A New Potent Antifungal 'Quinone Methide' Diterpene with a Cassane Skeleton from *Bobgunnia madagascariensis*

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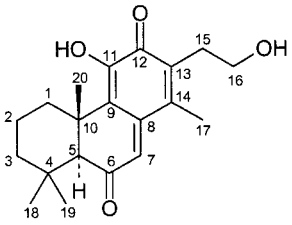
A new 'quinone methide' diterpene with a cassane skeleton was isolated from the root bark of *Bobgunnia madagascariensis* (DESV.) J.H. KIRKBR. et WIERSEMA (Leguminosae). Its structure was established as (4b*S*,8a*S*)-4b,5,6,7,8,8a-hexahydro-4-hydroxy-2-(2-hydroxyethyl)-1,4b,8,8-tetramethylphenanthrene-3,9-dione by spectroscopic methods including single-crystal X-ray analysis. This compound showed strong antifungal properties towards human pathogenic fungi, in particular the yeast *Candida albicans*.

Introduction. – *Bobgunnia madagascariensis* (DESV.) J.H. KIRKBR. et WIERSEMA (Leguminosae), which was known as *Swartzia madagascariensis* DESV. until April 1997 [1], is a tree widely distributed in tropical Africa. Several traditional medicinal uses have been documented [2]. The roots in particular are employed as a cure for leprosy and syphilis. Insecticidal activity against termites has been also reported [3]. *B. madagascariensis* has attracted much attention in the last decade, because of the molluscicidal properties of the fruits. Detailed phytochemical investigation of the pods afforded several strongly molluscicidal saponins [4]. Field trials carried out in Tanzania demonstrated the potency of water extracts for killing schistosomiasis-transmitting snails [5]. In fact, *B. madagascariensis* is considered one of the most promising plants for the vector control of this tropical parasitic disease. A few investigations have dealt with the other constituents of *B. madagascariensis*. A flavonoid has been purified from the seeds [6], and a series of pterocarpans have been isolated from the heartwood [7–9].

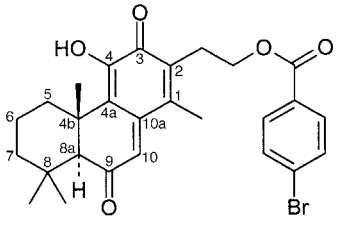
As part of our screening aimed at the discovery of new antifungal lead compounds, we observed that a lipophilic extract of *B. madagascariensis* root bark contained pale yellow metabolites that strongly inhibited growth of both *Candida albicans* and *Cladosporium cucumerinum* in TLC bioautography assays [10][11]. We report here on the isolation, structure elucidation, and antifungal properties of the main active constituent, which was shown to be a new 'quinone methide' diterpene with a cassane skeleton.

Results and Discussion. – Dried and powdered root bark of *B. madagascariensis* was extracted at room temperature with CH_2Cl_2. Fractionation of the CH_2Cl_2 extract by

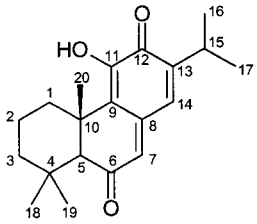
column chromatography (silica gel), followed by medium-pressure liquid chromatography (MPLC; *Diol* material), afforded compound **1** (see *Exper. Part*). The structure of **1** was assigned as (4b*R**,8a*R**)-4b,5,6,7,8,8a-hexahydro-4-hydroxy-2-(2-hydroxyethyl)-1,4b,8,8-tetramethylphenanthrene-3,9-dione from spectroscopic data and comparison with those reported for the '8,12-quinone methide' diterpene taxodione (**2**) isolated from *Taxodium distichum* (L.) L.C. RICH. (Taxodiaceae) [12].



1 (cassane numbering)



1a (phenanthrene numbering)



2

The EI-MS of **1** exhibited a molecular ion at *m/z* 330. The relative molecular mass was confirmed by $[M+H]^+$ and $[M+NH_4]^+$ pseudomolecular ions at *m/z* 331 and 348 in the D/CI-MS. The molecular formula deduced was $C_{20}H_{26}O_4$. In the UV spectrum, maxima were detected at 325 (22400), 335 (22400), and 415 (2100) nm, which suggested a 'quinone methide' chromophore. The UV data were, in fact, almost identical to those of **2**. The NMR data ((D_6)acetone) of **1** were consistent with a tricyclic diterpene skeleton and further revealed the close structural relationship to **2**. Values assigned to cycles A and B were in particular in excellent agreement. On the other hand, compared with **2**, no isopropyl group was present, but a hydroxyethyl chain was inferred from a *t* ($J=6.8$ Hz) at δ 3.62 and a br. *t* ($J=6.6$ Hz) at δ 2.83 in the ^{1}H-NMR spectrum. An additional quaternary Me group (δ(C) 15.9, δ(H) 2.34 ppm) was also apparent. The complete connectivity could be deduced from 2D-NMR experiments. In the gHMBC spectrum, couplings were observed between CH_2(15) and C(12), C(13), C(14), and C(16), which confirmed the attachment of the hydroxyethyl chain at C(13). At the same time, long-range correlations were detected from C(8), C(13), and C(14) to the methyl protons CH_3(17), demonstrating the Me group to be at C(14).

Definitive evidence and relative configuration of the structure of **1** were obtained from a single-crystal X-ray analysis. Crystals were grown from i-PrOH. The molecular structure and crystallographic numbering scheme are illustrated in *Fig. 1*, and the crystal packing is shown in *Fig. 2* [13]. Within experimental error, the extensive conjugated system, consisting of atoms C(6) to C(14), is planar. The fused saturated cyclohexane ring, atoms C(1) to C(5) and C(10), has a chair conformation. In the crystal symmetry, related molecules are H-bonded through OH–C(16) of the side chain and OH–C(11) to form a helix extending in the *b* direction. The O(21)H group of the i-PrOH molecule of crystallization is H-bonded to the O(6) atom at C(6). Compound **1** is, to our knowledge, the first 'quinone methide' diterpene with a cassane skeleton.

For the determination of the absolute configuration, an X-ray analysis of a halogenated derivative of compound **1**, the 16-*O*-(4-bromobenzoyl) derivative **1a**, was undertaken. The halogenated ester **1a** was prepared in pyridine and purified by column chromatography (silica gel), followed by recrystallization (see *Exper. Part*). The presence of the Br-atom was clearly visible in the D/CI spectrum ($[M+H]^+$ and $[M+NH_4]^+$ at *m/z* 513, 515 and 530, 532, resp.). The absolute configuration was determined based on the anomalous dispersion effect of the Br-atom. The absolute structure

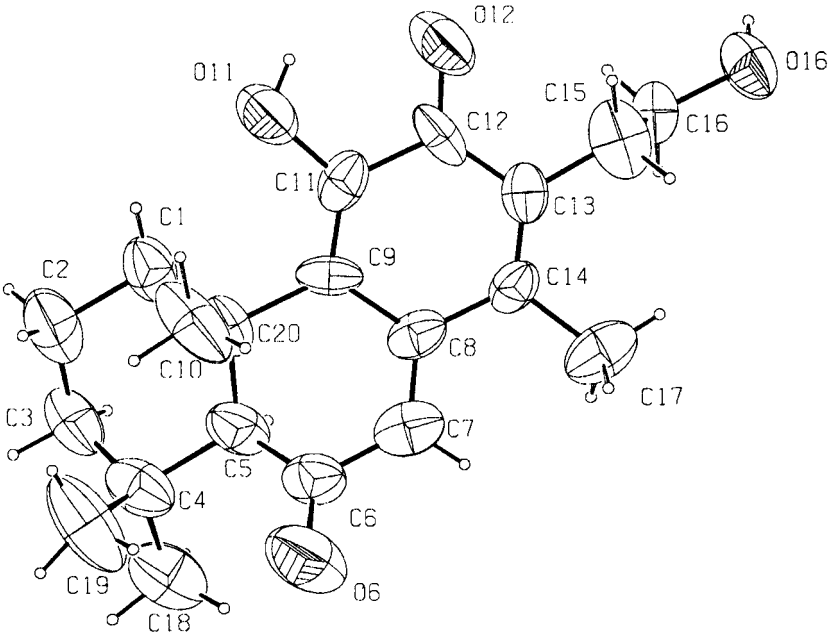


Fig. 1. *Perspective view of the molecule* **1** *showing the numbering scheme*

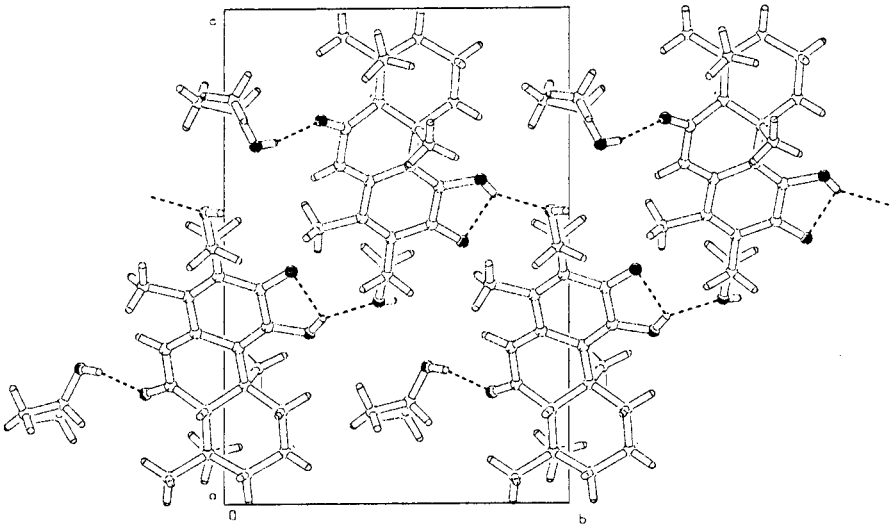


Fig. 2. *Crystal packing of* **1** *viewed down the* a *axis.* ··· H-Bonding. O(11)–H(11)···O(16^{i}): O(11)···O(16^{i}) 2.753 Å, O(11)–H(11)···O(16^{i}) 146.3°; O(21)–H(21)···O(6): O(21)···O(6) 2.846 Å, O(21)–H(21)···O(6) 162.8°; symmetry operation *i*: $-x, 0.5+y, -1-z$.

parameter had a value of $-0.012(7)$. The molecular structure and crystallographic numbering scheme of **1a** is illustrated in *Fig. 3* and the crystal packing in *Fig. 4* [14]. Thus, the absolute configuration of compound **1** was established as (4b*S*,8a*S*).

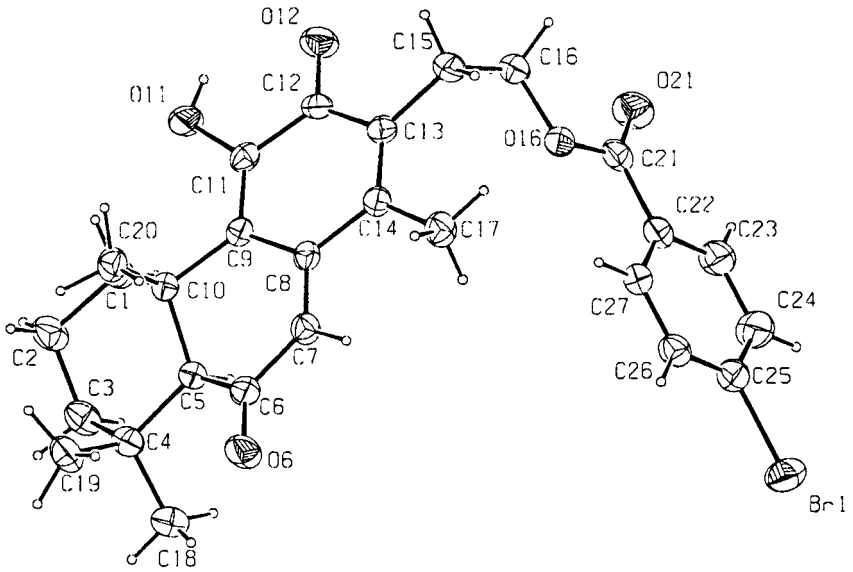


Fig. 3. *Perspective view of the molecule* **1a** *showing the numbering scheme*

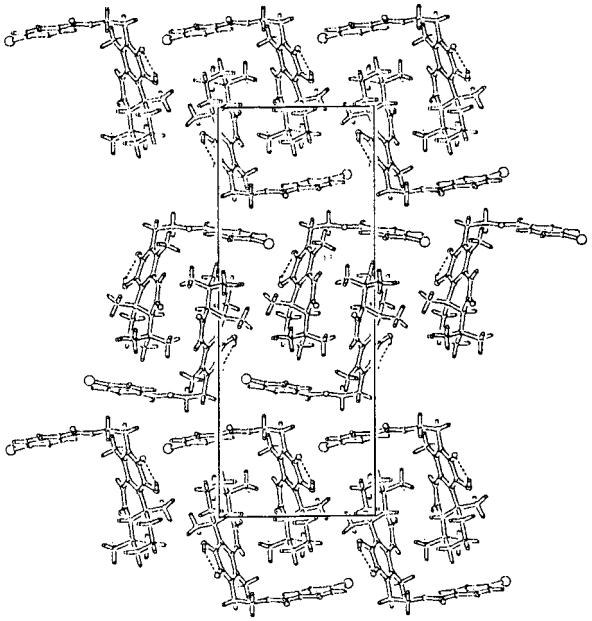


Fig. 4. *Crystal packing of* **1a**

With regard to biological activity, 0.1 μg of compound **1** was sufficient to inhibit the growth of *Candida albicans*, and 0.4 μg inhibited the growth of *Cladosporium cucumerinum* on TLC plates. This prompted us to compare the antifungal activity of **1** with that of commercial antifungal agents towards a panel of commercial and clinical

fungi, such as several *Candida* spp. or other human pathogenic fungi (*Aspergillus* spp.). Compound **1** was tested against each organism in a modification of the NCCLS microbroth assay using the tetrazolium salt XTT, to quantitate viable fungi cells [15][16]. Visual assessment of minimum inhibitory concentrations (*MIC*) and determination of 50-percent inhibition concentration (IC_{50}) were performed. Compound **1** was particularly active against the yeasts *Candida* spp., with *MIC* values lower than those of the reference compounds amphotericin B and fluconazole (*Table*).

Table. *Antifungal Spectrum of* **1** *towards* Candida *spp. Compared with That of Commercial Antimycotic Agents*

	MIC [μg/ml]		
	Candida albicans 90028	*Candida glabrata* 90030	*Candida krusei* 6258
1	0.19	0.19	0.19
Amphotericin B	0.50	0.39	0.78
Fluconazole	0.50	16.0	32.0

Compound **1** appears thus to be a promising lead for the development of novel antimycotic drugs and could soon enter clinical trials. Although **1** can be obtained in relatively large amounts from the root bark of *B. madagascariensis*, this will not be sufficient for an eventual commercial development. The synthesis of this compound should be possible, as a convenient method to obtain taxodione has been recently published [17][18].

Several diterpenes have been reported in the past to possess antifungal properties. Dihydrotanshinone in particular, a diterpene *o*-quinone isolated from *Salvia miltiorrhiza* BUNGE (Lamiaceae), was shown to be highly active against dermatophytic fungi [19].

Financial support was provided by the *Swiss National Science Foundation*. We are grateful to *Phytera*, *Inc.*, Worcester, MA, USA, for the evaluation of the antifungal properties and for financial support. The compound described in this publication is the subject of a patent, US 5.929.124 ('Antimicrobial Diterpenes'), issued on July 27, 1999.

Experimental Part

General. Atomic numbering: the cassane numbering (see **1**) is used, except in the systematic *IUPAC* name of **1** (phenanthrene numbering, see **1a**). TLC: silica gel *60* F_{254} Al sheets (*Merck*); petroleum ether/AcOEt 1:1; detection at 254 and 366 nm and with *Godin*'s reagent [20]. Medium-pressure liquid chromatography (MPLC): home-packed *LiChroprep Diol* (25–40 μm; 460 × 25 mm i.d.; *Merck*). M.p.: *Mettler-FP-80/82* hot-stage apparatus; uncorrected. $[\alpha]_D^{23}$: *Perkin-Elmer 241-MC* polarimeter (solvent, *c* in g/100 ml of solvent). UV: *Perkin-Elmer Lambda-20* UV/VIS spectrometer; in EtOH; λ_{max} [nm] (ε). NMR: *Varian Inova-500* spectrometer; ^{1}H/multinuclear inverse probehead at 499.870 MHz; ^{13}C, at 125.704 MHz, multiplicities from DEPT sequences; attributions by use of gradient double quantum filtered correlation spectroscopy (gDQCOSY), gradient heteronuclear single quantum coherence (gHSQC), and gradient heteronuclear multiple bond correlation (gHMBC) experiments; δ in ppm rel. to $SiMe_4$, *J* in Hz. EI- and D/CI-MS: *Finnigan-MAT-TSQ-700* triple-stage quadrupole instrument; EI: *m*/*z* (rel. intensity in %), ionization energy 70 eV; D/CI: NH_3, positive-ion mode.

Bioassays. Bioautography against *Cladosporium cucumerinum* and *Candida albicans* was carried out as previously reported [10][11]. *MIC* and IC_{50} values were determined in a modification of the NCCLS microbroth assay with the tetrazolium salt XTT [15][16]. Fungi were grown on *Sabouraud* dextrose agar plates and incubated overnight. On day 2, compound **1** was diluted (8 concentrations) in DMSO. Then, 2 μl of diluted solns. were added to test wells of a 96 well sterile culture plate, followed by addition of 98 μl of suspension of fungi to RPMI-MOPS [15] to a concentration of $5 \cdot 10^2$ cells/ml. After incubation, *MIC* values were determined,

followed by addition of XTT [16]. Plates were read at OD_{450}, and percent inhibition and IC_{50} values were then calculated.

Plant Material. Bobgunnia madagascariensis (DESV.) J. H. KIRKBR. et WIERSEMA was collected in 1996 near Murewa, Zimbabwe. A voucher specimen has been deposited at the National Herbarium and Botanical Garden of Zimbabwe, Causeway, Harare.

Fungal Strains. Human pathogenic fungal strains were commercially obtained from the *ATCC* and from *Chrisopee Technologies*, or from clinical isolates of Dr. *M. Rinaldi*, University of Texas, Dr. *A. Sugar*, Boston University, and Dr. *D. Grabill*, Audie Murphy Veteran's Administration Hospital.

Extraction and Separation. Dried, powdered root bark (1090 g) of *B. madagascariensis* was extracted at r.t. with CH_2Cl_2 (6.5 l) to give 65 g of extract. A portion of the extract (64.5 g) was fractionated by CC (silica gel, petroleum ether/AcOEt 2:1) to afford fraction *F* (5.90 g). Final purification of the main part of this material (5.88 g) by MPLC (*Diol*, petroleum ether/AcOEt 5:1) provided pure **1** (1757 mg).

(4bS,8aS)-4b,5,6,7,8,8a-Hexahydro-4-hydroxy-2-(2-hydroxyethyl)-1,4b,8,8-tetramethylphenanthrene-3,9-dione (**1**): Orange plates from i-PrOH. M.p. 64–66°. TLC (SiO_2, petroleum ether/AcOEt 1:1): R_f 0.25, brownish red with *Godin*'s reagent [20]. $[\alpha]_D^{23} = +63.7$ ($CHCl_3$, $c = 0.2$). UV (EtOH): 325 (22400), 335 (22400), 415 (2100). ^{1}H-NMR ((D$_6$)acetone): 6.50 (*s*, H–C(7)); 3.62 (*t*, $J = 6.8$, CH_2 (16)); 3.03 (br. *d*, $J = 11.8$, H_a–C(1)); 2.83 (br. *t*, $J = 6.6$, CH_2 (15)); 2.65 (*s*, H–C(5)); 2.34 (*s*, Me(17)); 1.73 (*m*, H_b–C(1), H_a–C(2)); 1.56 (*m*, H_b–C(2)); 1.38 (br. *d*, $J = 11.7$, H_a–C(3)); 1.26 (*s*, Me(19)); 1.26 (obscured, H_b–C(3)); 1.24 (*s*, Me(20)); 1.10 (*s*, Me(18)). ^{13}C-NMR ((D$_6$)acetone): 201.1 (C(6)); 182.3 (C(12)); 146.5 (C(14)); 145.1 (C(11)); 142.0 (C(8)); 134.8 (C(13)); 131.3 (C(7)); 127.0 (C(9)); 62.4 (C(5)); 61.3 (C(16)); 43.0 (C(4)); 42.9 (C(3)); 37.8 (C(1)); 33.4 (C(18)); 33.3 (C(10)); 31.0 (C(15)); 22.2 (C(19)); 21.6 (C(20)); 19.3 (C(2)); 15.9 (C(17)). EI-MS: 330 (100, M^+), 315 (51), 302 (18), 287 (31), 269 (22), 261 (27), 248 (50), 229 (67), 215 (50), 201 (46). D/CI-MS: 348 ($[M + NH_4]^+$), 331 ($[M + H]^+$).

Crystallographic Data of **1**. Suitable crystals of **1** were grown from i-PrOH. Orange plates. $C_{20}H_{26}O_4 \cdot [(CH_3)_2CHOH]_{0.75}$, monoclinic, space group $P2_1$, $a = 5.995(2)$, $b = 11.335(6)$, $c = 16.530(6)$ Å, $\beta = 95.53(3)°$, $Z = 2$; 2081 reflections measured, 2081 independent reflections, 626 observed reflections ($I > 2s(I)$), final $R_1 = 0.0985$, $R_{W2} = 0.2209$, goodness of fit 0.83, residual density max./min. 0.33–0.42 eÅ^{-3}. Absorption coefficient $\mu = 0.0777$ mm^{-1}; no correction for absorption was applied. Intensity data were collected at r.t. on a *Stoe-AED2* 4-circle diffractometer using MoK_α graphite monochromated radiation (λ 0.71073 Å) with $\omega/2\theta$ scans in the 2θ range 5–50°. The structure was solved by direct methods with the program SHELXS-86 [21]. The refinement and all further calculations were carried out with SHELXL-93 [22]. All of the H-atoms, including the OH protons, were included in calculated positions and allowed to ride on the corresponding C- or O-atom. The non-H-atoms were refined anisotropically, by means of weighted full-matrix least-squares on F^2.

In view of the limited number of observed reflections, the bond lengths and angles cannot be considered to be of a very high accuracy but are normal within experimental error. No attempt was made to determine the absolute configuration of the molecule. Full tables of atomic parameters and bond lengths and angles may be obtained from the *Cambridge Crystallographic Data Centre*, 12 Union Road, Cambridge CB2 1EZ (U.K.) on quoting the deposition No. CCDC-138890. The molecular structure and crystallographic numbering scheme (*Fig. 1*) and the crystal packing (*Fig. 2*) were drawn using PLATON and PLUTON [13], resp. Further details may be obtained from the author *H. St.-E.*

2-[(4bS,8aS)-3,4b,5,6,7,8,8a,9-Octahydro-4-hydroxy-1,4b,8,8-tetramethyl-3,9-dioxophenanthren-2-yl]ethyl 4-Bromobenzoate (**1a**). A soln. of **1** (40 mg) in pyridine (17 ml) was stirred at r.t. An excess of 4-bromobenzoyl chloride (280 mg) was added slowly (20 mg/5 min) to the sample. After 4.5 h (TLC monitoring), the mixture was poured into ice-water (60 ml) and then extracted with AcOEt. The mixture was purified by CC (silica gel, cyclohexane/^{t}BuOMe 10:1): 29 mg of **1a**. Orange rods from hexane/CH_2Cl_2. M.p. 151–153°. TLC (SiO_2, petroleum ether/AcOEt 1:1): R_f 0.60. $[\alpha]_D^{23} = +76.9$ ($CHCl_3$, $c = 0.065$). UV (EtOH): 244 (18600), 322 (20900), 333 (20400), 413 (1380). ^{1}H-NMR ($CDCl_3$): 7.85 (*dm*, $J = 9.0$, H–C(23), H–C(27)); 7.59 (*dm*, $J = 9.0$, H–C(24), H–C(26)); 7.50 (*s*, OH–C(11)); 6.49 (*s*, H–C(7)); 4.40 (*t*, $J = 6.8$, CH_2(16)); 3.07 (*m*, CH_2(15)); 2.96 (br. *d*, $J = 10.8$, H_a–C(1)); 2.58 (*s*, H–C(5)); 2.30 (*s*, Me(17)); 1.71 (*m*, H_b–C(1), H_a–C(2)); 1.60 (*m*, H_b–C(2)); 1.41 (br. *d*, $J = 11.7$, H_a–C(3)); 1.27 (*s*, Me(19)); 1.26 (*s*, Me(20)); 1.20 (*m*, H_b–C(3)); 1.12 (*s*, Me(18)). ^{13}C-NMR ($CDCl_3$): 201.1 (C(6)); 181.4 (C(12)); 165.8 (C(21)); 146.5 (C(14)); 143.8 (C(11)); 140.8 (C(8)); 132.5 (C(13)); 131.8 (C(23), C(27)); 131.5 (C(7)); 131.0 (C(24), C(26)); 128.9 (C(22)); 128.2 (C(25)); 127.0 (C(9)); 63.3 (C(16)); 62.3 (C(5)); 42.7 (C(4)); 42.4 (C(3)); 37.0 (C(1)); 33.2 (C(18)); 32.7 (C(10)); 26.2 (C(15)); 21.9 (C(19)); 21.7 (C(20)); 18.6 (C(2)); 15.8 (C(17)). D/CI-MS: 532, 530 ($[M + NH_4]^+$), 515, 513 ($[M + H]^+$).

Crystallographic Data of **1a**. Suitable crystals of **1a** were grown from hexane/CH_2Cl_2 20:1. Orange rods. $C_{27}H_{29}BrO_5$, orthorhombic, space group $P2_12_12_1$, $a = 7.1422(4)$, $b = 11.2433(9)$, $c = 29.5614(18)$ Å, $Z = 4$, $D_c = 1.437\ g \cdot cm^{-3}$, 16172 reflections collected, 4430 independent reflections ($R_{int} = 0.0451$), 3624 were considered ($I > 2s(I)$), final $R = 0.0287$ (obsd. data), $wR_2 = 0.0581$ (all data), goodness of fit 0.920, residual density max./min. 0.271/−0.398 e Å^{-3}. Absorption coefficient $\mu = 1.768\ mm^{-1}$; no correction for absorption was applied. Intensity data were collected at 193 K on a *Stoe-Image-Plate-Diffraction* system with MoK_α graphite monochromated radiation. Image plate distance 70 mm, ϕ rotation 0–200°, step $\Delta\phi = 1°$, 2θ range 3.27–52.1°, $d_{max} - d_{min} = 12.45 - 0.81$ Å. The structure was solved by direct methods with the program SHELXS-97 [21]. The refinement and all further calculations were carried out using SHELXL-97 [23]. The H-atoms were included in calculated positions and treated as riding atoms according to SHELXL-97 default parameters. The non-H-atoms were refined anisotropically by means of weighted full-matrix least-squares on F^2. Absolute structure parameter = −0.012(7).

The absolute configuration was based on the anomalous dispersion effects of the Br-atom. The bond lengths and angles are normal within experimental error. The molecular structure and crystallographic numbering scheme (*Fig. 3*) and the crystal packing (*Fig. 4*) were drawn according to [13][14]. Further details may be obtained from the author *H. St-E.* and the *Cambridge Crystallographic Data Centre* (see above; deposition No. CCDC-138891).

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Received October 14, 1999