Tricalysiolide G, and Tricalysiols A and B: rearranged e*nt*-Kaurane-Type and e*nt*-Kaurane-Type Diterpenoids from the Leaves of *Tricalysia dubia* (Lindl.) Ohwi

Dong-Hui HE,[a] Katsuyoshi MATSUNAMI,[a] Hideaki OTSUKA,*[,a] Takakazu SHINZATO,[b] Mitsunori ARAMOTO,[c] Masahiko BANDO,[d] and Yoshio TAKEDA[e]

[a]Department of Pharmacognosy, Graduate School of Biomedical Sciences, Hiroshima University; 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan: [b]Subtropical Field Science Center, Faculty of Agriculture, University of the Ryukyus; 1 Senbaru, Nishihara-cho, Nakagami-gun, Okinawa 903-0213, Japan: [c]Iriomote Station, Tropical Biosphere Research Center, University of the Ryukyus; 870 Aza Uehara, Taketomi-cho, Yaeyama-gun, Okinawa 907-1541, Japan: [d]Medicinal Chemistry Research Institute, Otsuka Pharmaceutical Co. Ltd.; 463-10 Kagasuno, Kawauchi-cho, Tokushima 771-0192, Japan; [e]Faculty of Integrated Arts and Sciences, The University of Tokushima; 1-1 Minamijosanjima-cho, Tokushima 770-8502, Japan:

*Corresponding author e-mail: hotsuka@hiroshima-u.ac.jp Tel. & Fax: +81-82-257-5335

Abstract Three rearranged *ent*-kaurane diterpenes having the cafestol-type framework were isolated from the leaves of *Tricalysia dubia*. Two of them were found to be known diterpenoids, tricalysiolides B and C. Tricalysiolide B was isolated as colorless prisms in this experiment and its three dimensional structure was determined by X-ray crystallography. The remaining one was a new compound and named tricalysiolide G. Two new *ent*-kaurane-type diterpenoids, to which tricalysiols A and B were given as trivial names, were also isolated. The structures of the new compounds were elucidated from spectroscopic evidence.

Key words *Tricalysia dubia*; Rubiaceae; rearranged *ent*-kaurane; *ent*-kaurane

Introduction

In previous papers [1, 2], rearranged *ent*-kaurane glycosides and *ent*-kaurane glucosides were isolated from a MeOH extract of leaves of *Tricalysia dubia* (Rubiaceae). From the wood part of the same plant, the isolation of five cafestol-type rearranged *ent*-kaurane diterpenoids has been reported [3]. In this experiment, relatively less polar fractions obtained on Diaion HP-20 CC were investigated, which yielded three new non-glycosidic diterpenoids, named tricalysiolide G (**1**), and tricalysiols A (**4**) and B (**5**), respectively, and two known non-glycosidic diterpenoids. The known compounds were identified with tricalysolides A (**2**) and B (**3**). This paper deals with structural elucidation of the new compounds and X-ray analysis of tricalysiolide B.

Results and Discussion

Air-dried leaves of *T. dubia* were extracted with MeOH three times and the concentrated MeOH extract was partitioned with solvents of increasing polarity. The *n*-BuOH-soluble fraction was separated by column chromatography (CC) on a highly porous synthetic resin (Diaion HP-20), and normal silica gel and reversed-phase octadecyl silica gel (ODS) CC, and droplet counter-current chromatography (DCCC) to afford three new diterpenoids, (**1**, **4** and **5**) (Fig. 1), along with two known compounds (**2**

and **3**) [3]. The details and yields are given in the Experimental section. The three dimensional structure of tricalysioside B (**3**) was confirmed by X-ray crystallographic analysis. The structures of the new compounds (**1**,**4** and **5**) were then elucidated from spectroscopic evidence (Fig. 1).

Compounds **2** and **3** were isolated as colorless needles and identified as tricalysiolides A and B, respectively, when their spectroscopic data were compared with those reported [3]. The orientation of the hydroxyl group at C-3 in tricalysiolide B (**3**) has been determined to be at the β-position, since NOE correlation was observed between the hydroxyl hydrogen atom and H-5 [3]. In the present study, since **3** was obtained as suitable crystals, its three dimensional structure was confirmed by X-ray analysis (Fig. 2). The assumption made on spectroscopic analysis was verified and two molecules were found to exist in an asymmetric unit as hydrates.

Tricalysiolide G (**1**), $[\alpha]_D$ –165°, was isolated as colorless crystals and its elemental composition was determined to be $C_{20}H_{28}O_5$ by negative-ion high-resolution (HR)-FAB-MS. The IR spectrum indicated that **1** possessed hydroxyl (3395 cm^{-1}) and lactone (1747 cm^{-1}) groups. In the ^{13}C-NMR spectrum, 20 signals, which implied that **1** was a diterpenoid, were observed. Together with ^{1}H-NMR data, characteristic signals [δ_C 81.4 (d) with δ_H 4.72 (ddd, *J*=10, 8, 1 Hz), 175.2 (s), 111.5 (d) with δ_H 5.74 (s) and

173.7 (s)] were assigned to an α,β-unsaturated γ-lactone. Other functional groups, deduced from NMR data, comprised one each of primary [δ_C 66.2 (d) with δ_H 4.11 (d, *J*=11 Hz) and 4.14 (d, *J*=11 Hz)], secondary [δ_C 82.6 (d) with δ_H 3.81 (br. s)] and tertiary [δ_C 81.3 (s)] carbinols. From these data, **1** was expected to be a cafestol-type rearranged kaurane and when its NMR data were compared with those reported, it was found to be an identical compound to the aglycone of tricalysioside A, which was obtained through acid hydrolysis [1]. From a diagnostically similar specific optical rotation value to that of the aglycone of tricalysioside A and the same sign, the absolute structure of **1** was confirmed to be of the *enantio*-series.

Tricalysiol A (**4**), $[\alpha]_D$ –17.5°, was isolated as colorless crystals and its elemental composition was determined to be $C_{20}H_{32}O_5$ by HR-FAB-MS. The IR spectrum indicated that **1** possessed hydroxyl (3367 cm^{-1}) groups. In the ^{13}C-NMR spectrum, together with ^{1}H-NMR, DEPT, and HSQC spectra, the 20 signals observed were comprised of those of two tertiary methyls, eight methylenes, one of which bears a hydroxyl group [δ_C 66.2 (t) with δ_H 4.10 (d, *J*=11 Hz) and 4.13 (d, *J*=11 Hz)], five methines, two of which bear a hydroxyl group [δ_C 76.4 (d) with δ_H 3.57 (dd, *J*=13, 4 Hz) and 82.7 (d) with δ_H 3.80 (s)], four quaternary carbons, one of which bears a hydroxyl group [δ_C 81.3 (s)], and an aldehyde functional group [δ_C 207.6 (d) with

δ_H10.44 (s)]. These functionalizations were similar to those of parts of tricalysiosides I and M [2]. The ^{13}C- NMR data for A and B rings were diagnostically superimposable on those of tricalysioside I (**6**) and those of the C and D rings of tricalysioside M (**7**). Therefore, the structure of tricalysiol A (**4**) was elucidated to be *ent*-3β,15β,16β,17-tetrahydroxykauran-19-al. Because of the aforementioned isolation of the rearranged *ent*-kaurane and the co-occurrence of *ent*-kaurane glucosides in the same plant, tricalysiol A (**4**) must be of the *enatio*-series.

Tricalysiol B (**5**), $[\alpha]_D$ –25.3°, was isolated as colorless crystals and its elemental composition was determined to be $C_{22}H_{36}O_6$ by HR-FAB-MS. The IR spectrum indicated that **1** possessed hydroxyl (3412 cm^{-1}) groups. From the ^{13}C-NMR spectral data together with the ^{1}H-NMR, DEPT and HSQC spectra, **5** was assumed to be a pentahydroxykaurane derivative with two primary alcohols at C-17 and 19, two secondary alcohols at C-3 and 15, one tertiary alcohol at C-16, and two extra carbons that must be an acetyl group [δ_C 20.8 (q) with δ_H 2.02 (3H, s) and 171.2 (s)]. The ^{13}C-NMR data for the A and B rings were essentially the same as those of tricalysioside L (**8**). Although the NMR data for the C and D rings closely resembled those for tricalysiol A (**4**), the C-17 carbon signal showed a significant downfield shift from δ_C 66.2 to 68.6, and an upfield shift was observed for C-16 from δ_C 81.3 to 79.2. In the

^{1}H-NMR spectrum, a downfield shift was also observed for H_2-17 from δ_H 4.10 and 4.13 to 4.49 and 4.74, respectively. Therefore, the acetyl group was located on the hydroxyl group at the C-17 position and this was further confirmed by the HMBC spectrum in which H_2-17 protons showed cross peaks with δ_C 171.2. Finally, for the same reason, tracalysiol B (**5**) must be of the *enantio*-series and was elucidated to be *ent*-3β,15β,16β,17,19-pentahydroxykaurane-17-*O*-acetate.

Experimental

General Experimental Procedures

Melting points were determined with a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured on a Union Giken PM-101 digital polarimeter. IR spectra were measured on a Horiba FT-710 Fourier transform infrared spectrophotometer and an UV spectrum on a JASCO V-250 UV/VIS spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM α-400 spectrometer at 400 MHz and 100 MHz, respectively, with tetramethylsilane (TMS) as the internal standard. HR-FAB-MS were taken on a JEOL JMS SX-102 spectrometer and PEG-400 was used as the calibration matrix.

A highly-porous synthetic resin (Diaion HP-20) was purchased from Mitsubishi Kagaku (Tokyo, Japan). Silica gel CC and reversed-phase [octadecyl silica gel (ODS)] open CC were performed on silica gel 60 (Merck, Darmstadt, Germany) and Cosmosil 75C_{18}-OPN (Nacalai Tesque, Kyoto, Japan) [Φ = 50 mm, L = 25 cm, linear gradient: MeOH-H_2O (1:9, 1 L) $\rightarrow$ (1:1, 1 L), fractions of 10 g being collected], respectively. Droplet counter-current chromatography (DCCC) (Tokyo Rikakikai, Tokyo, Japan) was equipped with 500 glass columns (Φ = 2 mm, L = 40 cm), the lower and upper layers of a solvent mixture of $CHCl_3$-MeOH-H_2O (5:6:4) being used as the stationary and mobile phases, respectively. Five-gram fractions were collected and numbered according to their order of elution with the mobile phase. HPLC was performed on an ODS column (Inertsil; GL Science, Tokyo, Japan; Φ = 6 mm, L = 25 cm), and the eluate was monitored with a UV detector at 254 nm and a refractive index monitor.

Plant Material

Leaves of *T. dubia* (Lindl.) Ohwi (Rubiaceae) were collected in Okinawa, Japan, in August 1990, and a voucher specimen was deposited in the Herbarium of Pharmaceutical Sciences, Graduate School of Biomedical Sciences, Hiroshima University (90-TD-Okinawa-0822).

Extraction and Fractionation

Dried leaves of *T. dubia* (6.04 kg) were extracted with MeOH and then concentrated to 6L. The extract was washed with *n*-hexane and then the MeOH layer was concentrated to a gummy mass. The latter was suspended in water and extracted with EtOAc to give 361 g of an EtOAc-soluble fraction. The aqueous layer was extracted with *n*-BuOH to give a *n*-BuOH-soluble fraction (290g), and the remaining water-layer was concentrated to furnish 325 g of a water-soluble fraction. The *n*-BuOH-soluble fraction was separated first by CC on Diaion HP-20 (Φ = 5.0 cm, L = 60 cm), with MeOH-H_2O [20% (8 l), 40% (8 l), 60% (8 l), and 80% (8 l) in water, successively], 500 ml factions being collected. The fraction eluted with 60% MeOH (41.2 g in fractions 19-22) was subjected to a column of silica gel (1.0 kg) using $CHCl_3$ (3 l) and $CHCl_3$-MeOH [99:1 (6 l), 49:1 (6 l), 97:3 (6 l), 24:1 (6 l), 19:1 (6 l), 47:3 (3 l), 23:2 (6 l), 9:1 (6 l), 7:1 (3 l), 17:3 (3 l), 33:7 (3 l), 4:1 (3 l), 3:1 (3 l), and 7:3 (3 l); fractions of 500 ml being collected] as the solvent system. The residue (982 mg) of fractions 24–26 (4% MeOH eluate) was subjected to ODS CC, fractions of 10 g being collected, and the residue (667 mg) of fractions 134–168 was purified by DCCC to afford 126 mg of crystalline compound **3** in fractions 150–200. The residue (47.4 mg in fractions 201–239) was further purified by HPLC (40 % MeOH) to give 14.7 mg of **4** in a crystalline state.

The fraction eluted with 80% MeOH (13.6 g in fractions 23-26) on Diaion HP-20

CC was subjected to a column of silica gel (300 g) using $CHCl_3$ (3 l) and $CHCl_3$-MeOH [99:1 (3 l), 49:1 (3 l), 97:3 (3 l), 24:1 (3 l), 19:1 (3 l), 47:3 (3 l), 23:2 (3 l), 9:1 (3 l), 7:1 (3 l), 17:3 (3 l), 33:7 (3 l), 4:1 (3 l), 3:1 (3 l), and 7:3 (3 l); fractions of 500 ml being collected] as the solvent system. The residue (1.12 g) of fractions 16–22 (3–4 % MeOH eluate) was subjected to ODS CC, fractions of 10 g being collected. From fractions 183–192 and 192–200, 51.2 mg and 7.3 mg of **2** and **5** were obtained in crystalline states, respectively. The residue (800 mg) of fractions 150–176 obtained on ODS CC was subjected to DCCC and fractions were collected until number 432. Evaporation of the remaining stationary phase gave a crystalline residue, which was then recrystallized from MeOH to afford 254 mg of **1.**

Tricalysiolide G (**1**)

Colorless crystals. Mp. 244–247 ºC, $[\alpha]_D^{21}$ –165° (*c* 1.02, pyridine). IR ν_{max} (film) cm^{-1}: 3395, 2936, 1747, 1644, 1456, 1083, 1046, 1027; UV λ_{max} (MeOH) nm (log ε): 218 (4.09); ^{1}H-NMR (pyridine-d_5) δ:0.69 (3H, s, H_3-20), 0.94 (1H, ddd, *J*=14, 14, 4 Hz, H-1a), 1.28 (1H, br. d, *J*=9 Hz, H-9), 1.47 (3H, m, H-2a, 11a and 12a), 1.52 (2H, m, H_2-6), 1.59 (1H, m, H-11b), 1.69 (1H, ddd, *J*=14, 4, 4 Hz, H-1b), 1.83 (2H, m, H-12b and 14a), 1.90 (2H, m, H_2-7), 2.00 (2H, m, H-5 and 14b), 2.22 (1H, dddd, *J*=10, 10, 4, 4

Hz, H-2b), 2.50 (1H, br. d, *J*=4 Hz, H-13), 3.81 (1H, br. s, H-15), 4.11 (1H, d, *J*=11 Hz, H-17a), 4.14 (1H, d, *J*=11 Hz, H-17b), 4.72 (1H, ddd, *J*=10, 8, 1 Hz, H-3), 5.74 (1H, s, H-18); ^{13}C-NMR (pyridine-d_5): Table 1; HR-FAB-MS (negative-ion mode) *m*/*z*:347.1839 $[M–H]^-$ (Calcd for $C_{20}H_{27}O_5$: 363.1858).

Tricalysiolides A (**2**) and B (**3**)

Colorless crystals. Mp. 245–247 ºC, $[\alpha]_D^{21}$ –168° (*c* 1.02, pyridine) and colorless crystals. Mp. 231–234 ºC, $[\alpha]_D^{21}$ –142° (*c* 1.29, pyridine), respectively.

Tricalysiol A (**4**)

Colorless crystals. Mp 145–148 ºC, $[\alpha]_D^{21}$ –17.5° (*c* 0.80, pyridine). IR ν_{max} (film) cm^{-1}: 3367, 2935, 2872, 1710, 1650, 1513, 1456, 1164, 1077, 1037; ^{1}H-NMR (pyridine-d_5) δ:0.94 (1H, ddd, *J*=13, 13, 4 Hz, H-7a), 0.95 (3H, *s*, H_3-20), 1.09 (1H, d, *J*=11 Hz, H-5), 1.16 (1H, br. d, *J*=5 Hz, H-9), 1.44 (3H, s, H3-18), 1.53 (3H, m, H-11a, 11b and 12a), 1.72 (1H, dd, *J*=13, 4 Hz, H-1a), 1.80 (2H, m, H-12b and 14a), 1.86 (1H, ddd, *J*=13, 4, 4 Hz, H-7b), 1.97 (3H, m, H-6a, 6b and 14b), 1.99 (1H, m, H-1b), 2.06 (1H, dddd, *J*=13, 4, 4, 4 Hz, H-2a), 2.27 (1H, dddd, *J*=13, 13, 13, 4 Hz, H-2b), 2.49 (1H, d-like, *J*= 4 Hz, H-13), 3.57 (1H, dd, *J*=13, 4 Hz, H-3), 3.80 (1H, s, H-15), 4.10 (1H, d, *J*=11 Hz, H-17a), 4.13 (1H, d, *J*=11 Hz, H-17b), 10.44 (1H, *s*, H-19); ^{13}C-NMR (pyridine-d_5): Table 1; HR-FAB-MS (negative-ion mode) *m*/*z*:351.2172 $[M–H]^-$ (Calcd for $C_{20}H_{31}O_5$:

351.2171).

Tricalysiol B (**5**)

Colorless crystals. Mp. 236–239 ºC, $[\alpha]_D^{21}$ –25.3° (*c* 0.49, pyridine). IR ν_{max} (film) cm^{-1}: 3412, 2940, 2871, 1715, 1447, 1425, 1378, 1253, 1166, 1079, 1045; ^{1}H-NMR (pyridine-d_5) δ:0.86 (1H, ddd, *J*=13, 13, 3 Hz, H-7a), 0.94 (1H, br. d, *J*=11 Hz, H-5), 1.00 (3H, s, H_3-20), 1.11 (1H, br. d, *J*=7 Hz, H-9), 1.45 (1H, m, H-12a), 1.50 (3H, s, H_3-18), 1.54 (2H, m, H_2-11), 1.72 (1H, dd, *J*=13, 4 Hz, H-14a), 1.75 (1H, m, H-7b), 1.84 (4H, m, H-1a, 6a, 6b and 12b), 1.93 (2H, m, H-1b and 14b), 1.97 (1H, m, H-2a), 2.02 (3H, s, C$\underline{H}_3$CO), 2.07 (1H, m, H-2b), 2.48 (1H, d-like, *J*=4 Hz, H-13), 3.59 (1H, dd, *J*=12, 6 Hz, H-3), 3.66 (1H, d, *J*=11 Hz, H-19a), 3.80 (1H, s, H-15), 4.49 (2H, d, *J*=11 Hz, H-17a and 19b), 4.70 (1H, d, *J*=11 Hz, H-17b); ^{13}C-NMR (pyridine-d_5): Table 1; HR-FAB-MS (negative-ion mode) *m*/*z*:395.2448 $[M–H]^-$ (Calcd for $C_{22}H_{35}O_6$: 395.2434).

X-ray Analysis of **3**

The crystal used for data collection was a colorless prism with approximate dimensions of 0.3 × 0.2 × 0.1 mm. All data were obtained Rigaku AFC-5S automated four circle diffractometer with graphite-monochromated Mo*K*α radiation. Crystal data: $C_{20}H_{28}O_5 \cdot H_2O$, *M*r =366.45, triclinic, space group *P*1, *a* = 11.73(2) Å, *b* = 11.954(7) Å,

c = 7.110(2) Å, α = 95.05(3)º, β = 101.58(4)º, γ = 104.18(6)º, V = 937(2) Å^3, Z = 2, Dc = 1.299 Mgm^{-3}, F(000) = 396, μ(Mo$K\alpha$) = 0.945 cm^{-1}. Unit cell parameters were determined by least squares refinement of the optimized setting angles of 25 reflections in the range of 7.8º<θ<12.5º. The intensities were measured using $\omega/2\theta$ scan up to 55º. Three standard reflections were monitored every 150 measurements. The data were corrected for Lorentz and polarization factors. Correction for secondary extinction was applied (coefficient = 0.10671 × 10^{-5}). The absorption correlation was applied (transmission factor = 0.850–0.944) [4]. Of the 4518 reflections which collected, 4307 unique ones were used for structure determination and refinement. The structure was solved by a direct method using a teXsan crystallographic software package [5]. All non-H atoms were found in the Fourier map. The refinement of atomic parameters were carried out by means of full matrix least-squares refinement, using anisotropic temperature factors for all non-H atoms. All H atoms, except for those attached to O atoms, were located geometrically and not refined. The H atoms attached to O atoms were not found in the differential Fourier map. The final refinement converged with R_1 = 0.048 and R_w = 0.139 for 464 parameters. Atomic scattering factors were taken from the ”International Table for X-ray Crystallography” [6].

Acknowledgement The authors are grateful for access to the superconducting NMR instrument in the Analytical Center of Molecular Medicine of the Graduate School of Biomedical Sciences, Hiroshima University. This work was supported in part by grant-in-aids from the Ministry of Education, Science, Sports, Culture and Technology of Japan (No. 18590117 and 18032050).

References

1. He D-H, Otsuka H, Hirata E., Shinzato T, Takeda Y, Bando M (2002) Tricalysiosides A-G: Rearranged *ent*-kauranoid glycosides from the leaves of *Tricalysia dubia*. J Nat Prod 64:685–688

2. He D-H, Matsunami K., Otsuka H, Shinzato T, Aramoto M, Bando M, Takeda Y (2005) Tricalysiosides H-O: *Ent*-kaurane glucosides from the leaves of *Tricalysia dubia*. Phytochemistry 6:2857–2864

3. Nishimura K, Hitotsuyanagi Y, Sugeta N, Sakakura K, Fujita K, Fukaya H, Aoyagi Y, Hasuda T, Kinoshita T, He D-H, Otsuka H, Takeda Y, Takeya K (2005) Tricalysiolides A-F, new rearranged *ent*-kaurane diterpenes from *Tricalysia dubia*. Tetrahedron, 61:1512–1519

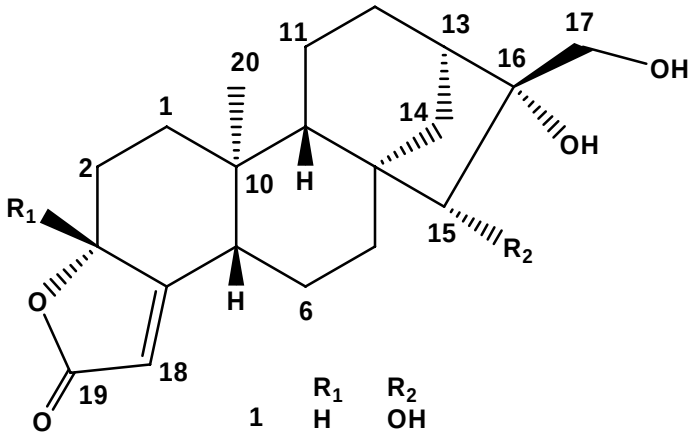
4. North ACT, Phillips DC, Mathews FS (1968) A semi-empirical method of absorption correction. Acta Crystallogr. A24:351–359.

5. “teXsan, Single Crystal Structure Analysis Software, Version 1.11,” Molecular Structure Corporation and Rigaku Corporation (2000). MSC, 3200 Research Forest Drive, The Woodlands, TX, 77381, USA, and RC, 3-9-12 Matsubara-cho, Akishima, Tokyo 196-8666, Japan, respectively.

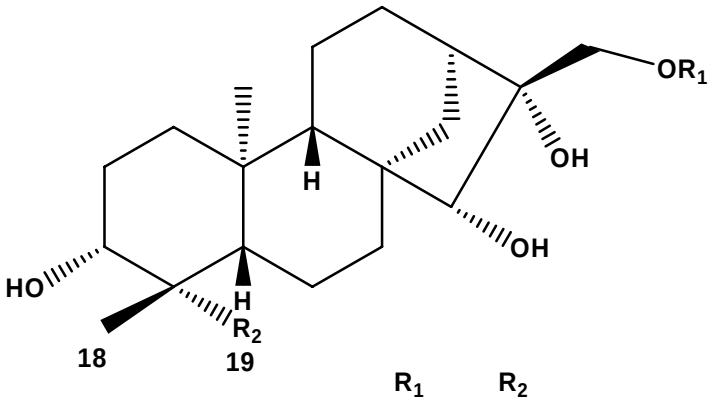
6. “International Tables for X-ray Crystallography,” Vol. C, Kynoth Press, Birmingham, 1992.

Table 1 ^{13}C-NMR data for tricalysiolide G (**1**), and trycalysiols A (**4**) and B (**5**) in d_5-pyridine

C	**1**	**4**	**5**
1	36.0	36.2	36.0
2	29.9	28.6	28.5
3	81.4	76.4	80.2
4	175.2	53.4	43.3
5	48.9	56.9	56.1
6	21.4	20.5	20.3
7	34.7	38.4	38.9
8	47.8	47.7	47.9
9	52.5	54.6	56.1
10	43.1	39.4	39.9
11	19.7	19.1	18.9
12	25.8	26.1	26.3
13	43.6	34.6	46.9
14	36.5	36.8	37.1
15	82.6	82.7	82.4
16	81.3	81.3	79.2
17	66.2	66.2	68.6
18	111.5	21.5	23.8
19	173.7	207.6	64.6
20	14.9	18.5	18.5
$\underline{C}H_3CO$			20.8
$CH_3\underline{C}O$			171.2



	R_1	R_2
1	H	OH
2	H	H
3	OH	H



	R_1	R_2
4	H	CHO
5	$COCH_3$	CH_2OH
6	Glc	CHO
7	H	CH_2OGlc
8	Glc	CH_2OH

Fig. 1

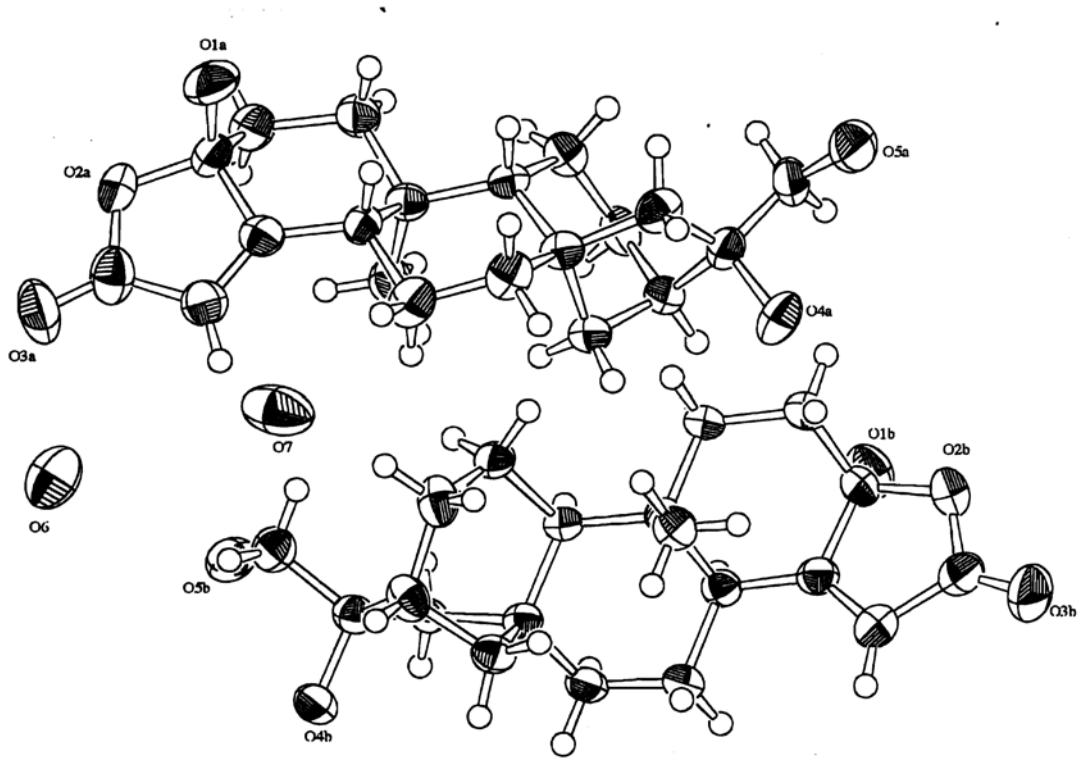


Fig. 2

Figure legend

Fig. 1

Structures

Fig. 2

ORTEP representation of tricalysiolide B (**2**), as determined an single-crystal X-ray analysis. Oxygen atoms have crystallographic numbering and simple circles denote hydrogen atoms.