Limnantheoside C (20-Hydroxyecdysone 3-*O*-β-D-glucopyranosyl-[1→3]-β-D-xylopyranoside), a Phytoecdysteroid from Seeds of *Limnanthes alba* (Limnanthaceae)

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A new ecdysteroid glycoside, limnantheoside C (20-hydroxyecdysone 3-O-β-D-glucopyranosyl-[→3]-β-D-xylopyranoside [**1**]), together with limnantheoside A (20-hydroxyecdysone 3-*O*-β-D-xylopyranoside [**2**]) and 20-hydroxyecdysone (**3**) have been isolated by bioassay/RIA-directed HPLC analyses of a methanol extract of the seedmeal of *Limnanthes alba* Hartw. ex Benth. The structure of the novel ecdysteroid glycoside (**1**) was determined unambiguously by UV, LSIMS and a combination of 1D- and 2D-NMR experiments. These three compounds are isolated from *Limnanthes alba* for the first time.

Introduction

Ecdysteroids are the steroid hormones of arthropods and perhaps of other invertbrate phyla too. Steroidal analogues of these compounds also occur in certain plants, where they are referred to as phytoecdysteroids, and are believed to contribute to the deterrence of invertebrate predators. Ecdysteroids are also credited with interesting, and mainly beneficial, pharmaceutical activities on vertebrates (Dinan, 2001). The ecdysteroid receptor protein (EcR: either intact, or the ligand-binding domain fused with appropriate domains of other regulatory proteins) is being actively studied as a component of a gene switching mechanism in vertebrate and plant transgenic systems (Fussenegger, 2001). Convenient sources of potent ecdysteroids are required to develop this approach to its full potential. As part of a search for novel ecdysteroid receptor agonists and antagonists (Dinan, 1995; Dinan *et al.*, 1999; Dinan *et al.*, 2001), we have surveyed 5000 species of terrestrial plants and identified many species not previously known to accumulate phytoecdysteroids. Amongst these were members of the Limnanthaceae, a small family consisting of only 8 species in 2 genera (Link, 1992).

Limnanthes alba Hartw. ex Benth. (Limnanthaceae), known as meadowfoam, is an oil seed crop (Jolliff and Seddigh, 1993) which has good commercial potential (Norberg *et al.*, 1993; Throckmorton *et al.*, 1982) and possible application in the cosmetic industry owing to the seeds' content of long-chain fatty acids (95% C_{20} – C_{22}; Isbell *et al.*, 1996). With regard to the chemical constituents of *L. alba*, so far only fatty acids (Hayes and Kleiman, 1993), free and esterified sterols (Lechner *et al.*, 1999) and glucosinolates (Bartelt and Mikolajczak, 1989; Vaughn *et al.*, 1996) have been published. Bioassay/RIA-guided examination of seeds of various members of the genus *Limnanthes* in our laboratory showed that there are moderate to

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Abbreviations: E, ecdysone; 20E, 20-hydroxyecdysone; PoA, ponasterone A; LSIMS, liquid secondary ion mass spectrometry; NP, normal-phase; RIA, radioimmunoassay; RP, reversed-phase; SPE, solid-phase extraction.

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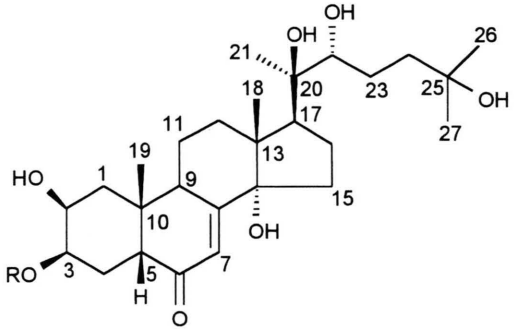
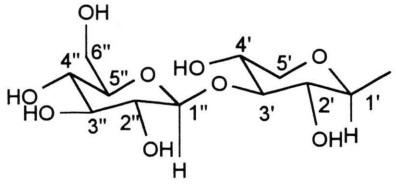
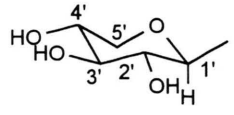
high levels of phytoecdysteroids in the seeds of all members of the genus *Limnanthes* (Sarker *et al.*, 1997). 20-Hydroxyecdysone (20E), ponasterone A (PoA) and two novel ecdysteroid xylosides (limnantheoside A [20E 3-*O*-β-D-xyloside] and limnantheoside B [PoA 3-*O*-β-D-xyloside]) were isolated from seeds of *L. douglasii* (Sarker *et al.*, 1997). The ready availability of large amounts of seedmeal of *L. alba* after extraction of the seed oil with non-polar solvents led us to believe that this could be a good potential source of ecdysteroids on a commercial scale. An investigation of ecdysteroids present in the seedmeal of *L. alba* under guidance of bioassay/RIA led to the discovery of a new ecdysteroid glycoside, limnantheoside C (Fig. 1, compound **1**), together with two known compounds: limnantheoside A (**2**) and 20E (**3**). Here we report the isolation and structural elucidation of these three compounds.







3. R= H

Fig. 1. Ecdysteroid structures: limantheoside C (**1**), limnantheoside A (**2**) and 20-hydroxyecdysone (**3**).

Materials and Methods

General experimental procedures

UV spectra were in MeOH. NMR spectra were obtained on a Bruker AVANCE DRX400 instrument using standard Bruker microprograms. The chemical shifts are expressed in ppm, LSIMS (+ve ion mode); glycerol matrix using a Cs^+ primary beam on a VG Quattro triple quadruple mass spectrometer (VG Biotech, Altrincham, UK); SPE C_{18} cartridges (Sep-Pak Vac 35cc [10 g], Waters/Millipore, Luton, Beds., U. K.) were used for pre-HPLC fractionation; HPLC: a) preparative/semi-preparative; Gilson Model 806 HPLC coupled with a Gilson UV-visible detector, b) analytical; Gilson model 811 HPLC coupled with a Gilson 160 diode-array detector and using Gilson Unipoint computer program. Technoprep 10C8 preparative C_8 (Phenomenex, Macclesfield, U. K.), Spherisorb semipreparative C_{18} and C_6 columns, Apex II Diol semiprep. column and Spherisorb 5 ODS(2) analytical C_{18} and C_6 columns (Jones Chromatography, Hengoed, Mid-Glamorgan, U. K.) were used. Chromatographic separations were monitored at 242 nm.

Radioimmunoassay

RIA was performed according to the procedure described previously (Dinan, 1992) using ecdysteroid-specific antisera, DBL-1 and Black, which were generously donated by Prof. Jan Koolman (University of Marburg, Germany). The cross-reactivities of these antisera with a range of phytoecdysteroids are given elsewhere (Dinan, 1995).

Bioassay

Ecdysteroid agonist activities of the extract, SPE fractions, HPLC fractions were assessed with a microplate-based bioassay using the *Drosophila melanogaster* B_{II} cell line (Clément *et al.*, 1993).

Plant material

Seeds, seedmeal and screenings of *Limnanthes alba* (cv. Floral) were donated by OMG/Natural Plant Products LLC (Salem, OR, U. S. A.). A voucher specimen of the seeds has been retained at the Department of Biological Sciences, University of Exeter.

Extraction

Seedmeal (50 g) of *L. alba* was extracted three times (3×24h) with 3×450 ml of MeOH at 55 °C with repeated agitation using an ultrasonic bath. Extracts were pooled and evaporated to 210 ml and this was made to a 70% aq. methanolic solution by adding 90 ml water. After being defatted with *n*-hexane (4×150 ml), the extract was concentrated using a rotary evaporator at a maximum temperature of 45 °C to give 4.7 g residue.

Analytical HPLC

A portion of the seedmeal extract of *L. alba* (equivalent to 5 mg seedmeal) was dissolved in 30% MeOH in H_2O (100 µl) and separated on a RP(C_{18})-analytical column eluted at 1 ml min^{-1} with a gradient from 30 to 100% MeOH in H_2O over 30 min, followed by elution with MeOH for a further 10 min. Fractions (1 ml) were collected and monitored by bioassay and RIA. Also, portions (100 µl) of each fraction were evaporated and hydrolysed with a mixture of *Helix pomatia* hydrolases (Sigma, Type H1; 10 mg/ml in 0.1 M sodium acetate buffer, pH 5.4) for 2 days at 37 °C, after which ethanol (0.8 ml) was added to precipitate protein and the supernatant was assessed by RIA and bioassay.

Isolation of the compounds

RP(C_{18})-SPE fractionation of the concentrated extract (redissolved in 10% MeOH in water) using a MeOH-H_2O step-gradient (10, 25, 80 and 100% MeOH), followed by bioassay/RIA test revealed the presence of ecdysteroids in the 80% MeOH/H_2O fraction which was then subjected to HPLC using a prep. RP(C_8)-column (linear gradient at 5 ml min^{-1} from 40–60% MeOH in H_2O over 60 min, followed by elution with MeOH for a further 10 min) to yield 5 fractions. Fractions 2 (15–20 min) and 3 (20–25 min) were found to be bioactive and RIA-positive. Fraction 2 was subjected to RP(C_6)-semiprep. column (isocratic elution with 40% MeOH in H_2O, 2 ml min^{-1}) to yield 3 fractions (2–1 [Rt = 11 min], 2–2 [Rt = 19.5 min] and 2–3 [Rt = 22.5 min]). Further purification of fractions 2–1, 2–2 and 2–3 on an NP(DIOL)-semiprep. column (isocratic elution with 7% dichloromethane in MeOH at 2 ml min^{-1}), respectively, produced **1** (2.8 mg), **3** (981 µg) and **2** (3.1 mg).

Limnantheoside C (20-hydroxyecdysone 3-*O*-β-D-glucopyranosyl-(1→3)-β-D-xylopyranoside) (**1**): gum; UV λ_{max} nm (log ε) 243 nm (4.12); LSIMS (+ve ion-mode) *m/z* 775[M+H]$^+$; ^{1}H NMR (Table I); ^{13}C NMR (Table II).

Limnantheoside A (20-hydroxyecdysone 3-*O*-β-D-xylopyranoside) (**2**): white amorphous; LSIMS (+ve ion-mode) *m/z* 613[M+H]$^+$; ^{1}H NMR in MeOH (Table I); ^{13}C NMR in MeOH (Table II); UV, ^{1}H NMR and ^{13}C NMR in D_2O: data as reported (Sarker *et al.*, 1997).

20-Hydroxyecdysone (**3**): amorphous; HPLC, UV, ^{1}H NMR (Table I) and ^{13}C NMR (Table II):

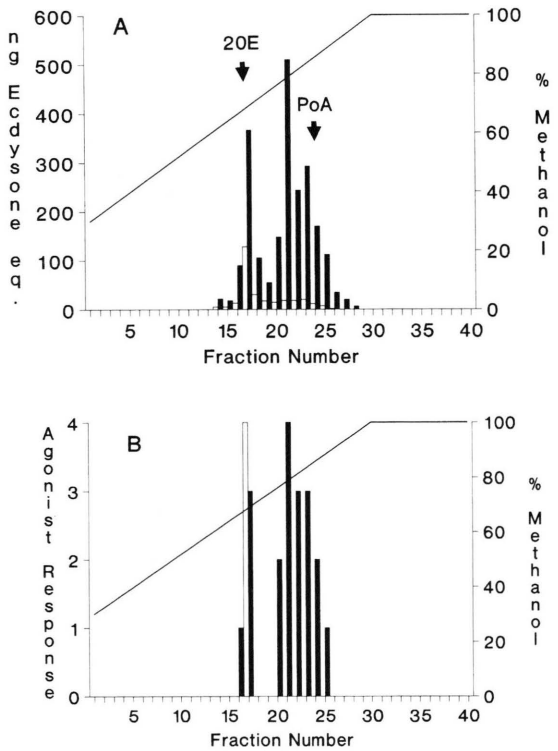


Fig. 2. RP(C_{18})-HPLC separation of a portion the methanolic extract of seeds of *Limnanthes alba* cv. Floral (equivalent to 0.5 µg E eq. with the DBL-1 antiserum before enzymic hydrolysis). Fractions of 1-min duration were collected and assessed for ecdysteroid content before (□) and after (■) enzymic hydrolysis with *Helix pomatia* hydrolases by (A) RIA using the DBL-1 antiserum and (B) the B_{II} bioassay. The retention times of 20-hydroxyecdysone (20E) and ponasterone A (PoA) are indicated.

Table I. ^{1}H NMR spectral data of compounds **1**, **2** and 20E (Lafont and Wilson, 1996) (constant *J* [Hz] in parentheses).

Proton	**1**	**2**	20E
1_{ax}	1.42	1.42	1.43
1_{eq}	1.84	1.84	1.78
2_{ax}	3.85 (m, w1/2 = 22)	3.84 (m, w1/2 = 22)	3.83 (m, w1/2 = 22)
3_{eq}	4.06 (m, w1/2 = 8)	4.05 (m, w1/2 = 8)	3.94 (m, w1/2 = 8)
4_{ax}	1.69	1.69	1.65
4_{eq}	1.88	1.88	1.75
5	2.43 (dd, 13, 3.8))	2.43 (dd, 13, 3.8)	2.38 (dd, l3, 4)
7	5.82 (d, 2.2)	5.82 (d, 2.2)	5.85 (d, 2.5)
9_{ax}	3.15 (m, w1/2 = 21)	3.15 (m, w1/2 = 21)	3.09 (m, w1/2 = 21)
11_{ax}	1.68	1.68	1.65
11_{eq}	1.82	1.82	1.78
12_{ax}	2.14 (ddd,13, 13, 5)	2.14 (ddd,13, 13, 5)	2.13 (ddd, 13, 13, 5)
12_{eq}	1.88	1.88	1.85
15a	1.96	1.96	2.00
15b	1.59	1.59	1.55
16a	2.00	2.00	1.95
16b	1.73	1.73	1.75
17	2.39 (m)	2.39 (m)	2.39 (m)
22	3.33*	3.32*	3.33 (dd, 11, 2)
23a	1.29	1.29	1.30
23b	1.67	1.67	1.65
24a	1.81	1.81	1.75
24b	1.42	1.42	1.45
18-Me	0.89 (s)	0.89 (s)	0.89 (s)
19-Me	0.97 (s)	0.97 (s)	0.96 (s)
21-Me	1.20 (s)	1.20 (s)	1.18 (s)
26-Me	1.19 (s)	1.19 (s)	1.19 (s)
27-Me	1.20 (s)	1.20 (s)	1.20 (s)
$1'_{ax}$	4.38 (d, 7.3)	4.32 (d, 7.2)	
$2'_{ax}$	3.49 (dd,7.3, 8.9)	3.27* (dd, 7.2, 8.9)	
$3'_{ax}$	3.54 (t, 8.9)	3.34* (t, 8.9)	
$4'_{ax}$	3.63 (ddd, 5.1, 8.9, 11)	3.51 (ddd, 5.1, 8.9 ,11)	
$5'_{ax}$	3.94 (dd, 5.1, 11.5)	3.89 (dd, 5.1, 11.5)	
$5'_{eq}$	3.26 (dd, 11, 11.5)	3.23 (dd, 11, 11.5)	
$1''_{ax}$	4.61 (d, 7.6)		
$2''_{ax}$	3.28* (dd, 7.6,9.0)		
$3''_{ax}$	3.40 (t, 9.0)		
$4''_{ax}$	3.29* (t, 9.0)		
$5''_{ax}$	3.32* (ddd, 2.0, 5.8, 9.0)		
$6''_{ax}$	3.64 (11.8, 5.8)		
$6''_{eq}$	3.88 (11.8, 2.0)		

$w_{1/2}$ = width at half-height in Hz; ax=axial; eq=equatorial; * signals shielded by MeOH peaks, but could be observed by HMQC and ^{1}H-^{1}H COSY.

data as reported (Lafont and Wilson, 1996; Píš *et al.*, 1994).

Results and Discussion

Initial bioassay/RIA-based screening of seed methanol extracts of various *Limnanthes* spp. (Sarker *et al.*, 1997) showed that *L. alba* contained RIA-positive material detected with ecdysteroid-specific antisera and showed moderate agonist activity in the *Drosophila melanogaster* microplate-based B_{II} cell bioassay (Clément *et al.*, 1993). Analysis of extracts of various batches of *L. alba* seedmeal, deriving from different farms in Oregon and New Zealand and from different years (1996–2000), revealed that they all contained ecdysteroids with levels varying from 203–439 μg E eq./g (mean [± SD] = 283.4 [± 65.6] μg E eq./g, n = 11). Screenings (senesced aerial plant parts), on the other hand, contained a much lower level of ecdysteroids (48.4 μg E eq./g). Initial chromato-

graphic analysis by RP-HPLC and bioassay/RIA examination of the methanol extract of seeds of *L. alba* (cv. Floral) revealed the presence of both ecdysteroids and ecdysteroid conjugates (Fig. 2). The RIA detects free ecdysteroids much more readily than conjugated ecdysteroids, so the presence of conjugates can be recognised after hydrolysis of the conjugates to release immunoreactive material. Subsequent RP- and NP-HPLC chromatography of the RIA-positive material of the methanol extract of the seedmeal of *L. alba*, guided by bioassay/RIA, resulted in the isolation of a novel ecdysteroid glycoside, limnantheoside C (20-hydroxyecdysone-3-O-β-D-glucopyranosyl-[1→3]-β-D-xylopyranoside [**1**]) and two known compounds: limnantheoside A (**2**) and 20E (**3**) (Fig. 1). The structures of **2** and **3** were readily identified by direct comparison of their HPLC and spectroscopic characteristics with those published in the literature (Lafont and Wilson, 1996; Sarker *et al.*, 1997). The structure of the novel compound (limnantheoside C [**3**]) was identified unambiguously by 1D- and 2D-NMR experiments.

Compound **1** was readily recognised as a phytoecdysteroid from its positive response in the bioassay and RIA and its UV maximum absorption at 242 nm, which is characteristic of ecdysteroids. LSIMS spectrum (+ve ion-mode) of **1** showed an $[M+H]^+$ ion at *m/z* 775 compatible with the molecular formula $C_{38}H_{62}O_{16}$. Its ^1H NMR (Table I) and ^{13}C NMR (Table II) spectra showed obvious features of an ecdysteroid glycoside with a series of oxymethine and oxymethylene proton signals at δ 3.2 – δ 4.8 and a series of oxygenated carbon signals at δ 60 – δ 104 (Agrawal, 1992) in addition to the signals from the steroidal part. The signals for the protons and carbons of the steroidal ring system were very similar to those of 20E (Tables I and II) (Lafont and Wilson, 1996; Píš *et al.*, 1994) except that the signals for H-3 (δ 4.06) and C-3 (δ 75.1) were much deshielded relative to those for 20E, thus suggesting the attachment of a sugar unit at C-3 of 20E. The ^1H NMR and ^{13}C NMR spectra reveal two characteristic anomeric proton signals (δ 4.38 [1H, d, 7.3 Hz] and δ 4.61 [1H, d, 7.5 Hz]) and two characteristic anomeric carbon signals (δ 101.8 and δ 103.6). Together with the signals from nine other oxymethine protons at δ 3.2 – δ 4.0 and nine other oxymethine carbon signals at δ 60 – δ 90, these signals strongly suggest the presence of two sugar units: one pentose and one hexose. The identities of the pentose and hexose were deduced as β-D-pyranopentose and β-D-pyranohexose readily by the characteristic chemical shifts of the two anomeric protons (δ 4.38 [1H, d, 7.3 Hz] and δ 4.61 [1H, d, 7.5 Hz]) and their coupling-constants (Agrawal, 1992). The ^1H-^1H COSY spectrum confirmed all the ^1H-^1H correlations of **1**, especially all the adjacent protons on the two sugar ring systems. Comparing the ^1H and ^{13}C chemical shifts and ^1H-^1H coupling patterns of the protons on the two sugar ring systems with those of published

Table II. ^{13}C NMR spectral data of compounds **1**, **2** and 20E (Píš *et al.*, 1994) (400 MHz, in CD_3OD).

Carbon	**1**	**2**	20E
1	37.2	37.3	37.4
2	66.6	66.5	68.7
3	75.1	75.2	68.5
4	29.0	29.0	32.9
5	50.3	50.3	51.8
6	204.9	*	206.5
7	120.7	120.7	122.1
8	166.9	167.1	168.0
9	33.7	33.7	35.1
10	37.8	37.8	39.3
11	20.2	20.2	21.5
12	31.1	31.1	32.5
13	**	**	**
14	83.8	83.8	85.2
15	30.4	30.4	31.8
16	20.1	20.1	21.5
17	49.1	49.1	50.5
18	16.6	16.6	18.1
19	22.8	22.8	24.4
20	76.6	76.5	78.4
21	19.6	19.6	21.1
22	76.8	77.0	78.4
23	25.9	25.9	27.3
24	41.0	41.0	42.4
25	69.9	69.9	71.3
26	28.3	28.3	29.7
27	27.6	27.6	29.0
1′	101.8	102.1	
2′	72.4	73.0	
3′	85.5	76.2	
4′	68.5	69.7	
5′	65.2	65.6	
1″	103.6		
2″	74.1		
3″	76.5		
4″	72.2		
5″	77.0		
6″	61.3		

* Could not be detected by ^{13}C NMR (DEPT) experiment.

** Overlapped by a strong solvent signal at ca 48.5 ppm.

data (Agrawal, 1992), the two sugar units were easily deduced as a β-D-pyranoxylose and a β-D-pyranoglucose. The signals for H-3′ (δ 3.54 [1H, t, 8.5]) and C-3′ (δ 85.5) on the D-pyranoxylose unit were much more deshielded, which suggested the interglycosidic linkage between the D-xylose and D-glucose units is 3′→1″. A ^{1}H-^{13}C HMQC spectrum confirmed all ^{1}H-^{13}C direct ^{1}J correlations and the ^{1}H-^{13}C HMBC spectrum confirmed all the ^{1}H-^{13}C long-range ^{2}J, ^{3}J correlations in the molecule of **1**, especially the connection of C-3-O-C-1′ and C-3′-O-C-1″ (Table III). Thus, the structure of **1** was unambiguously identified as 20-hydroxyecdysone 3-O-β-D-glucopyranosyl-(1→3)-β-D-xylopyronoside (**1**), which we have named limnantheoside C.

The potency of **1** in the *Drosophila melanogaster* B_{II} cell assay for ecdysteroid agonist activity ($EC_{50} = 1.3 \times 10^{-6}$M) was almost identical to that of **2** ($EC_{50} = 1.6 \times 10^{-6}$M), but considerably lower than that of **3** ($EC_{50} = 7.5 \times 10^{-9}$M).

Ecdysteroid glycosides (mainly glucosides and galactosides) have been frequently reported from plant and animal sources (Lafont and Wilson, 1996), but reports on ecdysteroid xylosides are few, even though xylose is a common plant sugar. So far, only limnantheoside A and limantheoside B were isolated from the seed of *Limnanthes douglasii* (Sarker *et al.*, 1997). An ecdysteroid glycoside with both xylose and glucose units in its glycosidic part, has not been reported from any plant source before.

As is apparent from Fig. 2, ecdysteroid conjugates predominate over free ecdysteroids in seeds of *L. alba* and other ecdysteroid conjugates in addition to **1** and **2** (which coelute with **3** in the HPLC system depicted in Fig. 2) are present. However, it has not yet been possible to identify the major conjugates eluting between 20 and 25 min in this reversed-phase gradient system, owing to their instability.

Table III. ^{1}H-^{13}C HMQC direct correlation (^{1}J) and ^{1}H-^{13}C HMBC long-range correlation (^{2}J and ^{3}J) in compound **1**.

	δ C		
Proton	^{1}J	^{2}J	^{3}J
H_2-1	C-1	C-2, C-10	C-3, C-5, C-9, C-19
H-2	C-2		
H-3	C-3		C-1′(w)
H_2-4	C-4		
H-5	C-5	C-4, C-6, C-10	C-9, C-19
H-7	C-7		C-5, C-9, C-14
H-9	C-9	C-11	
H_2-11	C-11	C-9	
H_2-12	C-12		C-17, C-18
H_2-15	C-15	C-14, C-16	C-17
H_2-16	C-16	C-15, C-17	C-20
H-17	C-17	C-16	C-18
H-22	C-22	C-20	
H_2-23	C-23	C-24	
H_2-24	C-24	C-25	
Me-18	C-18	C-13	C-12, C-14, C-17
Me-19	C-19	C-10	C-1, C-9
Me-21	C-21	C-20	C-17, C-22
Me-26	C-26	C-25	C-24, C-27
Me-27	C-27	C-25	C-24, C-26
H-1′	C-1′		C-3
H-2′	C-2′	C-1′, C-3′	
H-3′	C-3′	C-2′, C-4′	C-1″
H-4′	C-4′	C-3′, C-5′	
H_{ax}-5′	C-5′	C-4′	C-1′,C-3′
H_{eq}-5′	C-5′	C-4′	C-1′
H-1″	C-1″		C-3′
H-2″	C-2″	C-1″, C-3″	
H-3″	C-3″	C-2″, C-4″	
H-4″	C-4″	C-3″, C-5″	
H-5″	C-5″	C-4″	
H_{ax}-6″	C-6″	C-5″	
H_{eq}-6″	C-6″		

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