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THE CYTOTOXIC PRINCIPLES OF *HYPTIS CAPITATA* AND THE STRUCTURES OF THE NEW TRITERPENES HYPTATIC ACID-A AND -B*

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Key Word Index—*Hyptis capitata*; Labiatae; cytotoxicity; triterpenes; hyptatic acid-A; hyptatic acid-B; tomentic acid; maslinic acid; 2α-hydroxyursolic acid.

Abstract—Bioassay-directed fractionation of a methanolic extract of *Hyptis capitata* has led to the isolation and characterization of five triterpene acids which include the new hyptatic acids -A and -B in addition to the known 2α-hydroxyursolic acid, tormentic acid and maslinic acid. Spectral data in conjunction with X-ray analysis of the methanol solvate of hyptatic acid-A established the structures of these compounds. Hyptatic acid-A and 2α-hydroxyursolic acid demonstrated significant *in vitro* cytotoxicity in human colon HCT-8 tumour cells.

INTRODUCTION

We reported recently on the isolation of ursolic acid as a cytotoxic principle of *Hyptis capitata* [1]. Further investigation on the cytotoxic polar triterpene fraction of the same plant has led to the isolation of new hyptatic acid-A (**1**) and -B (**4**) as well as of three known triterpenes: 2α-hydroxyursolic (**2**), tormentic (**3**) and maslinic acid (**5**). Compounds **1** and **2** showed significant cytotoxicity against human colon HCT-8 and other tumour cells whereas **3**–**5** lacked such activity (Table 1). The structures of **1**–**5** were elucidated from spectral data and a single-crystal X-ray analysis of the methanol solvate of compound **1**.

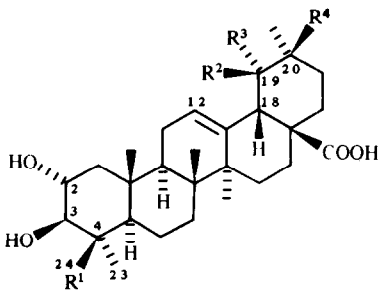
RESULTS AND DISCUSSION

The methanolic extract of the air-dried aerial part of *Hyptis capitata* was extracted with *n*-hexane. Separation of the methanol-soluble portion by repeated silica gel column chromatography (CC) and high performance liquid chromatography (HPLC) led to the isolation of compounds **1**–**5**.

Compound **1**, $C_{30}H_{48}O_5$, mp 298–304°, $[M]^+$ at *m/z* 488, was crystallized from methanol as colourless prisms. It gave a positive Liebermann–Burchard (LB) test for triterpenes. Its IR spectrum showed the presence of a carboxylic acid group. The ^1H NMR spectrum of **1** revealed the presence of six tertiary methyls (δ 0.81, 0.92, 0.95, 1.00, 1.17 and 1.24), one H-18 (δ 2.86, *dd*, *J* = 11.5 and 4.2 Hz), one olefinic and four carbinolic protons [δ 3.79 (*ddd*, *J* = 9.7, 9.7 and 4.0 Hz), 3.06 (*d*, *J* = 9.7 Hz), 3.39 (*d*, *J* = 11.0 Hz) and 4.03 (*d*, *J* = 11.0 Hz)]. These data indicated that **1** possesses an oleane skeleton. The large

Table 1. Cytotoxicities (ED_{50}, μg/ml) of compounds **1**–**5** against various tumour cells

Compound	KB	A549	HCT-8	P-388	L-1210
1	>4.0	5.9	4.2	6.7	>10
2	>4.0	4.9	2.7	6.1	>10
3	>4.0	>10	>10	>10	>10
4	>4.0	>10	>10	>10	>10
5	>4.0	>10	>10	>10	>10



1 R^1 = CH_2OH, R^2 = R^3 = H, R^4 = Me
2 R^1 = R^2 = Me, R^3 = R^4 = H
3 R^1 = R^2 = Me, R^3 = OH, R^4 = H
4 R^1 = CH_2OH, R^2 = Me, R^3 = OH, R^4 = H
5 R^1 = R^4 = Me, R^2 = R^3 = H

*Part 94 in the series 'Antitumour Agents', For part 93, see Fukamiya, N. Okano, M., Tagahara, K., Aratani, T. and Lee, K. H. (1988) *J. Nat. Prod.* **51**, 349.

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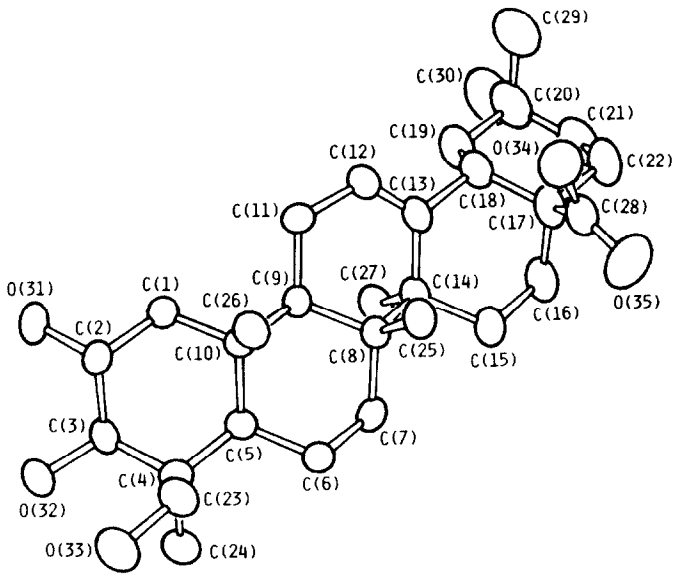

Fig. 1. Structure and solid-state conformation of one of the molecules of hyptatic acid-A (**1**) in the asymmetric crystal unit; hydrogen atoms have been omitted for clarity.

coupling constant ($J = 9.7$ Hz) between H-2 and H-3 pointed to their axial disposition, thereby indicating that the 2- and 3-hydroxyl groups are both equatorially oriented. The presence of a CH_2OH group attached to C-4 is substantiated by the appearance of the two doublets at δ3.39 and 4.03 ($J = 11.0$ Hz).

Unequivocal proof of the structure and complete stereochemistry of **1** as 2α,3β,24-trihydroxyolean-12-en-28-oic acid, i.e. hyptatic acid-A, was obtained by a single-crystal X-ray analysis of the methanol solvate. The crystal structure was solved by direct methods.* Full-matrix least-squares refinement of atomic positional and thermal parameters converged to $R = 0.052$ ($R_w = 0.073$)† over 4071 reflections. The asymmetric crystal unit comprises two molecules of **1** and a methanol molecule linked together by O-H. . . O hydrogen bonds. These units are further associated in the crystal to produce an arrangement which ensures that all OH groups participate in O-H . . . O hydrogen bonded interactions. A view of the solid-state conformation of one hyptatic acid-A molecule is presented in Fig. 1. The conformation of the other crystallographically independent molecule of **1** differs significantly only by a 180° rotation of the acid moiety about the C-17–C-28 bond.

Hyptatic acid-B (**4**), mp 210–211°, $C_{30}H_{48}O_6$, showed resonances in its ^{1}H NMR spectrum indicative of the presence of five tertiary (δ 0.78, 0.99, 1.19, 1.23 and 1.33) and one secondary (δ0.93) methyl groups, one olefinic proton (δ 5.29), two secondary [δ 3.79, (H-2β) and 3.05 (H-3α)] and one primary [δ3.40 (H-24$_a$ and H-24$_b$)] hydroxyl groups (Table 2). These data are similar to those for **1** except for the signals due to Hβ-18 and, to a lesser degree, the methyl groups, indicating that **4** possesses the same stereochemistry as **1**. The identical multiplicity (*s*) and the similarity of the chemical shifts for Hβ-18 [δ2.51 and 2.50, respectively, in **4** and **3** (Table 2)] led to the assignment of the 2α, 3β 19α, 24-tetrahydroxyurs-12-en-28-oic acid acid constitution to hyptatic acid-B (**4**).

The known compounds, 2α-hydroxyursolic acid (**2**), tormentic acid (**3**) and maslinic acid (**5**), were isolated and identified by IR, NMR and HPLC, and mixed melting point determinations with authentic samples.

EXPERIMENTAL

Mps: uncorr. ^{1}H NMR spectra are given in parts per million (δ) downfield from an internal standard (TMS). Silica gel (Kiesel gel 60, 230–400 mesh, Merck) was used for CC, and pre-coated silica gel plates (Kiesel gel 60 F254, 0.25 mm, Merck) were used for analytical TLC. Triterpenes were detected by spraying with 10% H_2SO_4 soln containing 1% $Ce(SO_4)_2$, followed by heating. HPLC was carried out on a Waters Associates Model 510 system using Model R401 differential refractometer and a Model 450 variable wavelength detector. The column used in this system was Partisil M9 10/50 ODS-2, 20 × 500 mm, Whatman. MeOH and MeCN–H_2O (80:20) were used as the mobile phase and the flow rate was 2–4 ml/min.

Plant material. The *Hyptis capitata* used was from a collection made in July 1979, in Shan-De-Mun, Taiwan, by the late Professor Huan-Chan Huang. A voucher specimen of this plant is kept at the School of Pharmacy, Kaohsiung Medical College, Taiwan.

Extraction and isolation. The powdered leaves and stems of *H. capitata* (3.18 kg) were extracted exhaustively with MeOH. The MeOH extract (171 g), after removal of fatty acids with *n*-hexane (5 × 4 l), was subjected to CC on silica gel (10 × 25 cm) eluted with a gradient of *n*-hexane (4.0 l), *n*-hexane–$CHCl_3$ (3:1, 4.5 l), *n*-hexane–$CHCl_3$ (2:1, 22.7 l), *n*-hexane–$CHCl_3$ (1:1, 9.0 l), $CHCl_3$ (5.0 l), $CHCl_3$–Me_2CO (2:1, 10.6 l), Me_2CO (8.0 l), MeOH–Me_2CO (1:1, 5.0 l), and MeOH (5.0 l) to give 10 fractions. Fractions 6 and 7 (9.5 g), resulting from elution with

*Crystallographic calculations were performed on a PDP11/44 computer by use of the Enraf–Nonius Structure Determination Package incorporating the direct methods programme MULTAN11/82.

†$R = \sum||F_o| - |F_c||/\sum|F_o|$; $R_w = [\sum w(|F_o| - |F_c|)^2/\sum w|F_o|^2]^{1/2}$.

Table 2. ^{1}H NMR spectral data* for compounds **1–5**

Compound	H-2	H-3	H-24$_a$, H-24$_b$	Hβ-18	H-12	Methyl groups
1	3.79 (*ddd*; 9.7, 9.7, 4.0)	3.06 (*d*; 9.7)	3.39, 4.03 (*d*; 11.0)	2.86 (*dd*; 11.5, 4.2)	5.26 (*t*, 3.2)	0.81 (*s*), 0.92 (*s*), 0.95 (*s*), 1.00 (*s*), 1.17 (*s*), 1.24 (*s*)
2	3.66 (*ddd*; 9.8, 9.8, 4.0)	2.95 (*d*; 9.8)	—	2.22 (*d*; 11.0)	5.27 (*t*, 3.6)	0.84 (*s*), 0.89 (*s*), 092 (*d*; 6.3), 1.06 (6H, *s*) 1.16 (*s*)
3	3.62 (*ddd*; 9.8, 9.8, 3.5)	2.91 (*d*; 9.8)	—	2.50 (*s*)	5.28 (*t*, 3.2)	0.80 (*s*), 0.81 (*s*), 0.95 (*d*; 6.1), 1.00 (*s*) 1.02 (*s*), 1.19 (*s*), 1.25 (*s*)
4	3.79 (*ddd*; 9.4, 9.4, 3.5)	3.05 (*d*; 9.4)	3.40, 4.40 (*d*; 11.0)	2.51 (*s*)	5.29 (*t*, 3.2)	0.78 (*s*), 0.93 (*d*; 5.8) 0.99 (*s*). 1.19 (*s*) 1.23 (*s*), 1.33 (*s*)
5	3.62 (*ddd*; 9.8, 9.8, 4.1)	2.90 (*d*; 9.8)	—	2.86 (*dd*; 14.0, 4.1)	5.25 (*t*, 3.4)	0.81 (*s*), 0.82 (*s*), 0.91 (*s*), 0.94 (*s*), 1.00 (*s*), 1.01 (*s*), 1.16 (*s*)

*Run in MeOH-d_4 at 400 MHz. Values are in ppm (δ). Coupling constants (J), in parentheses, are in Hz.

Table 3. Fractions of the triterpene mixture

Fraction Nos	Volume of total fraction (ml)	Yield (mg)	R_f value*
1–40	200	2,200	0.5 ~ 0.6
41–54	65	138	0.4 ~ 0.5
55–61	30	501	0.3 ~ 0.4
62–69	35	300	0.2 ~ 0.3
70–85	75	103	0.17 ~ 0.2
86–97	55	37	0.17
98–102	20	27	0.10 ~ 0.17
103–111	45	98	~0.10

*Kieselgel 60 F_{254}, 0.25 mm, $CHCl_3$–MeOH (10:1).

$CHCl_3$ (5.0 l) and $CHCl_3$–Me_2CO (2:1, 10.6 l), were found to show significant cytotoxicity in A-549 and HCT-8 systems, and were further chromatographed on silica gel (5 × 40 ml) and eluted with a gradient of $CHCl_3$ (1 l), EtOAc–Me_2CO (1:1, 6 l) and MeOH (2 l). The EtOAc–Me_2CO eluate, which contained the cytotoxic triterpenes, yielded eight fractions after one more series of separations by column chromatography on silica gel (3.5 × 40 cm) with elution by $CHCl_3$–MeOH (10:1) and collection of 5 ml eluates per fraction (Table 3).

Hyptatic acid-A (i.e. 2α,3β,24-*trihydroxyolean*-12-*en*-28-*oic acid*) (**1**). Fractions 62–69 afforded **1** after purification by HPLC. Compound **1**, R_t 7.0 min, was isolated as colourless prisms: $[\alpha]_D^{20}$ +57° (MeOH; *c* 0.2); IR ν_{max}^{KBr} cm^{-1}: 3410, 2920, 1675, 1440, 1370, 1040 and 1015; MS *m/z* 488 (M^+, $C_{30}H_{48}O_5$), 442, 393, 248, 233 and 203; ^{1}H NMR (CD_3OD): see Table 2.

2α-*Hydroxyursolic acid* (2α,3β-*dihydroxyurs*-12-*en*-28-*oic acid*) (**2**). Fractions 41–54 yielded **2** (R_t 10.8 min) as colourless amorphous powders (MeOH) after purification by HPLC: mp 241–245° (lit. [2] and [3] reported mp 244–246°), $[\alpha]_D$ +49° (MeOH; *c* 0.2); LB test positive; IR ν_{max}^{KBr} cm^{-1}: 3400, 2910, 1675, 1440, 1033 and 950; MS *m/z*: 472.3490 ($C_{30}H_{48}O_4$), 426, 408, 248, 223, 203 (base peak); ^{1}H NMR (CD_3OD): see Table 2.

Methyl 2α-*hydroxyursolate* (**6**). A soln of **2** in MeOH was methylated with CH_2N_2 at room temp. for 4 hr. The product was recrystallized from MeOH to give **6** as colourless needles: mp 211–213° (lit [3] reported mp 203–206°); MS *m/z*: 486, (M^+, $C_{31}H_{50}O_4$), 468, 450, 262 and 203. The retention time (HPLC), R_f value and MS of **6** were identical with those of an authentic sample.

Tormentic acid (2α,3β,19α-*trihydroxyurs*-12-*en*-28-*oic acid*) (**3**). Compound **3** was isolated from fractions 55–61 after HPLC separation. Compound **3**: R_t 6.0 min; colourless amorphous powders (MeOH); mp 265–268° (lit. [4] reported mp 266–267°); $[\alpha]_D^{20}$ +27° (MeOH; *c* 0.2); LB test positive; IR ν_{max}^{KBr} cm^{-1}: 3400, 2910, 1675, 1440, 1035 and 950; MS *m/z*: 488.3546 ($C_{30}H_{48}O_5$), 442, 370, 264, 210 and 146; ^{1}H NMR (CD_3OD); see Table 2.

Tormentic acid diacetate (**7**). Acetylation of **3** with acetic anhydride pyridine in the usual way yielded a diacetate (**7**) as colourless prisms (MeOH); mp 186–189° (lit [4] reported mp 194°); $[\alpha]_D^{20}$ +12° (MeOH; *c* 0.5); IR ν_{max}^{KBr} cm^{-1}: 3480, 1725, 1695 and 1640; MS *m/z*: 572 ($C_{34}H_{52}O_7$), 554, 526 454, 262, 246, 233, 231, 201 and 146. The identity of **7** with an authentic sample of tormentic acid diacetate was established by direct comparison [retention time (HPLC), R_f value, mmp. and MS).

Hyptatic acid-B (2α,3β,19α,24-*tetrahydroxyurs*-12-*en*-28-*oic acid*) (**4**). Fractions 62–69 furnished **4** as colourless amorphous powder after HPLC separation. Compound **4**: R_t 4.7 min; mp 225–228°; $[\alpha]_D^{20}$ +28° (MeOH; *c* 0.2); IR ν_{max}^{KBr} cm^{-1}: 3400, 2950, 1675, 1480, 1370 and 1040; MS *m/z* 504 ($C_{30}H_{48}O_6$), 458, 386, 264, 246, 201 and 146; ^{1}H NMR (CD_3OD): see Table 2.

Maslinic acid (2α,3β-*dihydroxyolean*-12-*en*-28-*oic acid*) (**5**). Compound **5** was obtained from fractions 55–61 by HPLC separation. Compound **5**: R_t 10.4 min; colourless amorphous powder (MeOH); mp 290–295° (dec.) (lit. [3, 4] reported mp 280–297°, dec.); $[\alpha]_D^{20}$ +34° (MeOH; *c* 0.2); IR ν_{max}^{KBr} cm^{-1}: 3400, 2920, 1680, 1455, 1375, 1040 and 945; MS *m/z*: 472.3490 ($C_{30}H_{48}O_4$), 426, 408, 393, 248, 223 and 203; ^{1}H NMR: see Table 2.

Methyl maslinate (**8**). Methylation of **5** in MeOH with CH_2N_2 at room temp. for 4 hr. yielded methyl ester **8** as colourless needles (MeOH): mp 254 ~ 260°; $[\alpha]_D^{20}$ +58° (MeOH; *c* 0.2); MS *m/z* 486, 468, 426, 409, 262, 249, 233, 223, 203, 189 and 133. The retention time (HPLC), R_f value and MS of **8** were identical with those for an authentic sample.

Biological assay. The *in vitro* cytotoxicity assay was carried out according to a National Cancer Institute protocol described in refs [1, 5]. In addition to the significant ($ED_{50} \leq 4.0$ μg/ml) cytotoxicity exhibited by hyptatic acid-A in human colon HCT-8 tumour cells ($ED_{50} = 4.2$ μg/ml), the present study revealed for the first time that the known 2α-hydroxyursolic acid also possesses significant cytotoxicity ($ED_{50} = 2.7$ μg/ml) (Table 1).

X-Ray analysis of hyptatic acid-A as its methanol solvate (**1**)·½MeOH. Crystal data: $C_{30}H_{48}O_5 \cdot \frac{1}{2}$MeOH, $M_r = 504.74$, monoclinic, $a = 14.302$ (3) Å, $b = 26.616$ (4) Å, $c = 7.431$ (2) Å, $\beta = 91.76$ (2)°, $V = 2827.4$ Å^3, $Z = 4$, $D_{calc} = 1.186$ g/cm^3, μ(CuKα radiation, $\lambda = 1.5418$ Å) = 6.0 cm^{-1}. Space group $P2_1$ (C_2^2) from the systematic absences, $0k0$ when k is odd, and **1** is chiral. Sample dimensions: 0.15 × 0.20 × 0.40 mm.

Preliminary unit-cell parameters and space group information were obtained from oscillation and Weissenberg photographs. Intensity data ($+h$, $+k$, $+l$) were recorded on an Enraf-Nonius CAD-4 diffractometer (CuKα radiation, incident-beam graphite monochromator; $\omega - 2\theta$ scans, $\theta_{max} = 67°$). From a total of 5022 independent measurements after averaging equivalent forms, those 4071 reflections with $I > 3.0\sigma(I)$ were retained for the structure analysis and corrected for the usual Lorentz and polarization effects. Refined unit-cell parameters were derived from the diffractometer setting angles for 25 reflections ($41° < \theta < 48°$) widely separated in reciprocal space.

The crystal structure was solved by direct methods. Approximate positions for the non-hydrogen atoms were obtained in part from an E-map and from subsequent weighted F_o Fourier syntheses. Hydrogen atoms were located in difference Fourier syntheses evaluated following several rounds of full-matrix least-squares adjustment of non-hydrogen atom positional and anisotropic thermal parameters. With the inclusion of the hydrogen atoms at their calculated positions, continuation of the least-squares refinement of non-hydrogen atom parameters led to convergence at $R = 0.052$ ($R_w = 0.073$). A view of the solid-state conformation of one of the molecules of **1** in the asymmetric crystal unit is presented in Fig. 1. Final atomic positional and thermal parameters, bond lengths and angles, hydrogen-bonded distances, torsion angles and a list of observed and calculated structure amplitudes have been deposited with the Cambridge Crystallographic Data Centre.

Neutral atom scattering factors used in the structure-factor calculations were taken from ref. [6]. In the least-squares iterations, $\sum w\Delta^2$ [$w = 1/\sigma^2(|F_o|)$], $\Delta = (|F_o| - |F_c|)$] was minimized.

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