Tetrahedron Vol. 47, No. 22, pp. 3545-3554, 1991
Printed in Great Britain

0040-4020/91 $3.00+.00
Pergamon Press plc

CHARACTERIZATION OF STEROIDAL GLYCOSIDES FROM TYLOPHORA SYLVATICA

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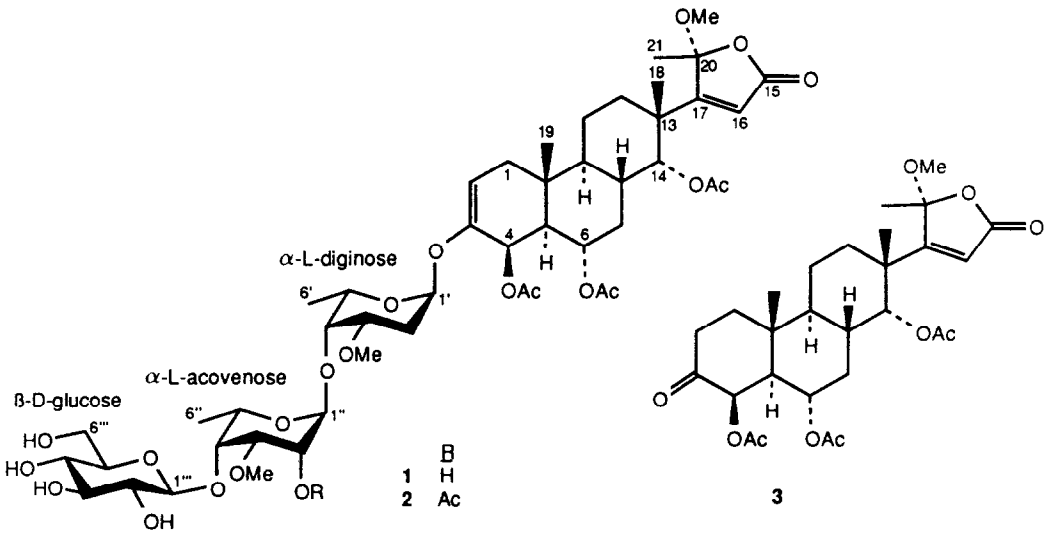
(*Received in USA* 1 *December* 1990)

Abstract: Two new steroidal glycosides (tylophoroside **1** and its monoacetate acetyltylophoroside **2**) from Tylophora sylvatica (Asclepiadaceae), a medicinal plant from the Ivory Coast, were characterized, largely by 2-D NMR methods. **1**, $C_{48}H_{72}O_{22}$, was found to have three sugars units connected by a vinyl ether linkage to the 3-position of a steroidal aglycone (a new feature in a natural product). The aglycone part contains a novel lactone ring attached at C-13, formed in nature after opening of the D-ring at the 14-15 bond. **1** and **2** have cardiotonic activity, and the aglycone tylogenin **3** is more active than the widely used dexamethasone in anti-allergic assays.

The new steroidal glycosides tylophoroside (**1**) and its monoacetate acetyltylophoroside (**2**) were isolated from Tylophora sylvatica (Asclepiadaceae), a medicinal plant from the Ivory Coast.[1] The former was the major substance isolated when the plant was collected during the dry season, and the latter predominated in a rainy season collection from a different location. Glycosides **1** and **2** are Na^+/K^+ ATPase inhibitors, which is reasonable in view of their structural resemblance to cardiac glycosides.[2] While glycosides **1** and **2** have mild anti-allergic activity, aglycone **3** exerts stronger anti-allergic activity in animal and human assay systems than the

widely used corticoids, dexamethasone and prednisolone. Most of the characterization work described below was done on tylophoroside (**1**), which was obtained first. Acetyltylophoroside (**2**) was later characterized very easily by comparison of its NMR parameters (Tables 1 and 2) with those of tylophoroside (**1**).



Fast atom bombardment mass spectrometry (FAB MS) on **1** indicated its molecular weight to be 1000 (m/z 1023 for $M+Na^+$), that of **2** to be 1042 (m/z 1065 for $M+Na^+$), and that of the aglycone tylogenin (**3**) obtained by mild acidic hydrolysis to be 534 (m/z 535 ($M+H^+$). Sodium ions, not purposely added, were picked up from the medium by the sugar portions of **1** and **2**. High resolution gave the molecular formulas of **1** and **3**: **1**, m/z 1023.443 (1023.441 calcd for $C_{48}H_{72}O_{22}Na$); **3**, m/z 535.261 (535.254 calcd for $C_{28}H_{39}O_{10}$).

The NMR parameters given in Tables 1 and 2 were derived from analysis of several types of NMR spectra as follows. Chemical shifts in the text will usually refer to the spectra in deuterochloroform, though some acetone-d_6 spectra were also measured. The ^1H, ^{13}C, ^1H-^1H correlated spectroscopy (COSY) and short-range (1-bond) ^1H-^{13}C COSY spectra (run only on **1** in $CDCl_3$ and **2** in acetone-d_6) led to a large fragment of coupled protons and carbons which could only be placed on a usual steroid or triterpenoid framework as shown in **4**, thus defining most of the substituents on the B and C rings of the aglycone. The relative configurations shown in **4** are clearly defined by the large (11.0-13.2 Hz) axial-axial ^1H-^1H coupling constants observed between H-5 and H-6, H-6 and H-7α, H-7α and H-8, H-8 and H-9, H-9 and H-11β, and H-11β and

Table 1. ^{1}H NMR Chemical Shifts (δ) and Coupling Constants (in Hz, in Parentheses) for 1-3.

	in $CDCl_3$			in acetone-d_6
Proton	1	2	3	2
1 alpha	1.92dd(16.8,2.0)	obscured	1.63m	1.95m
1 beta	2.22dd(16.8,6.5)	2.24dd(16.6,6.3)	2.05m	2.27dd(16.9,,6.7)
2 alpha	5.15dd(6.5,2.0)	5.17dd(6.5,2.0)	2.40m	5.19dd(6.7,2.0)
2 beta			2.72ddd(13.0,10.0,7.0)	
4	5.63d(4.9)	5.64dd(4.8,1.2)	5.33d(5.0)	5.60dd(4.9,1.5)
5	1.79dd(11.3,4.9)	1.80dd(11.2,4.8)	1.95dd(11.3,5.0)	1.86m
6	5.01td(11.3,4.8)	5.02td(11.2,4.8)	5.03td(11.3,4.8)	4.99td(11.2,4.8)
7 alpha	1.03q(12.0)	1.02q(12.0)	1.03q(12.0)	1.12q(11.2)
7 beta	1.96m	1.97m	1.97m	1.82m
8	2.09tt(12.0,2.3)	2.09m	2.11m	2.23m
9	1.26td(12.7,3.1)	obscured	1.26m	1.41m
11 alpha	1.61dq(13.2,3.1)	obscured	1.61m	1.65m
11 beta	1.49qd(13.2,3.6)	1.49qd(13.2,3.6)	1.49qd(13.0,3.6)	1.58qd(13.0,3.7)
12 alpha	2.07td(13.8,3.3)	2.06m	2.05m	2.16m
12 beta	1.70br d(13.8)	1.71br d(13.8)	1.70m	1.78m
1 4	5.03br s	5.04br s	5.04br s	5.13br s
1 6	5.94s	5.95s	5.95s	6.06s
1 8	1.31s	1.31s	1.32s	1.41s
1 9	1.04s	1.04s	1.18s	1.10s
2 1	1.73s	1.74s	1.74s	1.75s
4-OAc	2.01s	2.03s	2.07s	2.04s
6-OAc	1.96s	1.97s	2.04s	1.94s
14-OAc	1.97s	1.98s	1.98s	1.91s
20-OMe	3.19s	3.19s	3.20s	3.18s
1'	5.46br t(2.5)	5.51t(2.5)		5.50br d(2.1)
2'	1.86m	1.90m		1.90m(ax),1.76m(eq)
3'	3.51m	3.52m		3.52ddd(12.1,4.5,2.5)
4'	3.79d(2.2)	3.80d(2.9)		3.93d(2.5)
5'	3.75q(6.7)	3.76q(6.6)		3.80q(6.6)
6'	1.19d(6.7)	1.24d(6.6)		1.21d(6.6)
3'-OMe	3.36s	3.36s		3.36s

Table 1 continued.

	in $CDCl_3$		in acetone-d_6
Proton	1	2	2
1''	4.92s	4.90s	4.85d(1.4)
2''	4.01br s	5.32d(3.7)	5.16d(3.6)
3''	3.45m	3.47dd(3.7,1.8)	3.63t(3.4)
4''	3.95d(1.8)	3.94m	4.07m
5''	4.33q(6.7)	4.40q(6.6)	4.37qd(6.5,1.2)
6''	1.29d(6.7)	1.28d(6.6)	1.32d(6.5)
3''-OMe	3.47s	3.46s	3.44s
2''-OAc		2.13s	2.05s
1'''	4.41d(7.6)	4.55d(8.2)	4.42d(7.6)
2'''	3.48m	obscured	3.33m
3'''	3.52m	obscured	3.43m
4'''	3.60t(9.0)	obscured	3.43m
5'''	3.34m	obscured	3.33m
6'''	3.81m	3.76m	3.67dd(11.5,5.3)
		3.90dd(11.9,3.6)	3.82dd(11.5,2.9)

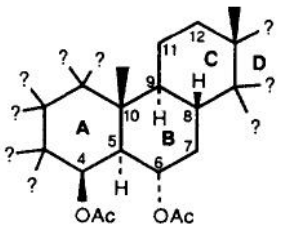
Table 2. ^{13}C NMR Chemical Shifts (δ) for 1-3.[3]

	in $CDCl_3$			in acetone-d_6	
Carbon	1	2	3	1	2
1	38.1	38.1	38.7	38.6	38.7
2	103.7	103.9	34.5	103.6	103.7
3	147.7	147.5	206.0	148.1	148.3
4	64.1	64.1	71.8	65.0	65.0
5	49.6	49.5	52.8	50.1	50.2
6	67.5	67.6	66.9	68.5	68.6
7	35.3	35.2	35.6	36.0	36.1
8	33.2	33.2	33.6	33.9	33.9
9	44.7	44.6	45.5	45.2	45.3
10	35.2	35.2	36.7	36.0	36.0
11	19.1	19.1	19.4	19.8	19.9
12	29.1	29.0	29.3	29.4	29.5
13	41.1	41.2	41.2	42.0	42.1
14	75.6	75.6	75.7	76.6	76.6
15	168.7	169.0	168.8	169.3	169.2

Table 2 continued.

	in $CDCl_3$			in acetone-d_6	
Carbon	1	2	3	1	2
16	120.2	120.2	120.4	121.0	121.0
17	172.2	172.4	172.2	173.4	173.3
18	20.2	20.4	20.5	20.6	20.8
19	13.9	14.0	14.9	14.2	14.3
20	109.8	109.9	109.9	110.5	110.5
21	24.0	24.2	24.1	24.7	24.7
4-OAc	20.8	21.0	20.6	20.9	21.0
	169.7	170.0	169.2	170.4	170.3
6-OAc	21.0	21.2	21.0	21.2	21.3
	169.9	170.2	169.9	170.8	170.7
14-OAc	20.8	21.0	20.9	21.0	21.1
	170.6	170.8	170.4	170.9	170.7
20-OMe	50.4	50.5	50.5	50.8	50.8
1'	94.4	94.3		94.6	94.7
2'	30.3	30.2		31.2	31.2
3'	76.0	76.0		76.1	75.7
4'	75.0	74.9		75.4	75.4
5'	67.4	67.3		68.2	68.2
6'	17.2	17.1		17.5	17.5
3'-OMe	56.4	56.6		56.5	56.5
1''	102.6	99.7		103.9	102.6
2''	66.9	67.3		67.8	68.3
3''	75.4	72.5		76.8	76.0
4''	75.7	75.2		76.6	73.7
5''	68.2	67.1		69.2	68.0
6''	16.7	16.9		17.2	17.4
3''-OMe	56.0	57.0		55.9	57.0
2''-OAc		21.2			21.2
		171.1			171.1
1'''	103.4	102.5		104.4	104.0
2'''	74.8	74.7		77.5	77.6
3'''	73.2	72.7		77.5	77.6
4'''	69.2	69.9		70.9	71.6
5'''	76.1	76.0		74.7	74.4
6'''	61.6	61.9		62.3	62.9

H-12α, and the small (4.9 Hz) coupling between H-4 and H-5. The absolute configuration in the aglycone portion of the molecule was assumed to be as usual for natural sterols. That substituents which shift methinyl proton and carbon absorptions downfield were attached at C-4 and C-6 was evident from their ^{1}H shifts (δ5.63 and 5.01, resp.) and ^{13}C shifts (δ64.1 and 67.5, resp.); these substituents proved to be acetates, as was considered likely from the start. The proton shift of H-4 is so far downfield that it might have been considered to be due to a vinyl proton, but the shift of the attached carbon indicated it to be in a methinyl group attached to oxygen.



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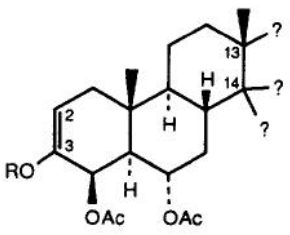

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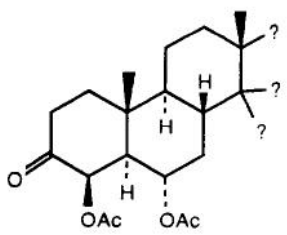

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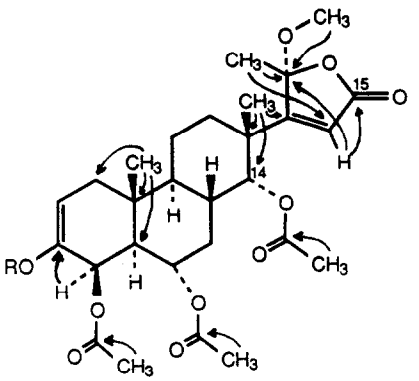
The downfield shift of H-4 was explained when a small group of coupled protons and carbons was added to complete the A ring, giving partial structure **5**: H-4 is allylic in addition to having an acetate attached. Sugars were presumed to be attached at C-3 as is very often the case. However, their attachment via a 2,3-vinyl ether appears to be new in natural steroidal glycosides, and on mild acidic hydrolysis results in a 3-ketone **6** (tylogenin, full structure **3**) rather than the usual 3-ol.

The determination of the arrangement of atoms in the vicinity of the D-ring was complicated by the lack of observable coupling between any of the protons in the region, even H-8 and H-14 (dihedral angle H8-C8-C14-H14 must be close to 90°). The problem was solved with the aid of a long-range (2- and 3-bond) ^{1}H-^{13}C COSY spectrum. The spectrum was not especially strong due to the small sample size (ca. 7 mg), but it showed the 2- and 3-bond (and one 4-bond) couplings shown by arrows on structure **7**, most of which involve methyl groups and thus give relatively large peaks. Hirundoside and relatives[4] are previously described natural derivatives of the steroidal aglycone hirundigenin (**8**) with the 14-15 bond cleaved and the same carbon skeleton as tylogenin (**3**), but with different oxygenation patterns.

The configuration at C-20 was tentatively assigned by comparison of the NMR shifts of the ca. 25% impurity present in **1-3** which is apparently the



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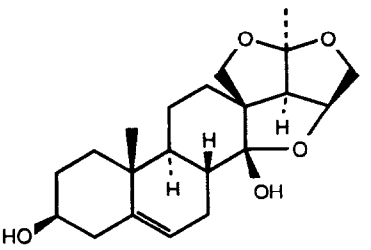

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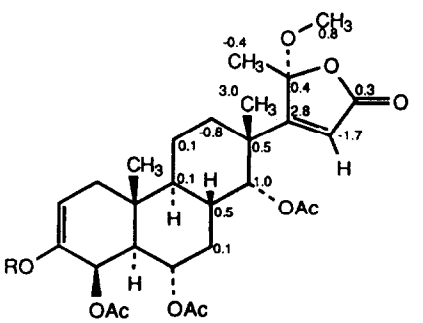
minor epimer at C-20, with those of the major epimer, and by noting that the major epimer is probably the more stable one. The separation of the epimers has not been accomplished, and the finding of the minor epimer in approximately the same relative amount in all three cases even after careful chromatography suggests that they equilibrate under ordinary handling. The ^{13}C NMR shifts of 20-epitylophoroside, 20-epiacetyltylophoroside, and 20-epitylogenin are given in Table 3, and the average difference in ^{13}C shifts between **1** and **2** and their 20-epimers are given on structure **9**. The locations and magnitudes of these differences are consistent with the view that these unseparated forms are the 20-epimers. Molecular mechanics calculations for conformations rotated at five-degree intervals about the 13-17 bond using the program PCMODEL[5] indicated the 20-(R) epimer to have a conformation 0.6 kcal/mole lower in energy than the lowest-energy conformation of the 20-(S) epimer; the major epimer found is thus presumed to have the 20-(R) configuration. This appears to be supported by the shift differences



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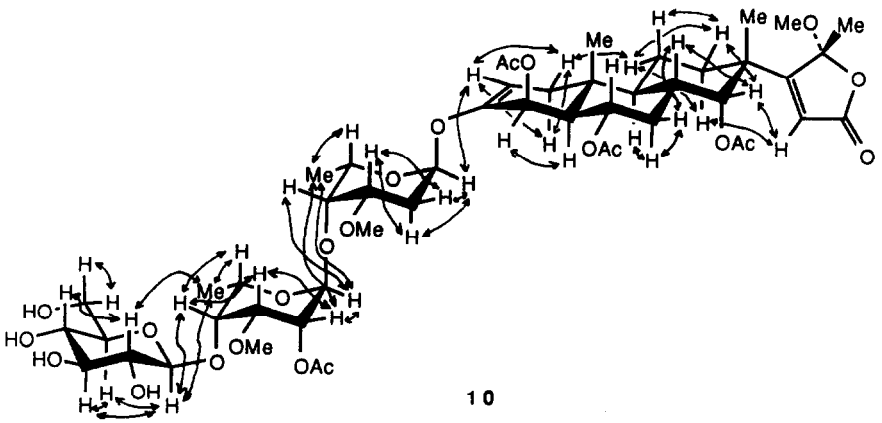
observed. In particular, the 2.8 ppm downfield shift observed for C-17 in the minor epimer as compared to the major epimer is due to this carbon being more in the plane of the 14-OAc carbonyl group in the former. Similarly, the 1.7 ppm upfield shift for C-16 in the minor epimer is due to this carbon being more above the plane of the 14-OAc in the minor epimer.

Table 3. ^{13}C NMR Chemical Shifts (δ) and ^{1}H Chemical Shifts (δ, in Parentheses) for 20-Epimers of **1-3**.[3]

	in $CDCl_3$			in acetone-d_6	
Carbon	**1**	**2**	**3**	**1**	**2**
7			35.7		
8	33.7	33.7	34.1	34.5	34.5(2.19)
9	44.8	44.8	45.7	45.4	45.5
11	19.2	19.2	19.5	19.9	19.9
12	28.3	28.2	28.5	28.6	28.6
13	41.6	41.7	41.8	42.5	42.6
14	76.4(5.12)	76.5(5.12)	76.6(5.13)	77.8	77.8(5.18)
15	169.0	169.0	169.1	169.5	169.5
16	118.5(5.88)	118.5(5.89)	118.6(5.88)	119.5	119.5(6.00)
17	175.0	175.2	174.9	176.3	176.2
18	23.1(1.28)	23.2(1.28)	23.3(1.29)	23.8	23.9(1.40)
20	110.1	110.3	110.2	111.0	111.0
21	23.5(1.73)	23.7(1.73)	23.7(1.73)	24.2	24.3(1.75)
14-C=O	169.7	169.9	169.8	170.6	170.5
20-OMe	51.3(3.33)	51.4(3.34)	51.4(3.35)	51.5	51.5(3.31)

The sugars remained to be elucidated. It was clear early on from its typical ^{1}H and ^{13}C NMR parameters that an unsubstituted β-glucopyranoside grouping was present (all of the hydrogens on the ring, H-1''' through H-5''', are axial in this monosaccharide, with definitive large vicinal axial-axial coupling constants between them), and this was thus the sugar furthest removed from the aglycone. The two other sugars were put together largely through ^{1}H COSY results and the consideration that none of the vicinal coupling constants in these two sugars except for that between H-2'(axial) and H-3' (12.1 Hz in **2**, where it is most visible) was large enough to be axial-axial. The position of attachment of substituents on these sugars was clear from the ^{13}C NMR shifts (the carbon absorbs about 8 ppm further downfield if a substituent other than H is attached to its oxygen). That the order of these two sugars follows the usual pattern for 6-deoxy sugars

joined to 2,6-bis-deoxy sugars was indicated by the mass spectrum of **1**, which shows a peak at m/z 323 for glucose (163) + acovenose (160) but none at m/z 307 for glucose + diginose (144). This was strongly confirmed by nuclear Overhauser spectroscopy (NOESY) on **2**, which showed H-6' and H-1'' to be close to one another, and H-6'' to be close to H-1'''. The interactions clearly visible in the NOESY spectrum of **2**, summarized on structure **10**, confirm many of the other structural assignments.



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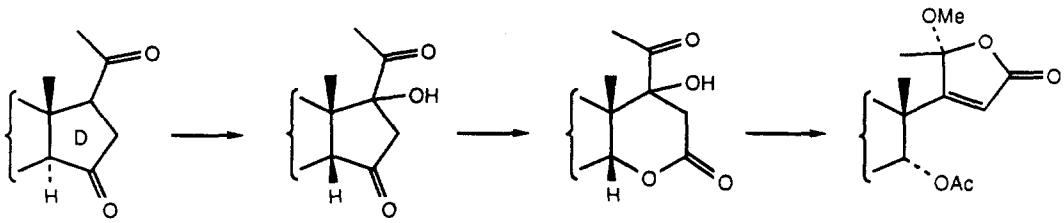
The glucose and acovenose are assumed to be D and L, respectively, because these are the only enantiomers which have been found in nature. Diginose, of which both enantiomers have been found in the closely related family Apocynaceae,[7] is tentatively assumed to be L here since it accompanies the very similar acovenose and may well come from a common biosynthetic intermediate.

The relative configurations of the diginose and glucose at the anomeric centers must be as shown from the small (2 x 2.5 Hz, axial-equatorial and equatorial-equatorial) and large (7.6 Hz, axial-axial) coupling constants, resp., between the anomeric proton and the proton on the adjacent carbon. The configuration at the anomeric carbon of acovenose is not similarly defined, as the observed near-zero coupling constant is consistent with either an axial-equatorial or equatorial-equatorial vicinal proton arrangement. It is assigned on the basis of Klyne's rule ("L-sugars are almost always alpha-anomers").[7]

1-3 are almost certainly biosynthesized via opening of a the D-ring of a steroid precusor. A possible sequence is shown from a 15-ketosteroid **11** which is epimerized at C-14 and oxidized at C-17 to give **12**. This is oxidized to **13**, which is hydrolyzed, acetylated, relactonized, dehydrated, and methylated to give **14**, as in **1-3**.



11 12 13 14

Experimental

NMR spectra were measured on Bruker AM-500 NMR spectrometers on $CDCl_3$ or acetone-d_6 solutions containing internal TMS. FAB MS were recorded on Varian MAT CH5 DF or VG 2AB-2F spectrometers in a matrix of 2-hydroxyethyldisulfide using Iontech guns and 8.0 KV xenon atoms for bombardment. Molecular mechanical calculations were performed using PCMODEL.[5]

Hydrolysis of **1** to **3**. After keeping **1** (50 mg) in 4 mL of 1N HCl for 1.25 h, extraction with two 4 mL portions of methylene chloride afforded 32.2 mg residue. TLC on C18 eluting with 70% methanol containing 1% ammonium hydroxide gave a major peak (220 nm detection) at Rf 0.36. The compound responsible partitioned approximately evenly (by TLC densitrometry) between the preequilibrated phases from ether-hexane-methanol-water (10:1:5:5) and that system was utilized for countercurrent chromatography in the Ito coil with the upper (aqueous) phase as the mobile phase, giving 22 mg (82%) of **3**.

Hydrolysis of **2** to **3**. After 2 min at 25°C, a solution of **2** (85 mg) in TFA (8.5 mg) was rapidly evaporated under vacuum and dried under a stream of N_2. Purification as above except using toluene-$CHCl_3$-CCl_4-methanol-water (8:2:2:8.5:1.5) gave 38 mg (87%) of **3**.

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1. The isolation of **1** and **2** will be described elsewhere.
2. Biological studies on **1-3** will be described elsewhere.
3. NMR assignments for Ac groupings may be interchanged, as may be methinyl carbon assignments in sugar parts of **1** and **2** in Tables 2 and 3.
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