Structure of the Ascarosides from *Ascaris suum*

J. P. Bartley*

School of Chemistry, Queensland University of Technology, GPO Box 2434, Brisbane 4001, Australia

E. A. Bennett and P. A. Darben

School of Life Science, Queensland University of Technology, GPO Box 2434, Brisbane 4001, Australia

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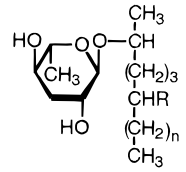
Six glycosides have been identified from the nematode *Ascaris suum*. The glycon part of all six glycosides is α L-3,6-dideoxymannose, previously known as ascarylose. The major components (**4** and **5**) and the minor components (**8** and **9**) have been shown by NMR and electrospray MS to involve a mixture of two homologous aglycons: the 2, ω-1 diols of hentriacontane and tritriacontane. Compounds **4** and **5** are glycosylated on only one of the hydroxy groups, while **8** and **9** are glycosylated on both. These compounds resemble ascarosides B and C previously isolated from *Parascaris equorum*. However, these aglycons are reported to be based on the 2,6-diol of hentriacontane. Compounds **6** and **7** are based on 2-hydroxy-nonacosane and 2-hydroxy-28-methylnonacosane glycosylated at C-2 with the same sugar. Although **6** and **7** are related to ascaroside A, previously isolated from *P. equorum*, these earlier reports suggest the chain in ascaroside A to be unbranched.

The ascarosides are a group of glycosides which are present in the neutral lipid fraction isolated from a range of nematode species.[1,2] They are particularly prevalent in nematodes from the superfamily Ascaridoidea, which are intestinal parasites of the higher vertebrates. Because ascarosides are characteristic of the metabolism of the oocyte, they are mainly recovered from eggs and tissues of the reproductive tract in female nematodes,[1] although some authors have also isolated them in small quantities from the tissues of male nematodes.[2] The ascarosides are located in the internal lipid layer of nematode eggshells and are believed to be responsible for the remarkable chemical resistance for which nematode eggs are renowned. The exact chemical nature of ascarosides is, therefore, of considerable interest, and they have been the target of efforts to develop disinfectants capable of destroying this barrier.

Using classical chemical procedures, Fouquey *et al.*[3,4] showed that the saponified lipid fraction of *Parascaris equorum* (from the horse) contained three ascarosides whose structures were closely related. All three were believed to be based on the glycon 3,6-dideoxy-L-arabinohexose (a sugar not known in other eukaryotes) to which they gave the name ascarylose. The most prevalent member of the ascaroside group, designated ascaroside B (**2**), was believed to involve the aglycon 2,6-dihydroxyhentriacontane, the C-2 hyroxy group of which was attached by a glycosidic link to a single molecule of ascarylose. A second member of the group, ascaroside C (**3**), was based on the same aglycon but contained two ascarylose units (attached at C-2 and C-6). The remaining member of the series, ascaroside A (**1**), also contained a single ascarylose molecule, but the aglycon was characterized as a mixture of L-2-alkanols containing between 26 and 30 carbon atoms. These aglycon chains were believed to be unbranched, and the ascarylose was attached at C-2, as in the other members of the group.

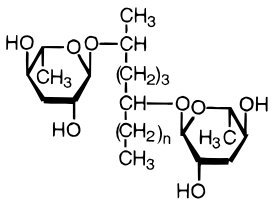


1: a) R=H n=20

b) R=H n=21

c) R=H n=22

2: R=OH n=24



3: n=24

Evidence for the location of the hydroxy groups in the aglycons was based largely on degradative chemistry. For example, dehydration of aglycon A followed by ozonolysis led to the production of ethanal and methanal from which it was concluded that the hydroxy group was at C-2. Similarly, ozonolysis of the diene from aglycon B produced succinic, malonic, and uncharacterized long-chain acids. The presence of malonic acid suggested the presence of a 2,6 diol. Furthermore, the UV spectrum of the dehydration products showed no conjugation and eliminated the possibility of the C-2,3; C-2,4; and C-2,5 isomers. Although, these chemical arguments seem valid, later workers have questioned the proposed structure of the aglycon. For example, Kirrman and Wakselman[5] synthesized 2,6-dihydroxyhentriacontane and demonstrated that it was not identical to the natural aglycon. Later, mass spectral analysis of the TMS ethers of the aglycon from ascaroside B led Tarr and Schnoes[6] to suggest that the hydroxy groups are on C-2 and C-(ω-1). However, no full spectroscopic examination of ascarosides has been undertaken, and no NMR analysis has previously been reported. In this paper we have used 2D NMR spectroscopy augmented

* To whom correspondence should be addressed. Phone: 07 3864 2266. FAX: 07 3864 1804.

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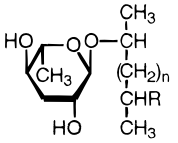
by electrospray MS to resolve this uncertainty and to establish unequivocally the structures of six ascarosides from *Ascaris suum* Goetze (class Nematoda, order Ascaridida, family Ascarididae), the pig roundworm. These spectra are reported for the first time and provide significant advantages over previously reported methods because they are recorded on the underivatized natural products and any uncertainty associated with possible isomerization or rearrangement during chemical manipulation is absent.

Results and Discussion

The unsaponifiable lipid fraction from the reproductive organs of *A. suum* was separated by preparative TLC on silica to give three major fractions, with the bulk of the material (73%) being present in fraction 2. The major components of these fractions are shown below to be closely related to the compounds designated by Fouquey *et al.*[3,4] as ascarosides A (**1**), B (**2**), and C (**3**).

HPLC−MS of fraction 2 carried out on a reversed-phase column, eluted with MeOH, indicated two main components in approximately equal quantities. Electrospray MS, which were acquired in both positive and negative modes (see Experimental Section), indicate molecular weights of 598.6 and 626.6 for the two components, suggesting that their structures differ by 2 CH_2 groups. These values are consistent with **4** (where $n = 27$) for the first component and **5** (where $n = 29$) for the second. The positive ion spectra also exhibited fragment ions at m/z 469 and 497, respectively, and this loss of 130 amu from the protonated molecular ion is consistent with loss of a dideoxy sugar moiety. Each of these ions then loses two molecules of H_2O, suggesting the presence of two hydroxy groups in the aglycon. The site of the glycosidic attachment and the position of the secondary hydroxy group is, however, unclear from the MS alone and can only be established from examination of the NMR data.



4: R=OH n=27

5: R=OH n=29

6: R=H n=25

7: R=CH_3 n=25

Even though HPLC−MS revealed two components in this fraction, NMR spectroscopy provided valuable structural information inasmuch as the homologous relationship between the two components (**4** and **5**) simply results in a variation of the intensity of the aliphatic CH_2 signal in the NMR spectrum. The ^{13}C-NMR spectrum indicated 14 different types of carbon, and the chemical shift information together with DEPT spectroscopy[7] suggested the presence of an anomeric CH, five other oxygenated CH groups, three CH_3 groups, and five different types of CH_2 groups. The spectrum is dominated by a strong CH_2 signal at δ 29.67, which clearly represents several equivalent aliphatic CH_2 groups (see Experimental Section).

The ^{13}C and the ^1H assignments were established using HMQC.[8] Of particular significance was the presence of five C−H correlations between signals in the ^{13}C region from δ 68 to δ 72 and 1H signals from δ 3.6 to δ 3.8. This confirms the presence of five oxygen-bearing methine carbons. It is significant that this information is not obvious from the 1D spectrum, as three of the ^1H signals and two of the ^{13}C signals are barely resolved even at 500 MHz.

The ^1H spectrum and the double quantum filtered homonuclear correlation spectrum (DQFCOSY)[9] provided information about the hydrogen connectivity and enabled the structure of the glycoside ring to be established unequivocally as follows. The anomeric hydrogen, H-1′ (δ 4.69) showed a cross peak to a signal at δ 3.80 assigned to H-2′. The chemical shift of this signal can only correspond to a hydrogen attached to an oxygenated carbon atom, establishing that C-2′ must be oxygenated. This H-2′ signal shows cross peaks to the two signals at δ 2.1 and 1.85, each of which integrates for one hydrogen and whose chemical shift is consistent with an $-OCCH_2CO-$ arrangement. There is also a cross peak between these two signals, and the HMQC spectrum has already shown that these two signals are on the same carbon atom. Thus, two hydrogens are attached to C-3′. Each of these signals then shows cross peaks to a signal centered at δ 3.60 (H-4′), and this in turn exhibits a cross peak to the signal at δ 3.70 (H-5′). Thus, both H-4′ and H-5′ must be attached to oxygenated carbons. Furthermore, H-5′ shows a strong cross peak to the envelope at 1.27. Although this region of the spectrum exhibits a strong signal corresponding to several equivalent CH_2 signals, both the DEPT and the HMQC spectra confirm the presence of a CH_3 signal in this region as well. The fine structure of the cross peak involved also indicates that only one coupling constant (with H-5′) is observed, and this absence of any further coupling suggests that H-6′ is a CH_3. These connectivities are consistent with the presence of a 3,6-dideoxyhexose ring.

The aglycon part of the molecule is clearly highly aliphatic as indicated by the size of the CH_2 signal at δ 1.27. The position of the substituents in the aglycon can be inferred from the two methyl doublets (integrating for 6 H's) at highfield (δ 1.1 and 1.2) in the ^1H spectrum. These signals are typical of an $-OCH(CH_3)-$ arrangement, a conclusion supported by the observation of DQFCOSY cross peaks from these methyls to hydrogens at about δ 3.8 (i.e., hydrogens attached to oxygenated carbons). This implies that the aglycon part of the molecule is oxygenated both at C-2 and on the penultimate carbon (i.e., C-30 in **4**). These structures differ from those isolated from *P. equorum* by Fouquey *et al.*, who found that in ascaroside B (**2**) oxygenation was at carbons 2 and 6.[4] If this were the case for our compounds, then only one methyl doublet would be observed in this region, while the remaining methyl would be a simple aliphatic one, which should resonate at about δ 0.9. Furthermore, this signal would not correlate to the oxygenated region at around δ 3.8.

Further support for the proposed structure is provided by the presence of long range heteronuclear correlations (Table 1 and Figure 1) observed using the HMBC[10] pulse sequence. For example, the following correlations—C2−H1, C2−H1′, C3−H1 and C3−H2—confirm the glyco-

Table 1. HMBC Data for Compounds **4, 6,** and **8**

	long-range correlated carbons		
H	**4**	**6**	**8**
1	C-2, C-3	C-2, C-3	C-2, C-3
2	C-3, C-4	C-3, C-4	C-3, C-4
28		C-27	
29		C-27, C-28	
30	C-29, C-28		C-28, C-29
31	C-30, C-29		C-30, C-29
1′	C-2, C-5′, C-3′	C-2, C-5′, C-3′	C-2, C-5′, C-3′
2′	C-1′, C-4′	C-1′, C-4′	C-1′, C-4′
3′e	C-1′, C-2′, C-4′, C-5′	C-1′, C-2′, C-4′, C-5′	C-1′, C-2′, C-4′, C-5′
3′a	C-1′, C-2′, C-4′, C-5′	C-1′, C-2′, C-4′, C-5′	C-1′, C-2′, C-4′, C-5′
4′	C-5′, C-6′	C-5′, C-6′	C-5′, C-6′
5′	C-4′	C-4′	C-4′

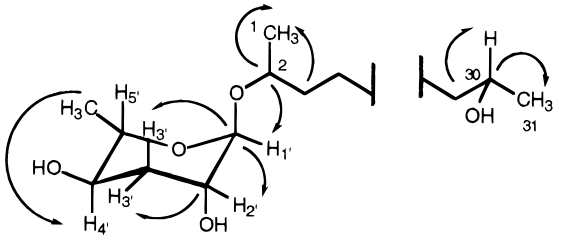


Figure 1. Selected HMBC correlations for compound **4**.

Table 2. Coupling Constants for two Conformations of **4**

	O-1′ eq		**O-1′ ax**		
hydrogens	angle (deg)	predicted *J* (Hz)	angle (deg)	predicted *J* (Hz)	observed *J* (Hz)
H1′−H2′	171	10.2	68	1.1	∼1
H2′−H3′(e)	53	3.3	52	3.4	3.8
H2′−H3′(a)	170	10.2	65	1.5	3
H3′(e)−H3′(a)	107	12	107	12	13.1
H3′(e)−H4′	69	2	55	2.9	4.6
H3′(a)−H4′	53	3.3	173	10.3	10.5
H4′−H5′	69	1.1	176	10.4	9.0
H5′−H6′				7	6.0

sidic attachment to C2 on the aglycon. Furthermore, the correlations C29−H31 and C30−H31 support the assignment of the hydroxy group in the aglycon to C-30 rather than C-6 as in ascaroside B (**2**).

Although the above arguments unequivocally establish the positions of the hydroxy groups in the dideoxyhexose, determination of the stereochemistry requires a closer examination of the coupling constants. These are reported in Table 2 and were measured from the 1D spectrum and confirmed by spectral simulation.[11] An analysis of the dihedral angles using the Karplus equation[12] suggests that the only structure with the correct combination of angles is the 2′-*R*,4′-*R*,5′-*S* isomer, L-dideoxymannose (or L-arabinohexose). Furthermore, the small coupling constant between H-1′ and H-2′ suggests either an eq−eq or an eq−ax relationship between these two protons. The predicted coupling constants for the two conformations of the β-anomer are shown in Table 2, and it is clear that only the O-1′ axial conformation (Figure 2) is consistent with the observed values. These observations are also consistent with predictions based on the anomeric effect,[13] which suggests a preference for the glycosidic oxygen to adopt the axial conformation as observed. Such an analysis also suggests a lower stability for the β form because the glycosidic oxygen in this anomer is required to adopt the equatorial conformation (Figure 2) in order to be consistent with the observed coupling constants (Table 2). The arguments used by Fouquey *et al.*[4,14] to establish the structure of ascarylose as an L-arabinohexose are based on measurements of optical rotation and, although adequate at the time, do depend on the purity of the samples, particularly because low values of molar rotations are involved. Nevertheless, the evidence that C-2′ is in the *R* configuration in ascaroside B seems strong, as it is based on the synthesis of the other likely possibilities. Thus, it appears that the aglycon of the ascarosides isolated by us is identical to the compound ascarylose reported by Fouquey *et al.* to be involved in the ascarosides of *P. equorum*.

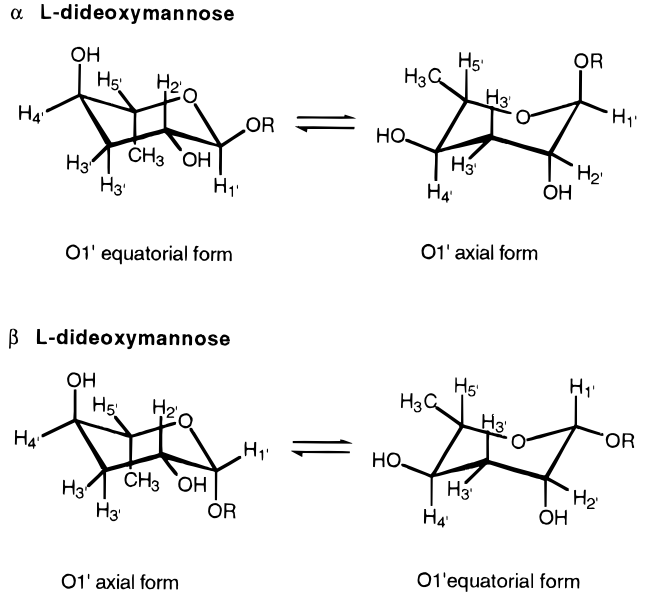


Figure 2. Conformers of the α- and β- forms of L-dideoxymannose (ascarylose).

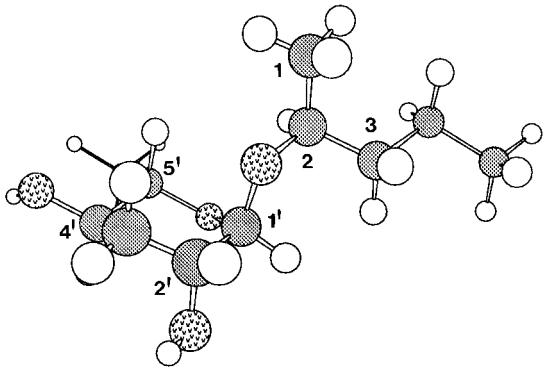


Figure 3. 3D representation of the fully energy-minimized structure of compound **4** (the aglycon chain is terminated after C-5 for clarity).

The empirical conclusions above are further supported by molecular mechanics calculations that were conducted using a strategy based on alternating steps of dynamics and minimization until a root mean square deviation of 0.0003 was obtained. The axial form was found to have a total energy of −26.46 kcal/mol and was favored by 2.17 kcal/mol over the equatorial form. The equatorial conformer could also be shown to converge to the more stable axial form after extensive minimization. A 3D representation of the fully energy-minimized axial form of **4** is shown in Figure 3 (the aglycon chain has been truncated after five carbons in order to simplify the diagram).

HPLC−MS analysis of fraction 1, carried out as described above, revealed the presence of two compo-

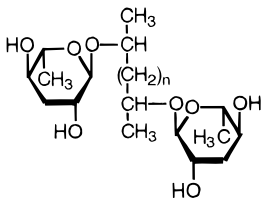
nents in about 4:1 ratio. Electrospray MS were acquired in both positive and negative modes and indicate molecular weights of 554.6 and 568.6, respectively, for the two components. These values are consistent with compounds **6** and **7** (where $n = 25$).

The ^{1}H-NMR spectrum shows considerable similarity with that of fraction 2, indicating that the structures of the compounds present are closely related to **4** and **5**. The most significant difference is the absence of the methyl doublet at δ 1.19 in the ^{1}H spectrum and its replacement by an overlapping doublet (δ 0.85) and a triplet (δ 0.87). This observation is consistent with the absence of oxygenation on the penultimate carbon of the aglycon, a conclusion further supported by the HMQC spectrum, which clearly shows only four cross peaks corresponding to hydroxylated carbons in the region between δ 68 and 74. However, the presence of two groups of signals between δ 0.84 and 0.88 suggests the presence of two compounds in one of which the aglycon contains an unbranched aliphatic chain (**6**) and in the other of which the aglycon terminates in an isopropyl branch (**7**). Once again these structures differ from those previously isolated in which the aliphatic chain in all three of the components of ascaroside A is unbranched.[14] Because the hydroxylated ascarosides **4** and **5** contain an odd number of carbons in their aglycons, it is tempting to suggest that **7** arises by reductive methylation of a hydroxylated C-29 precursor. This also implies that the C-28 compound (**1b**) reported by Fouquey *et al.*[14] might also be branched.

HPLC−MS analysis of fraction 3, carried out as described above, once again indicated the presence of two main components in about 2:1 ratio. Electrospray MS were acquired in both positive and negative modes and indicated molecular weights of 728.6 and 756.6, respectively, for the two components. These values are consistent with compounds **8** and **9** (where $n = 27$ and 29, respectively).

The NMR spectra of fraction 3 (see Experimental Section) were almost identical to those obtained for fraction 2 reported above, the most obvious difference being the absence of both the methyl signal at 23.43 in the ^{13}C spectrum and the methyl doublet at 1.19 in the ^{1}H spectrum. These signals were attributed to the C-31 and C-33 methyls in **4** and **5**, respectively. This observation suggests that the compounds in fraction 3 are closely related to the structure previously proposed by Fouquey[3,4] for ascaroside C (**3**) in which the hydroxy group in **2** is replaced by a second sugar moiety. However, the arguments presented above suggesting that the hydroxy group in our compounds is on the penultimate carbon would result in a structure that is now symmetrical about the central carbon of the aglycon chain, and the terminal methyl groups (C-1 and C-31 or C-33) become chemically and magnetically equivalent, as do the two sugar rings. This is consistent with the observed spectral data and confirm that fraction 3 contains a mixture of diglycosides **8** and **9**.

The earliest structural studies of ascarosides were carried out by Fouquey *et al.*[3,4] on the nematode *P. equorum* (a horse parasite). Subsequent studies have mostly involved the pig parasite *Ascaris lumbricoides* and have assumed the structure of the ascarosides in this species to be identical to those in *P. equorum*. The ascarosides identified by us in *A. suum* (a pig parasite)



8: n=27

9: n=29

resemble those found by Fouquey *et al.* in *P. equorum* in that three classes of ascarosides are present: diol monoglycosides, diol diglycosides, and monol glycosides. We also present here for the first time conclusive spectral evidence that the glycon in all three classes is α L-3,6-dideoxymannose as proposed by Fouquey *et al.* and referred to by them as ascarylose. On the other hand, the aglycons in ascarosides from *A. suum* are hydroxylated in all cases at the 2,ω-1 positions rather than the 2,6 positions, as in *P. equorum*. Our observations match those of Tarr and Schnoes,[6] who suggest that the ascarosides from *A. lumbricoides* and *Ascaris columnaris* also involve aglycons with hydroxy groups on C-2 and C-(ω-1). We also note, however, that 20% of the total monol aglycons in *A. suum* terminate in an isopropyl branch, a fact not reported for other species.

There is no evidence to suggest that this structural variation between the nematode species in the different animal hosts is not real, but it seems unlikely that the basic structure would vary widely between species of the same family, given that all ascarosides are characteristic of oocyte metabolism. It might be expected that the physiological requirements of the ascaroside layer would restrict the variation that can occur. In fact, Tarr[15] showed by TLC that the ascarosides of four nematodes from the order Ascaradida were comparable and were based on the same glycon. He concluded that they differed only in the relative proportions of the three types of ascarosides and in the their aliphatic chain lengths. No structural studies were carried out in this work, however, so the evidence is somewhat circumstantial. Unfortunately, this and all subsequent studies, have centered on the pig nematode, *A. lumbricoides*, so no direct comparison with *P. equorum* is possible. Our results, together with those of Tarr[15] and Fouquey *et al.*,[14] do suggest, however, that the glycon structure is preserved between species and that the C-2, C-(ω-1) substitution of the aglycon is at least consistent in nematodes of the pig.

Experimental Section

General Experimental Procedures. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Unity spectrometer (Varian, Palo Alto) at 7.02 tesla or on a Bruker AMX spectrometer (Bruker, Karlsruhe) at 11.7 tesla. Noise reduction was carried out when required using Aurelia software (Bruker, Karlsruhe). Samples were dissolved in $CDCl_3$ in 5-mm tubes, and 2D spectra were acquired without spinning at 37 °C. All chemical shifts are referenced to TMS as internal standard.

Animal Material. Adult female *Ascaris suum* were recovered from the small intestines of pigs slaughtered for human consumption at three abattoirs (Zwickers Smallgoods, Kingaroy, Toowoomba Abattoir, and Bris-

bane Central Abattoir). Species identification was confirmed by Prof. John Welsh, parasitologist in the School of Life Science, Queensland University of Technology, and a voucher specimen is lodged in his collection (AS0511993).

Extraction and Isolation. Suitable nematodes (172 g, wet wt) were washed in tap water, placed in 1 L of phosphate-buffered saline and maintained at 4 °C for a maximum period of 24 h before dissection. Each female nematode was dissected under a solution of 0.05 M H_2SO_4 to separate the reproductive organs from the rest of the tissues. These were homogenized in a Waring blender in 50 mL of $CHCl_3$−MeOH (2:1), and extraction was continued for 24 h. The suspension was then filtered (Whatman 541 paper) to remove larger particulate matter and evaporated under vacuum. The dried extract of the reproductive tract was redissolved in 200 mL of 2% methanolic NaOH and heated at 100 °C in a H_2O bath for 30 min. The volume was then reduced by heating under a stream of nitrogen and dissolved in 200 mL of distilled H_2O. This was heated to 100 °C for 30 min and then cooled to room temperature. $CHCl_3$ (200 mL) was then added and the solution extracted with occasional shaking for 24 h. The $CHCl_3$ phase, containing the unsaponifiable lipids (including the ascarosides) was then removed from the aqueous phase, washed with an aqueous 2 M NH_3, and evaporated under vacuum. The unsaponifiable residue was dissolved in 10 mL of 2:1 $CHCl_3$−MeOH.

Purification of the crude extract was accomplished by TLC (Riedel de Haen 37587 Si gel $60F_{254}$ special plates, 200 × 100 mm, 0.25-mm film thickness) developed with $CHCl_3$−MeOH (95:5). The ascarosides were located as pink/violet bands with modified Molisch reagent (50 mL of 1.0 M H_2SO_4 and 1 mL of 5% EtOH α-naphthol) sprayed onto a thin strip cut from the plate and heated for 1 h at 100 °C.[16] Once the ascaroside bands had been located, the remainder of the plate was sprayed with 0.5% dichlorfluorescein in EtOH, and the bands were identified by their yellow fluorescence under UV light. The regions corresponding to the ascaroside bands were removed and extracted with 2 mL $CHCl_3$−MeOH (2:1). The dichlorfluorescein was removed by washing with 1% solution of $NaHCO_3$ until the nonaqueous phase showed no sign of fluorescence (normally 10 extractions of 5 mL each). The non-aqueous phase was then dried under vacuum, and the purity of each ascaroside fraction was checked using analytical-scale TLC.

Liquid Chromatography−Mass Spectrometry. HPLC−MS was carried out on a VG Quattro II triple quadrupole mass spectrometer (Fisons Instruments) interfaced to a Hewlett-Packard HP1050 liquid chromatograph equipped with a PhaseSep Spherisorb S ODS2 column (250 × 46 mm). The chromatographic conditions were as follows: the elution solvent was MeOH; flow rate, 1 mL min^{-1}; injection volume, 20 μL. The eluent from the column was split before entering the mass spectrometer to give a flow of 150 μL min^{-1} through the electrospray probe into the atmospheric pressure ionization source. N_2 was used as both the nebulizing gas (80 L h^{-1}) and as the drying gas (400 L h^{-1}), and the source temperature was 100 °C. The cone voltage of 25 V was chosen by optimizing to minimize fragmentation and to produce maximal intensity in the molecular ion region. For calibration of the mass scale in the positive ion mode, a mixture of polyethylene glycols 300/600/1000 was used, with NH_4OAc (20 mM) to optimize the production of ammoniated adducts at the expense of sodiated and protonated species. Calibration in the negative ion mode was achieved using a sugar mixture consisting of raffinose, maltose, and corn syrup. MS were collected at a scan rate of 200 amu s^{-1} from *m/z* 100−600 amu in both positive and negative ion modes, and data were acquired in multi-channel analysis mode onto a DEC 486 computer using MassLynx software (VG Biotech). MS−MS was carried out by selection of the parent ion in the first quadrupole and passing the selected ion through a hexapole collision cell (160-mm long) operating in the RF only mode and pressurized with argon (0.38 Pa). A collision energy of 30 eV was used.

Compounds 6 and 7: fraction 1 (14.7 mg, 0.009%) containing a mixture of compound **6**, elution time 19.6 min; ESI−MS (positive) *m/z* (rel int) 407 $[M + H - C_6H_{10}O_3 - H_2O]^+$ (18), 555 $[M + H]^+$ (35), 572 $[M + NH_4]^+$ (100), 577 $[M + Na]^+$ (52); ESI−MS (negative) *m/z* (rel int) 553 $[M - H]^-$ (10), 589 $[M + Cl]^-$ (25), 613 $[M + OAc]^-$ (100); ^1H NMR ($CDCl_3$, 300 MHz) δ 4.69 (1H, d, *J* = 1 Hz, H-1′), 3.80 (1H, m, H-2), 3.70 (1H, dd, *J* = 9, 6 Hz, H-5′), 3.79 (1H, m, H-2′), 3.60 (1H, ddd, *J* = 4.5, 10.5, 9 Hz, H-4′), 1.58 (1H, m, H3a) 1.42 (1H, m, H-3b), 1.86 (1H, ddd, *J* = 13, 10.5, 3 Hz, H-3′a), 2.06 (1H, ddd, *J* = 13, 4, 4.5 Hz, H-3′e), 1.27 (nH, brs, H-5−H-27), 1.42 and 1.32 (2H, m, H-4,), 1.20 (2H, m, H-28), 1.10 (3H, d, *J* = 7 Hz, H-1), 1.28 (3H, d, *J* = 6 Hz, H-6′), 0.85 (3H, d, *J* = 7 Hz, H-29); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 96.4 (d, C-1′), 71.9 (d, C-2), 69.9 (d, C-5′), 69.4 (d, C-2′), 68.19 (d, C-4′), 37.25 (t, C-3), 35.34 (t, C-3′), 31.92 (t, C-27), 29.67 (t, C-5-C26), 25.73 (t, C4), 22.69 (t, C-28), 18.99 (q, C-1), 17.63 (q, C-6′).14.10 (q, C-29); and compound **7**, elution time 21.5 min; ESI−MS (positive) *m/z* (rel int) 421 $[M + H - C_6H_{10}O_3 - H_2O]^+$ (20), 569 $[M + H]^+$ (10), 586 $[M + NH_4]^+$ (100), 591 $[M + Na]^+$ (90); ESI−MS (negative) *m/z* (rel int) 567 $[M - H]^-$ (10), 603 $[M + Cl]^-$ (30), 627 $[M + OAc]^-$ (100); ^1H NMR ($CDCl_3$, 300 MHz) δ 4.69 (1H, d, *J* = 1 Hz, H-1′), 3.80 (1H, m, H-2), 3.70 (1H, dd, *J* = 9, 6 Hz, H-5′), 3.79 (1H, m, H-2′), 3.60 (1H, ddd, *J* = 4.5, 10.5, 9 Hz, H-4′), 1.58 (1H, m, H3a) 1.42 (1H, m, H-3b), 1.86 (1H, ddd, *J* = 13, 10.5, 3 Hz, H-3′a), 2.06 (1H, ddd, *J* = 13, 4, 4.5 Hz, H-3′e), 1.27 (nH, brs, H-5−H-27), 1.42 and 1.32 (2H, m, H-4,), 1.50 (1H, m, H-28), 1.10 (3H, d, *J* = 7 Hz, H-1), 1.28 (3H, d, *J* = 6 Hz, H-6′), 0.87 (6H, t, *J* = 7 Hz, H-29, H30); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 96.4 (d, C-1′), 71.9 (d, C-2), 69.9 (d, C-5′), 69.4 (d, C-2′), 68.19 (d, C-4′), 37.25 (t, C-3), 35.34 (t, C-3′), 29.67 (t, C-5-C26), 29.51 (t, C-27), 28.20 (d, C-28), 25.73 (t, C4), 18.99 (q, C-1), 17.63 (q, C-6′).14.11 (q, C-29, C-30).

Compounds 4 and 5: fraction 2 (89.3 mg, 0.052%) containing a mixture of compound **4**, elution time 9.0 min; ESI−MS (positive) *m/z* (rel int) 433 $[M + H - C_6H_{10}O_3 - 2\ H_2O]^+$ (100), 469 $[M + H - C_6H_{10}O_3]^+$ (60), 599 $[M + H]^+$ (12), 616 $[M + NH_4]^+$ (12), 621 $[M + Na]^+$ (28); ESI−MS (negative) *m/z* (rel int) 597 $[M - H]^-$ (25), 633 $[M + Cl]^-$ (75), 657 $[M + OAc]^-$ (100); and compound **5**, elution time 11.5 min; ESI−MS (positive) *m/z* (rel int) 461 $[M + H - C_6H_{10}O_3 - 2\ H_2O]^+$ (100), 497 $[M + H - C_6H_{10}O_3]^+$ (52), 627 $[M + H]^+$ (11), 644 $[M + NH_4]^+$ (14), 649 $[M + Na]^+$ (20); ESI−MS (negative) *m/z* (rel int) 625 $[M - H]^-$ (25), 661 $[M + Cl]^-$

(70), 685 $[M + OAc]^-$ (100); the NMR data are reported for **4** only, but assignments for **5** are analogous; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.69 (1H, d, J = 1 Hz, H-1′), 3.80 (1H, m, H-2), 3.70 (1H, dd, J = 9, 6 Hz, H-5′), 3.79 (1H, m, H-2′), 3.80 (1H, m, H-30), 3.60 (1H, ddd, J = 4.5, 10.5, 9 Hz, H-4′), 1.42 (2H, m, H-29), 1.58 (1H, m, H3a) 1.42 (1H, m, H-3b), 1.86 (1H, ddd, J = 13, 10.5, 3 Hz, H-3′a), 2.06 (1H, ddd, J = 13, 4, 4.5 Hz, H-3′e), 1.27 (nH, brs, H-5−H-27), 1.42 and 1.32 (4H, m, H-4, H-28), 1.19 (3H, d, J = 7 Hz, H-31), 1.12 (3H, d, J = 7 Hz, H-1), 1.28 (3H, d, J = 6 Hz, H-6′); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 96.4 (d, C-1′), 71.9 (d, C-2), 69.9 (d, C-5′), 69.4 (d, C-2′), 68.23 (d, C-30), 68.19 (d, C-4′), 39.43 (t, C-29), 37.25 (t, C-3), 35.34 (t, C-3′), 29.67 (t, C-5 - C27), 25.73 (t, C4, C28), 23.43 (q, C-31), 18.99 (q, C-1), 17.63 (q, C-6′).

Compounds 8 and 9: fraction 3 (17.9 mg, 0.01%) containing a mixture of compound **8**, elution time 5.5 min; ESI−MS (positive) m/z (rel int) 729 $[M + H]^+$ (35), 746 $[M + NH_4]^+$ (100), 751 $[M + Na]^+$ (52); ESI−MS (negative) m/z (rel int) 727 $[M - H]^-$ (10), 763 $[M + Cl]^-$ (25), 787 $[M + OAc]^-$ (100); and compound **9**, elution time 6.0 min; ESI−MS (positive) m/z (rel int) 757 $[M + H]^+$ (10), 774 $[M + NH_4]^+$ (100), 779 $[M + Na]^+$ (90); ESI−MS (negative) m/z (rel int) 755 $[M - H]^-$ (10), 791 $[M + Cl]^-$ (30), 815 $[M + OAc]^-$ (100); the NMR data are reported for **8** only, but assignments for **9** are analogous; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.69 (1H, d, J = 1 Hz, H-1′), 3.80 (1H, m, H-2), 3.70 (1H, dd, J = 9, 6 Hz, H-5′), 3.79 (1H, m, H-2′), 3.60 (1H, ddd, J = 4.5, 10.5, 9 Hz, H-4′), 1.42 and 1.58 (4H, 2m, H-3, H-29), 1.86 (1H, ddd, J = 13, 10.5, 3 Hz, H-3′a), 2.06 (1H, ddd, J = 13, 4, 4.5 Hz, H-3′e), 1.27 (nH, brs, H-5−H-27), 1.42 and 1.32 (4H, m, H-4, H-28), 1.12 (3H, d, J = 7 Hz, H-1), 1.28 (3H, d, J = 6 Hz, H-6′); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 96.21 (d, C-1′, C-1″), 71.77 (d, C-2, C30), 69.81 (d, C-5′, C-5″), 69.39 (d, C-2′, C-2″), 68.25 (d, C-4′, C-4″), 37.21 (t, C-3, C-29), 35.34 (t, C-3′, C-3″), 29.61 (t, C-5-C27), 25.71 (t, C4, C28), 18.96 (q, C-1, C-31), 17.62 (q, C-6′, C-6″).

Molecular Mechanics. Calculations were carried out with Discover 95.0 (Biosym/MSI, San Diego) using the cff91 forcefield. The strategy involved short periods of dynamics (1ps, 1fs steps) from 900 °K and decreasing to 300 °K. Annealing was performed after each step by minimization for 1000 steps.

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