X-Ray crystal structure analysis and ^{13}C NMR investigation of estriol 16- and 17-monoglucuronide derivatives

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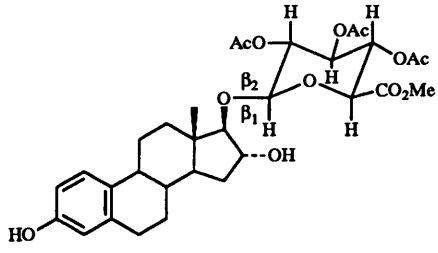
The crystal structure of methyl 1-*O*-[3,16α-dihydroxy-estra-1,3,5(10)-trien-17β-yl]-2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate, 1, clearly shows the β-linkage of the carbohydrate moiety with estriol and unambiguously shows the *trans* relationship of the 17α- and 16β-protons. ^{13}C NMR studies on 1 and on other estriol 16α- and 17β-glucuronide derivatives show differences between the two types of isomer based on the chemical shift values for C-16 and C-17 which are diagnostic. For C-17 derivatives with the β configuration, the chemical shift difference between the C-16 and C-17 signals was about 20 ppm, whereas the difference between the resonances for the 16α-derivatives was only about 5 ppm. The correlation of the 17α-proton with the anomeric proton (H-1′) from estriol 17β-glucuronide derivatives, based on 2D-NOESY spectra, was also useful in distinguishing the estriol 17β-derivatives from the 16α-isomers.

Estriol monoglucuronides are important human metabolites of estradiol, and have been synthesised by various methods.[1–7] The usual syntheses of estriol 16α-glucuronide and estriol 17β-glucuronide involve many steps including the glucuronidation of 16α-hydroxy-17-oxo-derivatives and reduction of the 17-carbonyl group. These synthetic routes therefore introduce the possibility of isomerisation and epimerisation at the 16 and 17 carbon centres. However, unlike the situation for six-membered rings, the determination of the relative stereochemistry at C-16 and C-17 in the five-membered D-ring of steroids is difficult to judge solely from the ^1H chemical shift data and the magnitude of the coupling constant (J) for the C-16 and C-17 protons (H_{16}–H_{17}). For example, there are only minor differences in the ^1H NMR spectra[7] for the estriol 16α- and 17β-glucuronides and hence identification of the exact position and configuration of the glycosidic linkage between estriol and the carbohydrate moiety is difficult to determine. Although the β orientation of the glucuronide ring is shown clearly by the fact that β-glucuronidase is active towards the β_2-linkage of compounds **1** and **2**,[8] no such simple test is available to confirm the orientation or position of the β_1-linkage. This linkage is of vital importance in the chemical synthesis of steroids for use in the analysis of biological fluids since if this stereochemistry is incorrect, recognition by binding proteins is seriously impaired.

We have developed a synthetic procedure for the preparation of estriol glucuronides starting from estriol and utilising selective protection and deprotection of the three hydroxy groups of estriol.[7] In this method the stereochemistry at the C-16 and C-17 positions of the D-ring should be preserved and hence the compounds synthesised serve as standards for the assignment of ^1H and ^{13}C NMR chemical shifts. It is difficult to obtain suitable crystals of estriol glucuronides in the free acid form hence an X-ray structure analysis was undertaken for compound **1** to confirm that the absolute configuration at all stereocentres was maintained during synthesis. In the present work, we report also ^{13}C NMR spectroscopic data, in conjunction with the 2D-NOESY technique, to establish the general spectroscopic differences between the estriol 17β- and 16α-glucuronide derivatives.



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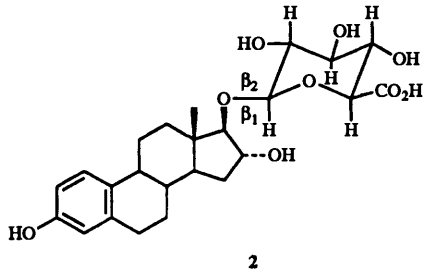
Hydrolysis



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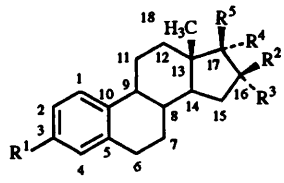
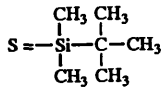
Results and discussion

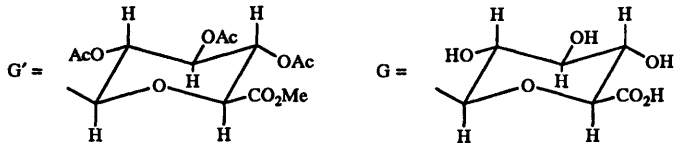
The structures of the compounds with known stereochemistry at carbons 16 and 17 are given in Table 1. Compounds **1**–**4**, **6** and **7** were previously synthesised from estriol during the synthesis of estriol 16α- and 17β-glucuronides[7] by initial protection of the 16α-hydroxy group with a silyl ether according to Laurent *et al.*[9] Compounds **5** and **8** were prepared by $NaBH_4$ reduction of methyl 1-*O*-[2,4-dibromo-3-hydroxy-17-oxoestra-1,3,5(10)-trien-16α-yl]-2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate according to the procedure of Numazawa *et al.*[5] The standards, compounds **9**–**11**, were all commercially available. The structures of all the derivatives were confirmed by ^1H and ^{13}C NMR spectroscopy together with ^1H–^1H COSY and 2D-NOESY spectra. This is important since, as is clear from previous work[7] and the coupling constants in Table 1, there is no way to distinguish the 16-glucuronides from the 17-glucuronides or the α-isomers from the β-isomers on the basis of the ^1H chemical shifts or the coupling constants alone. For example, there was no clear trend in the magnitude of the coupling constants to distinguish a *cis* arrangement from a *trans*

Table 1 Structures of estriol monoglucuronide derivatives together with H_{16}–H_{17} coupling constants



Compound	R^1	R^2	R^3	R^4	R^5	$J(H_{16}$–$H_{17})$/Hz
1	OH	H	OH	H	OG′	5.22[7]
2	OH	H	OH	H	OG	4.76[7]
3	$OCOCH_3$	H	OH	H	OG′	5.13[7]
4	$OCOCH_3$	H	OS	H	OG′	5.50[7]
5	OH	H	OG′	H	OH	5.13[a]
6	OH	H	OG	H	OH	5.49[7]
7	$OCOCH_3$	H	OG′	H	$OCOCH_3$	—[7]
8	OH	H	OG′	OH	H	4.39[a]
9	OH	H	OH	H	OH	6.0[b,5]
10	OH	H	H	H	OH	—[b]
11	OH	H	H	H	CH_2OH	—[b]





[a] Present work. [b] Commercially available compounds.

arrangement of the C-16 and C-17 hydrogen atoms. The *trans* arrangement ($R^2 = R^4 = H$) in compounds **1**–**4**, **6**, **7** and **9** gave a range of coupling constants from 4.76–6.0 Hz[7] whereas the *cis* arrangement ($R^2 = R^5 = H$) of compound **8** gave a similar value (4.39 Hz).

Fortunately, all of the estriol 17β-glucuronide derivatives show a cross correlation of the 17α-proton with the anomeric proton (1′) of the glucuronide ring in the 2D-NOESY spectra. No cross correlations were observed for any of the 16α-glucuronide derivatives between the 17α- or 16β-protons and the protons of the glucuronide ring. This difference serves to distinguish between the estriol 16- and 17-glucuronide derivatives. However, there still remains an ambiguity over the orientation (α or β) of the 17-glucuronide moiety since it is possible that the 17α-isomer, **8**, could also give the observed cross correlation in the 2D-NOESY spectrum. Although the stereochemistry of **1** is regarded as known, since the synthesis started from the 16α-silyl ether of estriol,[7] a crystal structure determination of compound **1** was carried out for confirmation of the absolute stereochemistry.

Crystal and molecular structure of 1

The molecular structure of **1** was determined by the X-ray technique and the numbering system used is shown in Fig. 1. The structure clearly confirms that the stereochemistry at C-17 is β as anticipated from the synthetic route adopted and as indicated by its 2D-NOESY spectrum. Ring A has the expected aromaticity with bond distances of 1.37(1)–1.38(1) Å, all the torsion angles (ranging from −4.9° to 4.2°) are close to the ideal value of zero and there are no significant deviations from the plane of 'best fit' through atoms C(1)–C(5) and C(10). The non-planar geometries associated with rings B, C and D are also as expected by comparison with other similar steroid molecules (**9**–**11**). The torsion angles calculated for these rings are all very close to, or lie within, the ranges reported for the two crystallographically independent molecules found in each of estriol (**9**),[11] estradiol 17β (**10**)[12] and compound (**11**).[13] The torsion angles and planes of 'best fit' data support the description of the chair arrangement for ring C and a geometry similar to that of the half-chair for ring B. The chair nature of the carbohydrate ring is also established by the crystal structure data. All the hydrogen substituents on this ring occupy axial sites so that those attached to adjacent carbon atoms adopt *trans* positions with respect to one another. The non-hydrogen ring substituents lie in the equatorial positions. The *trans* relationship of all adjacent protons observed in the latter ring is in accordance with the large coupling constants (> 6.0 Hz) obtained by ^1H NMR spectroscopy.[7,14]

Two intermolecular approaches are observed between O(1) and O(11) of a second molecule and between O(8) and the partially weighted water molecule O(14) at distances of 3.23 and 3.13 Å, respectively. These may be contrasted with other similar steroid molecules[11–13] which lack the bulky substituent at C(17) but where shorter H-bonding contacts, in the range 2.62–2.98 Å, are observed.

Of greatest interest are the arrangements adopted by the substituents on ring D. The carbohydrate moiety is attached in the β position so that O(3) is in the *trans* position relative to O(2). Steric pressures between H(17) and H(1′), which are *cis* to one another, are relieved by slight twists about the O(3)–C(17) and O(3)–C(1′) bonds so that a contact distance of 2.24 Å is achieved. This close approach is reflected in the cross-correlation of H(17) and H(1′) in the 2D-NOESY spectrum. Atoms H(16) and H(17) take up β and α positions respectively and give rise to a torsion angle of 137° for H(16)–C(16)–C(17)–H(17). The torsion angles for ring D show that it adopts an arrangement intermediate between the C(13) envelope and half chair conformations. The phase angle of pseudorotation (Δ) of +18.2° and maximum torsion angle (φ_m) of +47.7° which may be calculated from the torsion angles according to Altona *et al.*[15] for ring D, are both typical of other C-17 substituted steroids. This is also true for the ring C–D torsion angles about C(13)–C(14) of 60.1 and 47.1° respectively. These observations are in agreement with the suggestion that the nature of the

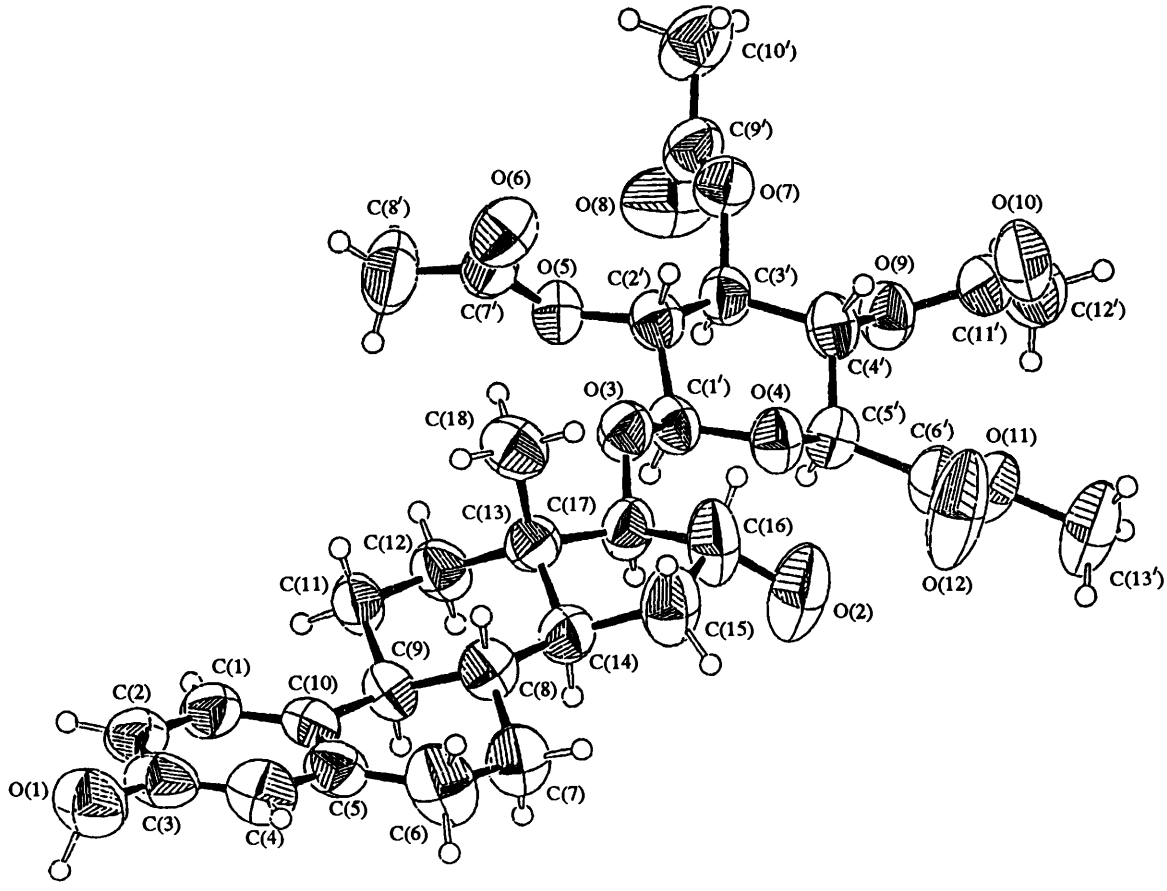


Fig. 1 Zortep[20] diagram of $C_{31}H_{40}O_{12}\cdot H_2O$ (**1**) showing the numbering system. Hydrogen atoms are labelled according to the atoms to which they are attached. Thermal ellipsoids have been drawn at the 50% probability level except for hydrogens where arbitrary levels have been used.

C(16) or C(17) substituent has no discernible influence on the actual geometry adopted for ring D despite the fact that the presence or absence of a substituent does.[15]

Glycosidic linkage-induced chemical shifts for C-16 and C-17 from ^{13}C NMR spectra

The ^{13}C NMR chemical shift values for compounds **1**–**9** are given in Table 2. The carbon atoms from the estriol skeleton (C-1, 2, 4, 16, 17 and 18) and carbon atoms from the carbohydrate moiety (C-1′, 2′, 3′, 4′ and 5′) were assigned unambiguously by a combination of ^1H–^1H 2D-COSY and ^{13}C–^1H HETCOR. The assignment of the remainder of the carbon atoms of estriol glucuronide derivatives was made by comparison of their ^{13}C NMR spectra with those of similar steroid derivatives reported in the literature.[8,16] The chemical shifts observed (Table 2) show good agreement with literature values.

The principal difference between estriol 16- and 17-glucuronide derivatives is reflected in the ^{13}C chemical shift values for the C-16 and C-17 atoms. With estriol as a reference compound (**9**), the introduction of a glycosidic linkage at either the C-17β-OH or the C-16α-OH position produced a downfield shift on the ring carbon atom. For example, after introduction of a 17β-glucuronic acid moiety, C-17 was deshielded for compounds **1**–**4** by about 8–9 ppm whereas all the C-16 atoms were shielded (Table 2). On the other hand for the 16α-glucuronide derivatives (**5** and **6**), the C-16 atoms were now deshielded by 8–9 ppm and the C-17 atoms were shielded. In all cases the upfield shift on the neighbouring carbon atom was between −1 and −4 ppm. The two exceptions to this pattern were compounds **4** and **7**, for which the downfield chemical shift at the point of attachment of the carbohydrate moiety was reduced by *ca.* 40%, although the upfield shift was the same as for the other compounds. In these two latter cases there were substituents on both the 16 and the 17 carbon atoms.

Thus, both C-16 and C-17 were significantly affected so that a glycosidic linkage to C-17β-OH caused the C-17 signals to move further downfield and those due to the neighbouring C-16 to move upfield. Since the C-17 resonance was further downfield than the C-16 resonance for estriol (compound **9** in Table 2), the overall substituent effect made a relatively large difference (over 20 ppm) to the chemical shifts between the C-16 and C-17 atoms for all of the estriol 17β-glucuronide derivatives. In contrast, however, the glycosidic linkage to the C-16α-OH group resulted in the opposite effect with a significant increase in the chemical shift for C-16, and a decrease for C-17, resulting in small chemical shift differences (less than 5 ppm) for all the estriol 16α-glucuronide derivatives. This major difference is thus important in distinguishing between the estriol 16- and 17-glucuronide derivatives. It was noted also that for all the *trans*-compounds (16β-H,17α-H) (**1**–**7**, **9**), the chemical shift values of C-17 were larger than for those of C-16, but that for the one 17α-compound (**8**) with a *cis*-arrangement of the hydrogens (16β-H,17β-H), this difference was reversed ($\delta_{C16} > \delta_{C17}$). Thus, the ^{13}C NMR chemical shift data unambiguously identifies both the point of attachment of the carbohydrate moiety and the absolute stereochemistry at C-17.

Despite the fact that the nature of the C(16) and C(17) substituents has little effect on the overall geometry of ring D, there are significant substituent-dependent differences in the ^{13}C NMR shift positions. For example, the C(17) ^{13}C NMR shift for estra-1,3,5(10)-triene-3,17β-diol (estradiol-17β) is 81.93 ppm[17] and the corresponding shift for the 3,17β-bis(tetrahydropyranyl) ether derivative of estradiol-17β is 84.24 ppm.[16] When compared with the larger C(17) ^{13}C NMR chemical shift differences for the bulkier substituents in compounds **1**–**4** and **9** in the present work (Table 2) it is clear that steric interactions play an important role in controlling the substituent effects in the ^{13}C NMR spectra. In steroids, the ring D substituents are

Table 2 ^{13}C NMR chemical shift values (ppm) of steroid compounds

	Compounds[a]								
C	1	2	3	4	5	6	7	8	9
1	126.7	127.9	126.2	121.6	127.9	127.9	126.3	126.5	127.9
2	112.7	114.5	118.6	—	114.5	114.5	118.6	112.7	114.5
3	153.6	156.7	—	—	156.8	156.7	148.4	153.5	156.8
4	115.3	116.8	121.6	118.7	116.8	116.8	121.5	115.2	116.9
5	138.1	139.5	148.5	138.2	—	139.5	137.9	138.1	139.6
6	47.54	—	47.63	47.52	—	—	47.86	—	—
7	44.03	46.29	44.03	—	46.00	46.06	44.49	46.19	46.14
8	43.77	45.94	44.03	43.63	45.65	45.60	43.63	43.37	45.83
9	37.98	40.59	37.64	37.78	40.64	40.70	37.69	38.62	40.82
10	132.1	133.2	138.2	121.6	133.1	133.2	137.5	132.6	133.3
11	36.77	39.03	36.77	34.35	39.66	38.80	38.89	31.88	38.83
12	32.22	34.54	32.31	—	38.68	33.30	31.39	31.01	35.72
13	29.49	31.10	29.40	26.90	31.40	31.43	29.31	29.63	31.46
14	27.01	29.24	26.90	25.89	29.38	29.35	26.89	27.99	29.35
15	25.74	27.89	25.57	25.72	27.94	27.97	25.60	25.66	28.00
16	76.60	78.60	75.99	76.30	88.68	88.39	84.43	83.50	79.55
17	100.2	99.82	100.3	96.67	89.14	89.48	87.22	78.67	91.41
18	12.64	13.95	12.64	12.61	13.66	13.72	12.99	17.16	13.66
	101.7	105.6	101.8	100.7	101.8	104.7	100.5	100.7	—
	71.15	75.69	71.19	72.42	73.67	75.63	71.27	71.10	—
	71.96	78.40	71.96	71.73	77.27	78.42	72.02	72.00	—
	68.74	73.79	68.77	69.70	71.69	74.02	69.40	69.29	—
	71.27	76.32	71.33	73.00	74.25	76.41	72.85	73.54	—
	170.2	173.6	170.5	—	172.1	172.2	170.2	170.2	—
OAc	20.62	—	20.62	—	21.44	—	20.85	20.62	—
	20.62		20.62		21.29		20.62	20.62	
	20.60		20.11		21.23		20.44	20.50	
C=O	169.5	—	169.4	—	—	—	170.1	169.4	—
	169.1						169.8	169.3	
	167.0						3 × 169	167.0	
OCH_3	53.21	—	53.19	52.70	—	—	52.84	53.13	—

never truly axial or equatorial but for all the estriol 16- and 17-glucuronide derivatives in the present work, the large carbohydrate moiety is likely to take up a more equatorial position than does a normal OH group. The large carbohydrate group also forces the neighbouring OH group to a more axial position than a normal OH group. Consequently the effect of the bulkier substituents on the ring geometry gives rise to the differing shielding or deshielding effects in ^{13}C chemical shifts of C-16 and C-17.[17] Because of the flexibility of ring D the precise details of the steric effects leading to the individual shifts are unclear and await further three dimensional structure determinations.

Experimental

Nuclear magnetic resonance investigation

^1H and ^{13}C NMR spectra were recorded at 270 and 67.8 MHz, respectively, on a JEOL GX 270 spectrometer. Chemical shifts were measured using $CDCl_3$ or CD_3OD as solvent and tetramethylsilane as internal standard. Values are given in ppm (δ) (s, singlet; d, doublet; t, triplet; m, multiplet). J values are given in Hz.

Synthesis of estriol derivatives 5 and 8

Estriol (**9**) was purchased from Sigma Chemical Co. and used without purification. The syntheses of the estriol 16- and 17-glucuronide derivatives (**1–4**, **6**,**7**) have been described in an earlier paper.[7] Compounds **5** and **8** were prepared from the reduction of methyl 1-*O*-[2,4-dibromo-3-hydroxy-17-oxo-estra-1,3,5(10)-trien-16α-yl]-2,3,4-tri-*O*-acetyl-β-D-glucopyranosuronate (175 mg, 0.23 mmol) with $NaBH_4$ (50 mg, 1.35 mmol) in the presence of $PdCl_2$ (100 mg, 0.376 mmol) as described in reference 5. After reaction, TLC (CH_2Cl_2 : EtOAc, 2 : 1) showed no starting material (R_F = 0.86) and two products **5** (R_F = 0.54) and **8** (R_F = 0.68). Separation of the reaction mixture by flash chromatography, using CH_2Cl_2 : EtOAc (2 : 1) as solvent gave pure compound **5** (96 mg, 70%) and pure compound **8** (40 mg, 30%). For **5** (white solid); mp 229–231 °C (from methanol) (lit.,[5] 229–231 °C); high EIMS m/z, 604.2529 (M^+) (Calc. for $C_{31}H_{40}O_{12}$, 604.2520); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3423 (OH), 1745 (CO); δ_H(270 MHz; $CDCl_3$–CD_3OD, 20 : 1) 0.80 (3 H, s, 18-Me), 2.04 (3 H, s, OCOMe), 2.05 (3 H, s, OCOMe), 2.06 (3 H, s, OCOMe), 3.67 (1 H, d, J 5.13, 17α-H), 3.78 (3 H, s, COOMe), 3.93–3.98 (1 H, m, 16β-H), 4.12 (1 H, d, J 9.5, 5′-H), 4.61 (1 H, d, J 7.69, 1′-H), 5.01 (1 H, t, J 8.06 and 9.15, 2′-H), 5.17–5.32 (2 H, m, 3′ and 4′-H), 6.55–7.13 (3 H, m, 1, 2 and 4-H). For **8** (white solid); mp 216–219 °C; high EIMS m/z, 604.2563 (M^+) (Calc. for $C_{31}H_{40}O_{12}$, 604.2520); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3423 (OH), 1721 and 1755 (CO); δ_H(270 MHz; $CDCl_3$) 0.72 (3 H, s, 18-Me), 2.04 (6 H, s, 2OCOMe), 2.06 (3 H, s, OCOMe), 3.73 (1 H, d, J 4.39, 17β-H), 3.77 (3 H, s, COOMe), 4.07 (1 H, d, J 4.39, 5′-H), 4.38 (1 H, m, 16β-H), 4.46 (1 H, d, J 8.06, 1′-H), 5.05 (1 H, t, J 8.43 and 8.42, 2′-H), 5.18–5.33 (2 H, m, 3′ and 4′-H), 6.55–7.17 (3 H, m, 1, 2 and 4-H).

Crystal structure determination of 1

Colourless crystals of **1** were obtained by the slow evaporation of methanol.

Crystal data

$C_{31}H_{40}O_{12}H_2O$, M = 623.68, orthorhombic, space group $P2_12_12_1$, a = 13.712(2), b = 13.961(2), c = 17.019(2) Å, U = 3257.8(7) Å (from the least-squares setting angles of 23 reflections; 16.97 < θ < 29.31°), Cu-Kα-radiation, λ = 1.5418 Å, Z = 4, D_c = 1.233 g cm^{-3}, F(000) = 1288, μ(Cu-Kα) = 7.6 cm^{-1}, colourless crystals; size 0.36 × 0.23 × 0.21 mm.

Data collection and processing

Enraf-Nonius CAD4 diffractometer at 293 K, ω–2θ scan mode, ω scan angle (1.30 + 0.14 tan θ)°, variable scan speed (2.06–8.24° min^{-1}), maximum count time 90 s, 3859 reflections measured (1.0 < θ < 22.0°, ±h, ±k, +l; 22.0 < θ < 52°, +h,

$+k, +l$; $52 < \theta < 65$, $-h, -k, -l$). The intensities of three standard reflections were monitored every two hours of X-ray exposure time and showed a maximum loss in intensity of 3.7%. Of the reflections measured 2774 were unique [merging R_{int} = 0.025] after data were corrected for Lorentz and polarisation effects, crystal decay and absorption effects (psi-scans;[10] maximum and minimum transmission factors 0.9998 and 0.9187, respectively).

Structure analysis and refinement

Structure solution was by direct methods.[19] Full-matrix least-squares refinement with anisotropic thermal motion was assumed for all non-hydrogen atoms except those of the water molecule. Hydrogen atoms were in calculated positions (C–H 0.96 Å). At convergence R and R_w were 0.066 and 0.078, respectively, for the 396 parameters refined using the 1989 data for which $F^2 > 2\sigma(F^2)$. The function minimised was $\Sigma w(|F_o| - |F_c|)^2$ with $w^{-1} = [\sigma^2(F_o) + 0.004\,071F_o^2]$. Computations were performed with the MolEN package,[10] SHELXS-86[18] and SHELX-76[19] programs.

Additional material, available from the Cambridge Crystallographic Data Centre (CCDC), comprises non-hydrogen atom coordinates, thermal parameters, H-atom coordinates, a full listing of bond distances and angles, selected torsion angles and deviations of atoms from planes of 'best fit' for compound **1**. For details of the deposition scheme, see 'Instructions for Authors', *J. Chem. Soc., Perkin Trans. 2*, 1996, Issue 1. Any request to the CCPC for this material should quote the full literature citation and reference number 188/6.

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