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Crystal structures of ecdysteroids: the role of solvent molecules in hydrogen bonding and isostructurality

László Fábián,[a] Gyula Argay,[a] Alajos Kálmán[a]* and Mária Báthori[b]

[a]Institute of Chemistry, Chemical Research Center, Hungarian Academy of Sciences, Budapest 114, PO Box 17, H-1525, Hungary, and [b]Department of Pharmacognosy, University of Szeged, Szeged, Hungary

Correspondence e-mail: akalman@chemres.hu

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Dedicated to Dr K. Sasvári on the occasion of his 90th birthday.

Three crystal forms of the steroid 20-hydroxyecdysone were identified by single-crystal X-ray diffraction analysis: a solvent-free modification, a methanol solvate hydrate and a trihydrate. The structure of a closely related steroid, polypodine B (the 5,20-dihydroxy derivative of ecdysone), was determined in its monohydrate form. Since the unit-cell volume of unsolvated 20-hydroxyecdysone was found to be considerably smaller than that of ecdysone [Huber & Hoppe (1965). *Chem. Ber.* **98**, 2403–2424], a new structure determination of ecdysone was performed, which confirmed the unexpected difference between the unit-cell volumes. The crystals of ecdysone and 20-hydroxyecdysone are isostructural, while the mixed solvate of 20-hydroxyecdysone is homostructural with the hydrate of polypodine B. A detailed analysis of the hydrogen-bond networks in these closely related crystal pairs highlights their packing similarities, demonstrates the role of solvent molecules, and explains the unexpectedly small cell volume of 20-hydroxyecdysone.

1. Introduction

The ecdysteroids, which include insect molting hormones and are also widespread in plants, affect various biological functions. Their effects on mammals include the increasing of protein synthesis, the decreasing of cholesterol levels, the normalization of hyperglycemia and the stimulation of the immune system. 20-Hydroxyecdysone (also termed as polypodine A) is a major insect molting hormone, but its relatively high abundance in some plants makes it advantageous for studies in vertebrates too.

The separation of 20-hydroxyecdysone from other steroids (*e.g.* ecdysones or triterpenes) requires various efficacious chromatographic techniques carefully controlled by spectroscopic measurements (Báthori, 1998). Crystallization was earlier applied to improve its purity (Báthori *et al.*, 2000) and, depending on the conditions applied, three colorless crystalline materials with different melting points were obtained. Their crystal structures determined by single-crystal X-ray diffraction analysis revealed 2β,3β,14α,20*R*,22*R*,25-hexahydroxy-5β-cholest-7-en-6-one (2), a mixed methanol solvate monohydrate [(2)·MeOH·H_2O] and a trihydrate [(2)·3H_2O]. The solvent-free structure proved to be identical to that reported by Dammeier & Hoppe (1971), but the new data collection permitted a more elaborate treatment of the alkyl-chain disorder. The similarity of the crystal structure of (2) to that of ecdysone [2β,3β,14α,22*R*,25-pentahydroxy-5β-cholest-7-en-6-one, (1)], published by Huber & Hoppe (1965), led us to check whether the two crystals were isostructural. In fact, they were found to exhibit a moderate degree of isostructurality. However, since the unit cell of (2) with the additional 20-

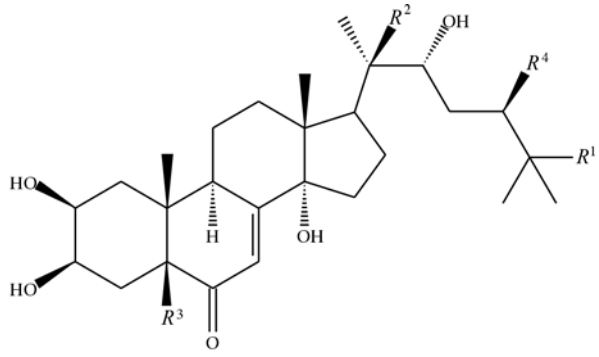
OH moiety was found to be *ca* 50 Å^3 *smaller* than that reported for (1), it was necessary to check the unit-cell parameters. The agreement between the calculated and observed densities (1.126 Mg m^{-3} and 1.127 Mg m^{-1}, respectively) of (1) (Huber & Hoppe, 1965) seemed to exclude substantial error in the measurement of the 'large' unit cell (V = 2725.3 Å^3). To shed light on this puzzle, two sets of new X-ray data were collected simultaneously from ecdysone crystals (a few mg of which were kindly provided by Professor R. Lafont, Ecole Normale Superieure, Paris, France), one by Cu $K\alpha$ and the other by Mo $K\alpha$ radiation. The new unit-cell volumes are 2745.5 (5) and 2740.8 (7) Å^3, the mean of which is even larger than that reported by Huber & Hoppe (1965). Again, while in agreement with the published structure, the new data also revealed conformational disorder of the alkyl chains.

A crystalline hydrate of ecdysone was identified with a unit-cell volume of 2807.2 Å^3. From density measurements, its unit cell was estimated to contain four steroid and two water molecules. The *ca* 80 Å^3 increase as compared with solvent-free ecdysone is accounted for by the presence of solvent molecules and does not suggest an exceptionally loose or unfavorable packing in ecdysone. Unfortunately, the structure of the hydrate has not been determined and the small amount of available material did not allow us to grow single crystals of this form.

In parallel with the above measurements, crystallization experiments with 2β,3β,5β,14α,20R,22R-25-heptahydroxy-5β-cholest-7-en-6-one (polypodine B) were performed. While 20-hydroxyecdysone contains an additional 20-OH group as compared with ecdysone, polypodine B contains a further 5-OH group. A single crystal of polypodine B obtained from aqueous methanol solution was subjected to X-ray diffraction. The crystal was found to be a monohydrate [(3)·H_2O] with a unit-cell volume of 2749.4 (5) Å^3, which, despite the two additional (5β,20R) hydroxy groups and one water molecule per asymmetric unit (Table 1), is still scarcely larger than that of (1). These crystals exhibit the same arrangement of the steroid molecules as observed in (2)·MeOH·H_2O, but the solvent molecules are located in different regions in the two structures.

The crystal structure of another closely related steroid, 24-epi-pterosterone [20R,22R,24R-2β,3β,14α,20,22,24-hexahydroxy-5β-cholest-7-en-6-one, (4)] was reported by Ohta *et al.* (1996). The crystals obtained from a methanol–water mixture contain one steroid, one methanol and two water molecules in the asymmetric unit. This steroid differs from 20-hydroxyecdysone only in the position of a hydroxy group: the alkyl chain has a 24-OH substituent in the former and a 25-OH substituent in the latter. Because of the molecular similarity, (4)·MeOH·2H_2O was included in our analysis.

Overall, a set of six crystal structures incorporating closely related steroids was available. A comparative analysis of these structures may shed light on factors influencing the crystallization behavior and pseudo-polymorphism (Haleblian, 1975) of ecdysteroids, a knowledge of which is an important prerequisite of their pharmaceutical utilization.



	R^1	R^2	R^3	R^4
ecdysone (**1**)	OH	H	H	H
20-hydroxyecdysone (**2**)	OH	OH	H	H
polypodine B (**3**)	OH	OH	OH	H
24-epi-pterosterone (**4**)	H	OH	H	OH

2. Experimental

2.1. Crystal preparation

All crystals were grown by slow solvent evaporation under ambient conditions. The composition of the solvent mixture was varied to test the solvation preferences of 20-hydroxyecdysone and polypodine B. The first single crystals of 20-hydroxyecdysone were obtained from a 9:1 methanol–water mixture and were found to be of composition (2)·MeOH·H_2O. From the same solvent mixture, polypodine B yielded a monohydrate, (3)·H_2O. From solutions in pure water, 20-hydroxyecdysone crystallized as a trihydrate [(2)·3H_2O], whereas polypodine B again formed the monohydrate. Solvent-free crystals of 20-hydroxyecdysone (2) were grown from a 4:1 mixture of ethyl acetate and methanol. We were unable to grow single crystals of polypodine B from ethyl acetate and methanol mixtures. The compositions of the solvate crystals were determined from the results of crystal structure refinements (see below).

2.2. Data collection, structure solution and refinement

Details of cell data, data collection and refinement are summarized in Table 1.[1] Each data set was collected on a CAD-4 diffractometer with graphite monochromated Cu $K\alpha$ or Mo $K\alpha$ radiation. Lattice parameters were refined by least-squares fit for 25 reflections. To check the variance in the unit-cell volume, ecdysone was subjected to two independent data collections on two CAD-4 diffractometers, equipped with an Mo and a Cu tube, respectively. The standard reflections for each data collection were checked every hour. When check reflections indicated a decay, it was appropriately corrected for. All reflections were corrected for Lorentz and polarization effects. $P2_12_12_1$, the common space group of these ecdysteroid structures, was uniquely determined from systematic absences. The crystallographic phase problems

[1] Supplementary data for this paper are available from the IUCr electronic archives (Reference: DE0015). Services for accessing these data are described at the back of the journal.

Table 1
Experimental details.

	(1)	(2)	(2)·MeOH·H_2O	(2)·3H_2O	(3)·H_2O
Crystal data					
Chemical formula	$C_{27}H_{44}O_6$	$C_{27}H_{44}O_7$	$C_{27}H_{44}O_7·CH_4O·H_2O$	$C_{27}H_{50}O_{10}$	$C_{27}H_{44}O_8·H_2O$
Chemical formula weight	464.62	480.62	530.68	534.67	514.64
Cell setting, space group	Orthorhombic, $P2_12_12_1$	Orthorhombic, $P2_12_12_1$	Orthorhombic, $P2_12_12_1$	Orthorhombic, $P2_12_12_1$	Orthorhombic, $P2_12_12_1$
a, b, c (Å)	7.750 (1), 9.935 (1), 35.657 (1)	7.663 (1), 10.541 (1), 33.084 (2)	7.167 (1), 9.703 (1), 41.412 (4)	11.293 (2), 14.202 (2), 18.721 (3)	7.163 (1), 10.303 (1), 37.254 (2)
V (Å^3)	2745.5 (5)	2672.4 (5)	2879.8 (6)	3002.5 (8)	2749.4 (5)
Z	4	4	4	4	4
D_x (Mg m^{-3})	1.124	1.195	1.224	1.183	1.243
Radiation type	Cu $K\alpha$	Cu $K\alpha$	Cu $K\alpha$	Mo $K\alpha$	Cu $K\alpha$
No. of reflections for cell parameters	25	25	25	25	25
θ range (°)	20.07–23.74	30.60–34.55	20.11–23.88	11.53–16.92	25.0–29.01
$F(000)$	1016	1048	1160	1168	1120
μ (mm^{-1})	0.624	0.686	0.734	0.089	0.756
Temperature (K)	293 (2)	293 (2)	293 (2)	293 (2)	293 (2)
Crystal form, color	Block, colorless	Block, colorless	Prism, colorless	Prism, colorless	Block, colorless
Crystal size (mm)	0.25 × 0.16 × 0.12	0.45 × 0.30 × 0.20	0.25 × 0.14 × 0.10	0.6 × 0.6 × 0.4	0.50 × 0.15 × 0.10
Data collection					
Diffractometer	Enraf–Nonius CAD-4	Enraf–Nonius CAD-4	Enraf–Nonius CAD-4	Enraf–Nonius CAD-4	Enraf–Nonius CAD-4
Data collection method	ω–2θ scans	ω–2θ scans	ω–2θ scans	ω–2θ scans	ω–2θ scans
Absorption correction	ψ-scan	ψ-scan	ψ-scan	ψ-scan	ψ-scan
T_{min}	0.8576	0.7477	0.8380	0.8984	0.896
T_{max}	0.9302	0.8750	0.9296	0.9718	0.927
No. of measured, independent and observed reflections	6982, 5410, 3946	5611, 4542, 4155	6775, 5774, 4751	13105, 11655, 6759	6667, 5553, 4972
Criterion for observed reflections	$I > 2\sigma(I)$	$I > 2\sigma(I)$	$I > 2\sigma(I)$	$I > 2\sigma(I)$	$I > 2\sigma(I)$
R_{int}	0.0134	0.0130	0.0120	0.0106	0.0087
θ_{max} (°)	74.74	74.99	74.99	33.42	74.85
Range of h, k, l	$-8 \rightarrow h \rightarrow 9$ $-12 \rightarrow k \rightarrow 12$ $-44 \rightarrow l \rightarrow 44$	$-8 \rightarrow h \rightarrow 9$ $-13 \rightarrow k \rightarrow 11$ $-35 \rightarrow l \rightarrow 41$	$-8 \rightarrow h \rightarrow 8$ $-12 \rightarrow k \rightarrow 12$ $-51 \rightarrow l \rightarrow 51$	$-17 \rightarrow h \rightarrow 17$ $-22 \rightarrow k \rightarrow 22$ $-29 \rightarrow l \rightarrow 29$	$-8 \rightarrow h \rightarrow 8$ $-12 \rightarrow k \rightarrow 12$ $-46 \rightarrow l \rightarrow 46$
No. and frequency of standard reflections	3 every 60 min	3 every 60 min	3 every 60 min	2 every 60 min	3 every 60 min
Intensity decay (%)	9	4	10	0	6
Completeness of 2θ	0.990	1.000	0.993	0.996	0.999
Refinement					
Refinement on	F^2	F^2	F^2	F^2	F^2
$R[F^2 > 2\sigma(F^2)]$, $wR(F^2)$, S	0.0353, 0.1088, 0.735	0.0363, 0.1036, 1.056	0.0382, 0.1213, 0.858	0.0535, 0.1422, 0.92	0.0362, 0.1042, 1.065
No. of reflections, restraints and parameters used in refinement	5410, 290, 376	4542, 244, 375	5774, 3, 356	11655, 0, 366	5553, 5, 338
H-atom treatment	Mixed	Mixed	Mixed	Riding	Mixed
Weighting scheme	$w = 1/[\sigma^2(F_o^2) + (0.1P)^2]$ where $P = (F_o^2 + 2F_c^2)/3$	$w = 1/[\sigma^2(F_o^2) + (0.0671P)^2 + 0.236P]$ where $P = (F_o^2 + 2F_c^2)/3$	$w = 1/[\sigma^2(F_o^2) + (0.1P)^2]$ where $P = (F_o^2 + 2F_c^2)/3$	$w = 1/[\sigma^2(F_o^2) + (0.0829P)^2]$ where $P = (F_o^2 + 2F_c^2)/3$	$w = 1/[\sigma^2(F_o^2) + (0.0701P)^2 + 0.1608P]$ where $P = (F_o^2 + 2F_c^2)/3$
$(\Delta/\sigma)_{max}$	0.001	0.003	0.014	0.001	0.001
$\Delta\rho_{max}$, $\Delta\rho_{min}$ (e Å^{-3})	0.152, −0.138	0.253, −0.206	0.225, −0.152	0.256, −0.184	0.184, −0.201
Extinction method	*SHELXL*	None	*SHELXL*	None	None
Extinction coefficient	0.0014 (2)	0	0.0017 (3)	0	0
Flack parameter	0.18 (17)	−0.19 (18)	0.09 (17)	−0.1 (7)	0.03 (14)

Computer programs used: *CAD*-4 *EXPRESS* (Enraf–Nonius, 1992), *XCAD*-4 (Harms, 1996), *SHELXS*97 (Sheldrick, 1997*a*), *SHELXL*97 (Sheldrick, 1997*b*).

were solved by direct methods, using the program *SHELXS*97 (Sheldrick, 1997*a*). The atomic positions for each structure were refined with anisotropic displacement parameters in F^2 mode with the program *SHELXL*97 (Sheldrick, 1997*b*). The H atoms pertaining to hydroxy groups and solvent molecules were located in difference Fourier maps and refined isotropically in riding mode. The H atoms of hydroxy groups (including methanol solvent) were allowed to rotate around the C—O bond with an idealized C—O—H angle. For the H atoms belonging to water molecules, the same shifts were applied as for the O atom of the molecule. In all structures except (2)·3H_2O, O—H distances were refined. Other H atoms

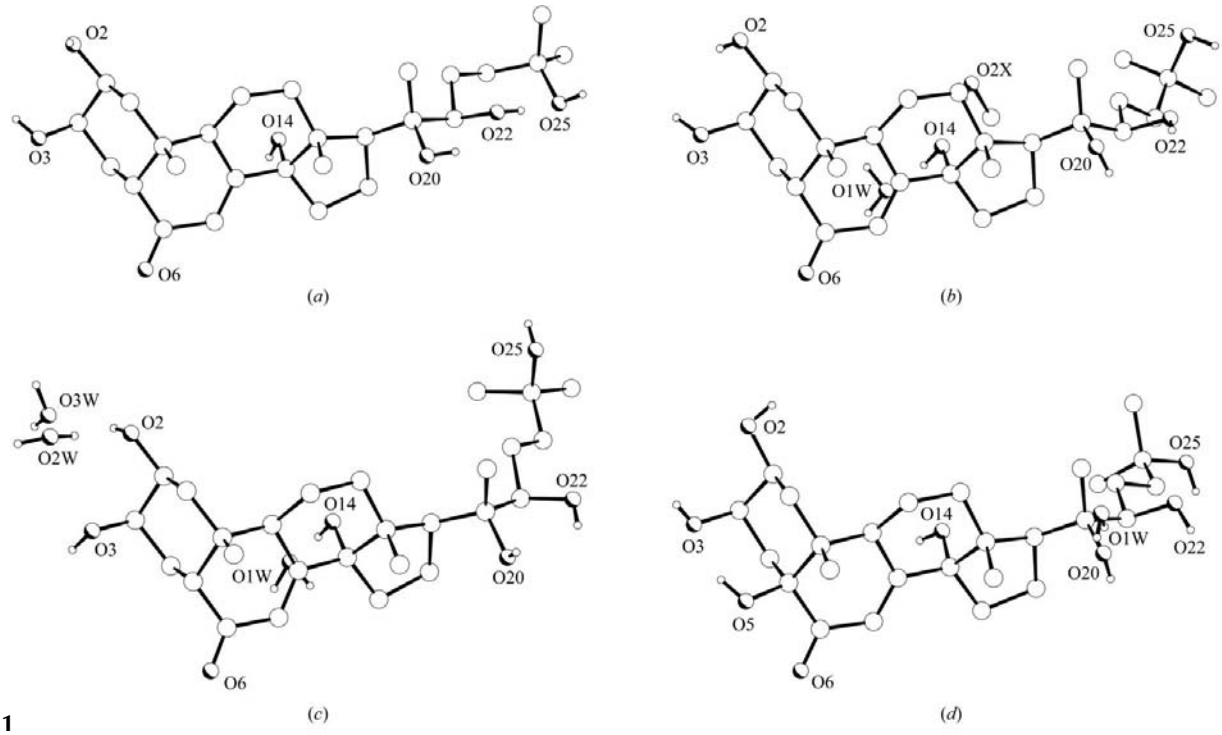


Figure 1
Asymmetric unit of (*a*) solvent-free 20-hydroxyecdysone, (2), (*b*) 20-hydroxyecdysone methanol solvate hydrate, (2)·MeOH·H_2O, (*c*) 20-hydroxyecdysone trihydrate, (2)·3H_2O and (*d*) polypodine B hydrate, (3)·H_2O. H(—C) atoms are omitted for clarity. Solvent H-atom positions on (2)·3H_2O were calculated to reflect probable hydrogen-bonding directions. Only main disorder components are shown.

were generated from assumed geometry and were refined isotropically in riding mode.

Two water molecules in the asymmetric unit of (2)·3H_2O were found to be disordered. The unconstrained refinements of the site occupation factors of the disordered positions indicated that both molecules are present in an approximately stoichiometric ratio, so final refinements were constrained to total occupancies of one. [The small amount of the chromatographically isolated 20-hydroxyecdysone did not permit us to develop (2)·3H_2O crystals for accurate elemental analysis.] The H atoms bound to the disordered water molecules could not be located in difference maps and were not included in the refinement. However, to simplify the analysis, hydrogen bonds were identified from short O···O contacts, and H atoms were generated along these bonds. Since each disorder component forms short contacts only with ordered atoms, the assignment of donors and acceptors was straightforward.

The absolute structures, except for that of (2)·3H_2O, were determined by refinement of the Flack parameter (Flack, 1983). Since the side chains of both ecdysone (1) and 20-hydroxyecdysone (2) exhibit conformational disorder, this was checked for ecdysone using the reflections collected with Mo $K\alpha$ radiation. The independent refinements on ecdysone resulted in the same geometry (the occupancy factors vary around 50%), with very slight differences, so publication of the second set of data appears superfluous. To achieve stable refinement, bond distances and atomic displacement parameters were restrained in the disordered alkyl chains so that equivalent bonds have the same lengths and bonded atoms have similar displacement parameters.

3. Discussion

Our crystallization experiments revealed a fundamental difference between the closely related steroids 20-hydroxyecdysone (2) and polypodine B (3) with respect to the ability of various solvents to incorporate into their lattice. By contrast, despite the different compositions, their solvates (2)·MeOH·H_2O and (3)·H_2O are homostructural.[2]

The solvent-free modifications of ecdysone and 20-hydroxyecdysone are isostructural. One would expect solvent-free polypodine B to be isostructural with the latter two crystals. However, our efforts to produce this form failed, and the tendency of polypodine B to crystallize as a monohydrate became evident from these attempts.

The conformation of the steroid molecules varies little among the ecdysteroid structures. Only the alkyl chains display some variation in the rotations around bonds C23—C24 and C24—C25 (Fig. 1). This means that, except for 25-OH, the steroid hydrogen-bond functions have the same relative orientation in each crystal. Consequently, the observed difference in interaction with solvents cannot be ascribed to conformational changes.

The incorporation of solvent molecules does not lead uniformly to tighter packing either. The packing coefficient (Gavezzotti, 1983) of the solvent-free (2) (0.64) is intermediate between those of the solvates (2)·MeOH·H_2O (0.66)

[2] When the strict conditions of isostructurality (*e.g.* molecular isometricity, identical location of molecules in the unit cells) are not fulfilled but the overall arrangement of the molecules in two structures is identical, then these structures are considered homostructural.

Table 2
Hydrogen-bond dimensions in the isostructural crystals of ecdysone (1) and 20-hydroxyecdysone (2).

Bonds involving disordered atoms are listed only if occupancies of all atoms are at least 0.5.

Label	*D*	H	*A*	H···*A* (Å)	*D*···*A* (Å)	*D*—H···*A* (°)
(1)						
a	O14	H14	O22*B*^{i}	2.08	2.89 (3)	172
b	O2	H2	O3^{ii}	1.95	2.749 (2)	165
c	O3	H3	O6^{ii}	2.03	2.802 (2)	156
d	O25*B*	H25*B*	O2^{iii}	1.92	2.733 (9)	170
e	O22*B*	H22*D*	O25*B*	1.81	2.63 (2)	172
(2)						
a′	O14	H14	O20^{i}	2.03	2.852 (2)	175
b	O2	H2	O3^{ii}	1.94	2.759 (2)	179
c	O3	H3	O6^{ii}	2.31	3.048 (2)	150
d	O25*A*	H25*A*	O2^{iii}	2.07	2.842 (14)	157
	O25*B*	H25*B*		1.98	2.707 (13)	147
e	O22	H22	O25*A*	1.88	2.671 (10)	161
			O25*B*	1.84	2.645 (12)	165
f	O20	H20	O22	2.12	2.580 (2)	115

Symmetry codes: (i) $1-x, -\frac{1}{2}+y, \frac{3}{2}-z$; (ii) $-\frac{1}{2}+x, \frac{3}{2}-y, 2-z$; (iii) $-x, \frac{1}{2}+y, \frac{3}{2}-z$.

and (2)·3H_2O (0.63). This suggests that simple close-packing considerations are inadequate for the interpretation of the ecdysteroid structures.

Therefore, the packing arrangements and the role of the solvent molecules must be regulated by directed hydrogen-bonding interactions, and a detailed analysis of hydrogen-bond networks provides a tool for understanding the observed structures and crystallization behavior.

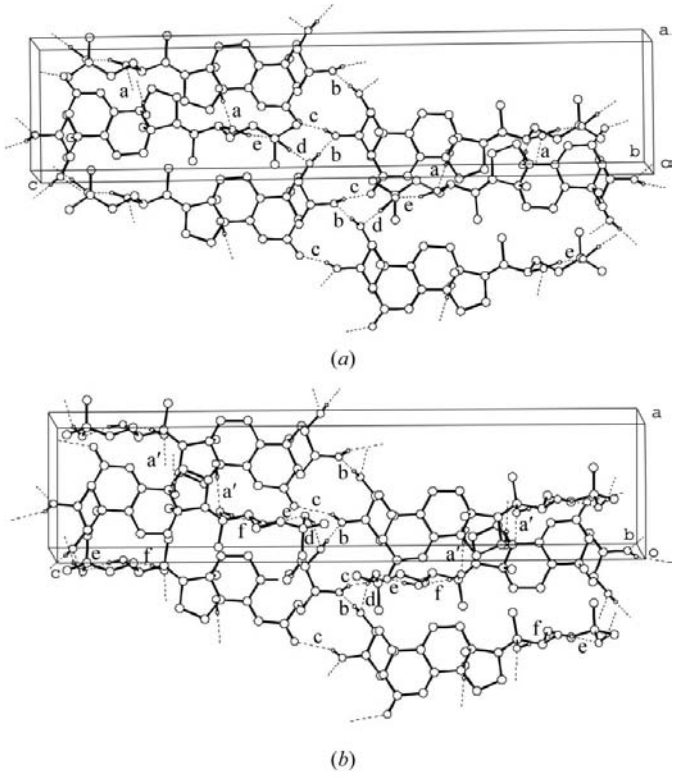


Figure 2
Crystal packing of solvent-free ecdysone (*a*) and 20-hydroxyecdysone (*b*).

Table 3
Isostructurality descriptors of the crystal structures.

Crystal structures	ϵ	*A*	I_v	I_v^{max}
(1)—(2)	0.0090	2.85	68%	95%
(3)·H_2O—(2)·MeOH·H_2O	0.0156	2.10	44%	97%

3.1. Isostructurality of ecdysone and 20-hydroxyecdysone

Although one would expect the introduction of new hydrogen-bond donors and acceptors to alter crystal packing, examples of isostructural steroid pairs with an H→OH replacement have already been reported. The isostructurality of cinobufagin and cinobufotalin was attributed to a special position of the hydroxy group in cinobufotalin that hinders intermolecular hydrogen bonding (Kálmán *et al.*, 1988). The crystal structures of 2-oxa-4-androstene-3,17-dione and its 6α-hydroxy analogue demonstrate interaction mimicry between C—H···O and C—O—H···O hydrogen bonds (Anthony *et al.*, 1998). The insertion of the O atom, as expected, led to an increased unit cell in both cases.

Compared with the above results, the apparent packing similarity (Fig. 2) of ecdysone (1) and 20-hydroxyecdysone (2) is surprising in two respects: (i) the 20-OH group introduces new hydrogen bonds into the latter crystal (Table 2) and (ii) the volume of the unit cell *decreases* on insertion of the additional O atoms.

Furthermore, the appearance of alkyl-chain disorder in both structures, besides underscoring their close relationship, suggests that the molecules are not confined tightly even in the more closely packed structure of (2). In (1), void channels at $(x, \frac{3}{4}, \frac{1}{2})$ were identified with a radius of *ca* 1.4 Å. These voids, surrounded by disordered alkyl chains, are too small to include solvent molecules. Hence, their presence is an indication of the poor fit of the ecdysone molecules. To find the reason for the unexpected difference in close packing, the hydrogen-bond networks of the two crystals were compared.

The numerical descriptors of isostructurality between (1) and (2) (Fábián & Kálmán, 1999; Rutherford, 1997) indicate a moderate degree of similarity with a relatively pronounced distortion of the unit-cell shape (Table 3).[3] The smaller unit-cell volume is achieved in (2) because the *a* and *c* axes are shorter and the *b* axis is longer than those in (1) (Table 1).

The hydrogen-bond interactions of the molecules are almost the same in the two structures. The most important difference is that the O14—H···O22 hydrogen bond of (1) is directed toward the 'new' O atom O20 in (2) (hydrogen bonds **a** and **a′** in Table 2). Together with the intramolecular O20—H···O22 hydrogen bond **f**, hydrogen bond **a′** replaces hydrogen bond **a**. The replacement of the hydrogen-bond sequence ···O22—H···O25—H··· with ···O20—H···O22—H···O25—H··· allows isostructurality to be maintained,

[3] The volumetric isostructurality index I_v gives a general description of packing similarity on the basis of overlapping molecular volumes, whereas the mean elongation ϵ and asphericity index *A* quantify the difference between the size and the shape of the unit cells, respectively. The more similar the unit cells are, the closer ϵ and *A* are to zero.

because both sequences contain one intermolecular donor and one intermolecular acceptor at similar locations.

In both structures, the molecules form layers parallel to plane *ac* of the unit cell (Fig. 2). These layers are held together by hydrogen bonds **b** and **c**, which form chains of twelve-membered rings along the screw axes parallel to the *a* axis. Adjacent layers are linked by hydrogen bonds **a** and **d**. These hydrogen bonds build antiparallel chains along independent screw axes parallel to the *b* axis (Fig. 3).

The similarity of the hydrogen-bond networks is also reflected by the graph-set descriptors (Etter, 1990; Bernstein *et al.*, 1995) assigned to them. Indeed, only hydrogen bond **a** and its combinations show any difference up to the second level of analysis. The chain motif $C(7)$ of hydrogen bond **a′** in (2) is shorter by one atom than the corresponding $C(8)$ chain of **a** in (1). The shorter chain pulls the molecules together more tightly along the *c* axis, so their centers come closer to each other (Fig. 2). In turn, this decreases the extent of the sheets in the direction of the *c* axis. Hence, the *ca* 2.5 Å decrease in the unit length *c* and the corresponding reduction in the unit-cell volume in 20-hydroxyecdysone are results of chain $C(\mathbf{a}')$ being shorter than chain $C(\mathbf{a})$ in ecdysone.

The possibility of explaining the difference in the two structures solely on the basis of hydrogen bonding suggests that directed hydrogen bonds are responsible for governing the packing of these hydrophilic steroid molecules.

3.2. Structural relationship of 20-hydroxyecdysone methanol solvate hydrate and polypodine B hydrate

Although the unit-cell shapes and unit-cell dimensions *a* and *b* of solvent-free 20-hydroxyecdysone (2) and the mixed solvate form [(2)·MeOH·H_2O] are similar, the relative arrangements of the steroid molecules in them are different. Indeed, the presence of two additional hydrogen-bonded solvent molecules accounts for a rearrangement. In contrast, the apparent packing similarity (Fig. 4) of (2)·MeOH·H_2O and polypodine B monohydrate [(3)·H_2O] is surprising. Polypodine B contains an additional hydroxy group as compared with 20-hydroxyecdysone, whereas the methanol molecules are missing from its crystal structure.

The quantitative descriptors (Fábián & Kálmán, 1999; Rutherford, 1997) of their similarity (Table 3) suggest that the unit cells of (2)·MeOH·H_2O and (3)·H_2O are almost as closely related as the unit cells of (1) and (2), though the difference is somewhat larger in size (ϵ) and somewhat smaller in shape (A). The isostructurality index I_v, however, is much smaller for these structures, indicating that, despite the similar overall packing arrangements, the positions of the molecules in their respective unit cells are altered significantly.

To facilitate the comparison of hydrogen-bond networks, the molecules are considered to consist of two fragments. The steroid skeleton and the hydroxy and oxo groups attached to it are collectively referred to as the 'head group', while the alkyl chain and its substituents are termed the 'tail group'.

In both solvated structures, head-to-head interactions connect the steroid skeletons and tail-to-tail hydrogen bonds link the alkyl chains of neighboring molecules. In contrast with the solvent-free structures (1) and (2), head-to-tail bonds are missing from these solvated structures. The solvent molecules are located in different regions of the two solvates: in (2)·MeOH·H_2O, they take part in head-to-head connections, whereas the water molecules of (3)·H_2O link adjacent tail groups (Fig. 4, Table 4).

Owing to the different roles of the solvents, there are no common hydrogen bonds in (2)·MeOH·H_2O and (3)·H_2O. Nevertheless, the general similarity of the intermolecular

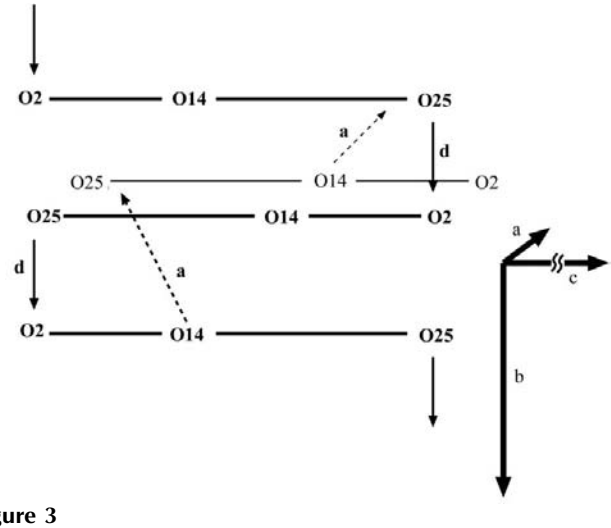


Figure 3
Schematic representation of hydrogen-bond chains along the *b* axis in ecdysone and 20-hydroxyecdysone. The molecule represented by a thinner line is shifted by a unit translation along the *a* axis. Bond **a** is directed to O22 in ecdysone and to O20 in 20-hydroxyecdysone, and it is followed by intramolecular bond(s) to O25 in both structures.

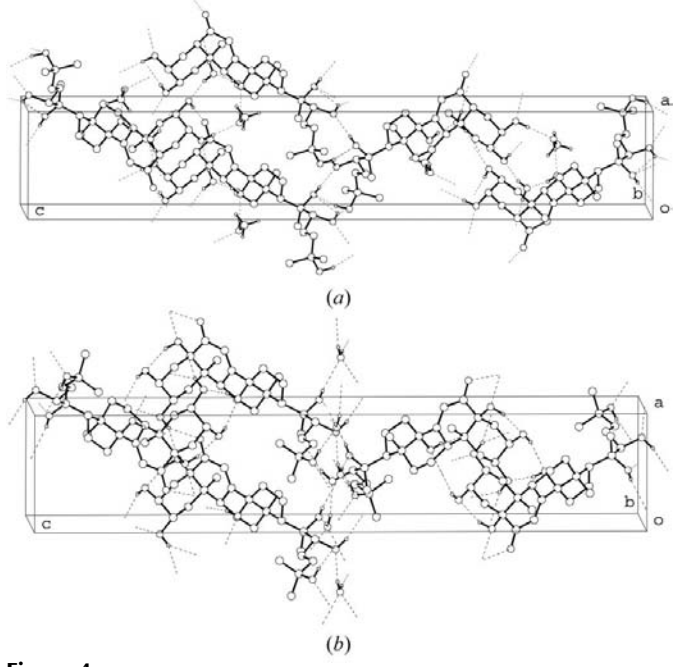


Figure 4
Crystal packing of 20-hydroxyecdysone methanol solvate hydrate (*a*) and polypodine B hydrate (*b*).

Table 4
Hydrogen-bond dimensions in 20-hydroxyecdysone methanol solvate hydrate [(2)·MeOH·H_2O] and polypodine B hydrate [(3)·H_2O].

The latter structure has been transformed to the same asymmetric unit as (2)·MeOH·H_2O.

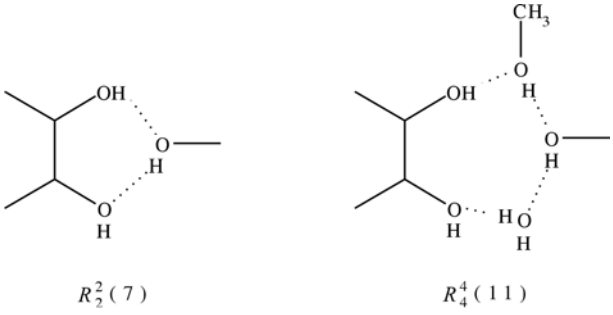
Label	*D*	H	*A*	H···*A* (Å)	*D*···A (Å)	*D*—H···*A* (°)
(2)·MeOH·H_2O						
a	O2	H2	O6^{i}	2.24	3.007 (2)	177
b1	3	H3	O2X^{ii}	1.92	2.794 (2)	171
b2	2X	H2X	O14	1.91	2.711 (2)	165
c1	14	H14	O1W	1.81	2.696 (2)	169
c2	O1W	H2W	O2^{iii}	1.97	2.775 (2)	165
d	O1W	H1W	O3^{iv}	2.03	2.815 (2)	171
e	O20	H20	O22^{v}	2.00	2.787 (2)	169
f	O22	H22	O25^{v}	2.50	3.204 (2)	144
g	O25	H25	O20^{vi}	1.94	2.871 (2)	161
h	O22	H22	O20	2.29	2.721 (2)	113
(3)·H_2O						
b	O3	H3	O14^{ii}	2.11	2.814 (2)	157
c	O14	H14	O2^{iii}	1.83	2.619 (2)	164
e1	O1W	H1W	O20	2.05	2.895 (2)	172
e2	O1W	H2W	O22^{v}	2.11	2.871 (2)	159
i	O2	H2	O5^{vii}	2.05	2.844 (2)	157
i	O2	H2	O6^{vii}	2.44	3.052 (2)	130
j	O20	H20	O1W^{v}	2.43	3.150 (2)	156
k	O22	H22	25$_{viii}$	1.96	2.828 (2)	159
l	O25	H25	O1W^{ix}	2.02	2.820 (2)	170
m	O5	H5	O3	1.89	2.676 (2)	149

Symmetry codes: (i) $1-x,\frac{1}{2}+y,\frac{3}{2}-z$; (ii) $2-x,\frac{1}{2}+y,\frac{3}{2}-z$; (iii) $2-x,-\frac{1}{2}+y,\frac{3}{2}-z$; (iv) $1-x,-\frac{1}{2}+y,\frac{3}{2}-z$; (v) $-\frac{1}{2}+x,\frac{1}{2}-y,2-z$; (vi) $\frac{1}{2}+x,\frac{1}{2}-y,2-z$; (vii) $1+x,y,z$; (viii) $-\frac{1}{2}+x,-\frac{1}{2}-y,2-z$; (ix) $x,-1+y,z$; (x) $-1+x,y,z$; (xi) $x,1+y,z$; (xii) $\frac{1}{2}+x,-\frac{1}{2}-y,2-z$.

interactions, which is to be expected from the analogous packing motifs, is revealed by a schematic representation of the hydrogen-bond networks (Figs. 5 and 6).

In (3)·H_2O, hydrogen bonds **b** and **c** connect the steroid skeletons related by the screw axis $2_1(0,y,\frac{3}{4})$ and its translation equivalents (Fig. 5*b*). Together, hydrogen bonds **b** and **c** form $R_2^2(7)$ rings. The three-center, bifurcated donor hydrogen bond[4] **i** (Jeffrey & Saenger, 1991) links molecules translated along the *a* axis and joins the helices of hydrogen bonds **b** and **c** into a two-dimensional network.

In (2)·MeOH·H_2O, the two solvent molecules are *inserted* into the $R_2^2(7)$ ring of (3)·H_2O, so that hydrogen bonds **b** and **c** are both split into sequences of two adjoining hydrogen bonds: **b1**, **b2** and **c1**, **c2**, respectively (Fig. 5*a*). As a result, the rings are enlarged to $R_4^4(11)$.



[4] The sum of the three O—H—O angles involving O2, H2, O5 and O6 is 358°, which proves the bifurcated nature of hydrogen bond **i**.

The inserted water molecules of (2)·MeOH·H_2O also provide an additional hydrogen bond, **d**. It links molecules related by the topologically independent screw axis $2_1(\frac{1}{2},y,\frac{3}{4})$. This interaction brings the reference molecule (label 0 in Fig. 5) close to the molecule with label i, and thus it assists in the formation of hydrogen bond **a**. The latter interaction can be regarded as a replacement of hydrogen bond **i** in (3)·H_2O, since both hydrogen bonds link O2 and O6 but on molecules with different relative positions. The 'flip' of hydrogen bond **i** in (3)·H_2O into hydrogen bond **a** in (2)·MeOH·H_2O may be a consequence of the changed steric conditions induced by hydrogen bond **d**, the lack of acceptor O5, the altered steric requirements of the enlarged $R_4^4(11)$ ring or, most probably, a combination of these factors.

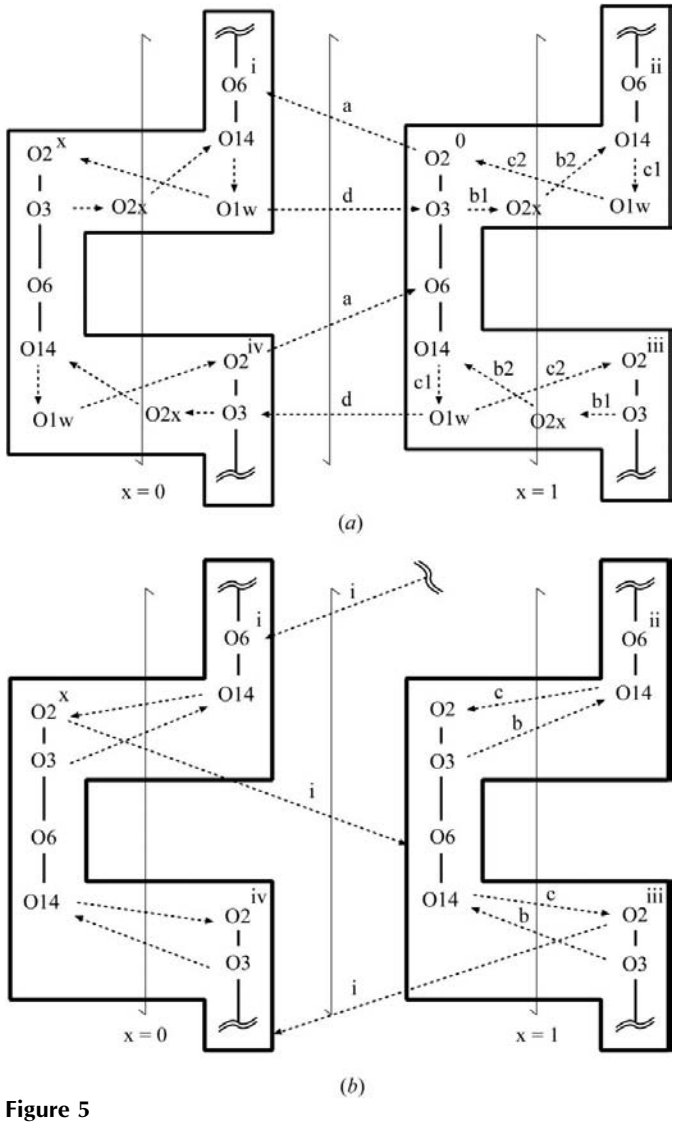


Figure 5
Schematic representation of hydrogen-bonding interactions between the steroid skeletons (head groups) in 20-hydroxyecdysone methanol solvate hydrate (*a*) and polypodine B hydrate (*b*). Atoms connected with a thick line belong to the same molecule. The plot is projected onto the *ab* plane with the *a* axis running right and the *b* axis running up in the plane of the paper. The bifurcated donor hydrogen bond **i** is shown as a single bond to O6 in (*a*). Symmetry codes given in label superscripts are listed in Table 4. Superscript 0 denotes the reference molecule.

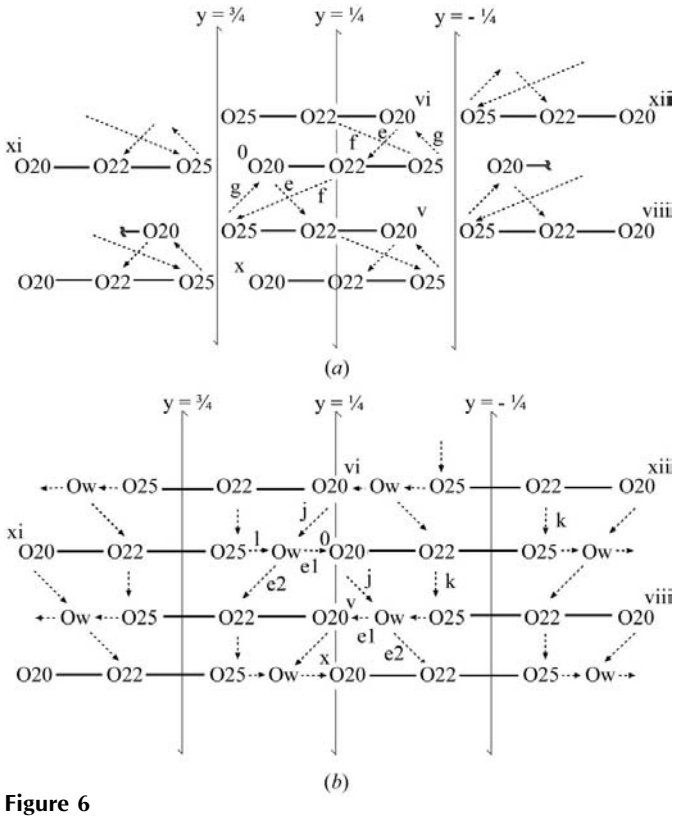


Figure 6
Schematic representation of hydrogen-bonding interactions between the alkyl chains (tail groups) in 20-hydroxyecdysone methanol solvate hydrate (*a*) and polypodine B hydrate (*b*). The plot is projected onto the *ab* plane with the *a* axis running up and the *b* axis running left in the plane of the paper. Symmetry codes are listed in Table 4.

While head-to-head interactions are governed by screw axes parallel to the crystallographic axis *b*, tail-to-tail interactions link molecules related by screw axes parallel to the *a* axis (Fig. 6). In (2)·MeOH·H_2O, hydrogen bonds **e**, **f** and **g** all connect molecules along the screw axis $2_1(x, \frac{1}{4}, 1)$. They maintain an antiparallel side-by-side arrangement of adjacent alkyl chains by linking two pairs of hydroxy groups on neighboring molecules (Fig. 6*a*).

Reflecting the flexibility of alkyl groups, the incorporation of solvent molecules has a much more pronounced effect on the tail-to-tail network than on the head-to-head interactions. Consequently, of the three hydrogen bonds **e**, **f** and **g**, only **e** has a counterpart in (3)·H_2O (Fig. 6*b*): hydrogen bonds **e1** and **e2** also connect O20 to $O22^{V}$ but now do so through a water molecule (Table 4). Another symmetry-equivalent water molecule provides a link between the same two molecules by connecting their 20-OH groups *via* hydrogen bonds **j** and **e1**. Thus, in the antiparallel side-by-side arrangement of the alkyl chains in (3)·H_2O, only O20 and O22 take part in hydrogen-bond formation along the screw axis $2_1(x, \frac{1}{4}, 1)$.

The inserted water molecules of (3)·H_2O mediate additional tail-to-tail connections (Fig. 6*b*) between the molecules related by the topologically independent screw axis $2_1(x, -\frac{1}{4}, 1)$ (hydrogen bonds **j** and **l**) and between the translated molecules along both the crystallographic *a* and *b* directions (hydrogen bonds **j**, **e2** and **e1**, **l**, respectively). Hence, all water molecules hold together four symmetry-related steroid molecules.

Besides the water-mediated interactions, a steroid-to-steroid hydrogen bond **k** forms a helix around the screw axis $2_1(x, -\frac{1}{4}, 1)$. Interestingly, while only the tail groups of molecules related by $2_1(x, \frac{1}{4}, 1)$ are connected in (2)·MeOH·H_2O, in (3)·H_2O these molecules are linked by water molecules and all the direct tail-to-tail bonds are governed by $2_1(x, -\frac{1}{4}, 1)$. Accordingly, the alkyl chains change not only their relative arrangements but also their positions with respect to the symmetry operators.

While hydrogen bonds interlink molecules parallel to the crystallographic axes *a* and *b*, the molecules themselves are approximately parallel to *c*, so the alternating hydrogen-bonded regions of the head and tail groups are joined by the steroid molecules into a three-dimensional network (Fig. 4).

From the above analysis, it is apparent that the isostructurality of (2)·MeOH·H_2O and (3)·H_2O is made possible by a special role of incorporated solvent molecules: they utilize their hydrogen-bond donor and acceptor functions simultaneously, which enables them to extend an existing pattern without changing its basic structure. Further, they can introduce additional links between the steroid molecules by using their remaining hydrogen-bond capacity.

Comparison of the structures hints at the tendency of polypodine B to crystallize as a monohydrate as well. Formation of the bifurcated hydrogen bond **i** with acceptors O6 and the additional O5 (Table 4) presumably hinders the integration of solvent molecules among head groups. The resulting closer approach of the steroid skeletons changes the relative position of the alkyl chains so that their donors and acceptors cannot match. Incorporated water molecules increase the range of tail-to-tail bonds and, in consequence of their tetrahedral coordination, afford more steric flexibility. This enhanced flexibility may considerably promote crystallization of the monohydrate, (3)·H_2O.

3.3. 20-Hydroxyecdysone trihydrate and 24-epi-pterosterone methanol solvate dihydrate

The unit-cell parameters (Table 1) of the trihydrate form of 20-hydroxyecdysone [(2)·3H_2O] show that the arrangement of the molecules in this structure is completely different (Fig. 7) from that in the methanol solvate hydrate form [(2)·MeOH·H_2O]. The packing coefficient (Gavezzotti, 1983) of 0.63 for (2)·3H_2O indicates the loosest packing among the three 20-hydroxyecdysone forms. Loose packing is also demonstrated by the disorder of water molecules O2W and O3W.

The steroid molecules form grid-like layers held together by O3—H···O22 and O20—H···O2 hydrogen bonds (**b** and **d** in Table 5, Fig. 7). These are the only intermolecular steroid–steroid bonds. They form perpendicular chains with *y* translation and screw axis $2_1(x, \frac{3}{4}, 0)$, respectively. Screw symmetry gives the layers a wavy shape. The solvent molecules are located at the concave face of each undulation. The hollow

Table 5
Hydrogen bonds and short O···O contacts in 20-hydroxyecdysone trihydrate [(2)·3H_2O].

Water molecules are represented by O1W, O2W and O3W with O2W*A*, O2W*B* and O3W*A*, O3W*B* being disordered positions.

Label	*D*	H	*A*	H···*A* (Å)	*D*···*A* (Å)	*D*—H···*A* (°)
a	O2	H2*A*	O3W*A*	1.92	2.655 (6)	149
a	O2	H2*A*	O3W*B*	1.87	2.685 (7)	174
b	O3	H3*A*	O22^{i}	1.92	2.731 (2)	167
c	O14	H14	O1W	2.00	2.811 (2)	168
d	O20	H20	O2^{ii}	1.87	2.667 (2)	165
e	O22	H22*A*	O20	2.20	2.640 (2)	114
f	O25	H25	O1W^{iii}	2.23	3.026 (3)	165
g	O1W	H1W*A*	O2W*A*^{iv}	2.56	2.858 (3)	103
g	O1W	H1W*A*	O2W*B*^{iv}	2.01	2.572 (6)	125
h	O1W	H1W*B*	O20^{v}	2.21	3.008 (2)	164
i	O2W*A*		O3		2.836 (3)	
i′	O2W*B*		O2		2.938 (5)	
j	O2W*A*		O6^{vi}		2.760 (3)	
j	O2W*B*		O6^{vi}		2.882 (4)	
k	O3W*A*		O25^{i}		2.815 (6)	
k	O3W*B*		O25^{i}		2.771 (8)	
l	O3W*A*		O3^{vi}		3.159 (6)	

Symmetry codes: (i) $x, 1+y, z$; (ii) $\frac{1}{2}+x, \frac{3}{2}-y, -z$; (iii) $1-x, -\frac{1}{2}+y, \frac{1}{2}-z$; (iv) $\frac{3}{2}-x, 2-y, \frac{1}{2}+z$; (v) $2-x, \frac{1}{2}+y, \frac{1}{2}-z$; (vi) $-\frac{1}{2}+x, \frac{5}{2}-y, -z$.

region that they occupy is covered by alkyl chains protruding from the layer.

Consecutive layers are related by screw axes parallel to *c*. Their protruding alkyl groups enter the hollows of the neighboring layer, forming side walls around the included water molecules.

The above picture could represent the typical case of space-filling guests in a hydrogen-bonded host lattice. However, the water molecules are also involved in hydrogen bonding (Table 5). Despite being disordered, each triplet of symmetry-independent water molecules is bound to six adjacent steroid molecules (Fig. 8). Five of them belong to the same layer, so they have direct steroid–steroid hydrogen bonds between them. Solvent-mediated interactions duplicate direct steroid–steroid bonds (see *e.g.* O3—H···O22, O2—H···O3W—H···O25 in Fig. 8) and provide additional links between steroid molecules (*e.g.* O2—H···O3W—H···O3). Hence, they have a stabilizing role in the formation of steroid layers. Moreover, water molecule O1W is involved in the only inter-layer hydrogen bond, **c** (Table 5).

A similar argument is valid for the methanol solvate dihydrate of 24-epi-pterosterone, (4)·MeOH·2H_2O (Ohta *et al.*, 1996). It crystallizes with the tetragonal space group $P4_12_12$ ($Z = 8$, $V = 5889.8$ Å). The failure of the authors to determine the H-atom positions for the solvent molecules suggests disorder (*cf.* $R = 0.049$) similar to that observed in (2)·3H_2O.[5]

In the structure of (4)·MeOH·2H_2O, ladder-like layers are also formed (Fig. 9). They are brought together by perpendicular hydrogen-bond chains with translation (hydrogen bond **g** in Table 6) and screw (hydrogen bonds **a** and **c**) symmetry, respectively. In contrast with (2)·3H_2O, the network of steroid–steroid hydrogen bonds is extended to three

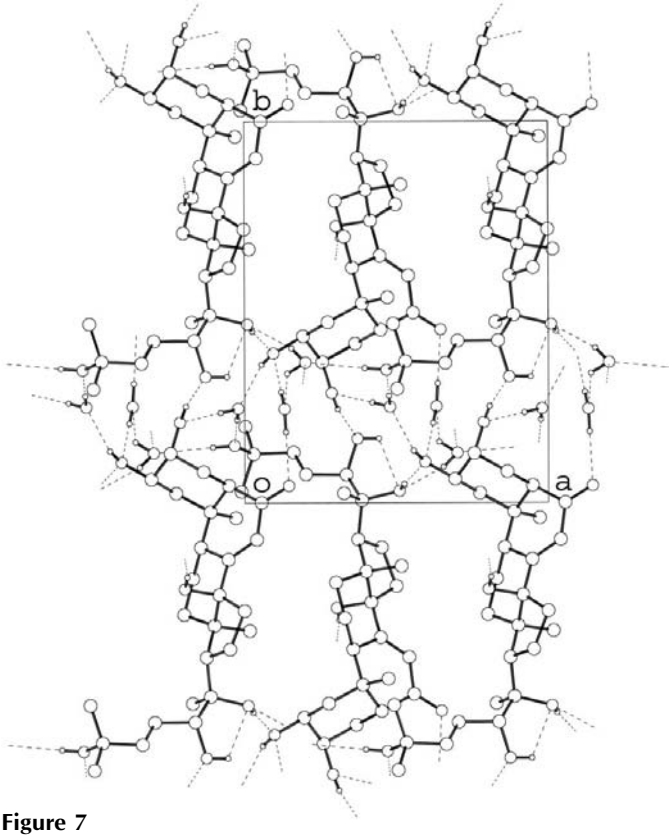


Figure 7
Layer structure of 20-hydroxyecdysone trihydrate. Only the main components of the disordered solvent molecules are shown with H atoms generated in hydrogen-bond directions.

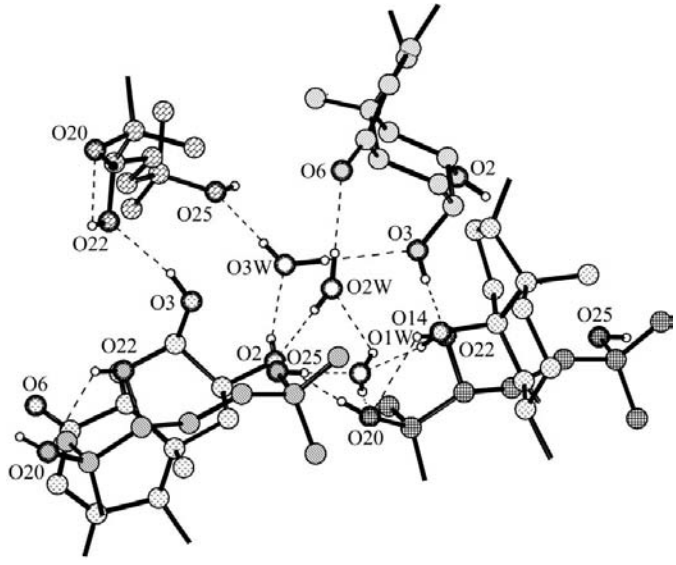


Figure 8
Hydrogen-bond environment of three symmetry-independent water molecules in the crystal structure of 20-hydroxyecdysone trihydrate. Only main components of the disordered solvent molecules are shown with H atoms generated in hydrogen-bond directions. The steroid molecules are truncated for clarity.

[5] As in (2)·3H_2O, the missing H atoms were generated on the basis of known hydrogen bonds and short O···O contacts to yield a consistent hydrogen-bond pattern.

Table 6
Hydrogen bonds and short O···O contacts in 24-epi-pterosterone methanol solvate dihydrate [(4)·MeOH·2H_2O].

Label	*D*	*A*	*D*···*A* (Å)	*D*—H···*A* (°)
a	O2—H	O22^{i}	2.841	155
b	O3—H	O2	2.801	104
c	O3—H	O6^{ii}	3.036	163
d	O14—H	O2W	2.762	156
e	O20—H	O1X	2.773	144
f	O20—H	O22	2.741	113
g	O22—H	O3^{iii}	2.939	168
h	O24—H	O2^{iv}	2.885	159
i	O1W	O14	2.774	
j	O1W	O24	2.885	
k	O2W	O1X^{v}	2.802	
l	O2W	O20^{i}	2.762	
m	O1X	O1W^{vi}	2.726	

Symmetry codes: (i) $-\frac{1}{2}-x,\frac{1}{2}+y,\frac{1}{4}-z$; (ii) $\frac{1}{2}-x,\frac{1}{2}+y,\frac{1}{4}-z$; (iii) $-1+x,y,z$; (iv) $-\frac{1}{2}-y,-\frac{1}{2}+x,\frac{1}{4}+z$; (v) $-\frac{1}{2}-y,\frac{1}{2}+x,\frac{1}{4}+z$; (vi) $-\frac{1}{2}+y,-\frac{1}{2}-x,-\frac{1}{4}+z$.

dimensions: adjacent layers related by fourfold screw axes are linked *via* hydrogen bond **h**.

Although the layers of (4)·MeOH·2H_2O undulate much less than those of (2)·3H_2O, the positions of solvent molecules with respect to them are similar in the two structures (Figs. 7 and 9): they occupy voids generated between adjacent layers. Interconnected solvent molecules provide additional steroid–steroid links in (4)·MeOH·2H_2O: between twofold screw-axis-related molecules within the same layer (hydrogen bonds **d** and **l**) and between fourfold screw-axis-related molecules of neighboring layers (hydrogen bond **e**).

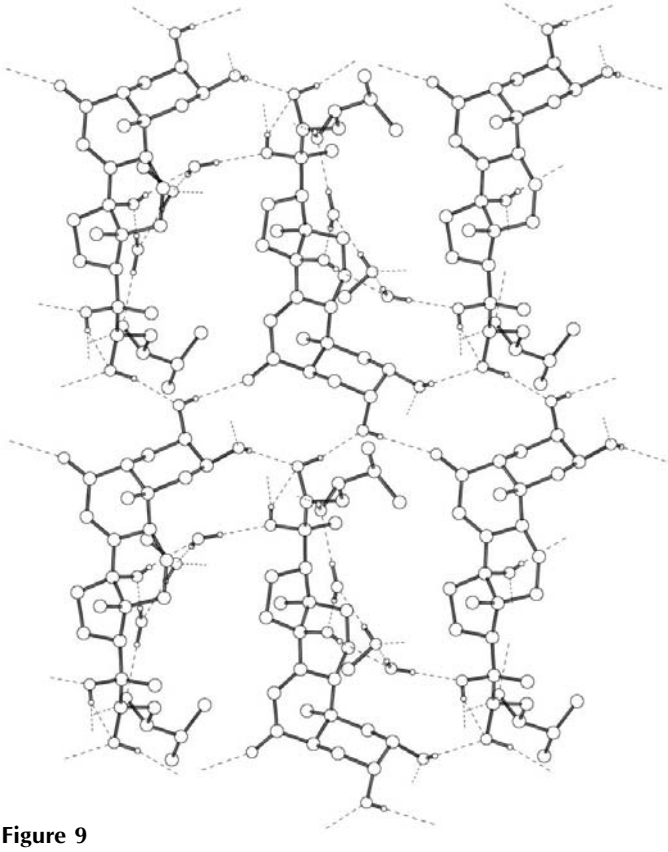


Figure 9
Layers in 24-epi-pterosterone methanol solvate dihydrate. H atoms of the solvent molecules were generated in probable hydrogen-bond directions.

Both in (2)·3H_2O and in (4)·MeOH·2H_2O, the solvent molecules occupy well defined regions of the structure. These are cavities formed in a hydrogen-bonded network of steroid molecules. Besides acting as space-filling guests, these molecules form hydrogen bonds with the host lattice. These bonds are similar to those formed in the solvates (2)·MeOH·H_2O and (3)·H_2O because they equally link hydrogen-bond functions of adjacent steroid molecules. Therefore, even the disordered solvent molecules in (2)·3H_2O and (4)·MeOH·2H_2O may have structure-cementing and structure-determining roles.

The formation of distinct solvent regions in these crystals allows high flexibility in the matching of donors and acceptors. Interconnected sets of solvent molecules are able to accept and donate a number of hydrogen bonds without strict geometrical requirements. This enables them to stabilize arrangements with a poor shape fit of steroid molecules by mediating hydrogen-bond interactions between them.

4. Conclusions

Ecdysteroid molecules consist of a rigid steroid skeleton and a flexible alkyl chain. Both fragments bear hydroxy groups, so the molecules are hydrophilic and can form hydrogen bonds with appropriate solvents. These relatively strong interactions allow solvent molecules to be incorporated into ecdysteroid crystals.

The structures described in this paper demonstrate a gradually increasing degree of crystal solvation. The solvent-free forms of ecdysone and 20-hydroxyecdysone [(1) and (2)] are isostructural. A careful comparison of their hydrogen-bond networks permits an explanation of the unexpectedly loose packing in (1). This emphasizes the importance of hydrogen bonds relative to anisotropic forces, which may be surprising in the packing of such big molecules.

The methanol solvate hydrate of 20-hydroxyecdysone [(2)·MeOH·H_2O] and the polypodine B hydrate [(3)·H_2O] comprise another isostructural pair. Since the solvent molecules are included in different parts of the two structures, they allow analysis of related 'solvated' and 'unsolvated' hydrogen-bond networks. They demonstrate that solvent molecules can be inserted into an existing network without breaking its structure by acting simultaneously as donors and as acceptors. Solvent molecules also mediate additional interactions. Apparently, they improve the matching of donors and acceptors with the additional hydrogen-bond functionality that they introduce into the crystal. The presence of solvent molecules among steroid molecules increases the range and relieves the orientational requirements of their interactions.

In 20-hydroxyecdysone trihydrate [(2)·3H_2O] and in 24-epi-pterosterone methanol solvate dihydrate [(4)·MeOH·2H_2O], the solvent molecules occupy voids in the steroid lattice, *i.e.* they act as space-filling guest molecules. However, they still form hydrogen bonds with neighboring steroid molecules, maintaining a similar hydrogen-bond mediating role to that

observed in (2)·MeOH·H_2O and (3)·H_2O. Such groups of solvent molecules in a cavity may easily adapt to the geometric requirements of adjacent donors and acceptors. This makes them an exceptionally capable 'glue' for the crystallization of ecdysteroids. The currently available data permitted an enumeration of the functions of the solvent molecules in ecdysteroid crystals and an interpretation of some structural features. More crystallization experiments and crystal structure analyses are needed for the identification of conditions leading to given forms and for the prediction of possible solvate structures.

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