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Phytochemistry 68 (2007) 591–595
PHYTOCHEMISTRY
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α-Glucosidase inhibitory pentacyclic triterpenes from the stem bark of *Fagara tessmannii* (Rutaceae)

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Received 8 September 2006; received in revised form 11 December 2006
Available online 31 January 2007

Abstract

In addition to fatty acids, a mixture of sterols (β-sitosterol, stigmasterol, campesterol and stigmastanol), lupeol, arctigenin methylether, sesamin, vanillic acid (**1**), 2,6-dimethoxy-1,4-benzoquinone (**2**), betulinic acid and two pentacyclic triterpene acetates were isolated from *Fagara tessmannii* Engl. They were identified as 3β-acetoxy-16β-hydroxybetulinic acid (**3a**) and 3β,16β-diacetoxybetulinic acid (**3b**), and their structures were established using 1 and 2D NMR spectra and by comparison with published data. Two derivatives of the compounds were prepared. Some isolated compounds were evaluated for their antifungal and antibacterial activities. Compounds **1** and **3a** showed significant inhibition of α-glucosidase.

Keywords: *Fagara tessmannii*; Rutaceae; Pentacyclic triterpene; Antifungal; Antibacterial; α-Glucosidase inhibition

1. Introduction

Fagara tessmannii Engl. (Rutaceae) (Syn. *Zanthoxylum tessmannii*) is a shrub of the African rainforests in South-West, Centre, South and East provinces in Cameroon. In the traditional medicine, it is used locally for the treatment of tumors, swellings, inflammation and gonorrhoea (Raponda-Walker and Sillans, 1961). The root bark of this plant is used in West Africa in traditional medicine as a toothbrush (Kerharo and Adam, 1971). Previous phytochemical studies of *F. tessmannii* collected at three different localities in Cameroon resulted in the isolation of alkaloids, lignans, triterpenes and steroids (Ayafor et al., 1984). The widespread use of *F. tessmannii* in indigenous medicine for different ailments, as well as the antifungal and antioxidant activities exhibited by the genus (Chaaib et al., 2003), justified further attempts to isolate and identify active compounds. In this paper, we report the isolation and structure elucidation of two new pentacyclic triterpene acetates, and their biological activities.

2. Results and discussion

The stem bark of *F. tessmannii* was extracted with MeOH. The extract was submitted to repeated column chromatography and preparative TLC (PTLC) to afforded fatty acids, a mixture of sterols (β-sitosterol, stigmasterol, campesterol and stigmastanol), lupeol, arctigenin methylether, sesamin, betulinic acid, vanillic acid (**1**), 2,6-dimethoxy-1,4-benzoquinone (**2**), and two new pentacyclic triterpene acetates (**3a**, **3b**). The ^{1}H and ^{13}C NMR, and

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doi:10.1016/j.phytochem.2006.12.015

MS of the known compounds were consistent with those reported in the literature.

Compound **3a** was obtained as a white powder from the hexane/ethyl acetate (7:3) extract B. The (+)-ESI HR mass spectrum indicated a pseudomolecular ion at *m/z* 532.39963 $[M + NH_4]^+$, corresponding to a molecular formula $C_{32}H_{54}NO_5$. The IR spectrum showed a broad signal at υ 3415 cm^{-1} indicating free hydroxyl group, and an ester signals at υ 1724 (C=O) and 1247 (C–O) cm^{-1}. Three absorptions at υ 2947, 1641 and 888 cm^{-1} indicated a vinylidene group (=CH_2), characteristic of lup-20(29)-ene (Roitman and Jurd, 1978).

The ^{1}H NMR data indicated that compound **3a** is a pentacyclic triterpenoid (Cheung and Williamson, 1969) with five methyl groups between δ 0.81–1.12 (*s*, 3H each), an olefinic methyl at δ 1.70, an acetyl methyl at δ 2.09, seven methine and nine methylene groups between δ 1.12 and 2.20. A methine signal at δ 3.20 (*ddd*, $J = 5.2$, 10.8, 12.8 Hz) was attributed to the proton at position C-19, and one proton signal at δ 3.74 (*dd*, 1H, $J = 4.4$ and 11.3 Hz) indicated the presence of oxygen and is characteristic of an equatorial (β) orientation at position C-16. A proton signal at δ 4.61 (*t br*, 1H) is due to the presence of an acetoxy group, two olefinic signals at δ 4.64 (*d br*, 1H, $J = 1.3$ Hz, Hb-29) and 4.76 (*d br*, 1H, $J = 1.3$ Hz, Ha-29) indicated an exomethylene group.

The ^{13}C NMR spectrum (Table 1) of compound **3** revealed the presence of 30 carbon atoms, which were in accordance with the proton data, and additionally a carboxylic acid signal at δ 177.9 and an acetate carbonyl at δ 171.0. The carbon atoms at δ 149.4 and 110.0 are characteristic for the carbons 20 and 29 of lup-20(29)-ene (Gunasekera et al., 1982). These data indicated that compound **3a** might be a lupan-type triterpene. The fragment at *m/z* 189 supported the presence of lup-20(29)-ene (Kumar et al., 1985).

In the HMBC spectrum (Fig. 1), correlations between the H-3 signal and carbons 1, 2, 4, 5 and 1′, between the proton H-16 and carbons 14, 15, 17, 18 and 28, and finally the correlation of H-19 with C-18, 20, 21, 22, 27 and 29 as well as the close similarity with the shifts of 16β-hydroxybetulinic acid (**3e**) (Table 1) indicated that compound **3a** is 3β-acetoxy-16β-hydroxybetulinic acid, which is described here for the first time.

Compound **3b** was isolated as amorphous solid from the same fraction B eluted with hexane/ethyl acetate (7:3). The (+)-ESI HR mass spectrum indicated a pseudomolecular ion at *m/z* 574.41021 $[M + NH_4]^+$, corresponding to the molecular formula $C_{34}H_{56}NO_6$. Comparison of the spectral data of compound **3a** and **3b** indicated an additional acetate group in **3b**, which was confirmed by the peak at *m/z* 496 $[M-CH_3CO-H_2O]^+$. The IR spectrum revealed the expected ester signals at υ 1729 cm^{-1} (C=O) and 1246 cm^{-1} (C–O).

The ^{1}H NMR spectra of **3a** and **3b** were nearly superimposable, the only difference being the presence of a further acetate group at δ 2.08 and the down-field shift of the H-16 signal from δ 3.74 in **3a** to δ 5.05 in **3b**. The main difference in the ^{13}C NMR spectrum (Table 1) was the presence of an additional ester carbonyl signal at δ 170.9.

Table 1
^{13}C NMR of compounds **3a, b, c and d** (75, 125 and 150 MHz, $CDCl_3$) in comparison with 16β-hydroxybetulinic acid (**3e**)

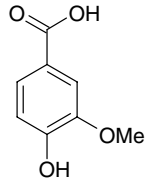
C Nr	3a	3b	3c	3d	3e
1	36.6	36.6	36.6	36.6	38.9
2	22.8	28.0	22.8	24.5	28.7
3	78.4	78.3	78.3	78.3	77.1
4	39.3	39.0	37.1	34.6	39.3
5	50.2	50.2	50.2	50.2	56.0
6	18.0	21.3	19.3	18.6	18.6
7	35.1	34.9	35.2	33.4	34.3
8	40.9	41.2	40.9	41.2	41.6
9	49.8	49.9	49.7	49.5	50.1
10	37.1	37.0	33.9	30.5	37.9
11	20.5	21.7	21.7	21.7	20.8
12	25.1	24.6	25.0	22.8	25.0
13	37.8	38.1	38.2	37.1	38.4
14	44.2	44.2	44.1	44.0	44.2
15	34.1	37.5	39.7	34.6	39.9
16	76.1	77.1	76.3	76.8	75.9
17	60.7	59.5	61.4	59.5	62.1
18	48.9	49.5	49.3	49.5	49.0
19	46.8	47.3	47.5	47.7	48.6
20	149.4	148.8	149.5	151.3	149.9
21	30.7	33.9	30.9	27.8	32.3
22	33.9	34.6	34.0	34.2	36.5
23	27.8	27.7	27.8	29.5	28.5
24	15.9	15.9	16.2	15.9	16.2
25	16.1	18.0	18.0	18.0	16.5
26	16.3	18.8	15.9	15.9	16.6
27	21.4	15.9	15.8	15.9	16.1
28	177.9	176.9	176.8	173.5	178.1
29	110.0	110.6	110.1	110.7	110.4
30	19.5	20.5	20.6	20.6	19.4
1′	171.0	170.3	170	170.6	–
2′	21.6	21.4	21.4	21.4	–
1″	–	170.9	–	170.9	–
2″	–	22.8	–	21.3	–
CH_3O	–	–	51.6	51.5	–

The analysis of the data and comparison with those of **3a** and 16β-hydroxybetulinic acid (**3e**) indicated that **3b** is 3β,16β-diacetoxybetulinic acid, which is described here for the first time.

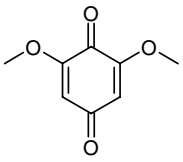
Methylation of compound **3a** with diazo methane yielded **3c** ($C_{33}H_{52}O_5$), and acetylation of **3c** gave **3d** ($C_{35}H_{54}O_6$). Their structures were confirmed by EI, HRESIMS, ^{1}H and ^{13}C NMR spectra (see Section 3).

The antifungal and antibacterial activities of **2** and **3a, b, c** and **d** were determined using the agar diffusion method with 9 mm paper disks loaded with 40 μg of each compound isolated from this plant. Only 2,6-dimethoxy-1,4-benzoquinone (**2**) showed activities against *Bacillus subtilis* (20 mm inhibition diameter), *Staphylococcus aureus* (17 mm), *Escherichia coli* (14 mm), *Streptomyces viridochromogenes* (Tü 57) (12 mm), *Mucor miehei* (12 mm), *Chlorella vulgaris* (10 mm) and *Scenedesmus subspicatus* (10 mm); 3β-acetoxy-16β-hydroxybetulinic acid (**3a**) showed weak activities against *Bacillus subtilis* (13 mm) and *Escherichia coli* (11 mm), and 3β,16β-diacetoxybetuli-

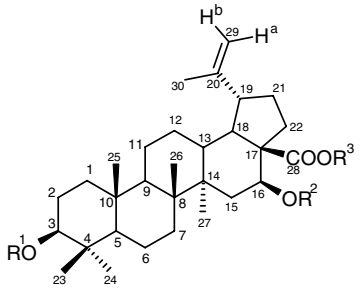
Structures of compound 1, 2, 3a, b, c, d and e



1



2



	R^1	R^2	R^3
3a	Ac	H	H
3b	Ac	Ac	H
3c	Ac	H	Me
3d	Ac	Ac	Me
3e	H	H	H

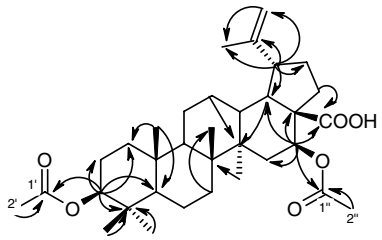


Fig. 1. Selected HMBC correlation in compound **3b**. Respective correlations were found also in compound **3a**.

nic acid (**3b**) showed weak activities against *Bacillus subtilis* (12 mm) and *Candida albicans* (14 mm).

When tested again three common glycosidases, compounds **3a** (7.6 ± 0.6 μM) and **1** (69.4 ± 0.8 μM), shown potent and selective inhibition of yeast α-glucosidase than against the other enzymes (Table 2). Compound **3a** showed significant higher inhibition of yeast α-glucosidase than deoxynojirimycin (425.6 ± 8.1 μM) and acarbose (780.0 ± 0.1 μM).

Table 2
Glycosidase inhibition of some isolated compounds

Compound	α-D-Glucosidase (yeast)	β-D-Glucosidase (sweet almonds)	α-D-Mannosidase (jack bean)
	$IC_{50} \pm SEM$	$IC_{50} \pm SEM$	$IC_{50} \pm SEM$
3a	7.6 ± 0.6	397.6 ± 0.8	NI
1	69.4 ± 0.8	295.8 ± 0.5	NI
2	900.0 ± 3.5	NI	NI
Arctigenin methylether	NI	NI	NI
Lupeol	NI	NI	NI
Mixture of steroids	NI	NI	NI
Sesamin	NI	NI	NI

NI, no inhibition at 800 μM concentration.

3. Experimental section

3.1. Materials and methods

NMR spectra were measured on Varian Unity 300 (300.145 MHz) and Varian Inova 500 (499.876 MHz) spectrometers. ESI MS was recorded on a Finnigan LCQ with quaternary pump Rheos 4000 (Flux Instrument). ESI HR mass spectra were recorded on A Bruker FTICR 4.7 T mass spectrometer. EI-MS spectra were recorded on a Finnigan MAT 95 spectrometer (70 eV) with perfluorkerosene as reference substance for HREI MS. IR spectra were recorded on a Perkin-Elmer 1600 Series FT-IR spectrometer from films. Flash chromatography was carried out on silica gel (230–400 mesh). R_f values were measured on Polygram SIL G/UV254 (Macherey-Nagel & Co.).

3.2. Plant material

The stem bark of *F. tessmannii* Engl. was collected in April 2005 at Limbe, South-West Cameroon. A specimen has been deposited in the National Herbarium, Yaoundé, Cameroon (Ref. No. 1490/SRFK).

4. Extraction and isolation

The powdered stem bark of *Fagara tessmannii* (1.5 kg) was extracted with MeOH at room temperature during 48 h. After removing the solvents by evaporation under reduced pressure, the crude extract (73.2 g) was chromatographed on silica gel. Using hexane/ethyl acetate of increasing polarity, a total of 125 sub-fractions (ca. 250 ml each) were collected and combined on the basis of TLC analysis leading to three main fractions A, B and C.

Fraction A (15.0 g) was chromatographed on silica gel and eluted with a mixture of hexane/ethyl acetate of increasing polarity to yield fatty acids (104.0 mg) (Andersen and Gorbert, 2002), mixture of sterols (GC showed the presence of β-sitosterol, stigmasterol, campesterol and stigmastanol) (102.0 mg) (Morris et al., 1984) and lupeol (69.0 mg) (Razdan et al., 1996).

Fraction B (7.0 g) was chromatographed on silica gel and eluted using hexane/ethyl acetate (7:3) to deliver betulinic acid (17.0 mg), 3β-acetoxy-16β-hydroxybetulinic acid **3a** (11.0 mg), 3β,16β-diacetoxybetulinic acid **3b** (4.0 mg), arctigenin methylether (6.0 mg) and sesamin (5.0 mg) (Ayafor et al., 1984).

Fraction C (11.0 g) produced in the same way vanillic acid **1**(12.5 mg) (Shchukin and Medvedeva, 1999) and 2,6-dimethoxy-1,4-benzoquinone (**2**) (15.0 mg).

4.1. 3β-Acetoxy-16β-hydroxybetulinic acid (**3a**)

White powder, $R_f = 0.53$ (CH_2Cl_2); m.p. 255–257 °C; IR (film): $\upsilon = 3415, 2947, 2873, 1724, 1641, 1451, 1375, 1247, 1183, 1040, 1020, 975, 888\ cm^{-1}$; ^1H NMR (300 MHz, $CDCl_3$): δ 4.76 (*d br*, 1 H, $J = 1.3$ Hz, Ha-29), 4.64 (*d br*, 1H, $J = 1.3$ Hz, Hb-29), 4.61 (*t br*, 1H, H-3), 3.74 (*dd*, 1H, $J = 4.4$, 11.3 Hz, H-16), 3.20 (*ddd*, 1H, $J = 5.2$, 10.8, 12.8 Hz, H-19), 2.15 (*m*, 2H, H-21), 2.13 (*m*, 2 H, H-15), 2.09 (*s*, 3H, Ac), 2.05 (*m*, 2H, H-12), 1.90 (*m*, 1H, H-18), 1.70 (*s*, 3H, H-30), overlapping multiplets at 1.72, 1.67, 1.62, 1.61, 1.60, 1.58, 1.50, 1.48, 1.40, 1.39, 1.38, 1.20, 1.10 (*m*, 15H, H-1, 2, 5, 6, 7, 9, 11, 13, 22), 1.10 (*s*, 3H, H-26), 0.98 (*s*, 3H, H-27), 0.87 (*s*, 3H, H-23), 0.83 (*s*, 3H, H-25), 0.82 (*s*, 3H, H-24); ^{13}C NMR (125 MHz, $CDCl_3$) (Table 1); EI MS (70 eV): *m/z* (%) 514 (M^+, 5), 454 (6), 189 (11), 145 (5), 107 (7), 81 (10), 55 (13), 43 (100); (+)-ESI HRMS: *m/z* 532.39963 ($[M+NH_4]^+$, calcd 532.39965 for $C_{32}H_{54}NO_5$).

4.2. 3β,16β-Diacetoxybetulinic acid (**3b**)

Amorphous solid, $R_f = 0.84$ (CH_2Cl_2); IR (film): $\upsilon = 3426, 3077, 2944, 2874, 1729, 1644, 1453, 1374, 1246, 1182, 1026, 985, 964, 920, 888, 757\ cm^{-1}$; ^1H NMR (300 MHz, $CDCl_3$): δ 5.08 (*dd*, 1H, $J = 4.9$ and 11.7 Hz, H-16), 4.78 (*d br*, 1H, $J = 1.4$ Hz, Ha-29), 4.62 (*m br*, 2H, Hb-29 and H-3), 2.78 (*ddd*, 1H, $J = 5.4$, 11.3 and 13.7 Hz, H-19), 2.40–2.12 (*m*, 4H, H-15 and H-21), 2.10 (*s*, 6H, H-2′ and H-2″), 1.98–1.70 (*m*, 3H, H-18 and H-22), 1.68 (*s*, 3H, H-30), 1.65-1.12 (*m*, 15H, H-1, H-2, H-5, H-6, H-7, H-9, H-11, H-12, H-13), 1.10 (*s*, 3H, H-25), 1.07 (*s*, 3H, H-26), 0.83 (*s*, 9H, H-23, H-24, H-27); ^{13}C NMR (150 MHz, $CDCl_3$) (Table 1); EI MS (70 eV): *m/z* (%) = 556 (M^+, 11), 496 (50), 481 (10), 452 (8), 436 (42), 421 (10), 392 (13), 246 (11), 213 (15), 199 (27), 190 (38), 189 (100), 190 (44), 173 (28), 135 (44), 95 (44), 81 (39), 43 (69); (+)-ESI HRMS: 574.41021 ($[M+NH_4]^+$, calcd 574.41074 for $C_{34}H_{56}NO_6$).

4.3. 3β-Acetoxy-16β-hydroxybetulinic acid methyl ester (**3c**)

Compound **3a** (5 mg), was dissolved in methylene chloride (2 ml) and an etherial diazomethane solution (2 ml) was added at −20 °C. Immediate evaporation to dryness gave **3c** (5 mg, 97%) as an amorphous solid with $R_f = 0.63$ (CH_2Cl_2); ^1H NMR (300 MHz, $CDCl_3$): $\delta = 4.90$ (*d br*, 1H, $J = 3.0$ Hz, Ha-29), 4.75 (*d br*, 1H, $J = 3.0$ Hz, Hb-29), 4.61 (*t br*, 1H, $J = 1.7$ Hz, H-3), 3.78 (*s*, 3H, MeO-), 3.60 (*t br*, 1H, $J = 2.4$ Hz, H-16), 2.99 (*ddd*, 1H, $J = 5.3$, 10.9 and 12.9 Hz, H-19), 2.15 (*m*, 1H, H-13), 2.08 (*s*, 3H, H-2′), 2.06–1.80 (*m*, 4H, H-12, H-21), 1.70 (*s*, 3H, H-30), 1.68–1.11 (*m*, 16H, H-1, H-2, H-5, H-6, H-7, H-9, H-11, H-15, H-22), 1.08 (*s*, 3H, H-26), 0.92 (*s*, 3H, H-27), 0.88 (*s*, 3H, H-23), 0.83 (*s*, 6H, H-24, H-25);^{13}C NMR (75 MHz, $CDCl_3$) (Table 1); EI MS (70 eV): *m/z* (%) = 528 (M^+, 13), 510 (100), 469 (10), 450 (30), 435 (9), 391 (9), 260 (10), 246 (13), 199 (24), 189 (38), 173 (11), 135 (18), 107 (18), 81 (20), 69 (17), 43 (20); (+)-ESI HRMS: 529.38875 ($[M + H]^+$, calcd 529.38928 for $C_{33}H_{53}O_5$), 551.3707 $[M+Na]^+$, calcd 551.37122 for $C_{33}H_{52}NaO_5$.

4.4. 3β,16β-Diacetoxybetulinic acid methyl ester (**3d**)

3-Acetoxy-16-hydroxybetulinic acid methyl ester (**3c**, 3 mg) was dissolved in pyridine (0.4 ml) and acetanhydride (0.9 ml). The solution was stirred for 6 h at 35 °C. Hydrolysis and usual work-up gave **3d** (2 mg, 62%) as an amorphous solid with $R_f = 0.81$ (CH_2Cl_2); ^1H NMR (300 MHz, $CDCl_3$): δ 5.1 (*dd*, 1H, $J = 5.0$ and 11.4 Hz, H-16), 4.70 (*d br*, 1H, $J = 0.8$ Hz, Ha-29), 4.60 (*d br*, 2H, Hb-29 and H-3), 3.72 (*s*, 3H, MeO), 3.06 (*m br*, 1H, H-19), 2.50 (*ddd*, 1H, $J = 5.2$, 10.7 and 11.3 Hz, H-13), 2.44–2.13 (*m*, 4H, H-15, H-21), 2.10 (*s*, 3H, H-2′), 2.03 (*s*, 3H, H-2″), 2.00–1.70 (*m*, 7H, H-1, H-12, H-18, H-22), 1.67 (*s*, 3H, H-30), 1.60–1.18 (*m*, 12H, H-2, H-5, H-6, H-7, H-9, H-11, H-21), 1.17 (*s*, 3H, H-26), 1.14 (*s*, 3H, H-27), 0.88 (*s*, 3H, H-23), 0.87 (*s*, 3H, H-24), 0.86 (*s*, 3H, H-25); ^{13}C NMR (125 MHz, $CDCl_3$) (Table 1); EI MS (70 eV): *m/z* (%) = 570 (M^+, 2), 510 (100), 495 (6), 450 (23), 435 (6), 391 (8), 260 (10), 246 (12), 199 (18), 187 (35), 185 (18), 135 (12), 107 (13), 81 (18), 43 (79); (+)-ESI HRMS: 588.42587 ($[M+NH_4]^+$, calcd 588.42639 for $C_{35}H_{58}NO_6$), 593.38126 ($[M+Na]^+$, calcd 593.38179 for $C_{35}H_{54}NaO_6$).

5. Biological activities

5.1. Antimicrobial assay

Agar diffusion tests were performed in the usual manner (Maskey et al., 2002) with *Bacillus subtilis* and *Escherichia coli* (on peptone agar), S*taphylococcus aureus* (Bacto nutrient broth), *Streptomyces viridochromogenes* (M Test agar), the fungi *Mucor miehei* and *Candida albicans* (Sabouraud agar), and three microalgae (*Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus subspicatus*).

Compounds were dissolved in an chloroform/MeOH (87:13) azeotrope and paper disks (Ø 9 mm) were impregnated with each 40 µg using a 100 µl syringe, dried for 1 h under sterile conditions and placed on the pre-made agar test plates. Bacteria and fungi plates were kept in an incubator at 37 °C for 12 h, micro algae plates for three days at room temperature in a day light incubator. The diameter of inhibition zones was measured.

5.2. Glycosidase inhibition

The glycosidase inhibition assay was performed according to the slightly modified method of Oki et al. (1999). Activity of the compounds has been determined against α-D-Glucosidase (E.C. 3.2.1.20), β-D-Glucosidase (E.C. 3.2.1.21), and α-D-Mannosidase (E.C. 3.2.1.24),

purchased from Wako Pure Chemical Industries Ltd. Osaka, Japan (Wako 076-02841). The inhibition was measured spectrophotometrically at 37 °C using 1 mM *p*-nitrophenyl α-D-glucopyranoside, and *p*-nitrophenyl β-D-glucopyranoside as a substrate at pH 6.9, then at pH 4.0 using 1 mM *p*-nitrophenyl α-D-mannopyranoside and 500 U/ml enzymes, in 50 mM sodium phosphate buffer containing 100 mM NaCl. 1-Deoxynojirimycin (0.425 mM) and acarbose (0.78 mM) were used as positive controls. The increment in absorption at 400 nm due to the hydrolysis of PNP-G by glycosidase was monitored continuously with the spectrophotometer (Molecular Devices, USA) (see Table 2).

Acknowledgements

We thank R. Machinek for the NMR spectra, H. Frauendorf for the mass spectra, and F. Lissy for microbiological work. H. M. P. P. is thankful to the DAAD (Deutscher Akademischer Austauschdienst) for a research grant J. D. W. thank the IFS (International Foundation of Sciences) for a providing financial support (F/3978-1).

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