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GHALAKINOSIDE, A CYTOTOXIC CARDIAC GLYCOSIDE FROM *PERGULARIA TOMENTOSA*

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Key Word Index—*Pergularia tomentosa*; Asclepiadaceae; calactin; ghalakinoside; cardiac glycoside.

Abstract—The isolation and structural elucidation using spectroscopic methods and X-ray analysis of ghalakinoside, a novel cytotoxic cardiac glycoside, containing a doubly linked sugar substituent are reported. Calactin, another related glycoside was also isolated and its structure similarly established.

INTRODUCTION

Pergularia tomentosa L., known locally as Ghalaka, is one of the wild plants belonging to the milkweed family (Asclepiadaceae). The plant is widely distributed in different zones in the Kingdom of Saudi Arabia [1]. Our previous work was concerned mainly with the preliminary phytochemical and biological screening of the plant [2], as well as the pharmacognostical study of the fruit [3].

In this article we report the isolation and characterization of a new cardiac glycoside, named ghalakinoside. This compound revealed a strong inhibitory activity *in vitro* against cells derived from human carcinoma of the nasopharynx (9 KB).

Calactin, another related glycoside previously reported in other Asclepiadaceae species [4–7] was also isolated for the first time from the title plant.

RESULTS AND DISCUSSION

Column chromatographic fractionation of the methanol soluble extractives of the defatted root materials afforded calctin (**1**) and ghalakinoside (**2**) in a yield of 0.07 and 1.0% w/w of the air-dried roots, respectively. The identity of **1** was based on the interpretation of its spectral data, ^{1}H NMR, ^{13}C NMR, EIMS, DCIMS, UV, IR (See Experimental and Tables 1 and 2) and by comparison with data reported in the literature on calactin diacetate and related glycosides [6, 8–10].

TLC of compound **2** yielded a purple colour when sprayed with Kedde reagent [11], indicating that it is a cardiac glycoside. The molecular ion $[M+1]^+$ at *m/z* 567 (DCIMS) and elemental analysis were in accordance with $C_{29}H_{42}O_{11}$. Examination of the ^{1}H NMR spectrum of **2** (Table 1) confirmed its steroidal nature and the presence of the characteristic α,β-unsaturated lactone of a cardenolide. The latter group was represented by a broad singlet at δ 5.85 (1H, 22-H) and an AB quartet further split by the 22-H at δ 4.84 (2H, 21-H_2). The steroidal ring system was suggested by the broad series of resonances between δ 0.7 and 2.3; a *dd* corresponding to H-17 was centered δ 2.3. Only one of the two commonly present methyl groups was observed as a singlet at δ 0.73 (3H). This resonance was assigned to the C-18 protons [12, 13], being shifted upfield relative to their position in most glycosides (δ 0.8–0.92). This indicates substitution in rings C and/or D of the steroid nucleus of **2**. A cardenolide with a 12β-hydroxy group C-18H had absorption at about the same position as for **2** [14]. An AB quartet ($J = 11$ Hz) at δ 3.5 and 3.88 indicates the presence of a-CH_2OH group at C-10 this being a typical absorption of other cardenolides with this substitution [12, 15–17].

This assignment was further confirmed by comparison of the ^{13}C NMR data of **2** (Table 2) with that of calactin and some related cardenolides. The data in Table 2 confirms the basic steroidal skeleton of the molecule. The resonance of C-5 at δ 45.14 proved this glycoside to be 5α-cardenolide [17,18]. Introduction of the 12 β-hydroxy group in **2** causes a long downfield shift of C-12 to δ 73.53. C-11 and C-13, both in the β-position from C-12, also move downfield by 9.69 and 8.07 ppm, respectively, relative to calactin. C-9, C-17 move upfield by 5.40 and 3.06 ppm, respectively, according to the 1,3-diaxial interaction with methine protons at C-9 and C-17, respectively [18]. The C-18 methyl protons are similarly shielded upfield by 6.56 ppm relative to calactin. A cardenolide with 12 β-hydroxy group had 18-H_3 absorption at about the same position [9]. On the other hand, in place of a resonance for the 19-H_3 at *ca* δ 12, there was a resonance at δ 58.19 which confirmed the presence of a hydroxyl group at position C-19.

The placement of **2** as closely related to calactin and other calotropin glycosides came from its resistance to acid hydrolysis; a character of certain cardiac glycosides isolated from *Asclepias*, *Calotropis* and *Pergularia* species [6, 10, 19–28]. The unique carbohydrate 4,6-dideoxy hexosulose and modified forms of it, was identified as the sugar of a number of these glycosides. The unusual stability to acids of the carbohydrate group in these compounds is due to its double attachment through acetal and hemiacetal links to positions 3β and 2α, respectively, of the cardenolide aglycone [29, 30].

The ^{1}H and ^{13}C NMR measurements have defined the

Table 1. ^{1}H NMR of Calactin (**1**) and ghalakinoside (**2**) in DMSO-d_6

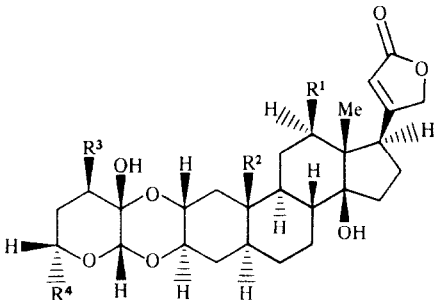
Assignment (number of H)	C-2 (1)	C-3 (1)	C-17 (1)	C-18 (3)	C-19	
					CHO	CH_2OH
Calactin (**1**)	3.70 (*m*)	4.15 (*m*)	2.73 (*dd*) (J = 10, 5 Hz)	0.72 (*s*)	9.97 (*s*)	
Ghalakinoside (**2**)	3.85 (*m*)	4.03 (*m*)	2.3 (*dd*) (J = 10, 5 Hz)	0.73 (*s*)		3.5, 3.88 (AB *quartet* J = 11 Hz)

stereochemistry of the carbohydrate of ghalakinoside (**2**) (Table 2). Instead of the signal at δ 1.15 (*d*, 3H) in ^{1}H NMR spectrum of calactin (**1**), a signal centred at δ 3.4 (*m*, 2H) corresponding to a CH_2OH group at C-5 was observed. This was confirmed by the signal in the ^{13}CNMR spectrum at δ 63.81 instead of 20.97 for the Me group in calactin (**1**) (Table 2). The sugar in **2** was thus identified as 4-deoxy hexosulose. This is the first report of its occurrence in Asclepiadaceae glycosides.

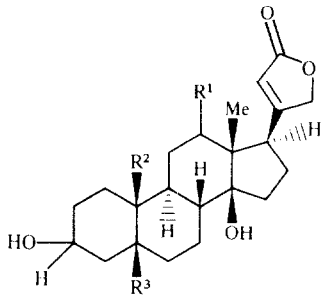
The mass spectrum of **2** (see Experimental) gave further confirmatory informations. The DCIMS of **2** exhibited an $[M+1]^+$ ion at *m/z* 567 corresponding to a molecular formula of $C_{29}H_{42}O_{11}$. Cleavage of the glycosidic linkage with concomitant transfer of hydrogens to C-2 and C-3 oxygens [31,32] resulted in ions for the aglycone + 1 at *m/z* 423 (100%) which corresponds to the genin $C_{23}H_{34}O_7$ (G). The major route of fragmentation of the aglycone involved four successive losses of 18 mass units to give ions *at m/z* 405, 387, 369 and 351. A loss of the CH_2O fragment from G-3 H_2O was shown by the ion at *m/z* 339. The latter loss, in conjunction with the lack of a methyl signal for C-19 in the ^{1}H NMR spectrum, suggested that C-19 was present as a hydroxymethylene group.

The EI mass spectrum, however, was characterized by the high relative intensity of a number of low mass ions which originate from the carbohydrate. Peaks at *m/z* 128 and 113 provided strong evidence on the presence of 4,6-dideoxyhexosulose moiety which is limited to calotropin sugars and sugars of certain Asclepiadaceae glycosides [6].

In the EI mass spectrum of ghalakinoside **2**, the base peak appears at *m/z* 144 ($C_6H_8O_4$) followed by an ion at *m/z* 113 corresponding to a loss of the CH_2OH group (31 mass units) which confirms the sugar to be 4-deoxyhexosulose in ghalakinoside (**2**) instead of 4,6-dideoxyhexosulose in calactin and in the reported Asclepiadaceae glycosides of the same nature [6, 7, 8, 10, 19-30].



	R^1	R^2	R^3	R^4
1	H	CHO	α-H, β-OH	Me
2	OH	CH_2OH	α-OH, β-H	CH_2OH
3	H	Me	α-H, β-OH	Me



	R^1	R^2	R^3
4	H	CH_2OH	α-H
5	OH	Me	β-H

The complete structures and stereochemistries of **1** and **2** were established unequivocally from single-crystal X-ray analyses of **1** · 1/2 CH_2Cl_2 and **2** · 5 H_2O, respectively. The crystal structures were solved by direct methods.* Full-matrix least-squares refinement of atomic positional and thermal parameters converged at R = 0.56 (2327 reflections) for **3** · 1/2 CH_2Cl_2 and R = 0.078 (2735 reflections) for 2 · 5H_2O. Views of the conformations of **1** and **2** in these crystals are presented in Figs 1 and 2, respectively.

On the other hand, the 9 KB in vitro cytotoxicity† of

*Crystallographic calculations were performed on PDP11/44 and MicroVAX II computers by use of the Enraf–Nonius Structure Determination Package incorporating the direct methods programme MULTAN11/82.

$$R = \Sigma||F_o| - |F_c||/\Sigma F_o|;\ R_w = [\Sigma w(F_o| - |F_c|)^2/\Sigma w|F_o|^2]^{1/2}$$

†Cytotoxicity (9 KB) was measured in the Cell Culture Laboratory, Cancer Research Center, Purdue University, West Lafayette, Indiana 47907, U.S.A.

C-21 (2)	C-22 (1)	C-1′ (1)	C-3′ (1)	C-4′ (2)	C-5′ (1)	C-6′	
						CH_3	CH_2OH
4.9 (*q*)	5.9 (*s*)	4.68 (*d*) ($J=4$Hz)	3.8 (*m*)	~2 (*m*)	3.95 (*m*)	1.15 (*d*) ($J=6$Hz)	
4.84 (*q*)	5.85 (*s*)	4.35 (*s*)	3.18 (*m*)	~2 (*m*)	3.48 (*m*)		3.4 (*m*)

Table 2. ^{13}C NMR chemical shifts of **1** and **2** and some related compounds

C	Calactin (**1**)	Ghalakinoside (**2**)	Di-*O*-acetyl Calactin [10]	Gomphoside **3** [28]	Coroglaucigenin (**4**) [18]	Digoxigenin (**5**) [9]
1	35.37[a]	36.08	35.7	41.9	33.0	30.0
2	68.40	67.85	70.8	68.1	32.7	27.9
3	69.74	72.22[a]	71.2	71.8	70.7	66.6
4	31.49	32.78[b]	32.4	32.0	39.8	33.3
5	41.76	45.14	43.6	44.3	45.2	36.4 (β)
6	27.17[b]	27.14[c]	27.7	27.6	28.8	26.9
7	27.4[b]	26.64[c]	27.4	27.1	28.1	21.9
8	42.43	40.15[d]	42.6	40.0	42.4	41.3
9	49.10[c]	44.55	48.6	49.0	50.8	32.6
10	52.29	40.55[d]	52.8	37.3	40.6	35.5
11	21.34	31.03	22.0	20.9	23.4	30.0
12	40.28	73.53	39.5	38.8	39.8	74.8
13	49.95[c]	55.49	49.4	49.3	50.2	56.4
14	83.31	84.25	85.0	83.5	84.8	85.8
15	32.99	32.29[b]	33.1	32.0	33.0	33.0
16	26.23	26.58[c]	26.9	26.4	27.3	27.9
17	47.42[c]	46.04	50.7	50.1	51.6	46.1
18	15.45	8.89	15.6	15.6	16.3	9.40
19	208.73	58.19	206.4	13.5	59.2	23.8
20	176.04[d]	176.52[d]	174.2	176.1	176.2	177.1
21	73.12	73.22	73.5	73.1	73.4	74.6
22	116.34	115.79	118.0	116.2	117.6	117.0
23	173.76[d]	174.12[d]	173.9	173.7	174.6	176.3
1′	93.8	95.72	93.2	93.9		
2′	90.24	91.22	95.6	90.1		
3′	71.11	71.83	70.5	69.8		
4′	37.36[a]	32.01[b]	35.0	39.7		
5′	65.2	72.40[a]	66.6	65.1		
6′	20.97	63.81	20.8	20.9		

[a–d]Values in any vertical column may be reversed.
Spectra of **1** and **2** were determined in DMSO-d_6.
The multiplicity determination using APT experiment [34].

ghalakinoside (**2**) showed strong activity (ED_{50}, 2.9×10^{-2}). This appeared to be the responsible factor for the previously mentioned inhibitory activity against KB cells of the $CHCl_3$ extract of the aerial parts of the plant [33]; ghalakinoside was also traced but in minute quantities in these organs. It is worthwhile to mention that some of the Asclepiadaceae glycosides [21, 23] including calotropin (the 3′epimer of calactin) were reported to be cytotoxic.

EXPERIMENTAL

The plant material was collected from the surroundings of Riyadh in the Central Zone of Saudi Arabia in November 1985. Identity was confirmed through the Taxonomy Section of the

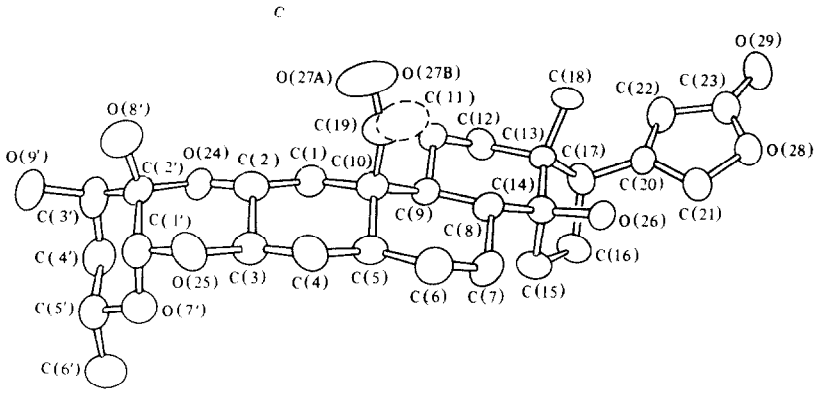


Fig. 1. Crystallographic numbering scheme and solid-state conformation of **1**; hydrogen atoms have been omitted for clarity. The aldehyde oxygen atom O(27) is disordered over two orientations [O(27A) and O(27B)].

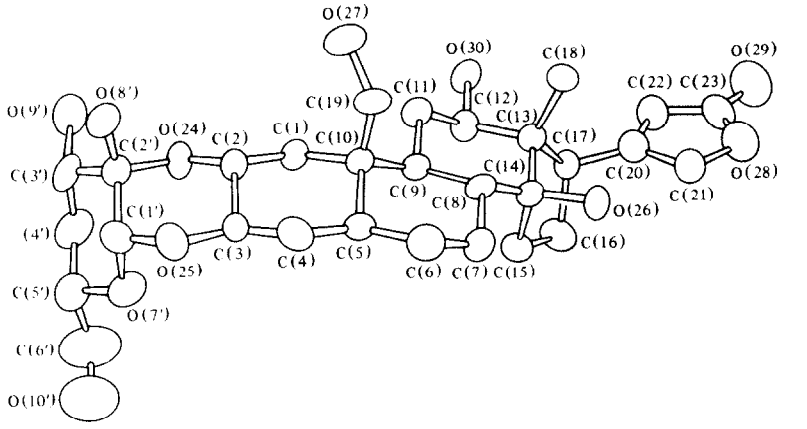


Fig. 2. Crystallographic numbering scheme and solid-state conformation of **2**; hydrogen atoms have been omitted for clarity.

Medicinal, Aromatic and Poisonous Plants Research Center College of Pharmacy, King Saud University. A voucher specimen was deposited at the herbarium of the pharmacognosy department, College of Pharmacy, King Saud University. Mps: uncorr.; IR, KBr; UV, MeOH; ^{1}H NMR, 90 MHz, DMSO-d_6, TMS as int. standard; ^{13}C NMR, 15.03 MHz, DMSO-d_6, TMS as int. standard. The carbons constituting the glycosides **1** and **2** were assigned according to published values for di-*O*-acetyl calactin [10], gomphoside [28], coroglaucigenin [18] and digoxigenin [9] with the use of APT experiments [34]; MS, EI and DCI.

Extraction and fractionation The air-dried powdered root (1 kg) was extracted sequentially with petrol (bp 60–80°) and MeOH (Soxhlet). After removal of the solvent *in vacuo*, the MeOH soluble residue (89.1 g) was found rich in Kedde positive compounds. Column chromatography of a portion (20 g) of the methanol-soluble residue over silica gel 60 (500 g; Merck, Darmstadt, F.R.G.) was undertaken using $CHCl_3$ and $CHCl_3$–MeOH mixtures of incresing polarity, 35 fractions (500 ml each) were collected. Elution by $CHCl_3$–MeOH (95:5) afforded a fraction containing calactin (**1**) from which this glycoside was crystallized (130 mg) using MeOH. Further elution of the chromatographic column by $CHCl_3$–MeOH (3:1) led to the isolation of ghalakinoside (**2**) which was crystallized from MeOH (2.245 g).

Calactin (**1**). White crystalline prisms and needles, 0.07% w/w of the dried roots, resistant to acid hydrolysis mp 262–267° (Lit [10]) $[\alpha]_D^{25}$ +57.3°, IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1820, 1780, 1755, 1735 (butenolide ring); 1720 (CHO) 1620, 1480, 1460, 1450, 1385, 1370, 1310, 1165, 1070, 1055 (*br* several) [4, 6]. UV λ_{max}^{MeOH} nm: 217, 310; ^{1}H NMR and ^{13}C NMR, see Tables 1 and 2; EIMS, *m/z* (rel. int.): 128 (100) ($C_6H_8O_3$ of 4,6-dideoxyhexosulose), 113 (75.8), 87 (33.3), 69 (33.3), 58 (93.9) [6]. *m/z* DCIMS *m/z* (rel. int.): 533 (34.8) $[M+1]^+$ ($C_{29}H_{40}O_9$), 51.5 (4.3) $[M+1-H_2O]^+$, 487 (7) $[M+1-H_2O-CO]^+$, 433 (62), 405 (50) [Genin + 1]$^+$ ($C_{23}H_{32}O_6$), 387 (28.3) $[G+1-H_2O]^+$, 369 (13.1) $[G+1-2H_2O]^+$, 341 (2.2) $[G+1-2H_2O-CO]^+$, 323 (3.2) $[G+1-3H_2O-CO]^+$, 257 (100).

Ghalakinoside (**2**) White radiating rods. 1% w/w of the air-dried roots, resistant to acid hydrolysis, mp 215–220° (from MeOH), $[\alpha]_D^{25}$ +60°, IR ν_{max}^{KBr} cm^{-1}: 3450 broad (OH), 1755, 1740, 1735 (butenolide ring), 1620, 1585, 1490, 1455, 1385, 1170, 1110, 1080, 1050, 1020, 980, UV λ_{max}^{MeOH} nm: 217; ^{1}H and ^{13}C NMR, see Tables 1 and 2; EIMS, *m/z* (rel. int.): 144 (100) for $C_6H_8O_4$ of 4-deoxyhexosulose, 126 (18.5) $[C_6H_8O_4-H_2O]^+$, 113 (28.6) $[C_6H_8O_4-CH_2OH]^+$, 97 (85.7), 87 (68.6), 85 (28.6), 80 (20), 71 (28.6), 69 (45.7), 59 (57), 57 (85.7), 55 (48.6); DCIMS *m/z* (rel. int.): 567 (21.7) $[M+1]^+$ ($C_{29}H_{42}O_{11}$), 549 (2.2) $[M+1-H_2O]^+$, 531 (1.1) $[M+1-134]^+$, 424 $[M+1-143]^+$, 423 (100) [Genin

$+1]^+$ ($C_{23}H_{34}O_7$), 405 (10.9) $[G+1-H_2O]^+$, 387 (43.5) $[G+1-2H_2O]^+$, 369 (10.9) $[G+1-3H_2O]^+$, 351 (3.7) $[G+1-4H_2O]^+$ 339 (3.3) $[G+1-3H_2O-CH_2O]^+$.

X-ray crystal structure analysis of calactin dichloromethane solvate, $1\cdot\frac{1}{2}$ CH_2Cl_2 *and ghalakinoside pentahydrate*, $2\cdot5H_2O$. Crystal data: $1\cdot\frac{1}{2}$ CH_2Cl_2, $C_{29}H_{40}O_9\cdot\frac{1}{2}$ CH_2Cl_2, $M=575.11$, orthorhombic, $a=12.737$ (5) Å, $b=14.737$ (6) Å, $c=29.395$ (10) Å, $V=5517.6$ Å^3, $Z=8$, $D_{calc}=1.385$ g cm^{-3}, μ(CuKα radiation' $=16.8$ cm^{-1}. Space group $C222_1(D_2^5)$ uniquely from the systematic absences: hkl when $h+k\neq2n$, $00l$ when $l\neq2n$. Crystal dimensions: $0.20\times0.20\times0.60$ mm. $2\cdot5H_2O$, $C_{29}H_{42}O_{11}\cdot5H_2O$, $M=656.73$, orthorhombic, $a=18.032$ (5) Å, $b=18.477$ (3) Å, $c=10.088$ (3) Å, $V=3361.1$ Å^3, $Z=4$, $D_{calc}=1.298$ g cm^{-3}, (CuKα radiation, $\lambda=1.5418$ Å) $=8.5$ cm^{-1}. Space group $P2_12_12_1$ (D_2^4) uniquely from the systematic absences: $h00$ when $h\neq2n$, $0k0$ when $k\neq2n$, $00l$ when $l\neq2n$. Crystal dimensions: $0.20\times0.25\times0.35$ mm.

Preliminary unit-cell parameters and space group information were obtained from oscillation and Weissenberg photographs. One octant of intensity data to $\theta=67°$ from each crystal recorded on an Enraf-Nonius CAD-4 diffractometer (CuKα radiation, incident-beam graphite monochromator; ω-2θ scans). From totals of 2753 and 3186 measurements for $1\cdot\frac{1}{2}$ CH_2Cl_2 and $2\cdot5H_2O$ respectively, those 2327 and 2735 reflections with $I>3.0\sigma(I)$ were retained for the structure analyses and the usual Lorentz and polarization corrections were applied.

Both crystal structures were solved by direct methods. Approximate coordinates for the majority of carbon and oxygen atoms were derived in each case from an E-map. Positions for the remaining non-hydrogen atoms of **1** and **2**, as well as for the carbon and chlorine atoms in the CH_2Cl_2 molecule in $1\cdot\frac{1}{2}$ CH_2Cl_2 and three water oxygen atoms in $2\cdot5H_2O$ were obtained from a series of structure-factor calculations and difference Fourier synthesis. Several rounds of full-matrix least-squares adjustment of non-hydrogen atom positional and antisotropic temperature factor parameters were followed by evaluation of difference Fourier synthesis. In the case of $1\cdot\frac{1}{2}$ CH_2Cl_2, calculated hydrogen atom positons, save those on the disordered aldehyde moiety, were all found to coincide with significantly positive regions. With the inclusion of hydrogen atoms at their calculated positions, continuation of the least-squares iterations led to convergence of $R=0.056$ ($R_w=0.080$). The corresponding difference Fourier synthesis for $2\cdot5H_2O$ revealed the presence of five maxima ($2.0-0.7$ eÅ^{-3}) located in a region surrounded by hydroxy and water oxygen atoms. These peaks were ascribed to two additional water molecules which were disordered over these sites with occupation factors ranging from 0.7 to 0.25. Following the inclusion of these additional water oxygen atoms into the structure-factor calculations, a further difference Fourier synthesis was evaluated and found to contain positive regions at positions calculated for all C-H hydrogen atoms. Not surprisingly, however, in view of the disordered arrangement of two of the water molecules, the hydroxy and ordered water hydrogen atoms were ill-defined but their probable positions could be derived on the basis of a hydrogen-bonding scheme. Inclusion of the latter hydrogen atoms as well as the C-H hydrogen atoms at their calculated positions in the subsequent least-squares iterations led to convergence at $R=0.078$ ($R_w=0.112$). Views of the structures of **1** and **2** are provided in Figs 1 and 2, respectively. Final atomic positional and thermal parameters, bond lengths, bond angles, torsion angles and lists of observed and calculated structure amplitudes for $1\cdot\frac{1}{2}$ CH_2Cl_2 and $2\cdot5H_2O$ have been deposited with the Cambridge Crystallographic Data Centre.

For the structure-factor calculations, neutral atom scattering factors and their anomalous dispersion corrections were taken from ref. [35]. In the least-squares iterations, $\sum w\Delta^2$ [$w=1/\sigma^2(|F_o|)$, $\Delta=(|F_o|-|F_c|)$] was minimized.

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