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TRITERPENOID SAPONINS FROM *BECIUM GRANDIFLORUM* VAR. *OBOVATUM*

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Key Word Index—*Becium grandiflorum* var. *obovatum*; Lamiaceae; triterpenoid saponins.

Abstract—Two new triterpenoid saponins, beciumecine 1 and 2, were isolated from the root bark of *Becium grandiflorum* var. *obovatum* and their structures established as 3-*O*-(β-D-glucopyranosyl) terminolic acid 28-*O*-β-D-apiofuranosyl(1–3)-[α-L-rhamnopyranosyl(1–3)-β-D-xylopyranosyl(1–4)]-α-L-rhamnopyranosyl(1–2)-α-L-arabinopyranoside and 3-*O*-(β-D-glucopyranosyl) 24-hydroxyterminolic acid 28-*O*-α-L-rhamnopyranosyl(1–3)-β-D-xylopyranosyl(1–4)-α-L-rhamnopyranosyl(1-2)-α-L-arabinopyranoside, respectively.

INTRODUCTION

A hot water extract of five plants, indigenous to Natal, South Africa, has reportedly been used by a traditional herbalist in the successful treatment of two cancer patients with an invasive breast and buccal cancer, respectively, during the first half of this century. Human lung carcinoma cells gave a pronounced *in vitro* response to an extract of one of these plants, known as umatanjane and identified as *Becium grandiflorum* var. *obovatum* by the National Botanical Institute, Pretoria. It was found that a 1:500 dilution of a hot water extract (1 g/10 ml) of the powdered root bark of this plant induces the formation of numerous intra-cytoplasmic vesicles in the cancer cells within 48 h. These vesicles tended to increase in size and eventually inhibited cell division and cell growth. Different combinations of hot water extracts of the five plants were also tested on melanoma cells and the combination of *B. grandiflorum* and a *Crinum* sp. showed synergistic cytotoxicity.

This paper deals with the isolation and characterization of two triterpenoid saponins, eliciting the typical response in cancer cells, from the root bark of *B. grandiflorum*. The two compounds were named beciumecine 1 and 2.

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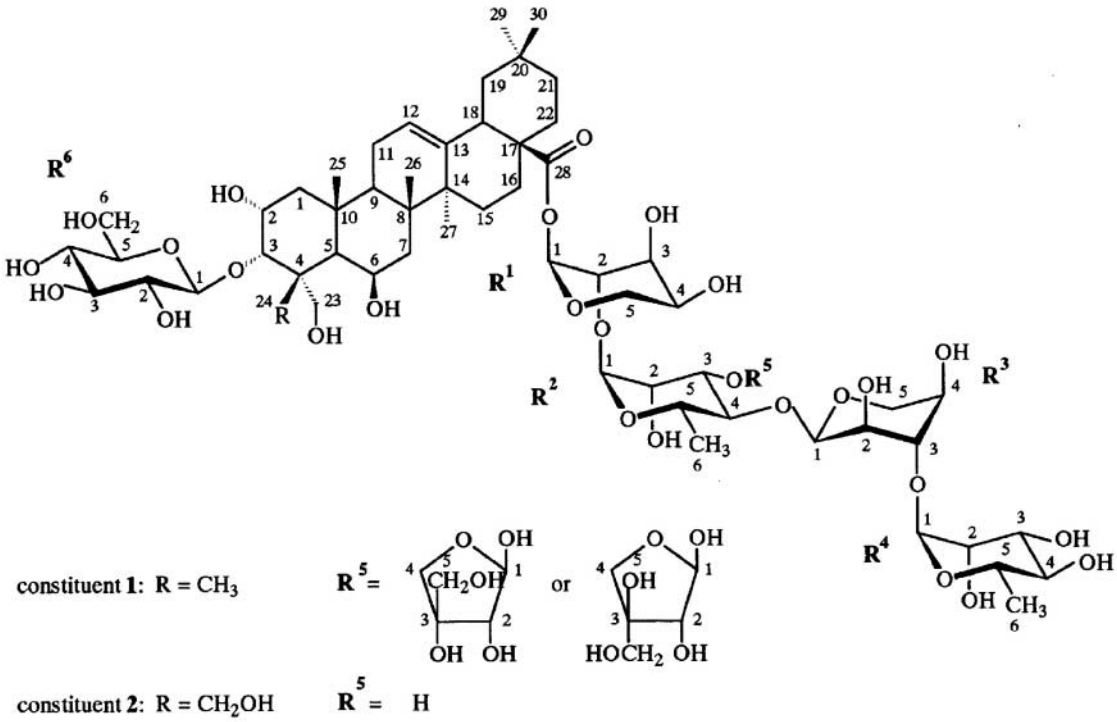
RESULTS AND DISCUSSION

The froth formed on the original hot water extract of the root bark suggested the presence of saponins in the extract and a positive Liebermann–Burchard test revealed that constituents **1** and **2** were both triterpenoid saponins.

Arabinose, xylose and glucose were identified in both constituents by GC-MS retention time comparison of their alditol peracetate and trimethylsilylether derivatives [1, 2] with the derivatives of authentic reference compounds. Using LC [3] with pulsed amperometric as well as refractive index detection, these sugars were found to be present in equimolar amounts in both **1** and **2**.

The complete structure of **1** was determined by a series of 500 MHz NMR experiments. The ^{1}H NMR chemical shifts, multiplicities and coupling constants in Table 1 and the ^{13}C NMR chemical shifts and multiplicities in Table 2 were determined by 2D HSQC, 2D HMBC and DEPT experiments and confirmed by 1D TOCSY, 2D COSY, 2D HSQC-TOCSY and 2D NOESY experiments.

The ^{1}H NMR spectrum of constituent **1** in $CDCl_3$ showed a triplet at δ 5.27, which correlated in the HSQC spectrum with an olefinic carbon doublet at δ 122.3. This proton was thus assigned to H-12. Six three-proton singlets at δ 1.19, 1.52, 0.93, 1.05, 0.88 and 0.90 could be assigned to the C-24, C-25, C-26, C-27, C-29 and C-30 methyl groups, respectively. The singlet at δ 175.5 in the



^{13}C NMR spectrum was assigned to the ester carbonyl group at C-28. The ^{13}C NMR chemical shifts of the aglycone of **1** were in good agreement with chemical shifts reported for the oleanane-type aglycone of tubeimoside [4], the only differences being the chemical shifts of C-6 and C-7. Both carbons resonated at a lower field in **1** (δ 65.6 instead of 18.7 for C-6 and δ 39.7 instead of 33.1 for C-7) due to the deshielding expected for a hydroxyl group on C-6.

Six CH carbons correlating with six anomeric protons indicated the presence of a maximum of six sugar units in **1**. The six sugars (R^1–R^6) were identified using the 1D TOCSY, as well as 2D HSQC-TOCSY spectra of **1**. Selective irradiation of, for example, the anomeric proton at δ 4.93 in a 1D TOCSY experiment, produced the complete ^{1}H spectrum of sugar R^4. The three-proton doublet observed at δ 1.08 (J = 6.2 Hz) in this spectrum and the doublet at δ 1.11 (J = 5.7 Hz) in the 1D TOCSY spectrum of sugar R^2, obtained by irradiation at δ 4.70, indicated that sugar units R^4 and R^2 were 6-deoxy sugars. 2D HMBC and 1D TOCSY spectra were used for the sequential assignment of the resonances for each monosaccharide, starting from the anomeric proton. In pyranosides, a 1,2-di-equatorial relationship between H-1 and H-2 leads to a small coupling constant of *ca.* 1.5 Hz, a 1,2-axial–equatorial relationship to a coupling constant of *ca.* 3.5 Hz and a large coupling constant of 7 to 9 Hz is expected as the result of a 1,2-diaxial relationship [5]. The anomeric coupling constants obtained for sugar units R^1 (J = 1.2 Hz), R^2 (J = 1.5 Hz) and R^4 (J = 1.6 Hz) were therefore indicative of an α-configuration and that of R^3 (J = 7.9 Hz) and R^6 (J = 7.6 Hz) of a β-configuration. In pyranosides, the mean $^1J_{CH}$ values for α and β anomers are 170 and 160 Hz, respectively [6]. The anomeric configurations of the pyranosides were therefore confirmed by the $^1J_{CH}$ coupling constants 173 Hz (R^1), 170.5 Hz (R^2), 163.5 Hz (R^3), 172 Hz (R^4) and 159 Hz (R^6), determined from the fully ^{13}C–^{1}H coupled HSQC spectrum with high resolution in the ^{1}H dimension. The $^3J_{HH}$ coupling constants determined from these 1D TOCSY and HSQC spectra allowed the assignment of the relative configurations of all the protons for each monosaccharide. However, sugar R^5 was a furanoside and its anomeric configuration could therefore not be determined by comparing its ^{1}H–^{1}H and ^{13}C–^{1}H coupling constants with those expected for pyranosides. The large ^{13}C chemical shift of the anomeric carbon at δ 110.2 was indicative of a fair amount of deshielding. The substituents at C-1 and C-2 are therefore expected to be in a configuration with the least sterical hindrance, i.e. *trans*-diaxial. R^5 is therefore present in **1** as one of the two isomeric forms of the β-anomer. In conclusion, sugar unit R^1 was identified as α-arabinopyranoside, R^2 and R^4 as α-rhamnopyranoside, R^3 as β-xylopyranoside, R^5 as β-apiofuranoside and R^6 as β-glucopyranoside.

Long-distance ^{13}C–^{1}H correlations in the 2D HMBC spectra of **1** indicated a glycosidic ester

linkage between H-1 of sugar unit R^1 and C-28 of the aglycone and a glycosidic bond between H-1 of sugar unit R^6 and C-3 of the aglycone. In the HMBC spectrum, the anomeric protons at δ 5.68 (R^1), 4.70 (R^2), 4.54 (R^3), 4.93 (R^4), 5.11 (R^5) and 4.29 (R^6) correlated with the carbons at δ 175.5 (C-28 of aglycone), 74.3 (C-2 of R^1), 75.9 (C-4 of R^2), 81.1 (C-3 of R^3), 79.8 (C-3 of R^2) and 80.7 (C-3 of aglycone), respectively.

The complete stereochemistry of constituent **1**, with the exception of the relative spatial orientation of the sugar units of the compound and the relative configuration of apiose, was determined by 2D HSQC-TOCSY and 2D NOESY experiments.

2D HSQC and 2D HMBC spectra enabled the assignment of the ^1H and ^{13}C signals of constituent **2** as listed in Tables 1 and 2. These assignments were confirmed by 1D TOCSY, 2D NOESY, 2D ROESY and 2D HSQC-TOCSY experiments. Comparison of the ^1H chemical shifts and coupling constants, as well as the ^{13}C chemical shifts of **2** with that of **1**, indicated that the only differences between the structures of these two components were the following:

(1) Sugar R^5, apiose, [δ 110.2 (*d*, C-1), 75.9 (*d*, C-2), 78.4 (*s*, C-3), 73.2 (*t*, C-4), 62.5, (*t*, C-5), and anomeric proton at δ 5.11] was not present in **2**.

(2) The total number of CH_2 triplets between δ 59.0 and 66.0 was unchanged although one CH_2 at δ 62.5 (C-5, R^5) was absent in **2**. The triterpenoid CH_3 at δ 15.6 (C-24 of aglycone) was not present in **2** and the singlet at C-4 in **1** was shifted to lower field (δ 42.3 to 48.2) in **2**, indicating that the CH_3 at C-24 in **1** has been replaced by a CH_2OH group in **2**. The additional triplet at δ 60.7 could thus be assigned to this CH_2OH group.

The question arose as to whether the biological activity of the saponins under discussion could be due to the presence of sugars with atypical absolute configuration in constituents **1** and **2**. Gas chromatographic comparison of the trifluoroacetylated sugars [7] from **1** and **2** with authentic optically pure reference sugars using a capillary column coated with heptakis(2,3-di-*O*-pentyl-6-*O*-methyl)-β-cyclodextrin showed that the hydrolysates of **1** and **2** contained D-glucose, D-xylose and L-rhamnose. Using hexakis(2,3,6-tri-*O*-pentyl)-α-cyclodextrin [8, 9] as stationary phase, arabinose from **1** and **2** was found to have the L-configuration. Trifluoro acetylation of apiose yielded fully derivatized isomers of both the D- and L-isomers and although these isomers and some partially derivatized isomers could be separated on the derivatized β-cyclodextrin column the absolute configuration of this sugar could not be unequivocally determined. The apiose of **1** was therefore assumed to have the D-configuration normally found in nature.

Based on the above evidence, constituents **1** and **2** were identified as, 3-*O*-(β-D-glucopyranosyl) terminolic acid 28-*O*-β-D-apiofuranosyl(1–3)-[α-L-rhamnopyranosyl(1–3)-β-D-xylopyranosyl(1–4)]-α-L-rhamnopyranosyl(1–2)-α-L-arabinopyranoside and 3-*O*-(β-D-glucopyranosyl) 24-hydroxyterminolic acid 28-*O*-α-L-rhamnopyranosyl(1–3)-β-D-xylopyranosyl(1–4)-α-L-rhamnopyranosyl(1–2)-α-L-arabinopyranoside, respectively. Beciumecine 1 and 2 are proposed as names for these constituents.

Based on the proposed structures, the molecular masses of **1** and **2** were calculated as 1354.6 and 1238.6 Da, respectively, which differs from the masses determined by TOF-MALDI, 1381.2 and 1262.4 Da, respectively, possibly due to the formation of Na^+ adducts with **1** and **2**.

EXPERIMENTAL

General

All glassware was heated to 500°C in an annealing oven to remove any traces of adsorbed organic material. Syringes and stainless-steel needles were cleaned by rinsing with CH_2Cl_2 (Merck, Residue Analysis Grade). Solvents were dried and distilled according to prescribed methods.

Analytical methods

Optical rotations were measured at 25°C in H_2O. ^1H and ^{13}C NMR spectra of **1** and **2** were recorded on a Varian UnityPlus 500 in DMSO-d_6 at 30°C at 499.9 and 125.7 MHz, respectively, using TMS at δ 0.0 as an internal reference. ^1H and ^{13}C NMR spectra of the synthesized sugars (in D_2O, at 25°C, using 2.5% w/v DSS at δ 0.015 as an external reference) and hexakis(2,3,6-tri-*O*-pentyl)-α-cyclodextrin (in $CDCl_3$ at 25°C with TMS as an internal reference) were recorded on a Varian VXR 300 at 299.91 and 75.42 MHz, respectively.

Kieselgel 60 F_{254} (Merck) and Kieselgel 60 (70–230 mesh ASTM) (Merck) were used for TLC and CC, respectively, and the spots on TLC were detected by spraying with H_2SO_4 (20%) and heating at 150°C. Plates coated with cellulose (Merck) were used for prep. TLC and the compound of interest was detected by spraying side strips of the plates with a mixture of orthophosphoric acid (1.3 ml, 85%), aniline (0.93 ml) and EtOH (100 ml, 70%) and heating at 100°C for 15 min. Compounds were extracted from the stationary phase with H_2O.

TOF-MALDI analyses were carried out on a Kratos KOMPACT MALDI III instrument, using 2,5-dihydroxybenzoic acid as matrix and bovine insulin as external calibrant. An accelerating voltage of 20 kV was used.

EIMS were recorded at 70 eV with a Carlo Erba QMD 1000 GC-MS system equipped with a capillary glass column (40 m × 0.3 mm) coated by the Laboratory for Ecological Chemistry (LECUS) with 0.25 μm PS-089, an apolar silanol-terminated

Table 1. ^{1}H NMR data of constituents **1** and **2** at 499.9 MHz in DMSO-d_6 at 30°C[a]

H	**1**	**2**
1a	1.82	1.78
1b	0.98	1.03
2	4.14 (1H, *bm*)	4.09 (1H, *bm*)
3	3.45 (1H, *d*, J_{H3H2} = 2.0 Hz)	3.57
5	1.17	1.36
6	4.32 (1H, *bm*)	4.26 (1H, *bm*)
7a	1.57	1.59
7b	1.35	1.47
9	1.45	1.48
11a	1.97	1.96
11b	1.87	1.88
12	5.27 (1H, *t*, $J_{H12H11a} = J_{H12H11b}$ = 3.4 Hz)	5.27 (1H, *t*, $J_{H12H11a} = J_{H12H11b}$ = 3.6 Hz)
15a	1.65	1.65
15b	0.96	0.98
16a	1.92	1.92
16b	1.60	1.58
18	2.87 (1H, *dd*, $J_{H18H19a}$ = 13.6, $J_{H18H19b}$ = 4.0 Hz)	2.86 (1H, *dd*, $J_{H18H19a}$ = 13.8, $J_{H18H19b}$ = 4.3 Hz)
19a	1.64	1.65
19b	1.06	1.06
21a	1.32	1.33
21b	1.16	1.14
22a	1.68	1.66
22b	1.42	1.44
23a	3.55	3.78
23b	3.19	3.69
24a	1.19 (3H, *bs*)	4.48
24b		3.55
25	1.52 (3H, *bs*)	1.53 (3H, *bs*)
26	0.93 (3H, *bs*)	0.93 (3H, *bs*)
27	1.05 (3H, *bs*)	1.06 (3H, *bs*)
29	0.88 (3H, *bs*)	0.88 (3H, *bs*)
30	0.90 (3H, *bs*)	0.89 (3H, *bs*)
R^1		
1	5.68 (1 H, *d*, J_{H1H2} = 1.2 Hz)	5.64 (1H, *d*, J_{H1H2} = 1.8 Hz)
2	3.61	3.62
3	3.81	3.79
4	3.70	3.68
5a	3.79	3.76
5b	3.32	3.32
R^2		
1	4.70 (1H, *d*, J_{H1H2} = 1.5 Hz)	4.77 (1H, *d*, J_{H1H2} = 1.4 Hz)
2	3.77 (1H, *dd*, J_{H2H1} = 1.5, J_{H2H3} = 2.4 Hz)	3.65
3	3.70 (1H, *dd*, J_{H3H2} = 2.4, J_{H3H4} = 9.0 Hz)	3.66
4	3.58 (1H, *t*, $J_{H4H3} = J_{H4H5}$ = 9.0 Hz)	3.45
5	3.53 (1H, *dd*, J_{H5H4} = 9.0, J_{H5H6} = 5.7 Hz)	3.50
6	1.11 (3H, *d*, J_{H6H5} = 5.7 Hz)	1.14 (1H, *d*, J_{H6H5} = 6.3 Hz)
R^3		
1	4.54 (1H, *d*, J_{H1H2} = 7.9 Hz)	4.44 (1H, *d*, J_{H1H2} = 7.7 Hz)
2	3.06 (1H, *t*, $J_{H2H1} = J_{H2H3}$ = 7.9 Hz)	3.11
3	3.33 (1H, *t*, $J_{H3H2} = J_{H3H4}$ = 7.9 Hz)	3.32
4	3.34 (1H, *dd*, J_{H4H3} = 7.9, J_{H4H5a} = 4.4 Hz)	3.33
5a	3.72 (1H, *dd*, J_{H5aH4} = 4.4, J_{H5aH5b} = 11.7 Hz)	3.71
5b	3.05	3.09
R^4		
1	4.93 (1H, *d*, J_{H1H2} = 1.6 Hz)	5.01 (1H, *d*, J_{H1H2} = 1.8 Hz)
2	3.67 (1H, *dd*, J_{H2H1} = 1.6, J_{H2H3} = 2.9 Hz)	3.68
3	3.48 (1H, *dd*, J_{H3H2} = 2.9, J_{H3H4} = 9.1 Hz)	3.49
4	3.18 (1H, *dd*, J_{H4H3} = 9.1, J_{H4H5} = 9.5 Hz)	3.18
5	3.91 (1H, *dd*, J_{H5H4} = 9.5, J_{H5H6} = 6.2 Hz)	3.88
6	1.08 (3H, *d*, J_{H6H5} = 6.2 Hz)	1.09 (1H, *d*, J_{H6H5} = 6.3 Hz)
R^5		
1	5.11 (1H, *d*, J_{H1H2} = 4.3 Hz)	
2	3.90	
4a	3.98 (1H,. *d*, J_{H4aH4b} = 9.3 Hz)	
4b	3.58 (1H, *d*, J_{H4bH4a} = 9.3 Hz)	
5a	3.37	
5b	3.33	
R^6		
1	4.29 (1H, *d*, J_{H1H2} = 7.6 Hz)	4.29 (1H, *d*, J_{H1H2} = 7.7 Hz)

Table 1 (*continued*)

H	**1**	**2**
2	3.05	3.04
3	3.13 (1H, *t*, $J_{H3H2} = J_{H3H4} = 8.3$ Hz)	3.13
4	3.07	3.04
5	3.07	3.05
6a	3.62 (1H, *bd*, $J_{H6aH6b} = 10.7$ Hz)	3.62
6b	3.41 (1H, *dd*, $J_{H6bH6a} = 10.7$, $J_{H6bH5} = 2.4$ Hz)	3.42

[a]Due to the complexity of the NMR spectra all multiplicities and coupling constants could not be determined.

95%-dimethyl–5%-diphenyl siloxane copolymer. He was used as carrier gas at a linear velocity of 28.6 cm s^{-1} at 40°C. The injector temperature was 250°C. Samples were injected with a split ratio of 1:6. Temperature program: 160 to 250°C at 2°C min^{-1}.

Two columns were used for chiral GC separations:

(1) Capillary glass column (30 m × 0.3 mm) coated by LECUS by the static procedure with 0.25 μm of a mixture of heptakis(2,3-di-*O*-pentyl-6-*O*-methyl)-β-cyclodextrin (Carganico and Burger, unpublished results) (5.1 mg) and OV-1701-OH (33 mg). Temperature program: 40 to 180°C at 1.7°C min^{-1}. H_2 was used as carrier gas at a linear velocity of 51.7 cm s^{-1} at 40°C.

(2) Fused silica column (30 m × 0.25 mm) coated by LECUS first dynamically with Silanox [8] and then by the static procedure with 0.125 μm hexakis(2,3,6-tri-*O*-pentyl)-α-cyclodextrin [9]. Temperature program: 40 to 160°C at 1.7°C min^{-1}. H_2 was used as carrier gas at a linear velocity of 47.6 cm s^{-1} at 40°C.

The FID was operated at 280°C and the injector temperature was 150°C. Samples were injected at a split ratio of 1:6.

HPLC separations were carried out on a Hewlett Packard HP 1092 liquid chromatograph equiped with a Phenomenex Partisil C_8 column (5 μm, 250 × 4.6 mm i.d.) using 0.05 M KH_2PO_4: acetonitrile–isopropanol (4:1) as the mobile phase at a flow rate of 1.5 ml min^{-1} and a diode array detector. Gradient 1: 90% A to 30% A in 20 min; gradient 2: 90% A to 30% A in 30 min.

LC analyses were carried out on two columns:

(1) Anion exchange column: Dionex Carbopac PA1 (250 × 4 mm) using NaOH (150 mM) as the mobile phase at a flow rate of 0.75 ml min^{-1} and pulsed amperometric detection.

(2) Cation exchange column: HPX 87H column (250 × 4 mm) using H_2SO_4 (0.01 N) as the mobile phase at a flow rate of 0.5 ml min^{-1} and refractive index detection.

Plant material

The plant collected in the New-Hanover district in Natal, South Africa, was identified as *B. grandiflorum* var. *obovatum* by the National Botanical Institute in Pretoria where a voucher specimen (J. Fortmann sn1) is kept.

Biological testing

H522 human lung carcinoma cells (NCI, Frederick, Maryland, U.S.A.) were grown in an automatic CO_2-controlled incubator for 24 h in RPMI 1640 medium containing 10% fetal calf serum. Cells were plated at 10 000 cells in 200 μl medium of each well of a 96 multiwell plate. After incubation for 24 h at 37°C, 20 ml of a 1:500 dilution of the extract (1 g 10 ml^{-1}) was added to each well.

Extraction and isolation

The powdered root bark (10 g) of *B. grandiflorum* was extracted with boiling hot, distilled H_2O (300 ml). The extract was fractionated by elution from a C_{18}-column (100 g) with different MeOH–H_2O concentrations. Each fraction was freeze-dried, dissolved in distilled H_2O and tested for biological activity. The biologically active 50% MeOH–H_2O fraction (547 mg) was further purified on an analytical HPLC column (gradient 1) by collecting the fraction eluting between 11.25 and 12.3 min. The eluates were combined, freeze-dried, dissolved in MeOH to precipitate salts and centrifuged. The supernatant was dried under N_2, dissolved in H_2O and freeze-dried to yield an amorphous powder (94 mg). This fraction was further separated into 4 constituents by HPLC (gradient 2). Only constituents **1** (29 mg) and **2** (32 mg) could be isolated in small amounts and in a relatively pure state by prep. HPLC on the analytical column.

Constituent 1

A white amorphous powder. R_t 18.8 min. $[\alpha]_D^{19}$ −31.76° (MeOH, *c* 1.7). TOF-MALDI *m/z*: 1381.2 $[M + Na]^+$. ^1H NMR (499.9 Hz, DMSO-d_6): see Table 1, ^{13}C NMR (125.703, DMSO-d_6): see Table 2.

Table 2. ^{13}C and HMBC NMR data of constituents **1** and **2** at 125.703 MHz in DMSO-d_6

C	**1**	HMBC	**2**	HMBC
1	45.7, *t*	1.52	46.1, *t*	
2	69.2, *d*	3.45	69.1, *d*	
3	80.7, *d*	1.19, 4.29, 3.19, 3.55	80.8, *d*	4.29
4	42.3, *s*	1.19, 1.17, 3.45, 3.55	48.2, *s*	4.48, 3.55, 3.78, 3.69, 1.36
5	46.9, *d*	1.52, 1.19, 3.55, 1.82	47.8, *d*	
6	65.6, *d*	1.17	64.7, *d*	
7	39.7, *t*	0.93	37.7, *t*	
8	38.1, *s*	1.87, 1.05, 0.93, 1.45	38.0, *s*	
9	47.7, *d*	1.52, 0.93, 1.17, 5.27, 1.35	48.0, *d*	
10	35.4, *s*	1.57, 1.52, 1.17, 1.45, 4.14	35.4, *s*	
11	23.0, *t*	5.27	23.0, *t*	
12	122.3, *d*	2.87	122.1, *d*	
13	142.6, *s*	2.87, 1.05	142.6, *s*	
14	41.8, *s*	1.05, 0.93, 5.27, 2.87	41.8, *s*	
15	26.9, *t*	1.05	27.0, *t*	
16	22.1, *t*	22.0, *t*		
17	46.3, *s*	2.87, 1.68, 1.42	46.2, *s*	
18	40.7, *d*	0.88, 1.65, 5.27, 1.06, 1.42	40.7, *d*	
19	45.3, *t*	0.88, 0.90, 2.87	45.2, *t*	
20	30.3, *s*	0.88, 0.90, 1.42, 1.64, 1.32	30.3, *s*	
21	33.2, *t*	0.88, 0.90, 1.68	33.1, *t*	
22	31.8, *t*	31.7, *t*		
23	63.4, *t*	1.19, 1.17, 3.45	59.4, *t*	
24	15.6, *q*	1.17, 3.45	60.7, *t*	
25	17.9, *q*	17.9, *q*		
26	17.4, *q*	1.57, 1.45	17.5, *q*	
27	25.6, *q*	5.27	25.5, *q*	
28	175.5, *s*	5.68, 1.68, 1.92	175.0, *s*	5.64
29	32.7, *q*	0.90	32.7, *q*	
30	23.3, *q*	0.88	23.3, *q*	
R^1				
1	90.9, *d*	3.79, 3.61, 3.32	91.1, *d*	
2	74.3, *d*	4.70, 5.68, 3.32, 3.81	73.9, *d*	4.77
3	67.1, *d*	5.68, 3.79, 3.61, 3.32	67.5, *d*	
4	63.5, *d*	3.61, 3.79, 3.32	63.9, *d*	
5	59.9, *t*	5.68, 3.70, 3.81	60.5, *t*	
R^2				
1	99.4, *d*	3.61	99.4, *d*	3.62
2	70.0, *d*	4.70, 3.70, 3.58	70.2, *d*	
3	79.8, *d*	5.11, 4.70, 3.58, 3.77	70.4, *d*	
4	75.9, *d*	1.11, 3.53, 3.70, 3.77, 4.54	80.5, *d*	4.44
5	67.1, *d*	4.70, 1.11	67.1, *d*	
6	17.5, *q*	3.58	17.4, *q*	
R^3				
1	102.9, *d*	3.72, 3.58, 3.06	104.5, *d*	3.45
2	74.3, *d*	3.33	74.8, *d*	
3	81.1, *d*	4.93, 3.72, 3.06, 3.34	80.8, *d*	5.01
4	68.2, *d*	3.33, 3.72, 3.06, 3.05	67.8, *d*	
5	65.8, *t*	4.54	65.9, *t*	
R^4				
1	100.6, *d*	3.91, 3.33, 3.67	100.2, *d*	3.32
2	70.5, *d*	4.93, 3.91, 3.48, 3.33, 3.18	70.4, *d*	
3	70.5, *d*	4.93, 3.91, 3.67, 3.18	70.4, *d*	
4	72.0, *d*	1.08, 3.91, 3.48, 3.67	71.9, *d*	
5	68.0, *d*	4.93, 1.08, 3.48, 3.18	67.9, *d*	
6	17.7, *q*	3.18, 3.91	17.8, *q*	
R^5				
1	110.2, *d*	3.98, 3.90, 3.70, 3.58		
2	75.9, *d*	3.58, 3.33, 5.11, 3.37		
3	78.4, *s*	5.11, 3.98, 3.90, 3.37, 3.33, 3.58		
4	73.2, *t*	5.11, 3.33, 3.37, 3.90		
5	62.5, *t*	3.98, 3.90		
R^6				
1	103.7, *d*	3.45, 3.05	103.9, *d*	3.57
2	73.8, *d*	3.13	73.7, *d*	
3	76.8, *d*	3.05, 3.07	76.7, *d*	
4	70.0, *d*	3.13, 3.62	70.0, *d*	
5	76.5, *d*	3.07, 3.41, 4.29	76.5, *d*	
6	61.0, *t*	3.07	61.0, *t*	

Constituent 2

A white amorphous powder. R_t 19.8 min. $[\alpha]_D^{20}$ −28.33° (MeOH, *c* 1.2). TOF-MALDI *m/z*: 1262.4 $[M + Na]^+$. ^1H NMR (499.9 Hz, DMSO-d_6): see Table 1, ^{13}C NMR (125.703, DMSO-d_6): see Table 2.

Reference compounds

D-Apiose and L-apiose as well as D-rhamnose are not commercially available and were synthesized. Reactions were monitored by TLC (EtOAc–hexane, 3:2).

D-Apiose. D-*Apiose* was synthesized according to the literature [10] with a few modifications. *p*-Toluenesulfonic acid hydrate (40 mg) was added with stirring to an ice-cold solution of D-xylose (10 g, 0.067 mol) and 2-methoxypropene (14.4 g, 0.2 mol) in DMF (130 ml). After 12 h at 0–5°C, the xylose had reacted and three reaction products were evident by TLC (R_f: 0.61, 0.3, 0.28). The acid catalyst was neutralized by stirring with dried Amberlite IRA-400 resin (OH^- form). The resin was filtered off and the filtrate was evaporated at oil pump vacuum (1 mm, < 40°C) to give a syrup (17.15 g) that was thoroughly extracted with dry pentane. Vacuum evaporation of the pentane-soluble fraction gave the aldehyde, 2,3:4,5-di-*O*-isopropylidene-D-xylose (6.15 g, 40%, R_f 0.61). This product was immediately subjected to Cannizzaro reduction with formaldehyde. Formaldehyde (5 ml of 35% aqueous soln, 0.056 mol) and NaOH (2.35 g in 33 ml of H_2O) were added in succession to a stirred soln of the aldehyde (5 g, 0.02 mol) in EtOH (50 ml). After stirring for 27 h at room temp, all of the starting material (R_f 0.49) had been converted into product, 2-(hydroxymethyl)-2,3:4,5-di-*O*-isopropylidene-D-*threo*-pentitol (R_f 0.24). The reaction mixture was neutralized with HCO_2H (2 ml, 96%), the EtOH removed under red. pres. and the residual water layer thoroughly extracted with $CHCl_3$. The combined extracts were dried ($MgSO_4$) and the solvent removed under vacuum to give crystalline 2-(hydroxymethyl)-2,3:4,5-di-*O*-isopropylidene-D-*threo*-pentitol, which was recrystallized from $CHCl_3$–hexane (1:1) to give the product as white needles (2.1 g, 37%). This compound (500 mg) was then hydrolyzed with 65% HOAc (70 ml) for 20 h at room temp until the starting material (R_f 0.52) had been converted into the glycol (R_f 0.1). HOAc and H_2O were removed at oil pump vacuum (< 40°C) to give a syrup which was dissolved in H_2O (45 ml). The pH was adjusted to 7 with Na_2CO_3 and the aq. soln was treated with $NaIO_4$ (675 mg in 45 ml of H_2O) at room temperature for 1.5 h. The excess of periodate was decomposed by adding a few drops of ethylene glycol and the reaction mixture was stirred for another hour at room temp. The reaction mixture was evaporated to dryness at oil pump vacuum and thoroughly extracted with $CHCl_3$. The solvent was evaporated under vacuum to give a crude, crystalline product. CC with $CHCl_3$–MeOH (9:1) yielded 2,3-*O*-isopropylidene-β-D-apiofuranose as a white powder (220 mg, 61% of 2-(hydroxymethyl)-2,3:4,5-di-*O*-isopropylidene-D-*threo*-pentitol). A soln of this compound (220 mg) in H_2O (10 ml) was treated with Dowex 50 W resin (H^+ form, 400 mg) for 5 h at 70°C. The resin was removed, washed with H_2O and the aq. extracts and filtrate evaporated at oil pump vacuum to give D-apiose as a syrup (120 mg); $[\alpha]_D^{25}$ + 5.45° (H_2O, *c* 1.1), lit. [11] $[\alpha]_D^{25}$ + 5.6° (H_2O, *c* 1.1). NMR analysis of D-apiose showed that a solution of this product in D_2O contained the four possible isomeric forms (a–d) of D-apiose. ^{13}C NMR (75.42 MHz, D_2O, 25°C); (a) δ 106.1 (*d*, C-1), 84.3 (*s*, C-3), 83.0 (*d*, C-2), 76.7 (*t*, C-4), 64.7 (*t*, C-5); (b) δ 104.4 (*d*, C-1), 81.6 (*s*, C-3), 79.9 (*d*, C-2), 75.7 (*t*, C-4), 66.1 (*t*, C-5); (c) δ 100.5 (*d*, C-1), 83.7 (*s*, C-3), 78.2 (*d*, C-2), 75.0 (*t*, C-4), 64.8 (*t*, C-5) and (d) δ 99.0 (*d*, C-1), 80.2 (*s*, C-3), 75.5 (*t*, C-4), 74.0 (*d*, C-2), 66.7 (*t*, C-5).

L-Apiose. L-*Apiose* (122 mg) was prepared by the same procedure starting with L-xylose; $[\alpha]_D^{25}$ −5.45° (H_2O, *c* 1.1), lit. [11] $[\alpha]_D^{25}$ −5.0° (H_2O, *c* 1.1). According to an NMR analysis of L-apiose a solution of this product in D_2O contained the four possible isomeric forms of L-apiose. ^{13}C NMR (75.42 MHz, D_2O, 25°C); (a) δ 106.1 (*d*, C-1), 84.3 (*s*, C-3), 83.0 (*d*, C-2), 76.7 (*t*, C-4), 64.7 (*t*, C-5); (b) δ 104.4 (*d*, C-1), 81.6 (*s*, C-3), 79.9 (*d*, C-2), 75.7 (*t*, C-4), 66.1 (*t*, C-5); (c) δ 100.5 (*d*, C-1), 83.7 (*s*, C-3), 78.2 (*d*, C-2), 75.0 (*t*, C-4), 64.8 (*t*, C-5) and (d) δ 99.0 (*d*, C-1), 80.2 (*s*, C-3), 75.5 (*t*, C-4), 74.0 (*d*, C-2) 66.7 (*t*, C-5).

D-Rhamnose. Methyl 2,3-*O*-isopropylidene-α-D-mannopyranoside was prepared as follows from methyl α-D-mannopyranoside [12]. Methyl α-D-mannopyranoside (10 g, 0.05 mol) was added to a soln of 2,2-dimethoxypropane (100 ml) and *p*-toluenesulfonic acid (2 g) in Me_2CO (100 ml) and the reaction mixture was stirred until the solid had dissolved (*ca.* 7 h) [R_f 0.22 (unreacted methyl α-D-mannopyranoside), 0.62 (methyl 2,3-*O*-isopropylidene-α-D-mannopyranoside, traces), 0.85 (methyl 2,3:4,6-di-*O*-isopropylidene-α-D-mannopyranoside, main product)]. The desired product, methyl 2,3-*O*-isopropylidene-α-D-mannopyranoside, was prepared in good yield from the main product, methyl 2,3:4,6-di-*O*-isopropylidene-α-D-mannopyranoside by selective hydrolysis, by adding H_2O (200 ml) to the reaction mixture and stirring it for another 3 h [R_f 0 (unreacted methyl α-D-mannopyranoside), 0.29 (methyl 2,3-*O*-isopropylidene-α-D-mannopyranoside, main product), 0.92 (methyl 2,3:4,6-di-*O*-isopropylidene-α-D-mannopyranoside, traces)]. A $NaHCO_3$ soln (40 ml, 1 M) was added to neutralize the catalyst and the reaction mixture was dried at oil pump vacuum. The residue was dissolved in H_2O (300 ml)

and the remaining traces of methyl 2,3:4,6-di-*O*-isopropylidene-α-D-mannopyranoside (R_f 0.92) was extracted from the soln with hexane (4 × 120 ml). The aq. layer was extracted continuously with $CHCl_3$ for 7 h. The $CHCl_3$ extracts were dried ($MgSO_4$) and vacuum evaporation gave the main product, methyl 2,3-*O*-isopropylidene-α-D-mannopyranoside (10.6 g, 90.5%, R_f 0.29), as a syrup. Attempts to recrystallize the product from propyl acetate was not successful. The product was therefore dried azeotropically with C_6H_6 to yield a dry product for tosylation.

Methyl 2,3-*O*-isopropylidene-α-D-mannopyranoside was tosylated selectively at C-6 at 0°C to yield methyl 2,3-*O*-isopropylidene-6-*O*-(*p*-tolylsulfonyl)-α-D-mannopyranoside [13]. Thus methyl 2,3-*O*-isopropylidene-α-D-mannopyranoside (10 g, 0.042 mol) was dissolved in dry pyridine (63 ml) in an ice bath and a soln of *p*-toluenesulfonyl chloride (10 g, 0.055 mol) in pyridine (42 ml) was added dropwise within 2 h with vigorous stirring. After a further 24 h of stirring at room temp, H_2O (4 ml) was added to terminate the reaction. The solvent was evaporated under vacuum, the residue was redissolved in CH_2Cl_2 (20 ml) and the soln was washed with HCl (5%) and saturated bicarbonate, then dried, and concentrated. The product was chromatographed with EtOAc–hexane (1:1) to yield methyl 2,3-*O*-isopropylidene-6-*O*-(*p*-tolylsulfonyl)-α-D-mannopyranoside (6.56 g, 40.5%, R_f 0.37) as a syrup.

D-Rhamnose was prepared in two steps from methyl 2,3-*O*-isopropylidene-6-*O*-(*p*-tolylsulfonyl)-α-D-mannopyranoside [14]. $NaBH_4$ (3.21 g, 85 mmol) was added in portions to a stirred soln of methyl 2,3-*O*-isopropylidene-6-*O*-(*p*-tolylsulfonyl)-α-D-mannopyranoside (6.56 g, 17 mmol) in DMSO (60 ml). The mixture was stirred at 80°C for 2 h and then poured into cold H_2O (300 ml). The product was extracted with Et_2O (4 × 150 ml) and washed with H_2O (225 ml). The extract was dried (Na_2SO_4) and the solvent was removed under red. pres. to yield a syrup. CC with CH_2Cl_2–EtOAc (1:1) yielded methyl 2,3-*O*-isopropylidene-α-D-rhamnopyranoside as a colourless syrup (2.3 g, 62.0%, R_f 0.18). Amberlite IR-120 (H^+ form, wet weight 8.5 g) was added to a suspension of this product (2.3 g, 10.5 mmol) in H_2O (85 ml). The mixture was boiled under reflux for 16 h with vigorous stirring to remove the isopropylidene group. The resin was filtered off and the filtrate was evaporated at oil pump vacuum to yield D-rhamnose (1.72 g, 95%) as an amorphous solid.

D-Rhamnose was purified by prep. TLC, using distilled H_2O–EtOAc–pyridine (25:100:35) as mobile phase [15] to yield pure D-rhamnose. $[\alpha]_D^{25}$ −13.38° (H_2O, *c* 1.57), lit. [16] $[\alpha]_D^{25}$ −6.13° (H_2O, *c* 1.4). ^{13}C NMR (75.42 MHz, D_2O, 25°C); α-anomer: δ 96.7 (*d*, C-1), 74.9 (*d*, C-2)[a], 73.5 (*d*, C-3)[a], 72.6 (*d*, C-4)[a], 71.0 (*d*, C-5)[a], 19.5 (*q*, C-6); β-anomer: δ 96.2 (*d*, C-1), 75.4 (*d*, C-5)[b], 74.7 (*d*, C-4)[b], 74.5 (*d*, C-3)[b], 74.0 (*d*, C-2)[b], 19.4 (*q*, C-6) ([a,b] interchangeable assignments).

*Hexakis(2,3,6-tri-*O*-pentyl)-α-cyclodextrin*

This chiral stationary phase was prepared according to the method used by Meier-Augenstein *et al.* [9] for the preparation of the corresponding derivatized β-cyclodextrin with a few modifications. Reactions were monitored by TLC.

A stirred soln of α-cyclodextrin (2 g, 2.06 mmol, dried for 2 days at 40–50°C under red. pres. in the presence of P_2O_5) in dry dioxane (28 ml) and dry DMSO (28 ml) was heated at an oil bath temperature of 50°C and treated with freshly powdered NaOH (1.5 g, 38.5 mmol) and 1-bromopentane (4.2 ml, 38.5 mmol) while stirring. Similar quantities of NaOH and 1-bromopentane were added after 2 and 4 h. After 18 h dioxane (7 ml), DMSO (7 ml) and 1-bromopentane (2.5 ml) were added. After a total reaction time of 40 h the mixture was poured into 400 ml of ice H_2O and the resulting mixture was extracted 5× with Et_2O. The combined extracts were washed with H_2O and dried (Na_2SO_4). The solvent was evaporated under vacuum and the reaction mixture was dissolved in hexane and a small amount of Et_2O. The soln was washed 3× with H_2O and dried (Na_2SO_4). Evaporation of the solvent and additional drying at oil pump vacuum, yielded a pale yellow viscous oil. Attempts to crystallize the product from MeOH were not successful. The reaction product was therefore dried azeotropically with C_6H_6 and further at oil pump vacuum. The product, hexakis(2,6-di-*O*-pentyl)-α-cyclodextrin, was a viscous oil and was used for the synthesis of hexakis(2,3,6-tri-*O*-pentyl)-α-cyclodextrin without further purification. The hexakis(2,6-di-*O*-pentyl)-α-cyclodextrin was dissolved in a mixture of dry THF (21 ml) and dry dioxane (7 ml) and alkylated with 1-bromopentane (14.4 ml, 139 mmol) in the presence of NaH (53%, 4 g, 166 mmol) in a N_2 atmosphere. The alkylation was conducted under absolutely anhydrous conditions at 80°C. Further NaH (2 g) and 1-bromopentane (14.4 g) were added to the reaction mixture after 4 days. After a total reaction time of 6 days the inorganic salts were centrifuged off, the organic layer concentrated under red. pres. and the reaction product dissolved in 100 ml Et_2O. This soln was washed with H_2O until neutral. The organic layer was dried (Na_2SO_4) and evaporation of the solvent at oil pump vacuum yielded a highly viscous yellow oil. The oil was further purified by CC using hexane, hexane–Et_2O (9:1) and hexane–Et_2O (4:1) successively. The desired product was eluted with hexane–Et_2O (4:1). Evaporation of the solvent yielded the product, hexakis(2,3,6-tri-*O*-pentyl)-α-cyclodextrin, as a highly viscous colourless oil (2.21 g, 54%); R_f 0.69, hexane-*tert*-butyl methyl ether (4:1). ^{13}C NMR (75.42,

$CDCl_3$, 25°C); δ 97.6 (C-1), 80.8 (C-3), 80.2 (C-2), 78.1 (C-4), 74.0 (C-1‴), 71.8 (C-1′), 71.5 (C-1″), 71.4 (C-5), 69.6 (C-6), 30.4 (C-2′)[c], 30.1 (C-2″)[c], 29.6 (C-2‴)[c], 28.5 (C-3′)[d], 28.4 (C-3″)[d], 28.3 (C-3‴)[d], 22.9 (C-4′)[e], 22.7 (C-4″)[e], 22.6 (C-4‴)[e], 14.1 (C-5′, C-5″, C-5‴) ([c,d,e] interchangeable assignments).

Hydrolysis of **1** *and* **2** *and derivatization of the resulting monosaccharides*

Small samples (200 μg) of **1** and **2** were hydrolyzed with TFA (2 M, 40 μl) at 120°C for 1 h in sealed tubes. The hydrolysates were evaporated to dryness at 50°C in a stream of purified (activated charcoal) N_2. The resulting monosaccharides as well as authentic reference sugars were derivatized for comparative GC and GC-MS analyses:

Alditol peracetates [1]. The monosaccharides were reduced to their respective alditols with 40 μl of a solution of $NaBH_4$ (10 mg) in NH_4OH (1 M, 0.5 ml). After 1 h at room temp. the excess of borohydride was decomposed by dropwise addition of glacial HOAc, until effervescence had ceased. H_3BO_3, produced on decomposition of the borohydride, was removed by adding MeOH (40 μl) to the reaction mixture, followed by evaporation to dryness at 50°C with N_2. The drying process was repeated with MeOH (4×40 μl) and CH_2Cl_2 (4×30 μl). Acetylation of the alditols was performed in a sealed tube by heating the alditols with Ac_2O (40 μl) at 120°C for 3 h. The acetylated products were dissolved in CH_2Cl_2 for GC-MS analysis. R_t (Ara), 17.4 min; R_t (Xyl), 18.3 min and R_t (Glu), 27.2 min.

Trimethylsilylethers [2]. Oximes of the monosaccharides were prepared by addition of a soln of hydroxylamine hydrochloride in pyridine (25 mg ml^{-1}, 20 μl) and heating the reaction mixtures at 70–75°C for 30 min. After cooling to room temp., hexamethyldisilazane (20 μl) and TFA (2 μl) were added. The reaction mixtures were shaken well and allowed to stand at room temp. for 30 min. The silylated products were diluted with CH_2Cl_2 for GC-MS analysis. R_t (Xyl), 22.3 and 22.6 min; R_t (Ara), 22.6 and 22.8 min and R_t (Glu), 34.3 and 35.1 min.

Trifluoroacetates [7]. The monosaccharides were treated with a mixture of CH_2Cl_2 (400 μl) and trifluoroacetic acid anhydride (100 μl) in sealed tubes at 100°C for 1 h. After cooling to room temp, the excess reagent was blown off with N_2. The resulting trifluoroacetates were dissolved in CH_2Cl_2 for GC analysis. Chiral column 1: R_t (L-Rha), 37.1 and 50.0 min; R_t (D-Xyl), 51.8 and 54.1 min; R_t (D-Glu) 58.8 and 64.71 min. Chiral column 2: R_t (L-Ara), 26.5 and 32.4 min.

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