(br s, 2, $H_{4'}$), 3.68 (t, 2, $H_{2''}$), 1.95 (s, 3, 5-CH_3), 0.94 (t, 2, $H_{3''}$), 0.01 (s, 9, Me_3Si); ^{13}C NMR δ 194.22 ($C_{2'}$), 107.26, 99.91 ($C_{1'}$ + $C_{3'}$), 70.59, 67.72 ($C_{1''}$ + $C_{2''}$), 60.20 ($C_{4'}$), 18.25 ($C_{3''}$), −1.39 (CH_3, Me_3Si); thymine peaks 163.23, 149.91, 134.79, 111.57, 13.16 (CH_3). Anal. Calcd for $C_{15}H_{24}N_2O_4Si$: C, 55.52; H, 7.45; N, 8.63. Found: C, 55.74; H, 7.45; N, 8.52.

(±)-N^1-(4-Hydroxy-1,2-butadien-1-yl)thymine (Thymallene, 1d). $SnCl_4$ (0.26 g, 1 mmol) was added with stirring and external ice cooling to a solution of compound **21** (0.32 g, 1 mmol) in CH_2Cl_2 (15 mL). The stirring continued for 2 h whereupon 4% NaOH in methanol (2.5 mL) was added. After 15 min, the reaction mixture was filtered through silica gel, which was then washed with CH_2Cl_2–MeOH (3 × 15 mL). The filtrate was evaporated, and the crude product was chromatographed on a silica gel column in CH_2Cl_2–MeOH (95:5). The appropriate fraction was evaporated to give thymallene (**1d**, 87 mg, 45%): mp 173–175 °C after crystallization from ethyl acetate–ether (4:1); UV (pH 7) max 287 nm (ε 8000), 225 (8000); IR (KBr) 1965 cm^{-1} (C=C=C); ^1H NMR δ 7.36 (d, 1, H_6), 7.19 (qt, 1, $H_{1'}$), 6.18 (q, 1, $H_{3'}$), 5.08 (t, 1, OH), 4.07 (t, 2, $H_{4'}$), 1.79 (d, 3, CH_3); ^{13}C NMR 194.11 ($C_{2'}$), 108.40, 98.35 ($C_{1'}$ + $C_{3'}$), 59.56 ($C_{4'}$); thymine peaks 164.18, 149.58, 136.51, 111.22, 12.54 (CH_3); EI-MS 194 (M, 26.0). Anal. Calcd for $C_9H_{10}N_2O_3$: C, 55.66; H, 5.19; N, 14.42. Found: C, 55.70; H, 5.35; N, 14.65.

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Registry No. **1d**, 120991-13-1; **3d**, 120991-05-1; **4**, 7288-28-0; **5**, 120991-04-0; **6**, 65-71-4; **7**, 120991-03-9; **8**, 40778-70-9; **9d**, 120991-06-2; **11**, 120991-07-3; **12**, 94426-69-4; **16**, 120991-08-4; **17**, 120991-09-5; **19**, 120991-10-8; **20**, 120991-11-9; **21**, 120991-12-0; 2′,3′-dideoxythymidine, 3416-05-5; 1,4-dichloro-2-butyne, 821-10-3.

Diosgenin-Bearing, Molluscicidal Saponins from *Allium vineale*: An NMR Approach for the Structural Assignment of Oligosaccharide Units

Shaoxing Chen and John K. Snyder*

Department of Chemistry, Boston University, 590 Commonwealth Ave., Boston, Massachusetts 02215

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An investigation into the molluscicidal substituents of field garlic, *Allium vineale*, has resulted in the isolation and identification of seven new saponins with up to six sugars. The isolations were accomplished by using a combination of countercurrent and adsorption chromatography. The aglycon of five of these natural products was shown to be diosgenin while the remaining two saponins bore nuatigenin and isonuatigenin aglycons, respectively. Extensive one- and two-dimensional NMR experiments were utilized to assign the structures.

Introduction

A great variety of *Allium* species (Liliaceae) have long been used in traditional medicines throughout the world for a great variety of medicinal purposes.[1] While the sulfur-bearing natural products of *Allium* spp. are well known for their biological activities,[2] interest in the saponin content increased during World War II as part of the search for alternative sources of diosgenin.[3] The important biological activities of saponins[4] are of increasing interest as recently illustrated by their potential value as antifungal agents.[4,5] A serious difficulty in assigning the structures of saponins is identifying the oligosaccharide unit.[4,6] In this paper we describe the isolation and structure assignment of nine saponins from field garlic, *Allium vineale*, seven of which were previously unreported.[7] Five of the new saponins have diosgenin as the aglycon, while the remaining two new natural products bear nuatigenin and isonuatigenin, respectively. The general approach to identifying the oligosaccharide units relies almost entirely on one- and two-dimensional NMR methods, supported by analyses of the mass spectral fragmentation patterns using negative ion FAB.

Results and Discussion

Isolation. Whole plants were divided into three parts (bulbs, stems and leaves, and flowers), extracted, and screened for activity. The methanol extract of the bulbs, which showed molluscicidal activity against the South American snail *Biomphalaria pfeifferei*,[8] was partitioned between water and ethyl acetate, and the active, aqueous fraction subsequently partitioned between water and 1-

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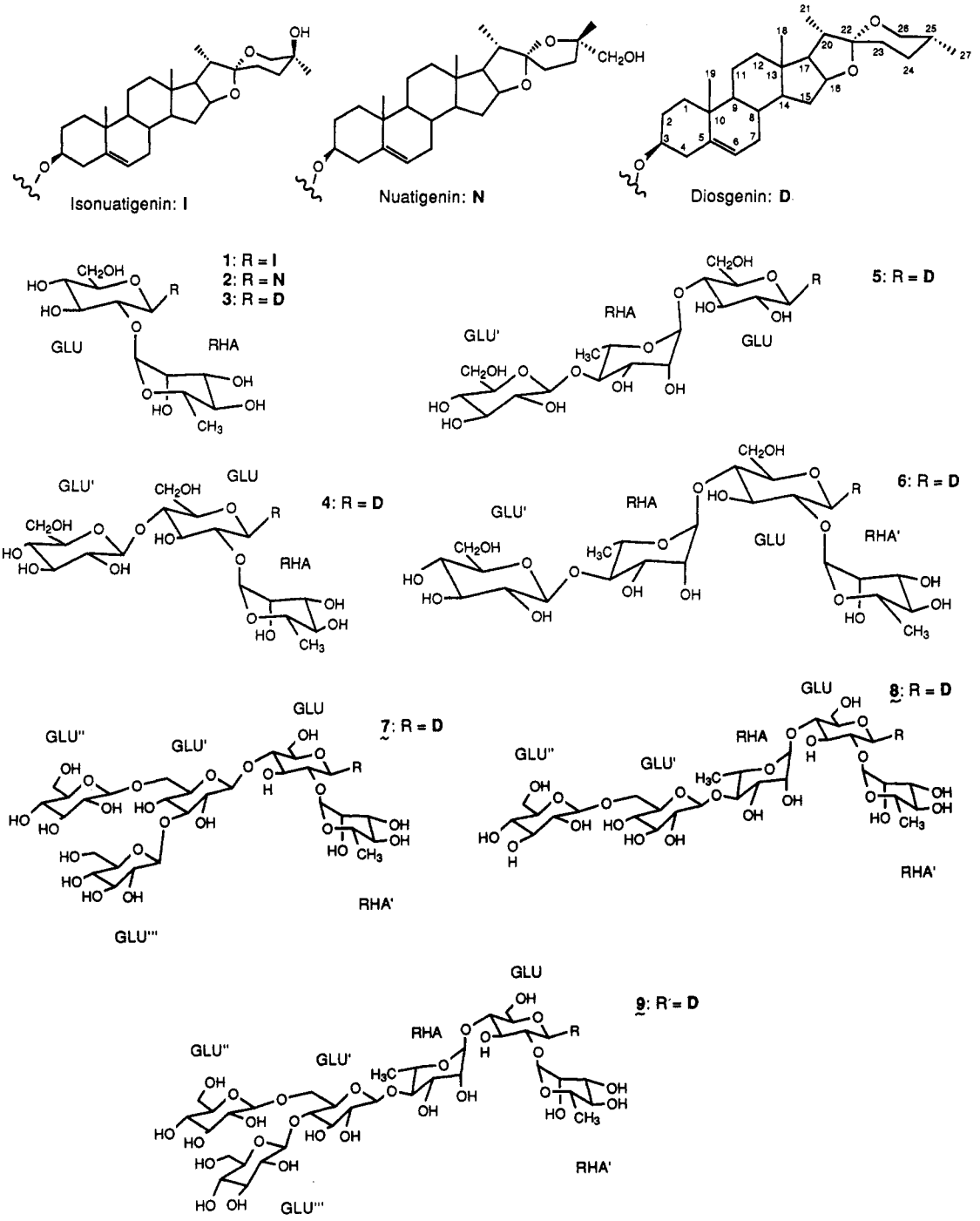
Chart I



butanol. The more active 1-butanol fraction was further fractionated by using rotation locular countercurrent chromatography (RLCC)[9] and then flash silica gel chromatography; saponins **1–6** (Chart I) were subsequently separated by reverse-phase HPLC (C_{18}). Saponins **7–9** (Chart I) could not be easily resolved by reverse-phase HPLC, but were separated into two mixtures containing the five and six sugar saponins, respectively (**7** + **8**, and **9** + minor saponins), by using droplet countercurrent chromatography.[10] Reverse-phase HPLC (C_{18}) resolved the saponins of each of these mixtures. While the aqueous fraction was also molluscicidal, the active components were ultimately shown to be the same saponins predominant in the 1-butanol fraction.

General Strategy for Structure Determination: Structure of 9. Examination of the ^{13}C NMR data, Table I, revealed that the aglycon of seven of the saponins, **3–9**, was diosgenin and the aglycons of **1** and **2** were isonuatigenin and nuatigenin, respectively.[6b,11] Two of the diosgenin saponins, **3** and **4**, proved to be identical with those previously isolated from *Ophiopogon planiscapus*, also of the Liliaceae family, upon comparison of the ^{13}C chemical shifts.[12] The number of sugars in each of the

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(11) While we cannot rule out the possibility of equilibration between **1** and **2** during isolation, we believe both to be natural products. See ref 6a, also: Tschesche, R.; Richert, K. H. *Tetrahedron* **1964**, *20*, 387.

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Table I. ^{13}C NMR Chemical Shifts for Aglycons 1–9[a]

sapogenin	diosgenin[b]	nuatigenin	isonuatigenin
C-1	37.4	37.5	37.8
C-2	30.1	30.2	30.2
C-3	78.2	78.3	78.3
C-4	38.9	40.5	39.0
C-5	140.7	140.8	140.8
C-6	121.7	120.2	121.7
C-7	32.2	32.2	32.3
C-8	31.7	31.6	31.7
C-9	50.2	50.2	50.4
C-10	37.0	37.1	37.0
C-11	21.0	21.1	21.2
C-12	39.8	38.9	40.0
C-13	40.4	39.8	40.5
C-14	56.5	56.5	56.8
C-15	32.0	32.3	32.2
C-16	81.0	80.8	81.4
C-17	62.7	62.6	63.0
C-18	16.3	16.1	16.4
C-19	19.4	19.4	19.6
C-20	41.9	38.4	42.0
C-21	15.0	15.2	15.2
C-22	109.3	121.7	109.6
C-23	31.6	32.6	27.9
C-24	29.2	33.8	33.8
C-25	30.4	85.6	65.9
C-26	66.8	70.1	69.8
C-27	17.3	24.1	27.0

[a] Spectra were recorded in pyridine-d_5 with one drop of D_2O at ambient temperature. All samples were exchanged with D_2O prior to recording the spectra. [b] Average values from **3**–**9** were used and the differences were within 0.7 ppm.

remaining saponins was determined by counting the resonances for anomeric carbons in the ^{13}C NMR spectra (99–107 ppm for these compounds), Table II, ultimately confirmed by the mass spectra. A HETCOR spectrum then enabled the one-bond correlation between the anomeric protons and carbons to be determined.

With the aglycons identified and the number of sugars established for each saponin, completion of the structure assignment required identifying the oligosaccharide units. Thus the individual sugars had to be identified, sequenced, and the linkage sites established. Since only a limited amount of material was available, we sought to accomplish this using spectroscopic techniques, particularly NMR. In order to minimize signal overlap in the ^1H NMR spectra, the hydroxyl protons were exchanged for deuterium prior to recording the spectra in pyridine-d_5. In addition, D_2O was added (one or two drops) to the NMR sample in order to eliminate any residual hydroxyl proton resonances, with the appearance of an HOD signal which did not overlap with any other resonances. The amount of D_2O added was adjusted to optimize signal dispersion; too much D_2O caused precipitation of the sample. The presence of rhamnose in the ^1H spectrum of each saponin was indicated by the characteristic methyl doublet (**9**: δ 1.61 and 1.76, J = 6.2 Hz for Rha and Rha′, respectively) and broad singlet of the anomeric proton (**9**: δ 5.79 and 6.38, $J_{1,2}$ = 1.3 and 1.0 Hz for Rha and Rha′, respectively). Other proton resonances useful in structural studies for all saponins are listed in Table III.

Assigning the saccharide structures was accomplished by a combination of techniques. The proton spin systems for each individual sugar was delineated and the exact frequencies for most nonexchangeable sugar proton chemical shifts assigned by using double quantum filtered,

Table II. ^{13}C NMR Chemical Shifts for Oligosaccharide Units 1–9[a]

		1	2	3	4	5	6	7	8	9
Glu	C-1	100.3	100.3	100.4	100.0	101.9	99.9	99.6	99.9	100.3
	C-2	79.6	79.7	79.7	78.3	76.5	78.7	77.3	78.1	78.1
	C-3	77.9	77.9	78.0	76.3	77.2	77.0	76.2	76.9	77.7
	C-4	71.8	71.8	71.9	81.8	78.6	77.9	80.5	77.5	78.1
	C-5	77.8	77.8	77.9	77.5	76.7	76.3	77.1	75.8	77.0
	C-6	62.6	62.5	62.7	61.7	62.5	60.9	60.9	62.2	61.4
Glu′	C-1				105.1	106.7	105.1	103.7	105.5	106.2
	C-2				75.1	75.6	75.7	73.0	74.8	75.6
	C-3				78.2	78.2	77.5	88.2	77.8	76.5
	C-4				71.3	71.4	70.8	69.0	71.3	81.2
	C-5				77.7	77.4	77.5	77.7	76.6	75.3
	C-6				61.7	61.4	61.9	69.5	69.7	68.6
Glu″	C-1							104.8	105.1	105.4
	C-2							74.6	74.8	75.1
	C-3							77.2	77.7	78.2
	C-4							71.1	71.3	71.6
	C-5							75.7	78.0	78.2
	C-6							61.9	60.9	62.4
Glu‴	C-1							104.0		104.8
	C-2							74.7		75.2
	C-3							77.2		78.3
	C-4							70.9		71.8
	C-5							76.9		78.1
	C-6							62.2		62.3
Rha	C-1	102.1	102.1	102.1	101.7	102.5	101.8	101.5	101.8	102.0
	C-2	72.6	72.6	72.7	72.4	72.1	71.7	71.8	71.6	71.9
	C-3	72.8	72.9	72.9	72.9	72.7	71.7	72.1	71.9	72.8
	C-4	74.2	74.2	74.3	74.3	85.2	83.0	73.4	83.7	84.9
	C-5	69.5	69.5	69.6	69.4	68.5	68.6	69.2	68.4	68.5
	C-6	18.7	18.7	18.9	18.6	18.4	18.0	18.2	18.4	18.6
Rha′	C-1						102.0		101.9	102.2
	C-2						71.4		71.0	72.4
	C-3						72.1		72.3	72.4
	C-4						73.2		73.7	74.0
	C-5						69.5		69.4	69.8
	C-6						18.2		18.3	18.8

[a] Spectra were recorded by pyridine-d_5 with one drop of D_2O at ambient temperature. All samples were exchanged with D_2O prior to recording the spectra.

Table III. Useful ^1H NMR Chemical Shifts 1-9[a]

	1	2	3	4	5
aglycon, vinyl	5.13 (d, 3.2)	5.29 (d, 3.3)	5.21 (d, 3.2)	5.27 (d, 3.1)	5.27 (d, 3.0)
methyl	1.22, 1.17 (d, 6.8), 1.03, 0.82	1.37, 1.10 (d, 6.9), 1.04, 0.80	1.12 (d, 6.8), 1.02, 0.80, 0.67 (d, 4.4)	1.13 (d, 6.8), 1.03, 0.81, 0.68 (d, 3.8)	1.09 (d, 6.8), 0.84, 0.77, 0.64 (d, 4.5)
Glu H-1	5.01 (d, 7.3)	5.03 (d, 7.3)	5.00 (d, 7.3)	4.95 (d, 7.0)	4.94 (d, 7.8)
H-2	4.25 (o)[b]	4.30 (o)			3.93 (t, 8.0)
H-3	4.25 (o)	4.30 (o)			4.15 (o)
H-4	4.13 (t, 9.4)	4.17 (t, 9.4)			4.35 (t, 9.7)
H-5	3.90 (o)	3.85 (o)			3.63 (m)
H-6	4.48 (d, 11.4), 4.29 (o)	4.51 (d, 11.4), 4.30 (o)			4.32, 4.05 (o)
Glu′ H-1				5.04 (d, 7.8)	5.20 (d, 7.8)
H-2					4.05 (o)
H-3					4.13 (o)
H-4					4.18 (o)
H-5					3.71 (m)
H-6					4.24 (m)
Rha H-1	6.37	6.38	6.34	6.25	5.79
H-2	4.79 (br)	4.82 (br)			4.51
H-3	4.60 (dd, 9.2, 3.2)	4.63 (dd, 9.2, 3.2)			4.63 (d, 8.7)
H-4	4.34 (t, 9.2)	4.37 (t, 9.2)			4.42 (t, 8.7)
H-5	4.96 (o)	5.00 (o)			4.93 (m)
H-6	1.75 (d, 6.2)	1.78 (d, 6.2)	1.75 (d, 6.1)	1.76 (d, 6.2)	1.69 (d, 6.0)

	6	7	8	9
aglycon, vinyl	5.33 (d, 3.4)	5.23 (d, 3.1)	5.28 (d, 2.8)	5.26 (d, 3.3)
methyl	1.13 (d, 5.8), 0.97, 0.79, 0.69 (d, 4.6)	1.06 (d, 6.8), 0.93, 0.74, 0.61 (d, 4.4)	1.08 (d, 6.8), 0.97, 0.76, 0.63 (d, 4.5)	1.13 (d, 6.9), 1.04, 0.82, 0.69 (d, 4.7)
Glu H-1	4.65 (d, 5.3)	4.82 (d, 6.0)	4.88 (d, 5.5)	4.93 (d, 5.4)
H-2	3.85 (o)	4.02 (o)	4.08 (o)	4.15 (o)
H-3	3.85 (o)	4.05 (o)	4.09 (o)	4.12 (o)
H-4	3.98 (o)	4.08 (o)	4.27 (o)	4.36 (o)
H-5	3.41 (d, 8.8)	3.71 (o)	3.54 (o)	3.50 (o)
H-6	4.36, 4.52 (o)	4.38 (o)	4.10, 4.25 (o)	4.13, 3.97 (o)
Glu′ H-1	4.99 (d, 7.9)	5.01 (d, 8.0)	5.14 (d, 7.8)	5.11 (d, 7.9)
H-2	3.57 (t, 8.0)	3.89 (o)	3.92 (o)	3.85 (o)
H-3	3.96 (o)	3.99 (o)	4.04 (o)	4.12 (o)
H-4	3.84 (o)	3.96 (o)	4.10 (o)	4.50 (o)
H-5	3.71 (m)	3.77 (o)	3.77 (o)	3.73 (o)
H-6	3.86, 3.96 (o)	3.92, 4.76 (o)	4.56, 4.19 (o)	4.56 (o)
Glu″ H-1		5.04 (d, 7.9)	4.96 (d, 7.9)	5.41 (d, 7.8)
H-2		3.89 (o)	3.93 (o)	4.00 (o)
H-3		4.15 (o)	4.15 (o)	4.23 (o)
H-4		3.89 (o)	4.08 (o)	4.14 (o)
H-5		4.00 (o)	3.80 (o)	3.99 (o)
H-6		4.27, 4.10 (o)	4.40, 4.23 (o)	4.41, 4.21 (o)
Glu‴ H-1		4.78 (d, 7.2)		5.56 (d, 7.9)
H-2		3.89 (o)		4.04 (o)
H-3		4.21 (o)		4.34 (o)
H-4		4.05 (o)		4.18 (o)
H-5		3.78 (o)		4.18 (o)
H-6		4.18, 4.38 (o)		4.41, 4.21 (o)
Rha H-1	5.36	6.05	5.72	5.76
H-2	4.37	4.64	4.57	4.54
H-3	4.37 (o)	4.45 (o)	4.56 (o)	4.54 (o)
H-4	4.16 (t, 8.9)	4.24 (o)	4.40 (o)	4.27 (o)
H-5	4.53 (o)	4.76 (o)	4.86 (o)	4.89 (o)
H-6	1.58 (d, 6.0)	1.66 (d, 5.9)	1.68 (d, 6.4)	1.61 (d, 6.2)
Rha′ H-1	5.83		6.25	6.30
H-2	4.60		4.78	4.80
H-3	4.31 (d, 10.0)		4.56 (o)	4.55 (o)
H-4	4.10 (t, 10.0)		4.30 (o)	4.26 (o)
H-5	4.58 (m)		4.87 (o)	4.85 (o)
H-6	1.60 (d, 5.9)		1.70 (d, 6.7)	1.76 (d, 6.2)

[a] Spectra were recorded in pyridine-d_5 with one drop of D_2O at ambient temperature. All samples were exchanged with D_2O prior to recording the spectra. [b] Overlapped signals are indicated by "(o)".

phase-sensitive COSY (DQCOSY) and relayed coherence transfer (RCT)[13] spectra with up to three relays. The approach is illustrated for the most complex of the saponins, **9**, in Figure 1. Beginning from the glucose anomeric protons, the glucose 2-position protons were easily assigned by the cross peaks in the DQCOSY spectrum. With the exception of proton Glu-H-3, assignment of the 3-position protons of the glucose residues were also discernable from cross peaks in the DQCOSY spectrum and by cross-sections in the COSY spectrum.[14] The assignments of these glucose 3-position resonances were confirmed by cross peaks in the single RCT spectrum to the anomeric protons (Figure 1B).

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(14) Though of lower resolution, we found it easier to use the absolute value COSY spectrum for examination of cross-sections rather than the phase-sensitive spectrum due to null points in the latter between the positive and negative signals of the cross peaks.

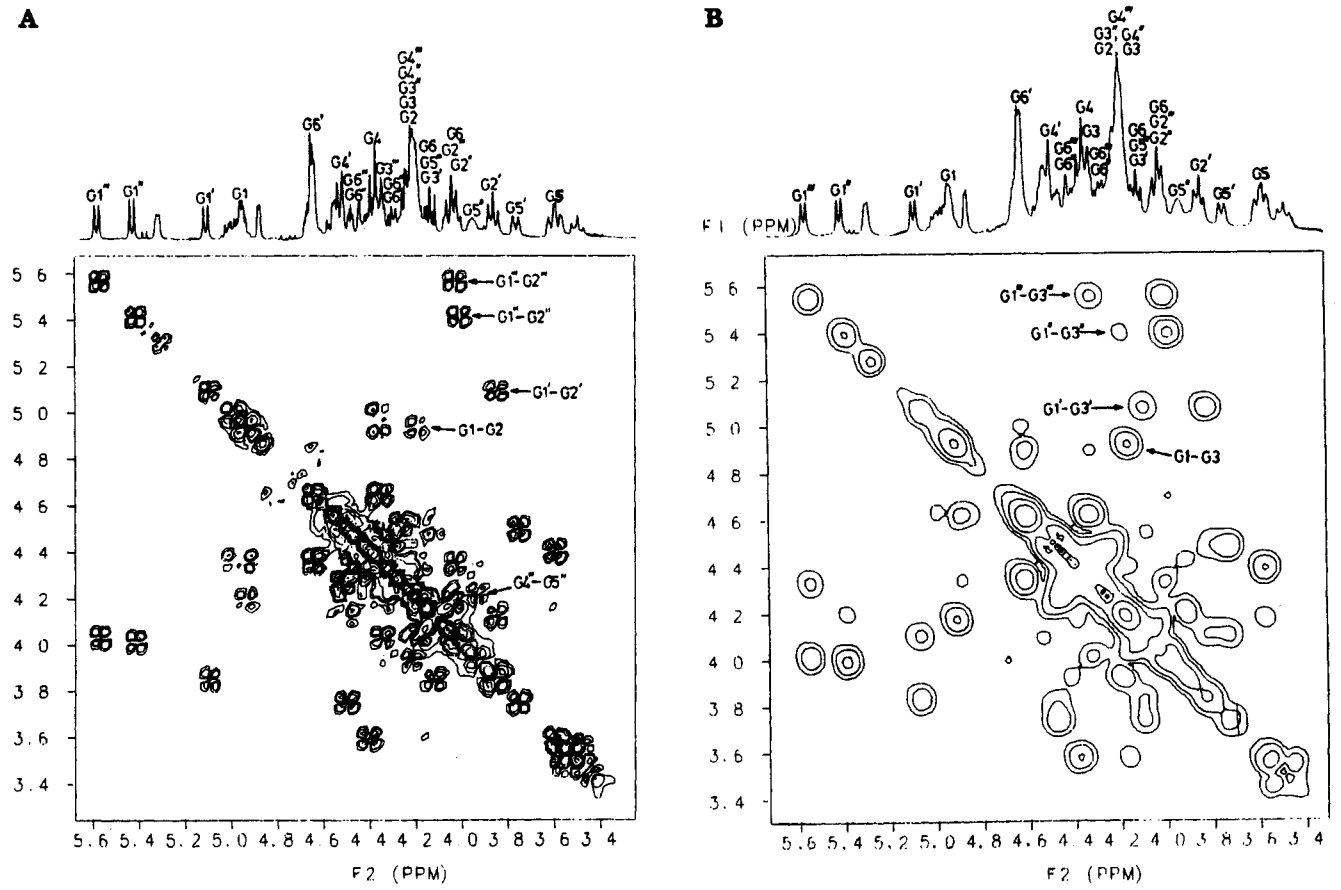

Figure 1. (A) Double quantum filtered, phase-sensitive COSY spectrum (DQCOSY) of saponin **9** with coherence between glucose anomeric and 2-position protons as well as coherence betwen Glu″-H-4 and Glu″-H-5 indicated by arrows. (B) Single relay coherence transfer spectrum (RCT) of saponin **9** with relayed coherence between glucose anomeric protons and 3-position protons indicated by arrows.

The sole exception to this simple approach using the DQCOSY and RCT spectra to assign the H-3 resonances occurred for the glucose residue attached to the aglycon (Glu) wherein Glu-H-2 and Glu-H-3 were overlapped. Furthermore, while coupling from anomeric Glu-H-1 to the 2-position proton (Glu-H-2) was observed in the COSY spectrum, it was apparent from the one-dimensional and homonuclear 2D J spectra that this coupling was somewhat smaller ($^3J_{H,H}$ = 5.4 Hz for Glu compared with $^3J_{H,H}$ = 7.8 Hz for the other glucose units) than anticipated for trans diaxial coupling in glucose. The anomeric proton was confirmed to be axially oriented by its heteronuclear one-bond coupling to the anomeric carbon ($^1J_{C,H}$ = 159.4 Hz).[15] A 2D NOE spectrum then revealed cross peaks between this anomeric proton and the 3- and 5-position protons (Glu-H-3 and Glu-H-5, respectively) (Figure 2A). One of these cross peaks appeared at the same location as assigned to Glu-H-2 by the DQCOSY experiment. That this peak was indeed due to an NOE between Glu-H-1 and Glu-H-3 (rather than Glu-H-5) was strongly supported by the lack of a new cross peak in the single RCT spectrum corresponding to relayed coherence between Glu-H-3 and Glu-H-1, since such a cross peak would be overlapped by the original coherence transfer between Glu-H-1 and Glu-H-2, which was not relayed. This was ultimately confirmed in the triple RCT experiment (Figure 3) by the cross peak due to Glu-H-1 to Glu-H-5 relayed coherence at the same frequency as the Glu-H-1/Glu-H-5 NOE cross peak (Figure 2A). The assignment of Glu-H-3 by the 2D NOE spectrum thus continued the mapping of the Glu spin system.

In a similar manner, assignments of the 3-position protons of the other glucose units were confirmed from the 2D NOE spectrum via dipolar couplings to the intraresidue anomeric protons (Table IV). The reduced coupling between Glu-H-1 and Glu-H-2 is most likely due to flattening of the pyranose ring attached to the aglycon. Indeed, this proved to be a common feature of all the saponins with the glucose attached to the aglycon as a branching sugar.

With assignment of Glu-H-3, mapping of the glucose spin systems continued to the 4-position protons. This was easily accomplished from the DQCOSY spectrum except for the Glu″-H-3, which was severely overlapped (δ 4.28). The location of the 4-position resonances of the Glu′ and Glu‴ units were confirmed by a double relay RCT spectrum wherein cross peaks between these respective 4-position protons and the corresponding anomeric protons appeared (Figure 2B). Of note in this double relay RCT spectrum was a lack of a new cross peak from the Glu″-H-1 of double relayed coherence to Glu″-H-4. The inference was that the Glu″-H-4 signal was overlapped by either Glu″-H-3 or Glu″-H-2. That the former possibility was correct (the Glu″-H-4 resonance was in the overlapped position also bearing the Glu″-H-3 signal, δ 4.28) was concluded by assigning the Glu″-H-5 signal (δ 3.91) from the 2D NOE spectrum, Figure 2B, via 1,3-diaxial dipolar coupling to the anomeric Glu″-H-1. With Glu″-H-5 located, the DQCOSY spectrum, Figure 1A, revealed the position of Glu″-H-4.

The 5-position protons of the glucose units were most easily assigned by the 2D NOE cross peaks showing 1,3-

(15) Bock, K.; Pedersen, C. *J. Chem. Soc., Perkin Trans. 2* **1974**, 293.

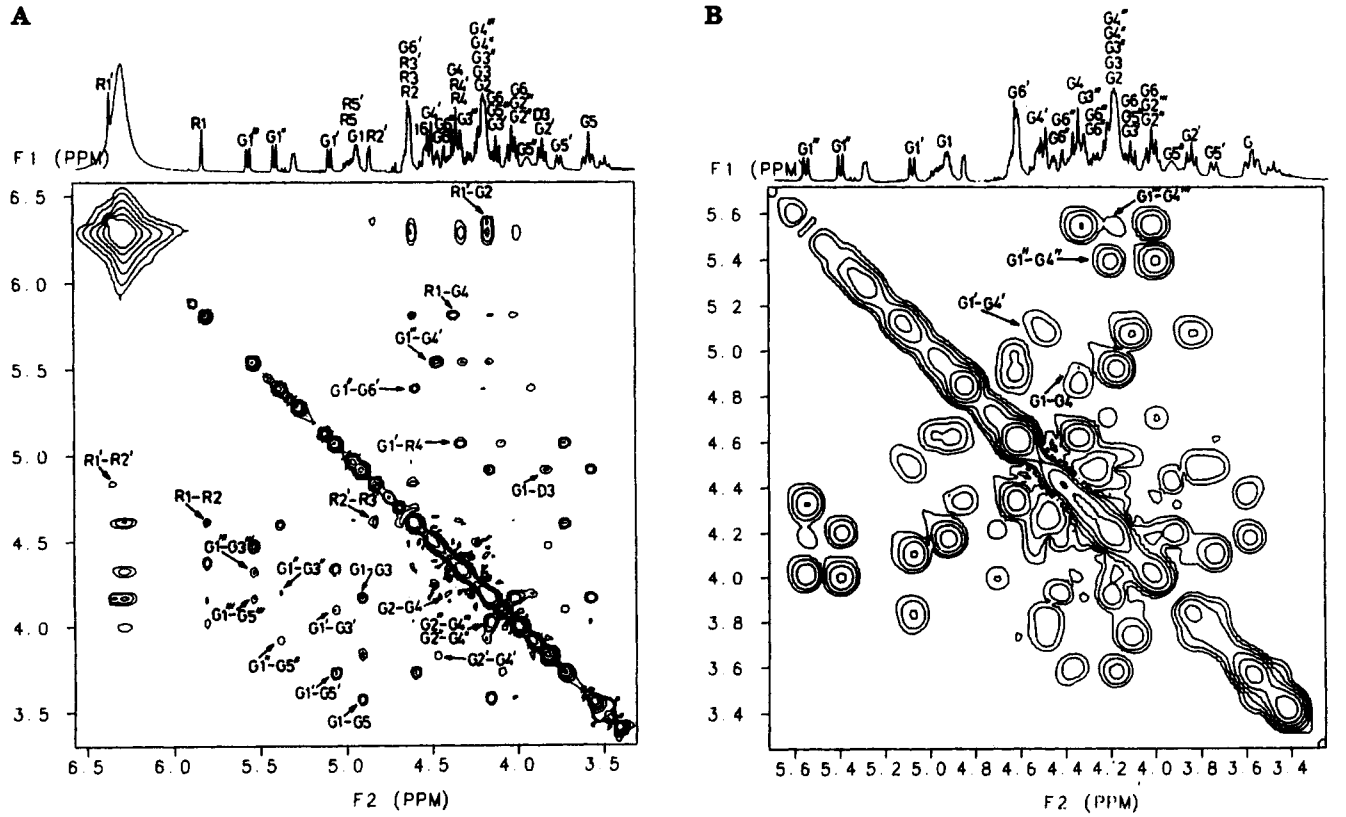

Figure 2. (A) Two-dimensional NOE spectrum (2D NOE) of saponin 9 with interresidue NOE's (those which cross the glycosidic bonds) labeled above the diagonal, intraresidue NOE's labeled below the diagonal. (B) Double relay coherence transfer spectrum (double RCT) of saponin 9 with relayed coherence between glucose anomeric protons and 4-position protons indicated by arrows.

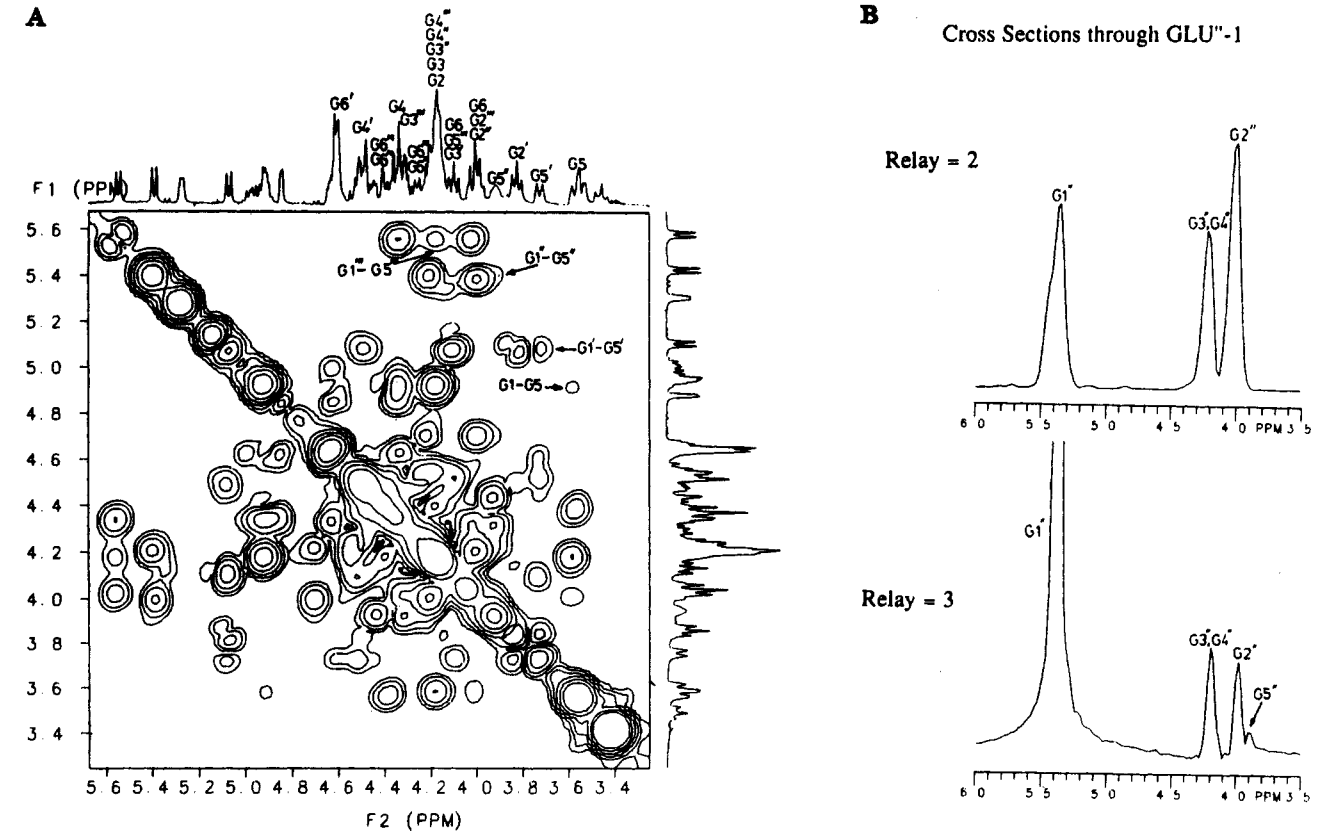

Figure 3. (A) Triple relay coherence transfer spectrum (triple RCT) of saponin 9 with relayed coherence between glucose anomeric protons and 5-position protons indicated by arrows. (B) Cross-sections at the resonance frequency of Glu''-H-1 in the double RCT and triple RCT spectra. Peak lebeled G1'' is the diagonal peak, other peaks are the coherence transfer and relayed coherence signals with Glu''-H-1 as indicated.

Table IV. NOE's of Protons of Oligosaccharide Units in 5–9[a]

saponin		interresidue NOE	intraresidue NOE
5	Glu H-1	Dio H-3	Glu H-3, Glu H-5
	Glu′ H-1	Rha H-4	Glu′ H-3, Glu′ H-5
	Glu H-2		Glu H-4
	Glu′ H-2		Glu′ H-4
	Rha H-1	Glu H-4	Rha H-2
6	Glu H-1	Dio H-3	Glu H-3, Glu H-5
	Glu′ H-1	Rha H-4	Glu′ H-3, Glu′ H-5
	Glu H-2		Glu H-4
	Glu′ H-2		Glu′ H-4
	Rha H-1	Glu H-4	Rha H-2
	Rha′ H-1	Glu H-2	Rha′ H-2
7	Glu H-1	Dio H-3	Glu H-3, Glu H-5
	Glu′ H-1	Glu H-4	Glu′ H-3, Glu′ H-5
	Glu″ H-1	Glu′ H-6	Glu″ H-3, Glu″ H-5
	Glu‴ H-1	Glu′ H-3	Glu‴ H-3, Glu‴ H-5
	Glu H-2		Glu H-4
	Glu′ H-2		Glu′ H-4
	Glu″ H-2		Glu″ H-4
	Glu‴ H-2		Glu‴ H-4
	Rha H-1	Glu H-2	Rha H-2
8	Glu H-1	Dio H-3	Glu H-3, Glu H-5
	Glu′ H-1	Rha H-4	Glu′ H-3, Glu′ H-5
	Glu″ H-1[b]	Glu′ H-6	Glu″ H-3, Glu″ H-5
	Glu H-2		Glu H-4
	Glu′ H-2		Glu′ H-4
	Glu″ H-2[b]		Glu″ H-4
	Rha H-1	Glu H-4	Rha H-2
	Rha′ H-1	Glu H-2	Rha′ H-2
9	Glu H-1	Dio H-3	Glu H-3, Glu H-5
	Glu′ H-1	Rha H-4	Glu′ H-3, Glu′ H-5
	Glu″ H-1	Glu′ H-6	Glu″ H-3, Glu″ H-5
	Glu‴ H-1	Glu′ H-4	Glu‴ H-3, Glu‴ H-5
	Glu H-2		Glu H-4
	Glu′ H-2		Glu′ H-4
	Glu″ H-2		Glu″ H-4
	Glu‴ H-2		Glu‴ H-4
	Rha H-1	Glu H-4	Rha H-2
	Rha′ H-1	Glu H-2	Rha′ H-2

[a] Spectra were recorded in pyridine-d_5 with one drop of D_2O at ambient temperature. All samples were exchanged with D_2O prior to recording the spectra. [b] NOE observed at 5 °C, but not at ambient temperature.

diaxial interactions with the intraresidue anomeric protons (Table IV). These assignments were corroborated by careful analysis of the DQCOSY, COSY (for cross-sections), single, double, and triple relay RCT spectra. For example, the assignment of Glu′-H-5 and Glu″-H-5 could be assigned directly from the triple relay RCT spectrum via cross peaks to their respective anomeric protons, Glu′-H-1 and Glu″-H-1. The cross peak to the latter anomeric proton appeared as a shoulder on the direct coherence transfer between Glu″-H-1 and Glu″-H-2 in the contour plot, Figure 3A, which was clearly resolved in the cross section (Figure 3B).

The 6-position protons of the glucose were most conveniently located from the HETCOR spectrum. The multiplicities of the carbons in the sugar region of the ^{13}C NMR spectrum (60–107 ppm) were established by using DEPT or an APT experiment.[16] Assignment of these proton resonances to individual sugars (and therefore the ^{13}C resonances as well) was then accomplished by careful examination of the DQCOSY and single, double, and triple relay RCT spectra.

(16) A DEPT experiment was used to assign the multiplicities of the saponin including the aglycon. When the assignments of the aglycon resonances are known, an APT experiment is sufficient to assign the sugar resonances since one is only concerned with distinguishing methylenes and methines.

Table V. T_1 Relaxation Times of 6–9[a]

	6 (at 30 °C)	7 (at 30 °C)	8 (at 35 °C)	9 (at 45 °C)
Glu H-1	0.159	0.203	0.187	0.226
Glu′ H-1	0.234	0.211	0.268	0.234
Glu″ H-1		0.223	0.418	0.256
Glu‴ H-1		0.266		0.305
Rha H-1	0.193	0.262	0.178	0.194
Rha′ H-1	0.247		0.194	0.274
Rha H-6	0.451		0.451	0.412
Rha′ H-6	0.466		0.584	0.552

[a] Spectra were recorded in pyridine-d_5 with one drop of D_2O at ambient temperature. All samples were exchanged with D_2O prior to recording the spectra.

The assignment of the rhamnose proton resonances to the individual sugar units proceeded analogously with a few points of note. Given the small values of the homonuclear proton coupling constants $^3J_{1,2}$ and $^3J_{2,3}$ in rhamnose, cross peaks could not be detected in the DQCOSY spectrum (supplementary material) for these connections. They were revealed, however, in a long-range COSY experiment (Δ = 300 ms).[17] While we were thus able to distinguish the 1- and 2-position protons of the two rhamnose spin systems, the two 3-position protons (Rha-H-3 and Rha′-H-3: δ 4.64 ppm) and two 4-position protons (Rha-H-4 and Rha′-H-4: δ 4.35 ppm) were nearly coincident. (In comparison, these four protons, Rha-H-3, Rha′-H-3, Rha-H-4, and Rha′-H-4, were cleanly resolved in saponin **6**.) The rhamnose 5-position protons were sufficiently distinct (δ 5.00 and 4.90 ppm) to enable cross peaks to the methyl groups (Rha-H-6 and Rha′-H-6) to be resolved. The 5- and 6-position protons of the rhamnose units were ultimately assigned to the interior and terminal rhamnose from the HETCOR spectrum after distinguishing the ^{13}C-signals on the basis of T_1 relaxation times, Table V.[18]

With the sugar protons assigned, a simple HETCOR spectrum then enabled assignment of most carbon resonance for each sugar. The only exceptions were correlations to proton resonances in severely overlapped regions of the spectrum. Assigning these carbons relied upon ^{13}C chemical shift analysis as well as relative T_1 relaxation times. The problem of reduced intensity of the methylene carbon signals with magnetically nonequivalent protons (C-6 of the glucose residues) often rendered these signals unobservable in the HETCOR spectra. Use of a fixed evolution period in the HETCOR sequence to minimize signal loss due to relaxation as described by Reynolds[19] overcame this problem, and all carbon signals could be assigned, Table II.

The coupling constants for the sugar protons measured directly from the one-dimensional spectra, from cross sections of the COSY spectra, or in the case of severe overlap, from a homonuclear 2D J spectra helped to es-

(17) In the RCT spectra cross peaks between the rhamnose 1- and 2-position protons appeared. These cross peaks result from direct coherence transfer (1–2-Hz couplings) rather than relayed coherence, which became observable due to the increased length of the RCT sequence, thus having the same effect as the delay in a long-range COSY. Distinguishing cross peaks as relayed coherence rather than direct coherence transfer from weak couplings was accomplished by comparing the RCT spectrum with a long-range COSY using a delay interval equivalent to the added time resulting from the relaying pulses in the RCT sequences.

(18) (a) Allerhand, A.; Doddrell, D. *J. Am. Chem. Soc.* **1971**, *93*, 2777. (b) Neszmely, A.; Tori, K.; Lukacs, G. *J. Chem. Soc., Chem. Commun.* **1977**, 613. (c) Yahara, S.; Kasai, R.; Tanaka, O. *Chem. Pharm. Bull.* **1977**, *25*, 2041. (d) Ishii, H.; Kitagawa, I.; Matsushita, F.; Shirakawa, K.; Tori, K.; Tozyo, T.; Yoshikawa, M.; Yoshimura, Y. *Tetrahedron Lett.* **1981**, *22*, 1529. (e) Hirai, Y.; Konishi, T.; Sanada, S.; Ida, Y.; Shoji, J. *Chem. Pharm. Bull.* **1982**, *30*, 3476.

(19) (a) Reynolds, W. F.; Hughes, D. W.; Perpick-Dumont, M.; Enriquez, R. G. *J. Magn. Reson.* **1985**, *64*, 304. (b) Perpick-dumont, M.; Reynolds, W. F.; Enriquez, R. G. *Magn. Reson. Chem.* **1988**, *26*, 358.

Table VI. $^1J_{C-H}$ (Hz) of 6-9 Anomeric Carbons[a]

	6	7	8	9
Glu H-1	159.4	160.1	159.2	159.4
Glu′ H-1	159.4	159.0	157.5	158.6
Glu″ H-1		161.6	161.8	158.2
Glu‴ H-1		161.6		159.6
Rha H-1	169.8	168.9	174.9	174.6
Rha′ H-1	169.8		174.9	172.8

[a] Spectra were recorded in pyridine-d_5 with one drop of D_2O at ambient temperature. All samples were exchanged with D_2O prior to recording the spectra.

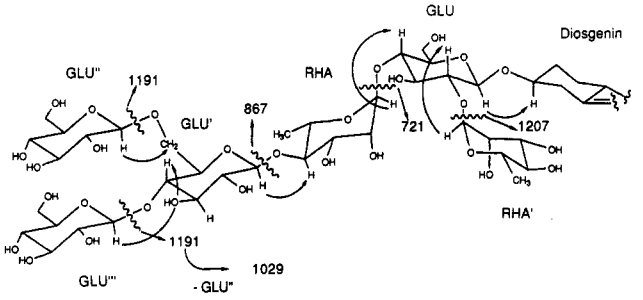

Figure 4. Mass spectral (negative ion FAB) fragmentations and interresidue NOE's of saponin **9**, which confirmed the sequence and linkage sites.

tablish axial–axial relationships between the glucose protons and thus identify each individual sugar. In many cases, however, the overlap was too overwhelming to enable unambiguous measurements of coupling constants. The proton stereochemical assignments based on coupling constants, and thus the glucose identities, were supported by the observed intraresidue NOE's revealing 1,3-trans diaxial interactions, thus requiring axial orientations of the protons. Furthermore, from the ^{13}C assignments sugar identities as well as branching sites were indicated by the characteristic downfield shift (3–10 ppm) induced by the formation of the glycosidic linkage. Ultimately, GC-FTIR analysis of the persilylated sugars in the hydrosylate of **9** showed only glucose and rhamnose to be present, in a 2:1 ratio.

Measurement of relative T_1 relaxation times of the anomeric carbons gave an indication of the sugar sequence, faster correlation times being manifested in longer T_1 times.[5a,18] While not a foolproof method, in each case, the slowest relaxation times of the anomeric carbons of a saponin were invariably those of the more mobile terminal sugars with the shortest relaxation times belonging to the more restrained, branched sugars attached to the aglycon, Table V. The T_1 measurements thus supported the assignment of terminal and branched sugars made on the basis of the ^{13}C chemical shifts. The stereochemistry of the anomeric center was assigned by the $^1J_{C-H}$ values as determined using heteronuclear 2D J spectra, Table VI.[20]

Final confirmation of the linkage sites and assignment of the sequence was accomplished by using 2D NOE spectra and analysis of the mass spectral fragmentation patterns in the negative ion FAB spectrum (Figure 4). The characteristic fragmentations of oligosaccharides, Table VII, confirmed the sequence assignments based upon relative T_1 relaxation times and chemical shift analysis. Nuclear Overhauser enhancements traversing the glycosidic linkages were invariably observed in the 2D NOE spectra, Figure 2A, confirming the linkage sites (and thus, the sequence). Other, intraresidue NOE's also observed in the 2D NOE spectra, particularly the 1,3-diaxial dipolar couplings in the glucose residues and the NOE's between H-1 and H-2 as well as H-2 and H-3 in the rhamnose units, also confirmed the ^1H assignments. The structure of **9** and all ^{13}C assignments were thus completed. To be sure, the assignment was aided considerably by having simpler saponins in hand for spectral comparison, yet we were able to complete the structure of **9** prior to that of simpler saponins due to the larger amount of sample.

Structures of the Remaining Saponins (1, 2, 5–8). A similar approach was used to assign the structures of the remaining saponins. The individual proton spin systems for each sugar was thus mapped out using various NMR techniques. The sequence of the sugars were resolved based upon relative T_1 relaxation times, mass spectral fragmentation patterns, and ultimately by interresidue NOE's crossing the glycosidic bonds. These NOE's also confirmed the linkage sites concluded on the basis of ^{13}C chemical shift analysis. The spectroscopic data used in these structural assignments are summarized in Tables I–VII.

Two specific problems deserve special note. The first illustrates the value of the fixed evolution time HETCOR for increasing the sensitivity in detecting correlations between a methylene carbon and the two directly bonded magnetically nonequivalent protons. Since the intensity of such cross peaks are only half of the intensity of methines and methyl groups, they can be difficult to observe with limited amounts of sample. In saponin **7**, such a problem was encountered for the Glu″ 6-position. Though numerous attempts were made with intervals optimized for $^1J_{CH}$ = 125, 135, and 140 Hz, the heteronuclear correlations were never observed for this position. With the fixed evolution HETCOR, however, the assignments were easy (supplementary material).

The second note of interest occurred in the 2D NOE spectra of saponin **8** (supplementary material). When this spectrum was run under ambient conditions, NOE's involving protons on the terminal glucose (Glu″) were not observed. We rationalized that the correlation times of these protons are such that $\tau_c\omega_c = 1$, thus the contributions of the double and zero quantum relaxation pathways cancel and no enhancements are observed.[21] Since the observed NOE's involving the remaining sugar protons were negative, the more rapid motion of this terminal glucose attached to the 6-position of Glu′ could indeed account for the increased contribution from the double quantum relaxation pathway compared to the other sugars. Lowering the temperature to 5 °C sufficiently increased the correlation times for the protons of this terminal sugar and the NOE's (negative!) were observed.[22]

Assignment of Absolute Stereochemistry

The optical rotations of the aglycons, (25*S*)-nuatigenin and (25*S*)-isonuatigenin isolated from the ethyl acetate fraction of the crude methanol extract, and (25*R*)-diosgenin isolated from the hydrosylate of **9**, confirmed the absolute

(20) Reported $^1J_{C-H}$ coupling constants for anomeric center of α-D-glucosides = 170 Hz, β-D-glucosides = 160 Hz: ref 15; of α-L-rhamnosides: 164–166 Hz, β-L-rhamnosides: 152–158 Hz. Kasai, R.; Okihara, M.; Asakawa, J.; Mizutani, K.; Tanaka, O. *Tetrahedron* **1979**, *35*, 1427.

(21) (a) Noggle, J. H. In *The Nuclear Overhauser Effect; Chemical Applications*; Academic Press: New York, 1971. (b) Bothnerby, A. A.; Stephens, R. L.; Lee, J.; Warren, C. D.; Jeanloz, R. W. *J. Am. Chem. Soc.* **1984**, *106*, 811. (c) Kessler, H.; Griesinger, C.; Kerssebaum, R.; Wagner, K.; Ernst, R. R. *J. Am. Chem. Soc.* **1987**, *109*, 607.

(22) The ROESY sequence would also enable observation of these NOE's under ambient conditions (for example: Kessler, H.; Bats, J. W.; Griesinger, C.; Koll, S.; Will, M.; Wagner, K. *J. Am. Chem. Soc.* **1988**, *110*, 1033). Unfortunately, due to hardware limitations, we were unable to use this pulse sequence.

Table VII. Mass Fragmentation of 1–9[a]

	1	2	5	6	7	8	9
$(M - H)^-$	737	737	883	1029	1207	1191	1353
$(M - Rha - H)^-$				883	1061	1045	1207
$(M - Glu - H)^-$			721	867	1045	1029	1191
$(M - Glu - Rha - H)^-$				721		883	1045
$(M - 2Glu - H)^-$					883	867	1029
$(M - 2Glu - Rha - H)^-$						721	883
$(M - 3Glu - H)^-$					721		867
$(M - 3Glu - Rha - H)^-$							721

[a] From negative ion FAB spectra.

stereochemistry as the (–)-enantiomers. We assumed that the absolute stereochemistry of the sugars in 1–9 would be the natural D-glucose and L-rhamnose on the basis of the molecular rotation differences.[6a,23] Furthermore, to the best of our knowledge, the "unnatural" enantiomers of these sugars have never been verified in plants. We confirmed this assumption for 7 by acid hydrolysis with subsequent glycosylation of the monomeric sugars with (+)-2-butanol, and pertrimethylsilylation of the resulting glycosides.[24]

Examination of this silylated glycoside mixture revealed three anomeric signals: δ 4.76 (d, J = 3.6 Hz) for 2,3,4,6-tetrakis(trimethylsilyl)-1-*O*-((*S*)-2-butyl)-α-D-glucopyranose, δ 4.24 (d, J = 7.6 Hz) for 2,3,4,6-tetrakis(trimethylsilyl)-1-*O*-((*S*)-2-butyl)-β-D-glucopyranose, and δ 4.56 (b s) for 2,3,4-tris(trimethylsilyl)-1-*O*-((*S*)-2-butyl)-α-L-rhamnopyranose, in accord with standard sugar derivatives prepared from D-glucose and L-rhamnose. The ratio of the anomeric signals, 2:2:1, agreed with the presence of four D-glucose units and one L-rhamnose. Addition of the standard D-glucose and L-rhamnose derivatives to the mixture derived from 7 increased the intensity of these signals without giving rise to new resonances which would be due to the diastereomeric L-glucose and D-rhamnose derivatives. Adding a diastereomeric mixture of the corresponding glucosides and rhamnosides (prepared from D-glucose and L-rhamnose with (±)-2-butanol) gave rise to three additional signals: δ 4.72 (J = 3.6 Hz) for 2,3,4,6-tetrakis(trimethylsilyl)-1-*O*-((*R*)-2-butyl)-α-D-glucopyranose, δ 4.23 (d, J = 7.6 Hz) for 2,3,4,6-tetrakis(trimethylsilyl)-1-*O*-((*R*)-2-butyl)-β-D-glucopyranose, and δ 4.60 (b s) for 2,3,4-tris(trimethylsilyl)-1-*O*-((*R*)-2-butyl)-α-L-rhamnopyranose. These latter three glycosides would have chemical shifts identical with those from the L-glucose and D-rhamnose derivatives with (+)-(*S*)-2-butanol. The persilylated glucoside of (+)-(*S*)-2-butanol and commercially available L-glucose confirmed these assignments. D-Rhamnose is unavailable.

Analysis of the persilylated 1-*O*-((*S*)-2-butyl)glycosides from the hydrolysis of 7 by GC–MS in comparison with the diastereomeric mixture prepared from D-glucose and L-rhamnose with (±)-2-butanol, and with the diastereomerically pure glycosides prepared from D-glucose, L-glucose, and L-rhamnose with (+)-(*S*)-2-butanol, confirmed the presence of natural D-glucose and L-rhamnose in 7 in a 4:1 ratio. The absolute stereochemistry of the sugars in the remaining saponins was assumed to be the natural D-glucose and L-rhamnose.

Comments on Biological Activity

We have used the molluscicidal bioassay as a simple isolation guide to biologically active natural products which may function as plant defensive compounds. As is well known, this bioassay is particularly effective for saponins.[8] While not rigorously quantitative, we have found that the molluscicidal activity increased with increasing number of sugars. The most active saponin, 9, was 100% lethal at 25 ppm in <24 h. Of the two-sugar saponins, 2 was active (50 ppm, <24 h) but the saponins 1 and 3 were inactive at this concentration.

The recent report of fungicidal activity in a related diosgenin saponin against *Candida albicans*[5b] led us to briefly examine the fungicidal activity of the saponin mixture against *Penicillium expansum*. The saponin fraction was found to inhibit the growth of this fungal species which causes storage rot in apples. The molluscicidal and antifungal activities of these saponins suggest that in addition to whatever other role these saponins play in the life history of *A. vineale*, they also have defensive capabilities. Indeed, the recent work by Crombie has clearly established the crucial defensive role the avenacins play as defensive compounds against the "Take All" fungus *Gaeumannomyces graminis*.[25] Moreover, they have shown that variants of *G. graminis* which are tolerant to these saponins quite likely neutralize these compounds via enzymatic cleavage of the glucose residues from the oligosaccharide unit. Thus the antifungal activity of saponins in a defensive role is precedented.[26]

Conclusion

In this work we have reported the isolation and structure determination of seven new saponins from *A. vineale*. In the course of resolving the structures of these natural products, we have used a strategy for assigning structures for oligosaccharides based primarily upon NMR spectroscopy. Particularly useful in this work was the relayed coherence transfer experiment in mapping out the individual spin systems for each sugar. In such a manner, permethylation/hydrolysis and partial hydrolysis studies which require relatively large amounts of material can be avoided. These diosgenin containing saponins of *Allium* are reminiscent of those commonly found in the Dioscoreaceae family, such as dioscin.[27]

Experimental Section

General. ^1H and ^{13}C NMR spectra were recorded on a Varian XL-400 (93.94 kG, 400 MHz for ^1H, 100 MHz for ^{13}C). Spectra were recorded in pyridine-d_5 (0.4 mL) with an optimal number of drops of D_2O (usually two) added as noted, after first exchanging hydroxyl protons for deuterons via a minimum of three D_2O wash/lyophilization cycles. The 7.55 ppm resonance of residual [4-^1H]pyridine and 135.5 ppm resonance of [2-^{13}C]pyridine-d_5 were used as internal references for ^1H and ^{13}C, respectively. All 1D and 2D pulse sequences were run using standard Varian software,

(23) Klyne, W. *Biochem. J.* **1950**, *47*, XLi.
(24) (a) Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1978**, *62*, 349. (b) Leontein, K.; Lindberg, B.; Lonngren, J. *Carbohydr. Res.* **1978**, *62*, 359.
(25) (a) Crombie, W. M. L.; Crombie, L. *Phytochemistry* **1986**, *25*, 2069. (b) Crombie, W. M. L.; Crombie, L.; Green, J. B.; Lucas, J. A. *Phytochemistry* **1986**, *25*, 2075.
(26) For another example: Schlosser, E. *Ber. Deitsch. Bot. Gesell.* **1983**, *96*, 351.
(27) Kawasaki, T.; Yamauchi, T. *Chem. Pharm. Bull.* **1962**, *10*, 703.

version 6.1c, except the fixed evolution HETCOR, which was added to the sequence library according to Reynolds' program.[19] ^{13}C multiplicities were assigned using the DEPT or APT sequences, and carbon assignments were completed using HETCOR, fixed evolution HETCOR, and T_1 relaxation times (Tables I and II). The T_1 relaxation times were measured using the inversion–recovery sequence at the indicated temperature with seven different time intervals between the 180° and 90° pulses bracketing the relaxation times themselves. The double quantum filtered phase-sensitive COSY (DQCOSY) and relayed coherence transfer spectra (RCT) in Figure 1 were recorded using spectral windows of 4000 Hz in both dimensions with acquisition times of 0.064 s, 256 increments with 80 and 64 transients per increment for DQCOSY and RCT, respectively. The intervals, τ, in the transfer sequence of the RCT spectra were set to 20 ms.[13] The data were processed as 1024 × 1024 matrices. The 2D NOE experiments were recorded under the same conditions (spectral windows of 4000 Hz in both dimensions, acquisition times of 0.064 s, 256 increments, data processed as 1024 × 1024 matrices) using a mixing time of 0.6 s, Figure 2B (Table IV). The HETCOR and fixed evolution HETCOR (supplementary material) were both recorded with spectral windows of 20 000 Hz in the carbon dimension and 4000 Hz in the proton dimension, acquisition times of 0.034 s, with 144 increments using 256 transients per increment. The fixed evolution HETCOR utilized an evolution time of 0.018 s and a refocusing interval Δ of 0.024 s. Mass spectra (medium and high resolution) were recorded on a Finigan MAT 8200 instrument using a thioglycerol matrix (1–5 $\mu g/\mu L$) negative ion FAB. GC-FTIR were recorded on a Perkin-Elmer 1800 instrument equipped with a Sigma 2000 capillary gas chromatograph. Optical rotations were measured in pyridine solution on a Rudolph Autopol III polarimeter with concentrations of sample reported in grams/100 mL. Rotation locular countercurrent chromatography (RLCC) was run on Model RLCC.A chromatograph (Eyela, Tokyo Rikakikai, Tokyo, Japan). Droplet countercurrent chromatography was run on Model DCC-300 chromatograph (Eyela, Tokyo Rikakikai, Tokyo, Japan).

Bioassays. The molluscicidal bioassay[8] with *Biomphalaria pfeifferei* and the antifungal bioassay with *Penicillium expansum*[28] were employed. The molluscicidal bioassay on each fraction following evaporation of the solvent in vacuo was used to guide the isolation.

Collection, Extraction, and Isolation. Whole plants of *A. vineale* (30 kg fresh wt) were collected in June 1986 in the Pottstown region of southeastern Pennsylvania, and the fresh bulbs, stems and leaves, and flowers were separated, washed with water, and extracted thrice with methanol. The active methanol extract of the bulbs was partitioned between water and ethyl acetate, and the active water fraction was further partitioned between 1-butanol and water. The 1-butanol-soluble material (30 g) was the most active fraction. This material was fractionated by rotation locular countercurrent chromatography, RLCC (4 g per run), with an iso-BuOH/*n*-PrOH/H_2O (5:3:10) solvent system, ascending mode. Chromatography of the active fraction on silica gel yielded four saponin mixtures: [1 + 2 + 3] eluted with $CHCl_3$/MeOH/H_2O (78:20:2); [4 + 5] and impure 6 eluted with the lower layer from $CHCl_3$/MeOH/H_2O (70:30:10); and [7 + 8 + 9 + minor saponins] eluted with the lower layer of $CHCl_3$/MeOH/H_2O (65:35:10). Final purification of saponins 1–6 was accomplished by C_{18} reverse-phase HPLC [Microsorb (5 μm), Rainin, 10 × 250 mm; UV detection, 210 nm]: 1 (20 mg), 2 (15 mg), and 3 (15 mg) using MeOH/H_2O (93:7), flow rate, 0.5 mL/min; 4 (20 mg) and 5 (20 mg) as well as 6 (40 mg) using MeOH/H_2O (95:5), flow rate 1 mL/min. The mixture of the four remaining saponins, 7–9, (170 mg) could not be separated by HPLC and was subjected to droplet countercurrent chromatography, DCCC, $CHCl_3$/MeOH/H_2O (7:13:8), descending mode, yielding mixtures [7 + 8] and [9 + minor saponins]. These saponins were subsequently purified by C_{18} reverse-phase HPLC as described above: 7 (40 mg) and 8 (20 mg) using MeOH/H_2O (90:10), flow rate, 1.5 mL/min; 9 (80 mg) using MeOH/H_2O (92:8), flow rate 1.4 mL/min. All saponins showed end absorbance only in the UV spectra; all saponins decomposed >220 °C without melting. For ^1H NMR data, see Table III. For ^{13}C NMR data, see Tables I and II. For mass spectral fragmentations, see Table VII.

Saponin 1: white powder; HRMS (negative ion FAB) *m/e* 737.411 85 $[M - 1]^+$, calcd for $C_{39}H_{62}O_{13}$ 737.411 22; $[\alpha]_D$ −64.3° (*c* = 0.25); NMR spectra recorded on 7 mg.

Saponin 2: white powder; HRMS (negative ion FAB) *m/e* 737.411 65 $[M - 1]^+$, calcd for $C_{39}H_{62}O_{13}$ 737.411 22; $[\alpha]_D$ −39.6° (*c* = 0.17); NMR spectra recorded on 7 mg.

Saponin 3: identical with saponin isolated from *Ophiopogon planiscapus*;[12] NMR spectra recorded on 7 mg.

Saponin 4: identical with saponin isolated from *O. planiscapus*;[12] NMR spectra recorded on 10 mg.

Saponin 5: white powder; HRMS (negative ion FAB) *m/e* 883.469 25 $[M - 1]^+$, calcd for $C_{45}H_{72}O_{17}$ 883.469 13; $[\alpha]_D$ −62.9° (*c* = 0.17); NMR spectra recorded on 15 mg.

Saponin 6: white powder; HRMS (negative ion FAB) *m/e* 1029.528 25 $[M - 1]^+$, calcd for $C_{51}H_{82}O_{21}$ 1029.527 04; $[\alpha]_D$ −69.4° (*c* = 0.11); NMR spectra recorded on 20 mg.

Saponin 7: white powder; HRMS (negative ion FAB) *m/e* 1207.579 40 $[M - 1]^+$, calcd for $C_{57}H_{92}O_{27}$ 1207.574 78; $[\alpha]_D$ −62.2° (*c* = 0.98); NMR spectra recorded on 20 mg.

Saponin 8: white powder; HRMS (negative ion FAB) *m/e* 1191.579 84 $[M - 1]^+$, calcd for $C_{57}H_{92}O_{26}$ 1191.579 86; $[\alpha]_D$ −65.0° (*c* = 0.59); NMR spectra recorded on 15 mg.

Saponin 9: white powder; HRMS (negative ion FAB) *m/e* 1353.633 00 $[M - 1]^+$, calcd for $C_{63}H_{102}O_{31}$ 1353.632 69; $[\alpha]_D$ −63.6° (*c* = 0.63); NMR spectra recorded on 30 mg.

GC–FTIR Determination of the Sugars of 9. Saponin 9 (13 mg) was refluxed in methanolic HCl (2 mL 5 N HCl + 3 mL of MeOH) for 4 h. After cooling, the solvent was removed in vacuo, the residue was dissolved in water (5 mL) and extracted with $CHCl_3$ (2 × 5 mL) to remove the aglycon diosgenin, and the aqueous layer was neutralized to pH 7 with NH_4OH and then lyophilized. To the residue was added anhydrous pyridine (1 mL), hexamethyldisilazane (0.2 mL), and trimethylchlorosilane (0.1 mL) in a screw-cap vial.[29] The vial was shaken vigorously for 30 s and allowed to stand at 50 °C in a water bath for 1 h. The reaction mixture was then concentrated in vacuo to about half its original volume and then subjected to GC-FTIR analysis for sugar identification. A standard sugar mixture of glucose (18 mg) and rhamnose (16.4 mg) was treated under identical hydrolytic and silylation conditions. The GC separation used an HP-1 methyl silicane capillary column (5 m × 0.53 mm × 2.65 μm film thickness; column temperature 225 °C, injector temperature 275 °C, interface temperature 235 °C) with nitrogen as a carrier gas: flow rate, 3 mL/min. The persilylated sugars in the hydrosylate of 9 were identified by comparison of their retention times and FTIR spectra with those of standard samples, relative areas of the peaks of the persilylated sugars from 9 was 2:1, glucose/rhamnose. Retention times of persilylated methylglycosides: from D-glucoside, 48.66 and 50.47 min (α- and β-anomers were not distinguished); from L-rhamnoside, 21.26 min (major, α-anomer, β-anomer not detected).

Determination of Absolute Stereochemistry: (25*R*)-Diosgenin. The methylene chloride extract from the acid hydrosylate of 9 as described above was dried over sodium sulfate and filtered, and the methylene chloride was removed in vacuo to yield crude diosgenin. Diosgenin was purified by flash chromatography on silica gel, eluting with CH_2Cl_2/MeOH (97:3) to yield white crystals (3.5 mg): mp 196–198 °C; $[\alpha]_D$ −119° (*c* = 0.35). Literature for (25*R*)-diosgenin: mp 200–202 °C; $[\alpha]_D$ −129° (*c* = 1.40).[6a,30]

(25*S*)-Nuatigenin and (25*S*)-Isonuatigenin. The ethyl acetate fraction (4.27 g) from the crude plant extract was partitioned between 5% aqueous methanol and petroleum ether (bp 35–60 °C), and the aqueous methanol soluble portion (3.60 g) was recovered by removal of the solvent in vacuo. The residue was subjected to flash chromatography on silica gel, eluting with CH_2Cl_2/MeOH (95:5), using ^1H NMR spectra to guide the isolation to the desired aglycons. Final separation of nuatigenin (2

(28) Experimental details for this bioassay were kindly provided by Dr. Zev Lidert of Rohm and Haas Co., Spring House, PA.

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mg) and isonuatigenin (3 mg) was accomplished by C_{18} reverse-phase HPLC [Microsorb (5 μm), Rainin, 10 × 250 mm; UV detection, 210 nm] using MeOH/H_2O (88:12), flow rate 1.7 mL/min, yielding isonuatigenin as a white powder: mp 212–215 °C; $[\alpha]_D$ −82° (c = 0.177) and nuatigenin as a white powder: mp 206–210 °C; $[\alpha]_D$ −62° (c = 0.092). Literature values for (25S)-isonuatigenin: mp 215–218 °C; $[\alpha]_D$ −140° (c = 2.00).[11] Literature values for (25S)-nuatigenin: mp 210–214 °C; $[\alpha]_D$ −94° (c = 2.0).[11] The lower optical rotations recorded on the aglycons isolated in this work in comparison with those reported in the literature are most likely due to concentration differences.[31]

D-Glucose and L-Rhamnose.[24] Saponin **7** (5.5 mg) was refluxed in aqueous HCl (1 N, 6 mL) for 7 h. After cooling, the reaction mixture was extracted with $CHCl_3$ (2 × 5 mL) to remove the diosgenin, and the aqueous layer was neutralized to pH 7 with Amberlite IRA68 ion-exchange resin and filtered, and the solution was transferred to a glass ampoule. The solvent removed by lyophilization, (+)-(*S*)-2-butanol (0.5 mL) and trifluoroacetic acid (5 drops) were added, and the ampuole was sealed. After standing overnight (12 h) at 100 °C in a steam bath, the solvent was removed in vacuo at 40 °C, and the residue was transferred to a screw-cap vial. To the residue was added anhydrous pyridine (1 mL), hexamethyldisilazane (0.2 mL), and trimethylchlorosilane (0.1 mL). The vial was shaken vigorously for 30 s and allowed to stand at 50 °C in a water bath. After cooling, the solvent was removed in vacuo, and the residue was dissolved in petroleum ether (bp 35–60 °C) and filtered, and the solvent was removed in vacuo for NMR analysis as described in the text, or used directly for GC–MS analysis as described below. Standard persilylated 2-butyl glycosides were prepared from commercially available D-glucose, L-glucose, and L-rhamnose with both (+)-(*S*)-butanol and (±)-2-butanol by a strictly identical procedure, which included exposure to the initial hydrolysis step.

GC–MS analysis was performed on the Finnigan MAT-90 using an HP-1 methyl silicane capillary column (30 m × 0.32 mm × 0.50 μm film thickness) with helium as carrier gas: flow rate of 6 mL/min. A temperaturee gradient was employed: 135 °C for 2 min, then a ramp of 1 °C/min to a final temperature of 200 °C. Retention times of persilylated glycosides: (+)-(*S*)-2-butyl D-glucoside, 33.91 min and 40.78 min (α- and β-anomers were not distinguished); (+)-(*S*)-2-butyl L-glucoside, 35.03 and 40.78 min (α- and β-anomers not distinguished); (+)-(*S*)-2-butyl L-rhamnoside, 16.61 min (major, α-anomer) and 19.08 min (minor, β-anomer); (+)-(*S*)-2-butyl D-rhamnoside, 16.08 min (major, α-anomer) and 18.72 min (minor, β-anomer).

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Supplementary Material Available: Double relayed coherence transfer spectrum of saponin **9** showing rhamnose H-6 through H-3 mapping, HETCOR and fixed evolution HETCOR spectra of **7**, and the 2D NOE spectra of saponin **8** at ambient and 5 °C (5 pages). Ordering information is given on any current masthead page.

(31) In support of this assumption, we measured the optical rotation of the diosgenin and isonuatigenin we had isolated at decreasing concentrations. In both cases, decreasing concentration led to a reduced specific rotation. Diosgenin: $[\alpha]_D$ −119° (c = 0.350), $[\alpha]_D$ −103° (c = 0.012). Isonuatigenin: $[\alpha]_D$ −82° (c = 0.177), $[\alpha]_D$ −71° (c = 0.021). For other examples of dramtic variation in optical rotation withh concentration, see: (a) Horn, D. H. S.; Pretorius, Y. Y. *J. Chem. Soc.* **1954**, 1460. (b) Horeau, A. *Tetrahedron Lett.* **1969**, 3121. (c) Kumata, Y.; Furukawa, J.; Fueno, T. *Bull. Chem. Soc. Jpn.* **1970**, *43*, 3920. (d) Meyers, A. I.; Roth, G. P.; Hoyer, D.; Barner, B. A.; Laucher, D. *J. Am. Chem. Soc.* **1988**, *110*, 4611.

Formation of 5,6- and 7,8-Dihydrohexahelicene: Mechanistic Details of the Rearrangement of the Primary Photocyclization Product of 2-Styrylbenzo[*c*]phenanthrene in the Presence of a Base

Wim J. C. Prinsen and Wim H. Laarhoven*,†

Department of Organic Chemistry, Catholic University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands

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Irradiation of 2-styrylbenzo[*c*]phenanthrene (**1**) in alkylamines or basic alcoholic solution results in the formation of a mixture of two dihydrohexahelicenes (5,6- and 7,8-dihydrohexahelicene, **5** and **6**). The ratio of **5** and **6** depends on the kind of solvent. In alkylamine **6** is the favored dihydrohexahelicene. In basic alcoholic solution **5** is the preferred product. Deuteration of the solvent causes a change in the ratio of **5** and **6** in favor of **5**. The reaction starts with the deprotonation of the primary formed, unstable 16d,16e-dihydrohexahelicene (**2**), followed by a protonation step. The site of this protonation determines the ratio of **5** and **6** and depends upon the acidity of the protonating agent, an alkylammonium cation or solvent molecule, and the electron densities at the various possible sites for protonation in the intermediate. Irradiation of **1** in several chiral alkylamines yielded optically enriched **6**.

Introduction

The photodehydrocyclization of 2-styrylbenzo[*c*]-phenanthrene (**1**) into hexahelicene (**3**) is a well-known photochemical reaction (Scheme I). *trans*-16d,16e-Dihydrohexahelicene (**2**) has been accepted as the primary photoproduct.[1-4] The oxidation of **2** occurs in the presence of O_2, I_2, TCNE, and other dehydrogenating reagents.[2-4]

† Address until September 1989: Department of Chemistry (c/o Dr. D. C. Neckers), Center for Photochemical Sciences, Bowling Green State University, Bowling Green, OH 43403.

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