (5% ethyl acetate in pentane). The ir and ^{1}H nmr spectra of the compounds indicated it to be a fatty acid ester of a triterpene alcohol. The high resolution ms molecular weight of 664.638 indicated a formula of C$_{46}$H$_{80}$O$_2$ demonstrating that the acid component of the ester must correspond to a formula of C$_{16}$H$_{32}$O$_2$, if the alcohol component corresponds to a formula of C$_{30}$H$_{50}$O. Basic hydrolysis of the compound followed by separation of the products into neutral and acidic fractions yielded 3β-hydroxy-9(11)-fernene (10) as the sole neutral component and palmitic acid as the only identifiable acid component, confirming that the parent structure of the ester must be 12. The acid was characterized as the methyl ester by comparison of ir spectra and glc retention times on two different columns to those of authentic methyl palmitate.

The final compound to be identified in the neutral pentane extract of the rhizomes was eluted by 50% ether in pentane (fraction G from the original Al_2O_3 column). Removal of solvent and subsequent crystallization of the resulting solid afforded pure 3β-hydroxy-9(11)-fernene (**10**) whose structure was established by the comparison of physical data (mp, rotation, and spectral) of **10**, its 3β-acetoxy, 3-keto, and 3β-methoxy derivatives, to data in the literature (5).

With the exception of 3β-acetoxy-9(11)-fernene (**11**) and 9(11)-fernenol palmitate (**12**), all identified compounds have been previously isolated from other ferns. The principal pentacylic triterpenes found in this species are those possessing the 9(11)-fernene skeleton. No free keto-steroids were discovered in the nonpolar extracts of this fern species.

EXPERIMENTAL[2]

PLANT MATERIAL.—Plant rhizomes were collected from natural sources. The plant specimens were identified and cultured by Dr. Ramón Riba y Nava E.[3] Preliminary treatment involved grinding of the waterwashed rhizomes in a Wiley Mill followed by air drying in a thin layer for 72 hr.

EXTRACTION OF PLANT MATERIAL AND ISOLATION OF ACIDIC, BASIC, AND NEUTRAL FRACTIONS (A, B AND C, RESPECTIVELY).—A total of 1.35 kg of ground dried *P. subpetiolatum* rhizomes was extracted 24 hr with pentane in a 40-liter continuous extractor. The concentrate (102 g, 7.56%) obtained by removal of solvent was dissolved in 1.5 liters of ether and extracted with 3 portions of 10% HCl (1 liter each) followed by two washings with distilled H_2O (1 liter each). The extracts were combined with the washings, neutralized with K_2CO_3, and extracted with three 1-liter portions of ether. The solutions were combined and concentrated *in vacuo* to yield basic substances (B, 1.6 g, 0.12%). Neutral substances (fraction C) were isolated by treatment of the acid-extracted ether solution with three 1-liter volumes of 10% KOH. After three 1-liter H_2O washings and one 1-liter washing with saturated NaCl, the ether solution was dried with anhydrous Na_2SO_4 and concentrated *in vacuo* to afford neutral compounds (C, 92 g, 6.84%). The basic aqueous extracts were combined with the water washings, neutralized with dilute HCl, and extracted with two 1-liter volumes of ether. Concentration of the combined ether extracts under reduced pressure yielded acidic substances (A, 6.7 g, 0.50%). Fractions A and B were stored for future analysis.

LARGE SCALE SEPARATION OF NEUTRAL FRACTION (C) ON AL_2O_3.—The neutral material (92 g) was separated into crude fractions by chromatography on a column containing 4.3 kg of Al_2O_3 (90 x 640 mm). Elution with pentane (9 liters) yielded fraction D (49 g, 3.63%); 3.5 liters 10% ether in pentane: fraction E (10 g, 0.74%); 4.0 liters 25% ether in pentane: fraction F (14 g, 1.04%); and 4.0 liters 50% ether in pentane: fraction G (9.0 g, 0.59%). Most of the more polar materials remaining on the column were removed with 5 liters of ethyl acetate (fraction H, 8.0 g, 0.59%). The last fraction (H) was stored for future analysis. More than 96% of the material originally introduced onto the column was accounted for.

ISOLATION AND CHARACTERIZATION OF $C_{30}H_{50}$ HYDROCARBONS.—Analysis by AgNO$_3$ tlc revealed fraction D from the above chromatography to be a mixture of eight unsaturated hydrocarbons. Of fraction D, 49 g was subjected to separation on a column of 20% AgNO$_3$ impregnated Al$_2$O$_3$ (1 kg, 40 x 1000 mm) with pentane as eluent. The first 2 liters of pentane contained nothing. The second 2 liters, on concentration, afforded 32 g (2.37%) of pure 9(11)-fernene (**3**, R$_f$(AgNO$_3$)=0.93), which crystallized as plates from methanol/CHCl$_3$. An additional 1.5 liters of pentane eluted an unseparated mixture of 5 hydrocarbons (Fraction I; 12 g; 0.89%: R$_f$(AgNO$_3$)=0.87, 0.67, 0.35, 0.32, 0.15). The next liter of pentane eluted 1.6 g (0.12%) of 13(18)-neohopene (**4**, R$_f$(AgNO$_3$)=0.10), which crystallized as needles from acetone. The final 1.5 liters of pentane yielded 1.3 g (0.10%) of diploptene (**5**, R$_f$(AgNO$_3$)=0.07), which also crystallized from acetone, but as plates. The mp, ir and rotation of the above natural products were identical in all respects to reference compounds **3**, **4**, and **5**, and they gave no depression on mmp.

A small quantity of Fraction I (300 mg) was cleanly separated by hplc. The instrument used was a Waters Associates ALC-202 with a 6 ft, ⅜ in. O.D. column packed with 20% AgNO$_3$ impregnated Silica Gel (150–170 mesh). Separation parameters were as follows: solvent hexane, temperature 30°, flow rate 0.4 ml/min @ 150 psi. Fractions (5 ml) were collected automatically and analyzed by AgNO$_3$ tlc. The appropriate fractions were combined and each crystallized from methanol-CHCl$_3$. Three additional hydrocarbons were identified as serratene (**8**, R$_f$(AgNO$_3$)=0.67), 7-fernene (**6**, R$_f$(AgNO$_3$)=0.35), and 17(21)-hopene (**7**, R$_f$(AgNO$_3$)=0.15) by comparison of mp(mmp), R$_f$(AgNO$_3$), and ir spectra to those of authentic samples. The two remaining hydrocarbons (R$_f$(AgNO$_3$)=0.87 and 0.32) were not present in sufficient quantities to be adequately characterized.

ISOLATION AND CHARACTERIZATION OF 3β-METHOXY-9(11)-FERNENE (**9**).—Fraction E eluted by 10% ether in pentane from the initial large scale Al$_2$O$_3$ column was shown to be a mixture of a major component (R$_f$=0.60) and two minor components (R$_f$=0.42, R$_f$=0.38) by tlc analysis (5% ethyl acetate in pentane). Fraction E was rechromatographed on 1 kg of Al$_2$O$_3$ on a 40 x 1000 mm column. Ether in pentane (5%) eluted 7.5 g (0.56%) of the major component (R$_f$= 0.60) as a crystalline solid, which was recrystallized from hexane to give 3β-methoxy-9(11)-fernene (**9**). The properties (ms, [α]D, mp, ir and ^{1}H nmr spectra) of authentic **9** prepared by treatment of 3βhydroxy-9(11)-fernene (**10**) with methyl iodide and base (5) were identical to those of the natural product. The two more polar components in fraction E (2 g) were identified in the following fraction (F) eluted from the large scale alumina column.

ISOLATION AND CHARACTERIZATION OF 3β-ACETOXY-9(11)-FERNENE (**11**) and 9(11)-FERNENOL PALMITATE (**12**).—The material eluted by 25% ether in pentane (Fraction F, 14 g) from the original Al$_2$O$_3$ column proved to be a mixture of the two compounds that were present as more polar minor components in the previous fraction (E, R$_f$=0.42, R$_f$=0.38, 5% ethyl acetate in pentane). The fraction from E containing the two more polar components was combined with F (total=16 g) and rechromatographed on 1 kg of Al$_2$O$_3$ (40 x 1000 mm). Elution with 1.5 liters of 5% ethyl acetate in pentane afforded the least polar component (fraction J, 6.5 g, 0.48%, R$_f$=0.42, 5% ethyl acetate in pentane). An additional 300 ml eluted an intermediate mixed fraction, and a final 500 ml eluted the more polar component, fraction K (8.0 g, 0.59%, R$_f$=0.38, 5% ethyl acetate in pentane).

The least polar component (J, R$_f$=0.42) was triturated with methanol, and the resulting solid crystallized as colorless needles from acetone. The compound was subsequently identified as 3β-acetoxy-9(11)-fernene (**11**). The mp, ir and, ^{1}H nmr spectra of the isolate and reference **11**, prepared by acetylation of 3β-hydroxy-9(11)-fernene (**10**), were indistinguishable. The more polar component K, (R$_f$=0.38) would not crystallized; however, on standing in acetone solution for 1 week, 4.5 g (0.33%) of a white amorphous solid was desposted whose spectral properties indicated it to be a fatty acid ester of a triterpene alcohol: mp 160–174° [α]^{26}D −10.5°; ν max 2930(s), 1730(s), 1645(w), 1430(s), 1375(s), 815(w), 790(w), cm^{-1}; pmr 5.38 J (1H, broad m), 2.2 δ (2H, t), 1.3–1.5 δ (>50 H, broad m), 1.1–0.75 δ (21 H, methyl groups); high resolution ms molecular weight 664.638 (calculated for $C_{46}H_{80}O_2$ 664.638); ms 664 (M$^-$, 5%), 649 (M$^-$ –CH$_3$, 8%), 515 (100%, characteristic of 9(11)-fernene derivatives (5), 409 (13%, M$^-$–C$_{16}$H$_{31}$O$_2$). Basic hydrolysis of the ester in ethanolic THF followed by a standard work-up afforded 3β-hydroxy-9(11)-fernene (**10**) as the neutral triterpene component, verified by comparison to authentic **10**, and an acid component identified as palmitic acid. The palmitic acid was characterized as the methyl ester prepared by treatment of the acid with methanolic BF$_3$. The ir spectra and glc retention times on 6 ft, ⅛ in. columns of 10% carbowax (2.6 min, 160°) and 10% SE 54 (2.0 min, 150°) were identical to spectra and retention times for methyl palmitate prepared from authentic palmitic acid by the method described above. The structure of the ester was, therefore, established to be **12**.

ISOLATION AND CHARACTERIZATION OF 3β-HYDROXY-9(11)-FERNENE (**10**).—The final fraction examined from the original Al$_2$O$_3$ column (G, 9 g), eluted by 50% ether in pentane, crystallized as needles from methanol and was subsequently identified as 3β-hydroxy-9(11)-fernene (**10**) by comparison of physical data (below) to literature values for **10**, its 3β-acetoxy, 3-keto and 3β-methoxy derivatives (5).

3β-hydroxy-9(11)-fernene: mp 190–192° (192–193°)[4]; $[\alpha]^{26}$D −18° (−19.4°, c=0.85); ir 3530, 2930, 1635, 1430, 1375, 1170, 820, 790 cm⁻¹ (3520, 2930, 1630, 1440, 1378, 1175, 820, 790 cm⁻¹); ms 426–23%, 411–90%, 273–18%, 259–100%, 241–50% (426–30%, 411–87%, 273–18%, 259–100%, 241–55%).

3β-acetoxy-9(11)-fernene: mp 218–220° (222–223°); $[\alpha]^{26}$D −9.5° (−8.9°, c=0.96); ir 2930, 1730, 1635, 1430, 1380, 1240, 1075, 820, 795 cm⁻¹; (2930, 1725, 1640, 1440, 1378, 1245).

3-keto-9(11)-fernene: mp 196–198° (198–199°) $[\alpha]^{26}$D −43° (−41.2°, c=0.39); ir 2930, 1710, 1635, 1470, 1445, 1380, 1115, 1010, 985, 815, 795 cm⁻¹; (2920, 1700, 1635, 1468, 1440, 1378, 1114, 1005, 980, 810, 790 cm⁻¹).

3β-methoxy-9(11)-fernene: mp 242–244° (242–243°); $[\alpha]^{26}$D −5.0 (−5.3°, c=0.79); ir 2920, 1635, 1430, 1380, 1260, 1185, 1103, 1020, 995, 820, 795 cm⁻¹ (2930, 1630, 1450, 1380, 1260, 1182, 1103, 1013, 993, 815, 790 cm⁻¹); ¹H nmr 5.35 δ-1H (5.33δ-1H), 3.35δ-3H, (3.35δ-3H), 2.65δ-1H, (2.65δ-1H), 1.05δ-3H, (1.06δ-3H), 1.0-0.72δ-21H (1.0–0.75δ-21H); ms 440–80% (440–78%), 425–100% (425–100%), 393–50% (393–40%), 373–75% (273–68%), 241–45% (241–47%).

ACKNOWLEDGMENTS

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[4]Values in parentheses indicate literature values from reference 5.