Antimicrobial Terpenoids from the Oleoresin of the Peruvian Medicinal Plant *Copaifera paupera*

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Abstract

Twelve known diterpenes **1 – 11** and **13**, and three known sesquiterpenes **14 – 16**, along with a new $C_{20}-C_{15}$ terpenoid **17**, with a structure based on an unprecedented skeleton in which a labdane diterpene is linked to a monocyclic sesquiterpene by an ester bridge, were isolated from the oleoresin of the Peruvian medicinal plant *Copaifera paupera* (Herzog) Dwyer (Leguminosae). Their structures were elucidated on the basis of spectral analysis, including homo- and heteronuclear correlation NMR experiments (COSY, ROESY, HMQC and HMBC), and by comparison with data in the literature. The leishmanicidal, antimicrobial, cytotoxic, and aldose reductase inhibitory activities were studied. Compounds **1** and **11** showed significant antimicrobial activity (MIC < 10 μg/ml) against Gram-positive bacteria, comparable with cephotaxime used as control. Compound **2** exhibited moderate cytotoxic activity against four cancer cell lines.

Key words

Copaifera paupera · Leguminosae · Copaiba oil · terpenoids · leishmanicidal · antimicrobial · cytotoxic · aldose reductase inhibitory activities

Introduction

Copaiba oils, also described as copaiba balsams or oleoresins, are produced by extraction of the trunk of trees belonging to the genus, *Copaifera*. These trees are largely distributed in northern South America, mainly in the Amazonas rain forest. Copaiba oils have been used for the treatment of cancer, ulcer, syphilis, bronchitis, and diarrhoea [1], and as bactericidal, anti-helminthic, analgesic, anti-inflammatory, gastro-protective, antitumor, and trypanocidal agents [2]. The oils are used in the flavour and cosmetics industry, and as diesel-like fuels [3]. In Peru, where resorting to medicinal plants represents a primary health care measure of the native population, the leishmanicidal properties of copaiba oils have been known for centuries, and at the present time they are used as plaster to threat leishmaniasis (Uta) [4].

The main constituents of these oils are diterpenes and sesquiterpenes [2]. Nevertheless, in spite of the existing abundant literature on copaiba oils properties, little is known of the relationship between the structures and the activities of the components.

During the search for new bioactive compounds from South American folk medicinal plants, this work was initiated to investigate the oil of *Copaifera paupera*. The present paper describes the isolation and identification of fifteen known terpenoids, and a new $C_{20}-C_{15}$ terpenoid. All compounds were identified by ex-

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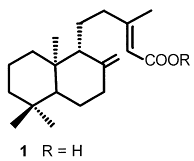
tensive analysis of spectroscopic data, and comparison with data in the literature. The leishmanicidal, antimicrobial, cytotoxic, and aldose reductase inhibitory activities of some of the compounds are described.



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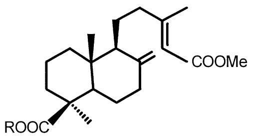

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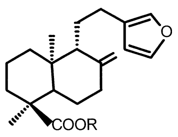

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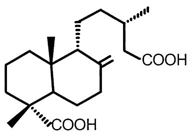

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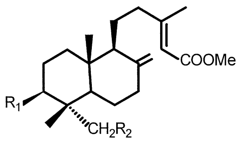

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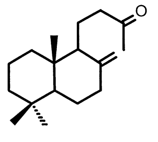

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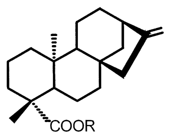

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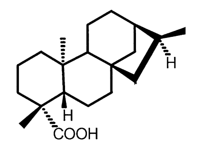

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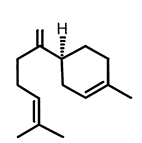

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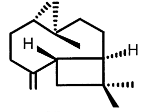

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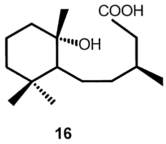

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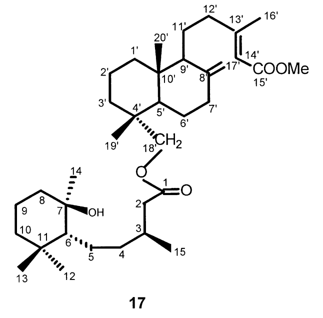

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Materials and Methods

General

Optical rotations were measured on a Perkin-Elmer 241 automatic polarimeter and the $[\alpha]_D$ are given in 10^{-1} deg cm^2 g^{-1}. IR spectra were recorded in $CHCl_3$ on a Bruker IFS 55 spectrophotometer and UV spectra were collected in absolute EtOH on a JASCO V-560. ^1H- and ^{13}C-NMR spectra were recorded on a Bruker Avance 400 NMR spectrometer at 400 MHz and 100 MHz, respectively. EIMS and HREIMS were recorded an a Micromass Autospec spectrometer. TLC 1500/LS 25 Schleicher and Schüell foils were used for thin-layer chromatography. Silica gel (particle size 40 – 63 μM, Merk) and Sephadex LH-20 (Pharmacia), were used for column chromatography, and semipreparative μ-porasil column (Waters, 10 μM, 19 mm × 25 cm) for HPLC.

Plant material and isolation

Copaifera paupera crude oil obtained from the trunk of the tree was collected in Madre de Dios, Provincia de Tambopata, Perú, in May 1997. A voucher specimen (No. CUZ-026 527) is deposited in the Vargas Herbarium, Facultad of Biology, Universidad Nacional de San Antonio Abad del Cusco, Perú.

Copaifera paupera oil (29.4 g) was chromatographed by dry flash chromatography on silica gel, using *n*-hexane-EtOAc mixtures of increasing polarity to afford 67 × 100 ml frs., which were reduced to 5 frs. by TLC: A (0 – 5 %, *n*-hexane-EtOAc), B (5 – 15 %), C (15 – 25 %), D (25 – 45 %), and E (45 – 100 %). A 40 mg sample of fraction A (16.2 g) was rechromatographed on semi-preparative HPLC using *n*-hexane as eluent to give **14** (t_R 22.6 min), and **15** (t_R 25.0 min). Fraction B (6.9 g) was rechromatographed on Sephadex LH-20, using *n*-hexane-$CHCl_3$-MeOH (2 : 1 : 1) as eluent to afford frs. 1 – 3. Fr. 1 (1.5 g) was then chromatographed on silica gel using Cl_2CH_2-acetone (from 10 : 0 to 5 : 5) as eluent to give **6** (R_f 0.61, *n*-hexane-EtOAc, 6 : 2). Fr. 2 (2.0 g) was subjected to a silica gel-column with *n*-hexane-EtOAc (from 10 : 0 to 5 : 5) as eluent to afford **13** and **1** (R_f 0.72, and 0.65, respectively, *n*-hexane-EtOAc, 6 : 4). Chromatography of fr. 3 (3.2 g) under the same conditions yielded **2**, **4**, and **10** (R_f 0.60, 0.55, and 0.42, respectively, *n*-hexane-EtOAc, 9 : 1). Fraction C (2.8 g), after chromatography on Sephadex LH-20 (*n*-hexane-$CHCl_3$-MeOH, 2 : 1 : 1), gave rise to compounds **5** and **11** (R_f 0.53, and 0.40, respectively, *n*-hexane-EtOAc, 7 : 3). Fraction D (2.1 g) was purified by silica gel column, using Cl_2CH_2-acetone (from 10 : 0 to 8 : 2) as eluent to give **3**, **8**, **9** and **17** (R_f 0.34, 0.60, 0.51, and 0.58, respectively, Cl_2CH_2-acetone, 9 : 1). Chromatography of fraction E (1.4 g) on Sephadex LH-20 (*n*-hexane-$CHCl_3$-MeOH, 2 : 1 : 1) afforded **7** (R_f 0.53, *n*-hexane-EtOAc, 6 : 4), and four additional fractions. Fr. 2 (50 mg) of these were further purified by silica gel column using *n*-hexane-EtOAc (from 10 : 0 to 0 : 10) yielded **16** (R_f 0.38, *n*-hexane-EtOAc, 7 : 3).

(–)-Copalic acid **1** (856.0 mg), $[\alpha]_D^{25}$: –15.3° (*c* 1.5, $CHCl_3$); (–)-methyl copalate **2** (15.4 mg), $[\alpha]_D^{25}$: –18.5° (*c* 1.8 , $CHCl_3$); agathic acid 15-methyl ester **3** (17.7 mg), $[\alpha]_D^{25}$: –18.5° (*c* 0.4, $CHCl_3$); agathic acid 15,19-dimethyl ester **4** (2.3 mg), $[\alpha]_D^{25}$: –15.6° (*c* 0.2, $CHCl_3$); (–)-polyalthic acid **5** (145.8 mg), $[\alpha]_D^{25}$: –34.3° (*c* 1.8, $CHCl_3$); (–)-methyl polyalthate **6** (8.8 mg), $[\alpha]_D^{25}$: –38.9° (*c* 2.4, $CHCl_3$); (–)-pinifolic acid **7** (51.7 mg), $[\alpha]_D^{25}$: –27.9° (*c* 2.1, $CHCl_3$); methyl 3*β*-hydroxy-labda-8(17),13-dien-15-ate **8** (4.3 mg), $[\alpha]_D^{25}$: + 24.8° (*c* 0.31, $CHCl_3$); (–)-methyl-18-hydroxy-copaiferolate **9** (12.4 mg), $[\alpha]_D^{25}$: –21.5° (*c* 0.2, $CHCl_3$); (+)-14,15-bisnorlabd-8(17)-en-13-one **10** (3.4 mg), $[\alpha]_D^{25}$: + 31.9° (*c* 0.4, $CHCl_3$); (–)-kaurenic acid **11** (25.5 mg), $[\alpha]_D^{25}$: –71.7° (*c* 6.7, $CHCl_3$); 16*β*-kauran-19-oic acid **13** (65.2 mg), $[\alpha]_D^{25}$: –51.7° (*c* 1.2, $CHCl_3$); *β*-bisabolene **14** (11.5 mg), $[\alpha]_D^{25}$: + 68.1° (*c* 0.5, $CHCl_3$); caryophyllene oxide **15** (5.5 mg), $[\alpha]_D^{25}$: –5.9° (*c* 0.2, $CHCl_3$); 3-methyl-5-(2,2,6-trimethyl-6-hydroxy-cyclohexyl)-pentanoic acid **16** (10.0 mg), $[\alpha]_D^{25}$: –1.9° (*c* 0.9, $CHCl_3$); with all other spectroscopic and chemical data identical with previously reported data [1], [5], [6], [7], [8], [9], [10], [11], [12], [13], [14], [15].

Pauperol (**17**). Colorless lacquer. $[\alpha]_D^{25}$: –7.4° (*c* 0.1, $CHCl_3$). UV (EtOH): λ_{max} = 220, 211, 202 nm. IR (film): λ_{max} = 3500, 2900, 2850, 1750, 1680, 1450, 1430, 1370, 1215, 1140, 1080, 880, 750 cm^{-1}. ^1H-NMR (400 MHz): *δ*, see Table **1**. ^{13}C-NMR (100 MHz): *δ*, see Table **1**. EI-MS: *m/z* (%) = 572 (M^+, 1), 556 (11), 554 (1), 524 (5), 317 (83), 285 (40), 257 (26), 203 (29), 147 (42), 95 (65), 69 (100); HR-EI-MS: calcd. for $C_{36}H_{60}O_5$ 572.44408, found 572.44621.

Table **1** ^{1}H-, ^{13}C- and HMBC NMR data for pauperol (**17**) and ^{13}C-NMR data for **9** and **16**

			17				***16***	***9***
	Unit I				***Unit II***			
Position	δ_H[a]	δ_C[b]	***HMBC***	δ_H[a]	δ_C[b]	***HMBC***	δ_C[b]	δ_C[b]
1		173.7 s			39.8 t		178.4 s	38.5 t
2	2.11, 2.30 dd (6.2, 14.4)	41.4 t	1[c], 15		18.4 t		41.3 t	18.6 t
3	1.98 m	31.0 d	2	1.4, 2.19 m	35.9 t	18, 19	30.8 d	39.7 t
4		38.4 t			35.4 s		41.4 t	35.3 s
5	1.34 m	23.2 t		1.27 m	49.5 d	10, 19	23.1 t	48.4 d
6	1.01 t	57.3 d	7, 13		21.5 t		57.0 d	21.5 t
7		74.2 s		1.60, 2.32 m	37.9 t	8, 17	74.9 s	37.9 t
8	1.35, 1.63 m	43.4 t			147.7 s		43.1 t	148.0 s
9		20.4 t		1.55 m	56.1 d	8	20.4 t	56.0 d
10		41.7 t			36.8 s		39.6 t	37.9 s
11		35.4 s		1.35 m	24.2 t	13	35.5 s	21.1 t
12	0.74 s	21.4 q	6, 10, 11[c], 13	1.89 m	39.7 t	9, 13[c], 14, 16	21.2 q	39.5 t
13	0.85 s	32.7 q	6, 10, 11[c], 13		161.0 s		32.8 q	161.1 s
14	1.10 s	23.2 q	6, 7[c], 8	5.58 s	114.9 d	12, 13[c], 15[c], 16	23.0 q	114.7 d
15	0.90 d (6.8)	19.9 q	2, 4		167.3 s		19.8 q	167.3 s
16				2.10 s	18.8 q	12, 13[c], 14		18.9 q
17				4.43 s 4.78 s	106.7 t	7, 8[c], 9		106.5 t
18				3.56, 3.79 d_{AB} (11.0)	72.8 t	3, 4[c], 5, 19, C = O		72.0 t
19				0.75 s	17.5 q	3, 4[c], 5, 19		17.6 q
20				0.65 s	14.9 q	1, 5, 9, 10[c]		14.9 q
OCH_3				3.69 s	50.8 q	15		

[a] δ, $CDCl_3$, *J* values in Hz.

[b] Data are based on DEPT and HMQC experiments.

[c] Two-bond coupling enhancement observed.

Leishmanicidal activity

The strain of *Leishmania* used in the presented study was MHOM/PE/95/LQ8 from *L. braziliensis*. Promastigotes were adapted for growth at 22 °C in RPMI 1640 modified medium (Gibco) and supplemented with 20% heat inactivated fetal bovine serum [17]. Logarithmic phase cultures were used for experimental purposes. Assays were performed in sterilized, 24-well microtiter plates (Corning™). To these wells were added 500,000 parasites, in promastigote form obtained from logarithmic phase cultures, and the test drug. The final volume was 500 μl in each well. Different concentrations were tested and parasite determination was performed after 48 h. by density determination on a Coulter Counter model Z1. Resistance was determined by measuring the IC_{50}. Tests were performed at least in triplicate on three different days in order to verify the results. The final IC_{50} was calculated as the arithmetic mean of the IC_{50} is obtained. Ketoconazol was used as positive control (IC_{50} 34.8 μg/ml).

Antimicrobial Assay

The activity was tested against Gram-positive (*Staphylococcus aureus* ATCC 6538, *S. epidermidis* CECT 232, *S. saprophyticus* CECT 235, *Bacillus subtilis* CECT 39) and Gram-negative (*Escherichia coli* CECT 99, *Pseudomonas aeruginosa* AK 958) bacteria, and yeast (*Candida albicans* UBC 1). Bacterial cultures were developed in Nutrient Broth (Oxoid) and the yeast culture in Sabouraud liquid medium. Inocula were prepared by diluting overnight cultures with the medium (10^5 u.v./ml). The *in vitro* evaluation of antimicrobial activity was performed following the two-fold dilution method [18] in the 0.1 – 50 μg/ml range. Results were expressed as minimal inhibitory concentration (MIC). Each MIC of the different compounds dissolved in DMSO was determined three times for all the strain tested. Cephotoxime was used as positive control.

Cytotoxic Assays

Cytotoxic activity was measured against the following cell lines: P-388 (ATCC CCL-46), suspension culture of a lymphoid neoplasm from a DBA/2 mouse; A-549 (ATCC CCL-185), monolayer culture of a human lung carcinoma; HT-29 (ATCC HTB-38), monolayer culture of a human colon carcinoma; MEL-28 (ATCC HTB-72), monolayer culture of a human melanoma. Cells were maintained, in logarithmic growth in EMEM, supplemented with 5% fetal calf serum (FCS), 10-2 M sodium bicarbonate, and 0.1 g/l penicillin G + 0.1 g/l streptomycin sulfate. The compounds assayed were dissolved in DMSO-MeOH (1 : 9) and tested following the method described previously [19]. Taxol was used as positive control (IC_{50} 0.59 and 0.012 μg/ml for P-388 and A-549, HT-29, and MEL-28 cell lines, respectively).

Assay of aldose reductase

Purification of the recombinant human aldose reductase used in the bioassay is based on the method described by Nishimura et al. [20]. The aldose reductase inhibitory activity *in vitro* was determined following a modification of the method reported above. Sorbinil was used as positive control (IC_{50} 0.8 μg/ml) [21].

Results and Discussion

Repeated chromatography on Sephadex HL-20 and silica gel of *Copaifera paupera* crude oil yielded twelve known diterpenes, **1 – 11** and **13**, and three known sesquiterpenes **14** and **16**, along with a new $C_{20}-C_{15}$ terpenoid **17**, with a structure based on the union of a labdane diterpene and a monocyclic sesquiterpene.

The known compounds were identified as (–)-copalic acid **1**, (–)-methyl copalate **2** [1], agathic acid 15-methyl ester **3** [5], agathic acid 15,19-dimethyl ester **4** [6], (–)-polyalthic acid **5**, (–)-methyl polyalthate **6** [7], (–)-pinifolic acid **7** [8], methyl 3β-hydroxy-labda-8(17),13-dien-15-ate **8**, (–)-methyl-18-hydroxy-copaiferolate **9**[9], (+)-14,15-bisnorlabd-8(17)-en-13-one **10** [10], (–)-kaurenic acid **11** [11], 16β-kauran-19-oic acid **13** [12], β-bisabolene **14** [13], caryophyllene oxide **15** [14], and 3-methyl-5-(2,2,6-trimethyl-6-hydroxycyclohexyl)-pentanoic acid **16** [15] by comparison of the NMR, IR, and mass spectra with data in the literature. Compounds **8** and **9**, were isolated from a natural source for the first time, and compound **12**, the methyl ester of (–)-kaurenic acid, was obtained by methylation of **11** with diazomethane in the usual way.

Compound **17** has the molecular formula $C_{36}H_{60}O_5$ by HREIMS and ^{13}C-NMR data. Its ^{1}H-NMR (Table **1**) spectrum revealed signals for seven methyl groups, five of them on quaternary saturated carbons (δ 0.65, 0.74, 0.75, 0.85, and 1.10), one on a tertiary carbon (δ 0.90, d, J = 6.8 Hz), and one on a vinyl carbon (δ_H 2.10). Two methylene protons of a primary alcohol (δ 3.56 and 3.79, d_{AB}, J = 11.0 Hz), a methoxy group (δ 3.69), and three vinyl protons (δ 4.43, 4.78, and 5.58) were also observed. These data were confirmed by ^{13}C-NMR (Table **1**) showing 36 carbons, and DEPT experiments revealed the presence of eight methyls, fifteen methylene, five methine, and five quaternary carbons, two carbonyl groups (δ 167.3 and 173.7) and one quaternary carbon bearing a hydroxyl group (δ 74.2). The IR spectrum corroborated the presence of the latter groups through the absorptions at 1680, 1750 and 3500 cm^{-1}. When **17** was treated with acetic anhydride in pyridine at room temperature it remained unaltered, which, in conjunction with the IR spectrum, attested to this molecule having a tertiary alcohol group. The analysis of its COSY, ROESY, HMQC, and HMBC spectra enabled the assignment of the signals of the two units I and II, which correspond to the monocyclic sesquiterpene **16** [15] and the labdane diterpene **9** [9], respectively.

The nature of the ester linkage connecting units I and II was confirmed through the HMBC (Table **1**) experiment, which showed a three-bond correlation between the carboxyl carbon of the unit I (C-1, δ_C 173.7) and H-18 of the unit II (δ_H 3.56 and 3.79), and by comparison with the ^{13}C-NMR data of 3-methyl-5-(2,2,6-trimethyl-6-hydroxycyclohexyl)-pentanoic acid **16** and the (–)-methyl-18-hydroxy-copaiferolate **9**. Thus the structure of **17**, for which the name pauperol is proposed, is that of a monocyclic farnesane and a labdane diterpene linked by an ester bridge. These two types of terpenes are present in the oil (**16** and **9**), and a biosynthetic route with participation of enzymes with acyl transferase activity could be put forward.

As a continuation of a program oriented towards the discovery of bioactive natural products, the isolated compounds were evaluate for their leishmanicidal, antimicrobial, cytotoxic, and aldose reductase inhibitory activities.

Compounds **1 – 3**, **5 – 7**, **9**, and **11 – 13** were assayed against *Leishmania braziliensis* promastigotes *in vitro*. All of the compounds were deemed inactive, with IC_{50} values up to 100 μg/ml. The crude oil showed a 20.1 % of inhibition at 300 μg/ml.

These compounds were also assayed to determine the minimal inhibitory concentration (MIC) against Gram-positive and Gram-negative bacteria, and the yeast *Candida albicans*. Compounds **1** and **11** showed significant activity against *B. subtilis*, *S. aureus* and *S. epidermidis*, comparable with cephotaxime used as control (Table **2**). On the other hand, compounds **2**, **7**, **9**, **10**, **12**, and **13** were inactive against all microorganisms assayed at a level of 50 μg/ml. On the basis of their chemical features, these results suggest that the presence of a carboxylic acid moiety could strongly affect the antimicrobial activity, as the masking of the carboxylic acid as its methyl ester fully eliminated the activity (**1** versus **2** and **11** versus **12**). In addition, the double bonds appear to induce some enhancement of the activity (**1** versus **7** and **11** versus **13**).

Compounds **1**, **2**, **5**, **7** , **11**, and **13** were tested for cytotoxic and aldose reductase [16] inhibitory (this enzyme appears to initiate the cataract development process in diabetics) activities. The cytotoxic activity was determined against the following tumor cell lines: P-388 (lymphoid neoplasm from a DBA/2 mouse), A-549 (human lung carcinoma), HT-29 (human colon carcinoma) and MEL-28 (human melanoma). Among these compounds, **2** exhibited moderate activity in the four tested cell lines, with IC_{50} values of 2.5, 5, 5, and 10 μg/ml against the P-388, A-549, HT-29 and MEL-28 cell lines, respectively. The remaining tested compounds showed no significant inhibitory activity (IC_{50} > 10 μg/ml). In the aldose reductase assay inhibition was observed only for diterpene **7**, which showed weak activity with an IC_{50} value of 25 μg/ml, while quercetrin used as control showed an IC_{50} of 0.9 μg/mL.

These results partially highlight the antimicrobial potential of the Copaiba oil as traditional remedy. However, its potential use for the treatment of the leishmanisis (Uta), could not be predicted, although the pure metabolites showed no activity

Table **2** Minimal inhibitory concentration (MIC, μg/ml) of selected terpenoids from *Copaifera paupera*

Bacteria	*1*	*5*	*9*	*11*	*Control* [a]
B. subtilis	6.3 – 3.1	30 – 20	30	5 – 2.5	8
S. aureus	10 – 8	50 – 40	>50	8 – 6	2.5 – 1.25
S. epidermidis	5 – 4	40	>50	6 – 4	2.5

[a] Control was cephotaxime.

against proliferation of *Leishmania tropica* promastigotes *in vitro*.

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