Structural elucidation of trichotetronines: polyketides possessing a bicyclo[2.2.2]octane skeleton with a tetronic acid moiety isolated from *Trichoderma* sp.

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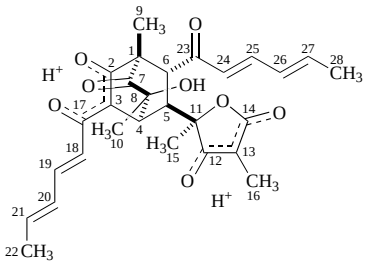
The isolation and structural elucidation of two novel fungal metabolites, trichotetronine and its dihydro congener, from *Trichoderma* sp. are described. The structures, including the relative and absolute stereochemistry, have been established using a variety of data including extensive NMR analyses and CD spectral studies. The compounds possess a bicyclo[2.2.2]octene system in conjunction with a tetronic acid moiety and two conjugated ketonic side chains. Along with them, trichodimerol, a known compound possessing an axis of symmetry, has been isolated. Both trichodimerol and the newly isolated trichotetronines appear to be derived from the condensation of two hexaketides having two extra methyl groups.

Introduction

Fungi of the genus *Trichoderma* are widely spread in soil, barks and sweet potatoes *etc.*, and their taxonomical classification is difficult. A wide variety of secondary metabolites has been isolated from *Trichoderma* sp.[1–4] In recent years, one kind of secondary metabolite the peptibols, *N*-acylated peptides having an amino alcohol as C-terminal residue, has been reported to show membrane-perturbing activity including antibiotic activity. [3,4] In addition, Ayer *et al.*[1,2] described the potency of *T. longibrachiatum* as a biocontrol agent for the American leaf spot disease of coffee, caused by the fungus *Mycena citricolor*. The isolation of several new compounds including trichodimerol **3**,[1] an interesting new compound possessing an axis of symmetry, were reported; however, the extracts of *T. longibrachiatum* grown on the liquid medium failed, unfortunately, to show antagonistic activity against *M. citricolor*.[1,2]



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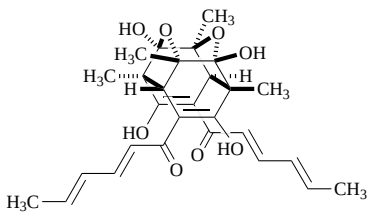

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During the course of our research on the chemistry of metabolites produced by plant-adhering fungi, a fungus belonging to *Trichoderma* sp. was separated from rice plant straw. From extracts of the fungus grown on a rice medium, two novel compounds along with trichodimerol **3** were isolated. The structure of new isolates, named trichotetronine **1** and dihydrotrichotetronine **2**, indicated the presence of a bicyclo[2.2.2]-octane system in conjunction with a tetronic acid moiety and two conjugated ketonic side chains in each of the two molecules. In this paper, we describe these structural studies, mainly based on extensive NMR analyses and CD spectral studies.

Results and discussion

Extraction and isolation of trichotetronines and trichodimerol

Extraction of the cultivated fungus together with its rice medium (150 g × 10 samples) with dichloromethane followed by ethyl acetate provided 10.0 g and 3.3 g, respectively, of extracted metabolites. These combined extracts were subjected to silica gel column chromatography to obtain 10 fractions. The fractions were further purified with silica gel and/or ODS columns (MPLC and HPLC) to isolate two novel compounds, trichotetronine **1** and dihydrotrichotetronine **2**, along with trichodimerol **3**, a known compound possessing an axis of symmetry.[1]

Structure of trichotetronine and dihydrotrichotetronine

Trichotetronine **1** was obtained as a yellow amorphous powder with $[a]_D$ +456 (*c* 0.08 in MeOH). The molecular weight of **1** was determined to be 496 from its positive FAB-MS spectrum with addition of 0.5 M aq. oxalic acid, which showed a $[M + Na]^+$ ion peak at *m*/*z* 519 and $[M + H]^+$ ion peak at *m*/*z* 497. The high-resolution FAB-MS indicated a molecular formula of **1** to be $C_{28}H_{32}O_8$ (Found: *m*/*z* 519.1982 $[M + Na]^+$. Calc. for $C_{28}H_{32}O_8Na$: 519.1995; Found: *m*/*z* 497.2148 $[M + H]^+$. Calc. for $C_{28}H_{33}O_8$: 497.2176). The IR absorption at around 3418br cm^{-1} was attributed to hydroxy groups, while peaks at 1723, 1622 and 1580 cm^{-1} implied the presence of ketones, a strongly chelated carbonyl, and conjugated double bonds. The UV spectrum showed an absorption maximum at 384 nm, suggesting a polyene conjugated carbonyl chromophore, and other peaks at 271 and 291 nm. The ^1H NMR spectrum of **1** displayed four singlet methyl signals, two doublet methyl signals, three methine proton signals, and eight olefinic methine proton signals. In the ^{13}C NMR spectrum, in addition to assignable carbon signals (equal to 6 sets of CH_3 and 11 sets

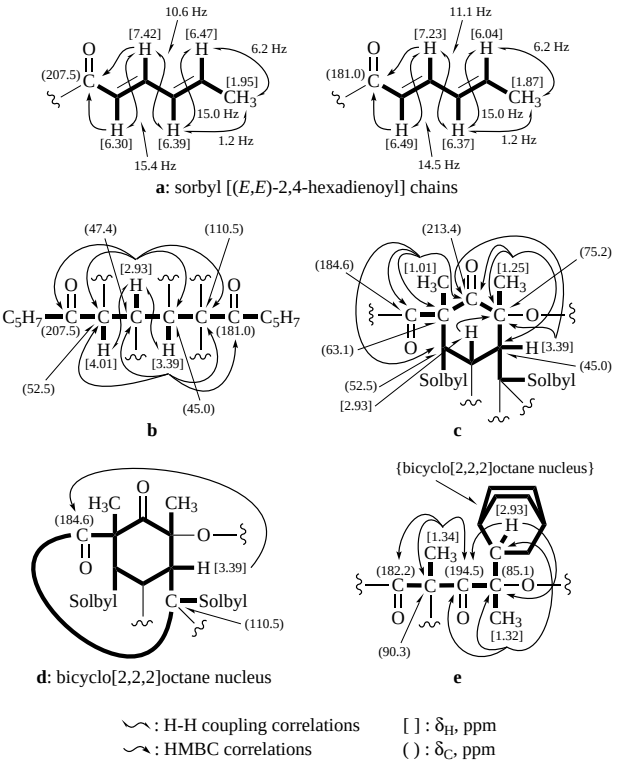


Fig. 1 NMR structure connectivities of **1** by H-H COSY and HMBC experiments

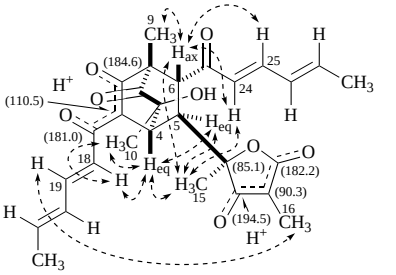


Fig. 2 Gross structure and relative stereochemistry of **1**

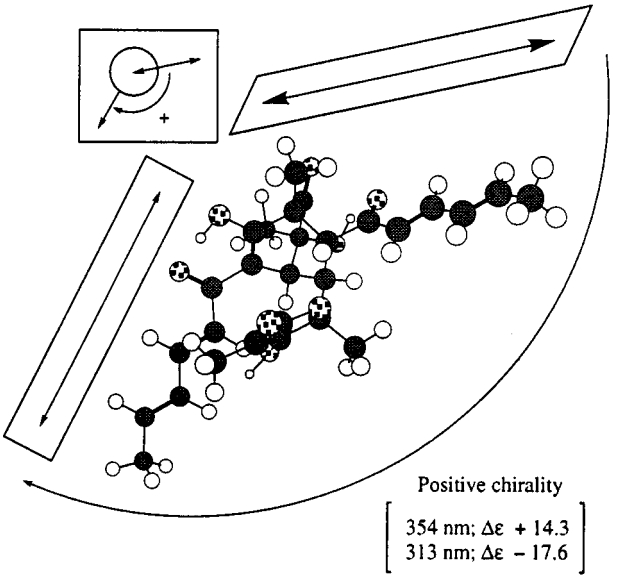


Fig. 3 Absolute stereochemistry of **1** by CD spectral analysis

of CH-), 11 quaternary carbons were observed at δ_C 63.1, 75.2, 85.1, 90.3, 110.5, 181.0, 182.2, 184.6, 194.9, 207.5 and 213.4 ppm. These NMR signals and the unsaturated ratio suggests the presence of three ring systems in the molecule. Further, the molecular formula of **1** shows three additional hydrogens, suggesting three exchangeable hydroxy groups. The construction of the molecular framework was based on H-H COSY and HMBC experiments. The H-H COSY spectrum of **1** showed coupling correlations between the doublet methyl signals and the olefinic proton signals and between the three methine proton signals; the former signal correlations were easily assigned as arising from two sets of (*E*,*E*)-buta-2,4-diene partial structures. HMBC correlations of these olefinic methine protons with carbonyl carbons at δ_C 207.5 (from δ_H 6.30 and 7.42) and δ_C 181.0 (from δ_H 6.30 and 7.42) established two sets of sorbyl [(*E*,*E*)-hexa-2,4-dienoyl] side chains,[1] which exhibited a MS fragment ion peak at *m*/*z* 95 (see Fig. 1a). In addition, the HMBC correlations of the methine protons, except the broadened signal at δ_H 4.01 which had no correlations, revealed the connections of the sorbyl side chains, one of which was directly linked to the methine carbon at δ_C 52.5 and the other linked to the methine carbon at δ_C 45.0 through the quaternary carbon at δ_C 110.5 (Fig. 1b). The methyl signal at δ_H 1.01 had HMBC correlations with carboxylic carbons at δ_C 213.4 and 184.6, the methine carbon at δ_C 52.5, and the quaternary carbon at δ_C 63.1. Also, the methyl signal at δ_H 1.25 had correlations with the carboxylic carbon at δ_C 213.4, the methine carbon at δ_C 45.0 and the oxy-quaternary carbon at δ_C 75.2. These correlations indicated the presence of a cyclohexanone ring linking the two sorbyl side chains (see Fig. 1c). Furthermore, the methine proton signal at δ_H 3.39 showed an additional HMBC correlation with the carboxylic carbon (δ_C 184.6) linked to the opposite side of the cyclohexanone ring. This correlation suggested the construction of an additional ring closure that resulted from a bond between the carbons at δ_C 110.5 and 184.6, and consequently, a bicyclo[2.2.2]octane system as a nucleus appeared in the molecule (Fig. 1d). Besides these units, a unit consisting of six carbons still remained. In this unit, HMBC correlations from the methyl signals at δ_H 1.34 and 1.32 revealed the preliminary formation of this unit and the connection to the bicyclo[2.2.2]octane nucleus (Fig. 1e). Thus, the connectivities of all carbons were made, and the postulated gross structure of **1** is shown in Fig. 2. One set of carbons at δ_C 181.0, 110.5 and 184.6 appeared to form an enolized *β*-diketone substructure. The other set of δ_C 182.2, 90.3 and 194.5 also appeared to be an enolized *β*-diketone substructure, which seemed to be constructed of a five-membered ring by a bond between the carbons at δ_C 182.2 and 85.1 through an oxygen. This unit, an enolized 3-oxo-2,4-dimethylbutanolide, is equal to a tetronic acid derivative, and the presence of this tetronic acid moiety in the molecule was deduced from the UV spectra, which showed pH dependent-shifted absorption maxima (+KOH: 257 nm for **1** and 258 nm for tetronic acid; +HCl: 235 nm for **1** and 233 nm for tetronic acid).[5] The relative stereochemistry of **1** was revealed by the analysis of the NOESY spectrum shown in Fig. 2. The equatorially orientated H-4 bridge-end methine proton showed strong NOE correlations with the H-5 methine proton and the H-18 olefinic proton of the enol-sorbyl side chain. The H-5 methine proton showed a strong NOE with the H-15 methyl protons, but none with the H-6 methine proton. The H-15 methyl protons based on the tetronic acid moiety showed a wide range of NOEs with the three methine protons of the bicyclo[2.2.2]octane nucleus, and the H-24 olefinic proton of the sorbyl side chain, due to the comparatively free rotation of the bond between C-5 and C-12. These correlations suggested the axial conjunction of the tetronic acid moiety at C-5 and the equatorial conjunction of the sorbyl side chain at C-6. Besides, the presence of NOEs between the H-10 methyl protons and H-18 olefinic proton of the enol-sorbyl side chain and between the H-16 methyl protons on the tetronic acid moiety and H-19 olefinic proton of the sorbyl side chain indicated the

configurations of the C-8 and C-15 shown in Fig 2. The absolute stereochemistry of **1** was elucidated by means of CD spectral analysis.[6] This spectrum exhibited a positive first maximum value at 354 nm ($\Delta\varepsilon$ + 14.3) and a negative second maximum value at 313 nm ($\Delta\varepsilon$ − 17.6) resulting from the chiral exciton coupling between the enol-sorbyl side chain (λ_{max} at 384 nm) and the sorbyl side chain (λ_{max} at 291 nm) shown in Fig 3. In the light of this spectroscopic evidence, the structure of trichotetronine **1** was established.

Dihydrotrichotetronine **2** was obtained as a pale yellow amorphous powder, with $[a]_D$ +354 (*c* 0.13 in MeOH). The molecular weight of dihydrotrichotetronine **2** was determined to be 498, and its molecular formula to be $C_{28}H_{34}O_8$ (Found: *m*/*z* 521.2131 $[M + Na]^+$. Calc. for $C_{28}H_{34}O_8Na$: 521.2152) based on positive FAB- and HRFAB-MS. The ^1H and ^{13}C NMR spectra of **2** were similar to those of **1**. The differences between **1** and **2** were observed by the disappearance of two olefinic protons and the alternative appearance of two sets of methylene protons [δ_H 2.22 (2H, dd, *J* 7.2, 14.7 Hz) and 2.63 (2H, br dd, *J* 7.0, 14.3 Hz)] and the corresponding carbons shifts [two olefinic methine carbons to δ_C 45.8 (t) and 25.9 (t)]. This indicated that one of the sorbyl side chains was partially hydrogenated. The position of saturation was assigned by analysis of the HMBC spectrum of **2**. This spectral analysis, for each set of methylene protons showed H–C long-range correlations with the carbonyl carbon at δ_C 45.8 that was assigned to be C-23, and indicated that the saturation was between C-24 and C-25. Thus, the structure of dihydrotrichotetronine **2** was assigned.

Biosynthetic route for trichotetronines

A tentative route for the biosynthesis of trichotetronines is illustrated graphically in Scheme 1. Namely, two 2,4-dimethylhexaketides, one being a sorbyl-cyclohexadienone type and the other a sorbyl-tetronic acid type,[7] approaches each other to form Diels–Alder type adducts.[8] On the other hand, trichodimerol is formed by the condensation of two sorbyl-cyclohexadienone type 2,4-dimethylhexaketides.

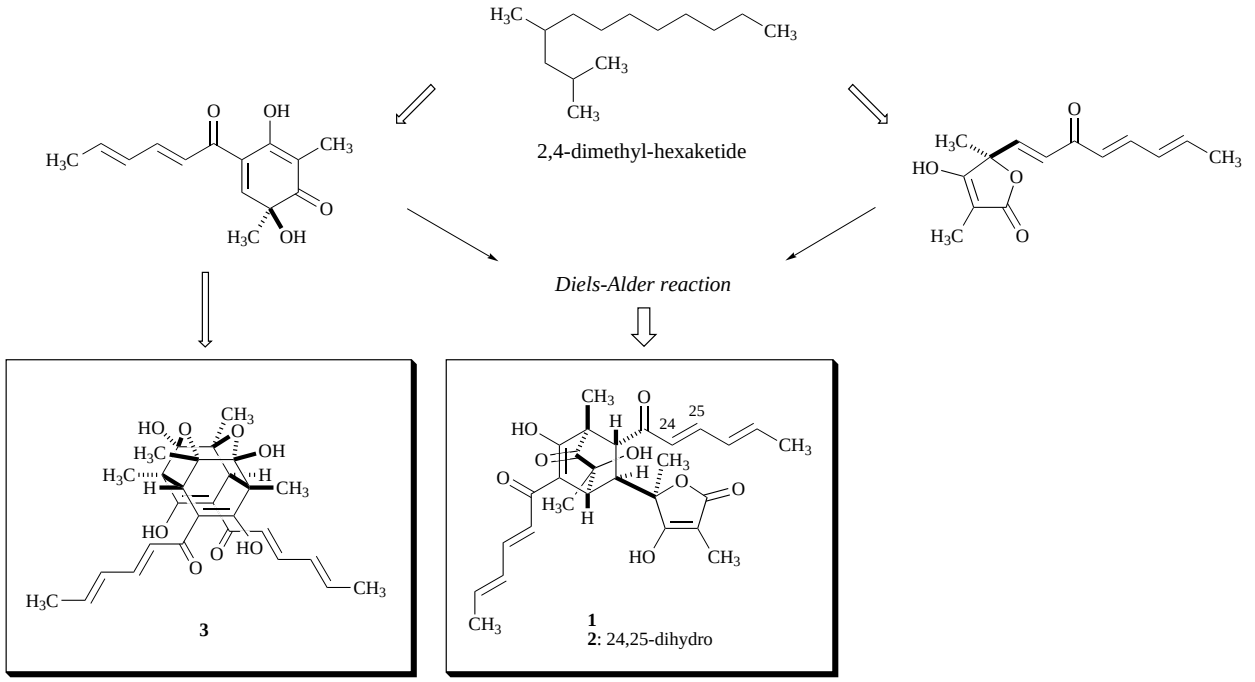
Experimental

General details

Silica gel (Si gel) open cc was performed on Silica gel 60 (Merck). Medium-pressure liquid chromatography (MPLC) was performed with a CIG column system (22 mm i.d. × 300 mm or 22 mm i.d. × 100 mm; Kusano Scientific Co., Tokyo) packed with 10 μm or 5 μm Si gel and/or octadecyl silica gel (ODS). HPLC was performed with an Inertsil PREP-ODS column (5 mm i.d. × 250 mm for analysis, 20 mm i.d. × 250 mm for preparative; GL Science Inc., Tokyo) packed with 10 μm ODS. TLC was conducted on pre-coated silica gel 60 F_{254} (Merck) and/or RP-18 F_{254}s (Merck) and the spots were detected by heating after spraying with 10% aq. H_2SO_4. Optical rotations were measured with a JASCO DIP-370 digital polarimeter and the $[a]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. FABMS and HRFABMS spectra were obtained on a JEOL AX-505H spectrometer. UV, IR and CD spectra were taken with a Hitachi U-2000 spectrophotometer, a JASCO FT/IR-5300 spectrophotometer and a JASCO J-720 spectropolarimeter, respectively. 1D and 2D ^1H and ^{13}C NMR spectra were recorded on a Varian spectrometer (Unity Plus 400) at 300 K using Bruker or Varian standard pulse sequences. NMR coupling constants (*J*) are given in Hz. Phase-sensitive ROESY experiments were conducted with a mixing time of 300 ms. A 150 ms delay was used to optimize one-bond correlation in HMQC and HSQC spectra and suppress them in HMBC spectra, and the evolution delay for long-range couplings in HMBC spectra was set to 63 ms.

Production, extraction and isolation of trichotetronines and trichodimerol

The metabolite-producing fungus was separated from rice plant straw at Ibaraki, Japan in 1995, and appears to have characteristics associated with a *Trichoderma* species. The fungus is deposited at Division of Microbiology, National Institute of Health Sciences, Japan. The fungus was grown on rice media (150 g × 10 bottles) for 3 weeks at 25 °C. The cultivated fungus together with its rice medium was extracted together with dichloromethane (×3; total 6 dm^3) followed by ethyl acetate (×3; total 6 dm^3) to provide 10.0 g and 3.3 g each of the extracted metabolites, which were of approximately similar constitution as checked by TLC. These extracts were subjected together to silica gel column chromatography eluting gradually with hexane–ethyl acetate (95:5–0:1), followed by ethyl acetate–methanol (9:1–0:1) solvent systems; 10 fractions were obtained. Fractions IX and X were fractionated by C18 reversed-phase silica gel (ODS) medium-pressure liquid chro-



Scheme 1 Possible biosynthetic route for trichotetronines and trichodimerol

matography (MPLC) independently, and derived fractions were further separated by ODS HPLC to obtain two novel compounds, trichotetronine **1** and dihydrotrichotetronine **2**. These new isolates were finally purified by ODS HPLC using acetonitrile–0.1 M aqueous ammonium acetate as eluent followed by neutralization with dilute aqueous HCl and extraction with dichloromethane. A known compound possessing an axis of symmetry, trichodimerol **3**, was isolated from the fraction V by silica gel MPLC, and was purified by ODS HPLC (acetonitrile–water). The isolation yields were approximately 20 mg for each of **1** and **2**, 55 mg for **3**; large losses and denaturation of **1** and **2** were observed.

Trichotetronine 1. Yellow amorphous powder (20 mg, 0.15 % from extracts); $[a]_D$ +456 (*c* 0.08 in MeOH); λ_{max}(MeOH)/nm 271, 291 and 384 (log ε 4.58, 4.63 and 4.29); $\Delta\varepsilon_{max}$(MeOH)/dm^3 mol^{-1} cm^{-1} +14.3, −17.6, +16.5, −18.4 (λ/nm 354, 313, 276, 243); ν_{max}(KBr)/cm^{-1} 3418, 2980, 2934, 1723, 1622, 1580, 1439, 1377, 1204, 1138, 1067 and 1001; positive FABMS *m*/*z* 541 (M^+ + 2Na − H, 24%), 501 (16) and 95 (59); positive FABMS (added 0.5 M aq. oxalic acid) *m*/*z* 519 (M^+ + Na, 25%), 497 (M^+ + H, 4), 479 (36) and 95 (100); *m*/*z* (HRFABMS) 519.1982 (M^+ + Na. Calc. for $C_{28}H_{32}O_8Na$: 519.1995) and 497.2148 (M^+ + H. Calc. for $C_{28}H_{33}O_8$: 497.2176); δ_H(400 MHz, $CDCl_3$) 1.01 (3H, s, H-9), 1.25 (3H, s, H-10), 1.32 (3H, s, H-15), 1.34 (3H, s, H-16), 1.87 (3H, br d, *J* 6.2, H-22), 1.95 (3H, br d, *J* 6.2,† H-28), 2.93 (1H, br dd, *J* 2.3 and 5.7, H-5), 3.39 (1H, br s, H-4), 4.01 (1H, br s, H-6), 6.04 (1H, dd, *J* 6.8 and 15.0, H-21), 6.30 (1H, d, *J* 15.4, H-24), 6.37 (1H, ddd, *J* 1.2, 11.1 and 15.0, H-20), 6.39 (1H, ddd, *J* 1.2, 10.6 and 15.0, H-26), 6.47 (1H, dd, 6.2 and 15.0, H-27), 6.49 (1H, d, *J* 14.5, H-18), 7.23 (1H, dd, *J* 11.1 and 14.5, H-19) and 7.42 (1H, dd, *J* 10.6 and 15.4, H-25); δ_C(100 MHz, $CDCl_3$) 6.2 (q, C-16), 12.9 (q, C-9), 18.7 (q, C-22), 19.1 (q, C-28), 24.2 (q, C-15), 24.3 (q, C-10), 45.0 (d, C-4), 47.4 (d, C-5), 52.5 (d, C-6), 63.1 (s, C-1), 75.2 (s, C-8), 85.1 (s, C-11), 90.3 (s, C-13), 110.5 (s, C-3), 126.8 (d, C-18), 129.3 (d, C-24), 131.8 (d, C-26), 132.9 (d, C-20), 136.8 (d, C-21), 141.4 (d, C-19), 144.8 (d, C-27), 148.4 (d, C-25), 181.0 (s, C-17), 182.2 (s, C-14), 184.6 (s, C-2), 194.9 (s, C-12), 207.5 (s, C-23) and 213.4 (s, C-7).

Dihydrotrichotetronine 2

Pale yellow amorphous powder (20 mg, 0.15% from extracts); $[a]_D$ +354 (*c* 0.13 in MeOH); λ_{max}(MeOH)/nm 257 and 368 (log ε 4.55 and 4.60); $\Delta\varepsilon_{max}$(MeOH)/dm^3 mol^{-1} cm^{-1} +32.0, –45.8, +0.4, –7.4 (λ/nm 363, 318, 254, 227); ν_{max}(KBr)/cm^{-1} 3434, 2930, 1734, 1632, 1605, 1561, 1447, 1383, 1314, 1256, 1138, 1055 and 997; positive FABMS (added 1 M NaI) *m*/*z* 521 (M^+ + Na, 29%), 481 (12) and 95 (68); positive FABMS (added 1 M KI) *m*/*z* 575 (M^+ + 2K − H, 17%), 537 (M^+ + K, 26), 499 (M^+ + H, 6), 459 (3) and 95 (38); *m*/*z* (HRFABMS) 521.2131 (M^++Na. Calc. for $C_{28}H_{34}O_8Na$: 521.2152); δ_H(400 MHz, $CDCl_3$) 1.15 (3H, s, H-9), 1.26 (3H, s, H-10), 1.48 (3H, s, H-15), 1.60 (3H, s, H-16), 1.62 (3H, dd, *J* 1.3 and 6.2, H-28), 1.90 (3H, br d, *J* 6.4, H-22), 2.22 (2H, br dd, *J* 7.0 and 14.3, H-25), 2.63 (2H, dd, *J* 7.2 and 14.7, H-24), 3.10 (2H, s, H-5 and H-6), 3.41 (1H, br s, H-4), 5.36 (1H, dddd, *J* 1.5, 6.6, 13.5 and 15.2, H-26), 5.47 (1H, tddd, *J* 1.2, 6.2, 12.3 and 15.2, H-27), 6.16 (1H, d, *J* 14.8, H-18), 6.23 (1H, ddd, *J* 6.4, 12.8 and 14.9, H-21), 6.32 (1H, ddd, *J* 1.1, 10.7 and 14.9, H-20) and 7.34 (1H, dd, *J* 10.7 and 14.8, H-19); δ_C(100 MHz, $CDCl_3$) 6.1 (q, C-16), 11.0 (q, C-9), 17.8 (q, C-28), 19.0 (q, C-22), 22.4 (q, C-15), 23.7 (q, C-10), 25.9 (t, C-25), 42.1 (d, C-4), 43.7 (d, C-5), 45.8 (t, C-24), 54.2 (d, C-6), 62.1 (s, C-1), 75.1 (s, C-8), 83.0 (s, C-11), 98.0 (s, C-13), 108.3 (s, C-3), 117.6 (d, C-18), 126.8 (d, C-27), 128.7 (d, C-26), 131.0 (d, C-20), 140.9 (d, C-21), 143.9 (d, C-19), 169.8 (s, C-17), 173.9 (s, C-14), 176.1 (s, C-12), 194.8 (s, C-2), 208.1 (s, C-7) and 213.8 (s, C-23).

† *J* values are given in Hz.

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