Improved microbiological hydroxylation of sesquiterpenoids: semisynthesis, structural determination and biotransformation studies of cyclic sulfite eudesmane derivatives†

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Two new cyclic sulfite eudesmane derivatives have been investigated. Their (R) and (S) sulfur configuration and the structural arrangement of their “A” rings have been assigned by means of their 1H and 13C NMR chemical shifts and have been confirmed by single-crystal X-ray analyses. Microbial transformation of these epimer cyclic sulfites and their dihydroy eudesmane precursor have been studied using the hydroxy- lating fungus Rhizopus nigricans. Increased biocatalysis rates and considerable differences in the biotransformation of both cyclic sulfite eudesmanes have been found. Promising 8α,11-dihydroxy derivatives have been isolated from the (S)-diastereomer bioconversion.

Introduction

Biocatalysis is increasingly becoming an important tool for organic synthesis. Thus, biotransformations with whole cells are currently accepted as methods to synthesise many fine chemicals, to prepare chiral building blocks or to modify natural products with biological activities. One of the most widespread enzymatic activities is the hydroxylation,6 but it is, perhaps, the least well understood. The main catalytic properties of these monoxygenase enzymes are their high non-activated carbon centres.7,9

Terpenes represent the largest family of natural products comprising over 30000 defined structures. In particular, sesquiterpene compounds with eudesmane skeletons are widely distributed in nature and are biologically quite active, having, for example, antimicrobial, antifeedant, cell-growth-inhibiting properties etc. Consequently, their synthesis has received particular attention in the past few decades,10,11 in addition to the fact that these compounds are often the starting materials for the semisynthesis of other products.12–14

Microbial hydroxylation of terpenoids21 has been used for the selective functionalisation of many of these compounds and constitutes an important alternative to chemical methods, enabling the specific access to remote positions on the molecule under mild reaction conditions. Therefore, we have previously described several biotransformations of diverse eudesmane substrates by different filamentous fungi, and we have isolated metabolites in which we observed both regio- and stereoselectivity in the hydroxylation of non-activated carbon centres.15,16

In the present paper, the synthesis of two new diastereomer eudesman-4β,6β-diylic sulfite derivatives has been investigated. The absolute configuration of the sulfur atom in this pair of compounds has been shown by the analysis of their experimental NMR chemical shifts and their 1JHH coupling constants. The half-boat conformation of the eudesmane “A” ring, due to the presence of the new sulfite cycle, has also been displayed. These results have been confirmed by X-ray crystallography. Finally, the different reactivity of both diastereomer sul- fites in the biotransformation processes with Rhizopus nigricans (CECT 2672), a synonym of Rhizopus stolonifer (ATCC 10404, IMI 061269), has also been studied, and promising hydroxylated metabolites have been isolated.

Results and discussion

The cyclic sulfite eudesmane derivatives 1 and 2 were derived from the natural compound 3, isolated from Sideritis leucantha Cav. subsp. meridionalis.13 Hydrolysis of the acetoxy group on C-6 of 3, and subsequent regioselective Jones’ oxidation of the alcohol on C-1 gave the diol 4. The diastereomer cyclic sulfites were synthesised by treatment of this diol (4) with thionyl chloride in methylene chloride, adding pyridine as base, to scaveng the hydrogen chloride released during the process (Scheme 1).14 The reaction was carried out at diverse temperatures between −45 and +45 °C – the reflux temperature of the reaction mixture – and different relative ratios of sulfate diastereomers 1 and 2 were found (Fig. 1, Table 1). Thus, when the reaction temper-
The existence of the cyclic sulfite affects the conformation of the “A” ring in these eudesmane derivatives. On comparing the 1H NMR spectra of both diastereomers and that of compound 4, we observed significant differences in the chemical shifts and in the coupling constants of different signal protons of that ring. A clear difference was found for the β-H signals. Thus, in the spectrum of 4, this proton appeared at δ 3.15, while in sulftes 1 and 2 the respective β-H signals were more shielded at δ 2.45 and 2.55, probably due to the absence of a 1,3-diaxial interaction with the oxygen atom on C-4. These discrepancies could be explained by a different conformation of the “A” ring in these compounds. Hence, in compound 4, these experimental values agreed with a half-chair conformation to this ring, and with a half-boat conformation in both cyclic sulfite derivatives (1 and 2).

These hypotheses and the configuration of the sulfur atoms in the sulftite rings were confirmed by the X-ray data of both derivatives (Figs. 2 and 3).

To explore the influence of the sulfur configuration in the biocatalysis reactions, shown in a previous work,18 we studied the biotransformation of these sulftite derivatives with the fungus R. nigricans. Incubation of the (S)-sulftite derivative (1) was maintained for 36 h, resulting in total consumption of substrate (1). On the other hand, the biotransformation of the (R)-diastereomer (2) was carried out for 72 h (double time), leaving 20% of unaltered substrate (2). For a better comparison of the different reactivity of the cyclic sulftites diastereomers, the sulftite derivative 1 was also incubated for 72 h.

Biotransformation of cyclic sulftite 1 by R. nigricans for 36 h yielded a mixture of metabolites from which, after treatment with Ac₂O-Py, compound 5 (20%) and metabolite 6 (45%) were isolated. In addition, metabolite 7 (20%) and a very polar metabolite 8 (3%) were also found (Scheme 2).

The 1H NMR spectrum of the first compound isolated (5) revealed a signal at δ 5.28 (ddd, J₈₋₇ = J₇₋₆ = 11.4, J₆₋₅ = 4.1) due to the geminal proton of an acetoxy group on C-8 with an equatorial arrangement. The 13C NMR data confirmed the new acetoxy group position, and therefore, compound 5 was 8α-acetoxy-1-o xo eudesman-4β,6β-diy1-S(S)-cyclic sulftite.

### Table 1

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<tr>
<th>Temperature/°C</th>
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<th>2</th>
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<tr>
<td>45</td>
<td>18.5 mg (37%)</td>
<td>28.5 mg (57%)</td>
</tr>
<tr>
<td>25</td>
<td>19.0 mg (38%)</td>
<td>28.0 mg (56%)</td>
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<tr>
<td>0</td>
<td>22.5 mg (45%)</td>
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<td>25.5 mg (51%)</td>
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<tr>
<td>−45</td>
<td>27.0 mg (54%)</td>
<td>20.5 mg (41%)</td>
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Table 1 Amounts and relative percentages of sulftites 1 and 2 at different temperatures.

![Scheme 1](image1.png)

**Scheme 1** Semisynthesis of cyclic sulftites 1 and 2.
Table 2 $^1$H NMR chemical shifts for compounds 1, 2, 4, 5, 6, 7 and 8

<table>
<thead>
<tr>
<th>Compounds</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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<tr>
<td>C-1</td>
<td>214.7</td>
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<td>216.3</td>
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</table>

Scheme 2 Biotransformation of 1 by Rhizopus nigricans cultures.

Table 3 Yields of compounds in the biotransformation of 1 at 36 and 72 h

<table>
<thead>
<tr>
<th>Yield of compounds (%)</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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<tr>
<td>t/h</td>
<td>36</td>
<td>20</td>
<td>45</td>
<td>20</td>
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<tr>
<td></td>
<td>72</td>
<td>15</td>
<td>29</td>
<td>20</td>
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</table>

The main metabolite isolated (6) had a new hydroxy group not acetylated, whose spectral data indicated a biodehydration on C-11. Consequently, metabolite (6) was 11-hydroxy-1-oxoeudesman-4$\beta$,6$\beta$-diyl-S($S$)-cyclic sulfite.

The comparison of the spectral data of metabolite 7 with those of compound 5 and metabolite 6 showed the presence of two new hydroxy groups at C-8 and C-11, which agreed with a structure of 8$\alpha$,11-dihydroxy-1-oxoeudesman-4$\beta$,6$\beta$-diyl-S($S$)-cyclic sulfite for this metabolite (7).

The last metabolite isolated (8) was considerably more polar than the others, due to the absence of the sulfite group, and with spectral data similar to those of metabolite 7. Hence, we deduced that metabolite 8 was 4$\beta$,6$\beta$,8$\alpha$,11-tetrahydroxy-eudesmane-1-one.

The same four compounds were isolated when the biotransformation of substrate 1 was maintained for 72 h. Nevertheless, the compound ratio presented substantial changes (Table 3). The whole percentage of 8$\alpha$,11-dihydroxylated compounds was almost doubled (23% for 36 h and 43% for 72 h), while the yield of metabolites without sulfite group also increased.

Biotransformation of the (R)-cyclic sulfite diastereomer (2) with R. nigricans for 72 h gave the metabolites 9 (5%), 10 (50%), 11 (2%) and 12 (10%), besides some 20% of unaltered substrate (2) (Scheme 3).

Metabolite 9 had an additional hydroxy group but without geminal proton. The $^1$C NMR spectral data positioned this new hydroxy group at C-7. Consequently, metabolite (9) had the structure of 7$\alpha$-hydroxy-1-oxoeudesman-4$\beta$,6$\beta$-diyl-S(R)-cyclic sulfite. The spectral data of the main metabolite isolated (10) indicated the presence of a new hydroxy group and the loss of the cyclic sulfite group. This compound was a 4-epi-cryptomeridiol derivative (4$\beta$,6$\beta$,11-trihydroxyeudesman-1-one), previously isolated from the incubation of different eudesmane derivatives with R. nigricans and Gliocladium roseum.

The spectral data of metabolite 11 indicated the presence of a carbon–carbon double bond placed between C-11 and C-12, probably formed by a dehydration reaction from an 11-hydroxy compound, and the absence of the sulfite group. Hence, this metabolite (11) was 4$\beta$,6$\beta$-dihydroxyeudes-11-en-1-one.

The most polar metabolite (12), which had lost the sulfite group, owned a new hydroxy group situated on C-8 in an equatorial arrangement, with a structure of 4$\beta$,6$\beta$,8$\alpha$-trihydroxy-eudesmane-1-one.
Steric hindrance, originated a (1

This reduction, which was achieved from the selective reduction of the carbonyl group at C-1 of substrate yielded the metabolite 4.

The versatile reactivity of this sulfite group and C-8. From the bioconversion of the (S)-sulfinyl derivatives seem more easily to lose the cyclic sulfinyl group and C-8. Microbial hydroxylation were detected. Also, the (R)-sulfinyl derivatives in these substrates (R. nigricans) were isolated but with poor yields, while, in the present work, the (R)-sulfinyl derivatives were obtained only from a C-8a and/or C-11 hydroxylations were detected, whereas, from the bioconversion of the (R)-sulfinyl (2), 7a-hydroxy and 11-ene derivatives were also found, but in limited proportion. Previously, 8a,11-dihydroxy derivatives have been isolated but with poor yields, while, in the present work, some 23% after only 36 h and 43% after 72 h were achieved. The versatile reactivity of this sulfite cyclic group and the 8a,11-dihydroxy derivatives isolated from the biotransformation of the (S)-diastereomer (1) offers new attractive possibilities to synthesise natural product derivatives such as 8,12-eudesmanolides.

**Experimental**

**General experimental procedures**

Measurements of NMR spectra (300.13 MHz 1H and 75.47 MHz 13C) were made in CDCl3, CD3COCD3 or CD3OD (which also provided the lock signal) with BRUKER spectrometers (AM-300 and ARX-400). The assignments of 13C chemical shifts were made with the aid of distortionless enhancement by polarization transfer (DEPT) using a flip angle of 135°. Bruker’s programs were used for 1H correlation (HMQC). IR spectra were recorded on a Nicolet 20SX FT-IR spectrometer. High-resolution mass spectra were made by LSIIMS ionization mode with a MICROMASS AUTOSPEC-Q spectrometer. X-Ray data were collected on a BRUKER P4 diffractometer equipped with graphite-monochromator Mo-Kα radiation (λ = 0.71073 Å). The crystal structures of compounds 1 and 2 were refined using the SHELXTL program package (ver. 6.10). Uncorrected melting points were determined using a Kofler (Reichert) apparatus. Optical rotations were measured on a Perkin-Elmer 241 polarimeter at 25 °C and are given in units of 10−1 deg cm2 g−1. Scharlau 60 silica gel (40–60 μm) was used for flash chromatography. CH2Cl2, CHCl3, or hexane containing increasing amounts of Me2CO, MeOH or 2-propanol were used as eluents. Analytical plates (silica gel, Merck 60 G) were rendered visible by spraying with H2SO4–AcOH, followed by heating to 120 °C. The identity of compounds 3 and 4 was confirmed by direct comparison with the authentic samples (IR, MS, NMR, etc.).

**Isolation of 6β-acetoxy-1β,4β-dihydroxyeudesmane (3)**

6β-Acetoxy-1β,4β-dihydroxyeudesmane was isolated from Sideritis leucantha Cav. subsp. meridianalis (Font Quer) O. Socorro.

**Synthesis of 4β,6β-dihydroxyeudesman-1-one (4)**

6β-Acetoxy-1β,4β-dihydroxyeudesmane (3, 1.90 g, 6.4 mmol) was dissolved into a MeOH–H2O (70%) solution (120 mL) containing KOH (5%) (6 g, 0.11 mol) and refluxed for 1 h. The reaction mixture was extracted with CH2Cl2, dried over anhydrous Na2SO4 and evaporated to dryness. Next, the resulting residue was stirred in acetone (50 mL) at 0 °C and Jones’ reagent was added dropwise until an orange-brown colour persisted (30 min), following the monooxidation by TLC. Methanol was then added and the reaction mixture was diluted with water (100 mL) and extracted with CH2Cl2 (3 × 100 mL). The organic layer was dried over anhydrous Na2SO4 and evaporated at reduced pressure. Chromatography on a silica-gel column yielded 4β,6β-dihydroxyeudesmane-1-one (4, 1.54 g, 95%).

**Formation study of sulfitic derivatives (1 and 2) at different temperatures**

Diol 4 (50 mg, 0.2 mmol) was dissolved in dichloromethane (1.5 mL) and pyridine (0.5 mL) in each experiment. The mixture was stirred at the adequate temperature and thionyl chloride (0.07 mL, 1 mmol) was added. After 5 min, water (4 mL) was cautiously added dropwise. The mixture was extracted with CH2Cl2 (3 × 4 mL), and the combined extracts were washed with saturated aqueous KH2SO4 (2 × 10 mL), dried with anhydrous Na2SO4 and evaporated under reduced pressure. Column chromatography over silica gel using hexane and increasing amounts of isopropanol as eluents gave 1-oxo-eudesman-4β,6β-diy-1(S)-cyclic sulfite (1) and 1-oxo-eudesman-4β,6β-diy-1(R)-cyclic sulfite (2) (yields in Table 1).
Crystal data for 1. Colourless solid, mp 113–115°C (from hexane); [α]D20 = −39 (c 1 in CHCl3); vmax (NaCl/cm−1) 2959, 1711, 1222 and 1192; δH (300 MHz; CDCl3); MeSi 0.95 and 0.97 (3 H each, d, J 6.5, 12-Me and 13-Me), 1.05 (1 H, ddd, J9,6 = 12.5, J11,9 = 9.1, J9,8 = 3.7, J6,7 = 3.3, 7-H), 1.20 (1 H, ddd, J11,9 = J9,13 = 13.6, J9,14 = 3.7, 9a-H), 1.44 (3 H, s, 15-Me), 1.48 (1 H, d, dddd, J6,7,8 = 2.9, J5,6 = 2.4, 5-H), 1.49 (1 H, ddd, J9,8 = 13.9, J9,13 = 13.6, J9,14 = 12.5, J3,4 = 3.3, 8B-H), 1.66 (1 H, m, J11,13 = 9.1, J9,13 = 13.6, 11,12-H), 1.80 (1 H, ddd, J9,13 = 13.9, J9,14 = 3.3, 8a-H), 1.87 (3 H, s, 15-Me), 2.00 (1 H, ddd, J9,8 = 13.6, J9,14 = 22.5, 3,3-H), 2.45 (1 H, ddd, J9,8 = 15.4, J9,14 = 7.3, J9,15 = 2.5, 2B-H) and 5.55 (1 H, dd, J9,13 = 3.3, J6,7 = 2.4, 6-H); δC(75.4 MHz; CDCl3; MeSi) Table 2; mlz (HRLSIMS) 323.1296 ([M + 23]+). C74H82SO9N6 requires 323.1293.

Synthesis of sulfites derivatives (1 and 2) For the biotransformation studies of these cyclic sulfite derivatives (1 and 2), their reaction formation was again carried out at 0°C. For this, 4β-6β-dihydroxydesman-1-one (4, 1.20 g, 4.7 mmol) was dissolved in dichloromethane (40 mL) and pyridine (11 mL), and then, thionyl chloride (1.67 mL, 23.5 mmol) was added dropwise. After previously indicated treatment, (S)-cyclic sulfite 1 (638 mg, 45%) and (R)-cyclic sulfite 2 (666 mg, 47%) were isolated.

Crystal structure determination of compounds 1 and 2 Crystals of compounds 1 and 2 suitable for X-ray diffraction were obtained by slow evaporation of hexane solution into a methylamethanol atmosphere.

Crystal data for 1. C74H82SO9N6. M = 300.44, monoclinic, a = 8.519(1), b = 9.782(1), c = 18.631(2) Å, U = 1552.6(2) Å3, T = 294(1) K, space group P21/c, Z = 4, Dcalc = 1.285 M g cm−3. F(000) = 648, µ(Mo-Kα) = 0.219 mm−1, 3332 reflections measured, 3129 unique (Rint = 0.0349, R = 0.0396) which were used in all calculations. R1 = 0.0409 (F > 4σ(F)) and 0.0535 (for all). Flack = −0.20 (9). The final wR2(F2) = 0.1086.

Crystal data for 2. C74H82SO9N6. M = 300.44, monoclinic, a = 9.321(2), b = 7.796(2), c = 11.132(2) Å, U = 1572.6(2) Å3, T = 294(1) K, space group P21/z, Z = 2, Dcalc = 1.253 M g cm−3. F(000) = 324, µ(Mo-Kα) = 0.213 mm−1, 3060 reflections measured. 3060 unique (Rint = 0.0000, R = 0.0330) which were used in all calculations. R1 = 0.0672 (F > 4σ(F)) and 0.1050 (for all). Flack = −0.18 (16). The final wR2(F2) = 0.1730.

Organism, media and culture conditions Rhizopus nigricans CECT 2672 was obtained from the Colección Española de Cultivos Tipo. Departamento de Microbio-

† CCDC reference numbers 203948 and 203949. See http://www.ccdc.cam.ac.uk/structures for crystallographic data in . cif or other electronic format.
Biotransformation of (S)-cyclic sulﬁte 1 (72 h)  

Substrate 1 (150 mg) was also dissolved in EtOH (3 ml), distributed among 3 Erlenmeyer flask cultures of *R. nigricans* and incubated for 72 h, after which the cultures were processed as indicated above to give a mixture of metabolites (137 mg, 44%) from which, after treatment with Ac₂O-Py, 5 (24 mg, 15% of overall yield) and 6 (46 mg, 29% of overall yield) were isolated. In addition, 7 (33 mg, 20%) and 8 (35 mg, 23%) were also found.

Biotransformation of (R)-cyclic sulﬁte 2  

Substrate 2 (375 mg) was dissolved in EtOH (5 ml), distributed among 5 Erlenmeyer flask cultures of *R. nigricans* and incubated for 72 h, after which the cultures were processed as previously indicated for the biotransformation of the substrate 1, to give a mixture (333 mg). The mixture was chromatographed on a silica gel column to provide the starting material 2 (75 mg, 20%), 7α-hydroxy-1-oxoexudesman-4β,6β-diy1-S(R)-cyclic sulﬁte (9, 20 mg, 5%), 4β,6β,11-trihydroxyexudesman-1-one (10, 169 mg, 50%), 23 4β,6β-dihydroxyexudesman-11-en-1-one (11, 6 mg, 2%), and 4β,6β,8α-trihydroxyexudesman-1-one (12, 34 mg, 10%).

7α-Hydroxy-1-oxoexudesman-4β,6β-diy1-S(R)-cyclic sulﬁte (9)  

Colourless solid, mp 141–143 °C; [α]D = +34 (c 1 in CHCl₃); νmax (NaCl/cm⁻¹) 3512, 2966, 1712, 1320 and 1154; δH (300 MHz; CDCl₃; Me₂Si) 0.93 and 0.94 (3 H each, d, J 6.8, 12-Me and 13-Me). 1.48 (3 H, s, 14-Me), 1.74 (3 H, s, 15-Me) and 4.58 (1 H, d, J 2.4, 6-H); δC (75.74 MHz; CDCl₃; Me₂Si) Table 4; m/z (HRLSIMS) 339.1243 ([M + 23]+). C₁₅H₂₀O₅Na requires 339.1242.

4β,6β-Dihydroxyexudesman-11-en-1-one (11)  

Colourless syrup; [α]D = +43 (c 1 in CHCl₃); νmax (NaCl/cm⁻¹) 3397, 3085, 1706, 1647, 1187 and 1130; δH (300 MHz; CDCl₃; Me₂Si) 1.33 (3 H, s, 15-Me), 1.60 (3 H, s, 14-Me), 1.79 (3 H, s, 13-Me), 3.17 (1 H, ddd, J 2.2, 6.3, 14.0) 6.5; 3.0 (1 H, br s, 7-H); 4.35 (1 H, br s, 6.2-H); 5.03 (1 H, m, 12-H) and 4.87 (1 H, br s, 8.2-H); δC (75.74 MHz; CDCl₃; Me₂Si) Table 4; m/z (HRLSIMS) 275.1625 ([M + 23]+). C₁₁H₁₀O₄Na requires 275.1623.

4β,8α-Trihydroxyexudesman-1-one (12)  

Colourless solid, mp 158–160 °C; [α]D = +53 (c 1 in CHCl₃); νmax (NaCl/cm⁻¹) 3396, 2924, 1691, 1135 and 1113; δH (300 MHz; CDCl₃; Me₂Si) 1.14 and 1.12 (3 H each, J 7.0, 12-Me and 13-Me). 1.39 (3 H, s, 15-Me), 1.64 (3 H, s, 14-Me), 3.13 (1 H, ddd, J 2.2, 6.3, 14.0); 4.19 (1 H, ddd, J 2.2, 6.3, 11.1) 6.5; 3.8 (4 H, 3.8-H) and 4.64 (1 H, br s, 6-H); δC (75.74 MHz; CDCl₃; Me₂Si) Table 4; m/z (HRLSIMS) 293.1727 ([M + 23]+). C₁₅H₂₁O₄Na requires 293.1729.

Biotransformation of diol 4  

Substrate 4 (90 mg) was also dissolved in EtOH (1 ml), added in 1 Erlenmeyer flask culture of *R. nigricans* and incubated for 6 days, after which the cultures were processed as indicated above to give the unaltered substrate (4, 11 mg, 12%), 6β-acetoxy-1α,4β-dihydroxyexudesmane (13, 18 mg, 20%) and compounds 10 (39 mg, 41%) and 12 (11 mg, 11%), previously isolated in the biocconversion of 2.

1α,4β,6β-trihydroxyexudesmane (13), Colourless syrup; [α]D = +32 (c 1 in CHCl₃); νmax (NaCl/cm⁻¹) 3418, 2912, 1136 and 1022; δH (300 MHz; CDCl₃; Me₂Si) 0.94 and 0.97 (3 H each, d, J 6.7, 12-Me and 13-Me), 1.30 (3 H each, s, 14-Me and 15-Me), 3.33 (1 H, br s, 1-H) and 4.57 (1 H, m, 6-H); δC (75.74 MHz; CDCl₃; Me₂Si) Table 4; m/z (HRLSIMS) 279.1934 ([M + 23]+). C₁₅H₂₁O₄Na requires 279.1936.

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References  