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The Isolation and *in vivo* Potent Antitumor Activity of Clerodane Diterpenoid from the Oleoresin of the Brazilian Medicinal Plant, *Copaifera Langsdorfii* Desfon.

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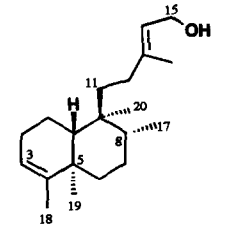
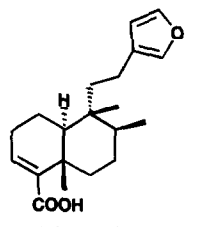
Abstract: An extremely potent antitumor *neo*-clerodane diterpene was isolated from the oleoresin of the Brazilian medicinal plant, *Copaifera langsdorfii* Desfon. This compound was identified as (-)-kolavenol **1**. The antitumor effect of **1** against IMC carcinoma as determined from the increase in life-span (I.L.S.) in mice was twice that of 5-FU. The structure elucidation and the antitumor activity of the other related compounds **2~6** in this oleoresin were also described.

During the search for new lead antitumor compounds from the South American folk medicinal plants, the oleoresin of "**Copaiba**", *Copaifera langsdorfii* Desfon.(*Leguminosae*), which has been used for the treatment of cancer, ulcer, syphilis, bronchitis, and diarrhea at the Amazonian region in Brazil, showed a potent antitumor activity against IMC carcinoma (murine tumor) in mice, without any notable cytotoxity against the same cells.

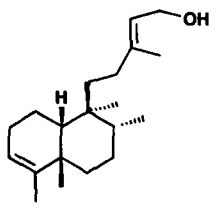
Bioassay-guided purification of the oleoresin of this plant resulted in the isolation of six related clerodane and labdane diterpenes , which were identified as (-)-kolavenol **1** [1a], (+)-hardwickiic acid **2** [2], *cis*-kolavenol **3**, *ent-neo*-4(18),13-clerodadien-15-ol **4**, (-)-copalic acid **5** [3], 8(17),13-labdadien-15-ol **6** [4]. Compounds **3** and **4** were first isolated as natural product.



(-)kolavenol 1



(+)hardwickiic acid 2



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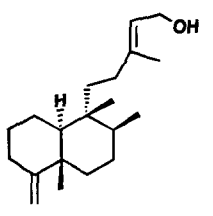

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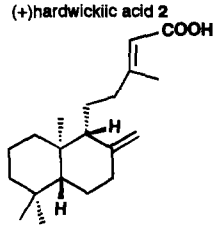
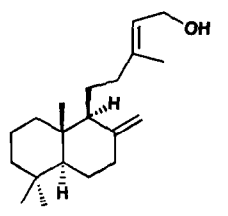
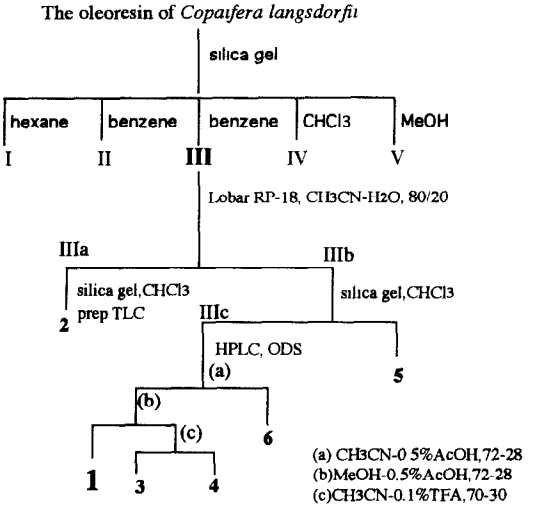


(-)copalic acid 5



6

The oleoresin of *C. copaifera* was fractionated by silica gel chromatography, by elution with hexane (fr.**I**), benzene (**II** and **III**), chloroform (**IV**), and methanol (**V**), successively. The bio-active fraction **III** was divided into fractions **IIIa** and **IIIb** depending on the retention time by the reversed-phase MPLC (Merck Lobar RP-18, 80% CH3CN in H2O). The former gave **2** by the isolation of silica gel chromatography and prep.TLC with chloroform. On the other hand, the latter gave **5** and **IIIc** . The antitumor activity was focused on **IIIc**, which was separated into **1** and minor constituents **3**, **4** and **6** by HPLC as shown in Scheme 1.



Scheme 1

All compounds were identified by the comparison of 500 MHz NMR spectra and IR ,MS[7] with the data in the literature and the extensive analysis of 2D NMR involving COSY, ^{13}C-^{1}H COSY, NOESY and HMBC spectra. Tables 1 and 2 show the assignments of ^{13}C and ^{1}H NMR . Compound **3**, having *cis*-ring fused A/B ring of kolavenol **1**, was first isolated from a natural source, but was reported as the synthetic intermediate[5] . Furthermore, **4** was revealed to be *ent-neo*-4(18),13-clerodadien -15-ol , because it was the antipode of the known compound, *neo*-4(18),13-clerodadien-15-ol.[6]

Table 1 ^{13}C NMR data of compounds **1~6** (125 MHz, CDCl3)

	1	**2**	**3**	**4**	**5**	**6**
1	18.25	17.45	17.74	21.73	42.07	39 15
2	26.86	27.47	24.06	28.69	19.34	19.40
3	120.47	140.29	123.13	33.11	39.02	42.23
4	144.47	141.55	139.89	160.70	33 55*	33 63
5	38.16	37.59	35.90	40 07	55 46	55 57
6	36 83	35 83	37.78	37 39	24.41	24.47
7	27.50	27 28	28.80	27.55	38.26	38.37
8	36.25	36.25	37 39	36.69	148.20	148.68
9	38 57	38 82	40.10	39.25	56.11	56 35
10	46.42	46.71	44.66	48 69	39 66	39.70
11	36 73	38 65	36.50	36 65	21.47	21 82
12	32.81	18.17	32.73	32 73	40.06	38.45
13	140.78	125.57	141.11	140.86	164.00	140 69
14	122.87	110.96	122.85	122 82	114 92	123 04
15	59 38	142.68	59.51	59 48	172.43	59.45
16	16.48	138.38	16 53	16.55	19 19	16 34
17	15 93	15.93	15.93	16.00	106 34	106 27
18	17 92	172.89	19 73	102.45	33.54*	33 61
19	19 91	20.53	33 08	20.89	21.68	21 72
20	18 32	18 24	17 28	18.18	14 44	14.49

*interchangeable

Table 2. ^{1}HNMR data of compounds **1~6** (500MHz, $CDCl_3$)

	1	2	3	4	5	6
1	1.42m	1.50m	1.81m	1.40-1.60m	1.18td(13.2,4.0)	1.00td(12.9,3.7)
	1.58m	1.67brdd(13.0,7.0)	1.99m	—	1.39brd(13.2)	1.77brd(12.9)
2	2.02m	2.18m	2.00m	1.28m	1.48m	1.47m
		2.33m	2.11m	1.87m	1.58m	1.60m
3	5.19brs	6.86dd(4.5,3.0)	5.27brs	2.10brd(13.4)	1.01td(12.8,4.0)	1.18td(13.4,4.0)
				2.30brtd(13.4,5.0)	1.73m	1.39brd(13.4)
5	—	—	—	—	1.09dd(12.8,2.7)	1.08dd(12.5,3.0)
6	1.18td(12.9,4.3)	1.17td(13.1,4.3)	1.07td(13.4,4.3)	1.52m	1.32qd(12.8,4.3)	1.32qd(12.5,4.3)
	1.71dt(12.9,3.4)	2.44dt(13.1,3.3)	2.00m		1.73m	1.72m
7	1.40m	1.44m	1.19m	1.45m	1.96m	1.97brdd(12.5,5.2)
		1.49m	1.24m		2.39ddd	2.39ddd
					(13.0,4.3,2.4)	(12.5,4.3,2.4)
8	1.47m	1.56m	1.44m	1.42m	—	—
9	—	—	—	—	1.58m	1.58m
10	1.34m	1.39brd(12.0)	1.36dd(5.7,2.0)	1.10dd(12.5,2.5)	—	—
11	1.36m	1.56m	1.33m	1.31m	1.51m	1.46m
	1.48m	1.66m	1.58m	1.45m	1.68m	1.60m
12	1.81td(13.4,4.9)	2.18m	1.88brt(8.5)	1.73m	1.98m	1.83m
	1.88td(13.4,4.3)	2.33m		1.84m	2.32ddd	2.16ddd
					(14.0,10.0,4.0)	(14.0,7.9,4.3)
14	5.39tq(6.7,1.3)	6.25brs	5.42tq(7.0,1.0)	5.37tq(6.7,1.2)	5.67q(1.2)	5.39tq(6.8,1.2)
15	4.13d(6.7)	7.34t(1.7)	4.15d(7.0)	4.13d(6.7)	—	4.15d(6.8)
16	1.67brs	7.20brs	1.70brs	1.66brs	2.17d(1.2)	1.67brs
17	0.80d(6.8)	0.83d(6.7)	0.77d(7.0)	0.81d(6.1)	4.49brs	4.51d(1.6)
					4.85d(1.2)	4.83d(1.6)
18	1.58q(1.8)	—	1.68q(1.6)	4.50brs	0.87s	0.87s
19	1.00s	1.26s	1.04s	1.05s	0.80s	0.80s
20	0.72s	0.76s	0.81s	0.73s	0.68s	0.68s

Coupling constants (J in Hz)are given in parentheses.

All compounds isolated from this oleoresin were subjected to the antitumor activity test on the increase in life-span (I.L.S.)[8] against IMC carcinoma in mice. 5-Fluorouracil (5-FU) was used as the positive control. The experiment was performed in three doses on the basis of **III**(240, 80, 26.7 mg/Kg)as summarized in Table 3. The dose of each compound described was determined from the content of the compounds **1~6** on the basis of **III**(240 mg/Kg), by the external standard method by HPLC. The most potent compound, (-)-kolavenol **1** was twice as effective (I.L.S. 98%, 41 mg/Kg/day, 4 days) as 5-FU (46%, 30 mg/Kg/day, 4 days). Since a difference between *in vivo* and *in vitro* experiments was observed in the activity, the antitumor effect of **Copaiba** and **1** was considered to be enhanced biological response modification (BRM).

Kolavenol **1** was reported as having biological activities against leaf cutter ants (*Atta cephalotes*), as well as their mutualistic attine fungus[1b]. Additionally, we report here the first evaluation against the antitumor properties of **1**. The oleoresin of **Copaiba** commercially available in bulk[9] without the environmental disruption will be a fascinating resource for the future.

Table 3. Antitumor effect of compounds **1~6** against IMC carcinoma in mice.

Sample	Content (%)	Dose(mg/Kg)	n	Body Wt. Change(g)	Life span(day) Mean±S.D.	I.L.S. (%)
III	100*	240.0	6	-0.7	23.2±5.3	82
1	17.1	41.1	6	0.1	25.2±3.8	98
2	32.3	77.6	6	1.6	14.8±8.5	17
3	0.17	1.8**	6	1.4	12.3±0.8	-3
4	0.017	0.27**	6	1.9	11.7±0.5	-8
5	8.6	20.7	6	1.5	14.0±2.8	10
6	3.9	9.0	6	1.4	13.0±2.1	2
5-FU	---	30.0	6	-1.8	18.5±5.3	46
control	---	saline	7	1.1	12.7±0.8	0

*13.8% from Copaiba oil ** excess

Tumor:IMC carcinoma 1 x 10^6 cells/mouse i.p., **Animal**: SLC:CDF1 mouse female 7w, **Administration**: d1~d4 i.p., **Vehicle**:0.1% Tween 80 saline (5-FU: saline)

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7) Physical data of compounds **1~6**

Compound **1**: $[\alpha]_D^{25}$=-50.9°(c 1.67, CHCl3); IR(KBr) 3346, 2930, 1665, 1454, 1381, 1000 cm^{-1}; HRMS M^+290.2581(C20H34O); Compound **2**: $[\alpha]_D^{22}$=+116.4°(c 0.53, CHCl3); m.p. 98.5-99.0℃ (MeOH-H2O); IR(KBr) 3444, 2962, 1686, 1620, 1386, 1261, 1025 cm^{-1}; HRMS M^+316.2011 (C20H28O3); Compound **3**: $[\alpha]_D^{25}$ =+27.8°(c 0.54, CHCl3); IR(KBr) 3362, 2938, 1671, 1450, 1383, 1002 cm^{-1}; HRMS M^+290.2651 (C20H34O) ; Compound **4**: $[\alpha]_D^{25}$-10.0°(c 0.80,CHCl3) [Lit., $[\alpha]_D$ +20.9° (c 0.7, CHCl3)]; IR(KBr) 3410, 2928, 1678, 1450, 1383, 998, 891 cm^{-1}; HRMS M^+290.2657(C20H34O); Compound **5**: $[\alpha]_D^{27}$-10.27°(c 1.07, CHCl3); m.p.91.5-92.0℃ (MeOH-H2O); IR(KBr) 2948, 1690, 1640, 1441, 1388, 1255, 1176, 890 cm^{-1}; HRMS M^+ 304.2357 (C20H32O2); Compound **6**: $[\alpha]_D^{25}$+5.24°(c 0.84, CHCl3); IR (KBr) 3400, 2910, 1640, 1450, 1390, 1100, 890 cm^{-1}; HRMS M^+290.2615 (C20H34O).

8) The antitumor activity was evaluated by the increase in life span(I. L. S.)
I.L.S=[treated group survival days—control group survival days]/control group survival days X 100

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