PERGAMON

Phytochemistry 59 (2002) 57–62

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Biotransformation of terpenes from *Stemodia maritima* by *Aspergillus niger* ATCC 9142

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Received 6 March 2001; received in revised form 7 August 2001

Dedicated to Professor Sir John Cornforth, University of Sussex as he enters his 85th year

Abstract

Incubation of stemodin (**1**) in cultures of *Aspergillus niger* ATCC 9142 resulted in the production of 2α,3β,13-trihydroxystemodane (**2**), 2α,7β,13-trihydroxystemodane (**3**) and 2α,13,16β-trihydroxystemodane (**4**), while stemodinone (**5**) afforded 13,18-dihydroxystemodan-2-one (**6**) and 13,16β-dihydroxystemodan-2-one (**7**). Four novel metabolites were obtained from the bioconversion of stemarin (**8**) by the fungus, namely 18-hydroxystemaran-19-oic acid (**9**), 7β,18-dihydroxystemaran-19-oic acid (**10**), 7α,18,19-trihydroxystemarane (**11**) and 1β-hydroxystemaran-19-oic acid (**12**). 19-*N,N*-Dimethylcarbamoxy-13-hydroxystemarane (**13**) was also transformed to afford 19-*N,N*-dimethylcarbamoxy-13,17ξ,18-trihydroxystemarane (**14**).

Keywords: *Aspergillus niger* ATCC 9142; Biotransformation; Stemodane; Stemarane; Diterpene; *Stemodia maritima*; Hydroxylation

1. Introduction

Aspergillus niger ATCC 9142 (IMI 41874, NRRL 599) has been exploited in the biotransformation of various substrates including steroids, terpenes and alkaloids. It has been used to reduce ketones to their corresponding alcohols (Fauvre and Veschambre, 1988) and oxidise heteroatoms (Auret et al., 1974). However, hydroxylation reactions on terpenes (Hoffmann et al., 1992; de Oliveira et al., 1999) and steroids (Auret and Holland, 1971; Holland and Thomas, 1982) are more common. Recently bioconversion of terpenoid pharmaceuticals by another strain of *A. niger* has been seen to parallel that of mammalian systems (Hashimoto et al., 1999; Lahlou et al., 2000; Hashimoto et al., 2001).

The plant *Stemodia maritima* has yielded a number of diterpenes including stemodin, stemodinone, stemarin and stemolide (Manchand et al., 1973; Manchand and Blount, 1976), some of which possess mild antiviral activity (Hufford et al., 1991). Stemodin and stemodinone have been previously biotransformed (Azerad, 2000) by *Cunninghamella echinulata* (Badria and Hufford, 1991; Hufford et al., 1991), *Polyangium cellulosum* (Badria and Hufford, 1991), *Rhizopus arrhizus* (Hufford et al., 1991) and *Cephalosporium aphidicola* (Hanson et al., 1994).

In an ongoing exercise to produce new analogues of natural products and their derivatives (Hanson et al., 1994; Buchanan and Reese, 2000), we now report the action of *A. niger* ATCC 9142 on stemodin (**1**), stemodinone (**5**), stemarin (**8**) and its dimethylcarbamate (**13**).

2. Results and discussion

Stemodin was isolated from *Stemodia maritima*. Incubation in cultures of *Aspergillus niger* ATCC 9142 afforded 2α,3β,13-trihydroxystemodane (**2**), 2α,7β,13-trihydroxystemodane (**3**) and 2α,13,16β-trihydroxystemodane (**4**). The latter was characterised as the diacetate **4a**. Terpene **2** has never been isolated as a product of microbial transformation, however, it is a

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0031-9422/02/$ - see front matter
PII: S0031-9422(01)00355-7

natural product (maristeminol) from the plant *S. maritima* (Hufford et al., 1992). Compound **3** has been isolated previously from bioconversion experiments using other fungi (Badria and Hufford, 1991; Hanson et al., 1994). The HREIMS spectrum of **4a** indicated that **4** was the product of monohydroxylation. Analysis of ^{13}C and DEPT NMR experiments revealed the appearance of a new methine resonating at δ 80.5. This was accompanied by the loss of a methylene at 30.1 ppm (C-16).

It is possible that **2** and **4** are produced by the same enzyme (enzyme 1). Preliminary molecular modelling studies using Chem3D software (CambridgeSoft Corporation) indicate that the distance between the potential binding group (2α-OH) and the hydroxylation site (3β-H) is approximately 2.7 Å in **1**. Similarly the 13-OH is 2.5 Å away from the 16β-H. A second enzyme (enzyme 2) could be involved in the formation of **3** as the distance between the 13-OH and the 7β-H in **1** is 6.9 Å (see later).

Since only small quantities were produced by the plant stemodinone (**5**) was synthesised via oxidation of stemodin (**1**) using Jones' reagent. Incubation of **5** with *A. niger* yielded 13,16β-dihydroxystemodan-2-one (**6**) and 13,18-dihydroxystemodan-2-one (**7**). These compounds have been isolated previously from fermentation of **5** with *Cephalosporium aphidicola* (Hanson et al., 1994).

The two enzymes proposed could be involved in the formation of **6** and **7**. The 13-OH- 16β-H distance in **5** is 2.5 Å (enzyme 1) while the 13-OH – 18-H minimum distance is 6.8 Å (enzyme 2).

The bioconversion of stemarin (**8**) led to the preparation of four new analogues. ^{13}C NMR data of **9** revealed a new nonprotonated carbon signal resonating at 183.4 ppm, which indicated the presence of a carboxylic acid functionality; this was accompanied by the loss the signal for the primary alcohol. Only two methyl resonances were seen in the ^{1}H NMR spectrum. There were two hydrogens coupled to each other resonating at 3.93 and 4.08 ppm. Analysis by ^{13}C NMR showed the loss of the methyl at 18.1 ppm and the appearance of a new signal at 62.0 ppm (CH_2–O). The mass spectrum of **9** had a peak at m/z 318.2189 which was assigned to [M–18^+]. Hence, the metabolite was assigned as 13,18-dihydroxystemaran-19-oic acid. Oxidation of a primary alcohol to the corresponding carboxylic acid by this fungus has not been reported previously. In the second metabolite (**10**) the primary alcohol had also been oxidised to the carboxylic acid. This was evident from the new signal at 184 ppm in the ^{13} C NMR spectrum. Two new signals were seen at 63.7 (CH_2–O) and 80.4 ppm (CH–O) while the resonances at 30.0 (C-7) and 18.1 ppm (C-18) had disappeared. The β stereochemistry of the newly inserted hydroxy moiety was determined based on the position and coupling constant data for the axial 7α-proton (δ 3.62, *m*. $^{w}/_{2} = 16$ Hz). The ESMS of **10** had a peak at m/z 375.2516 which was assigned to $M + Na^+$. The compound was identified as 7β,13,18-trihydroxystemaran-19-oic acid. Comparison of the ^{13}C NMR data of **11** with that from stemarin showed that the methylene signal at 71.5 ppm (C-19) was still present. The loss of peaks resonating at δ 18.1 (C-18) and 30.8 (C-7) was accompanied by the appearance of a methylene and methine at 64.4 and 66.9 ppm respectively. In this case the ^{1}H NMR data for the equatorial 7β-proton (δ 3.98, m, $^{w}/_{2} = 10.8$ Hz) indicated that the the hydroxy group had α stereochemistry. The metabolite was thus determined to be 7α,13,18,19-tetrahydroxystemarane. The ^{13}C NMR spectrum of the fourth metabolite (**12**) indicated the presence of the carboxylic acid (δ 182.7) and a signal at 76.2 ppm (CH-OH). Loss of the signal at 31.8 ppm suggested that hydroxylation had occurred at C-1. The equatorial nature of the hydroxyl was inferred from the position and multiplicity (δ 3.58, *dd*, $J = 5.1$, 11.1 Hz) of the H-1 signal. The HREIMS had a M^+ peak at m/z 336.2301. The metabolite was assigned as 1α,13-dihydroxystemaran-19-oic acid.

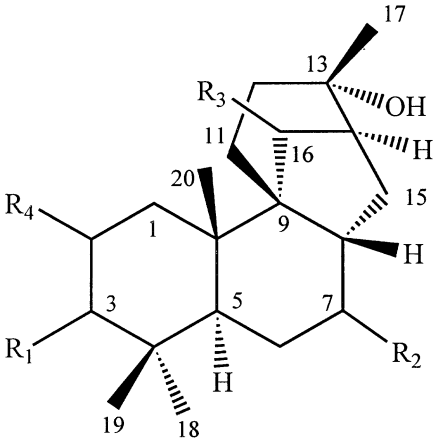
In an effort to block the oxidation of the alcohol, while still providing a docking group on the molecule, the dimethylcarbamate derivative of stemarin (**13**) (Vigne et al., 1986, 1991) was prepared by treatment of **8** with dimethylcarbamyl chloride (Jager et al., 1968; Agarwal and Khorana, 1972). HREIMS analysis suggested a molecular formula of $C_{23}H_{39}NO_3$ ($M^+ =$ 377.2930). The product of transformation (**14**) appeared to be a dihydroxylated compound. Comparison of the ^{13}C NMR spectra with that of the fed compound indicated that there was loss of a methyl signal at δ 17.8 (C-18) and a methylene signal at δ 26.8 (C-17). The new resonances at δ 61.8 and 65.8 were assigned to carbons 18 and 17 respectively. The stereochemistry of hydroxylation at C-17 could not be determined from the ^{1}H NMR data as the 17-proton signal was obscured by those of H-18 and -19. The metabolite was designated 19-*N,N*-dimethylcarbamoxy-13,17ξ,18-trihydroxystemarane.

In summary stemodin (**1**) was hydroxylated by the fungus to give two known analogues and one novel metabolite while stemodinone (**5**) was converted to two known analogues. Stemarin (**8**) gave four new compounds while its 19-dimethylcarbamate derivative (**13**) was hydroxylated to produce a single novel metabolite.

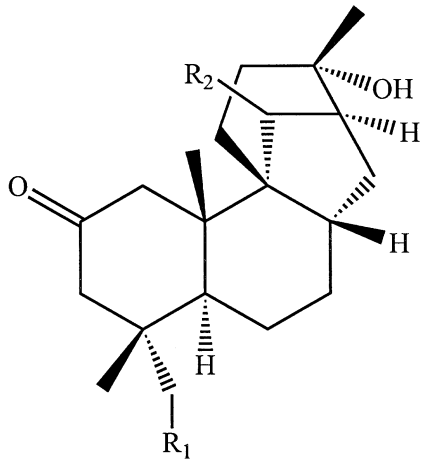
3. Experimental

3.1. General experimental

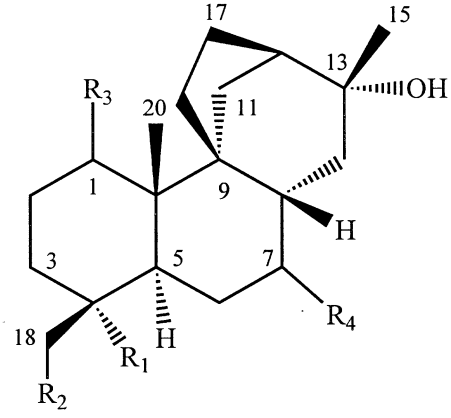
Melting points were recorded on a Thomas Hoover melting point apparatus and are uncorrected. IR spectra were recorded as KBr disks using a Perkin-Elmer 735B infrared spectrophotometer. ^{1}H and ^{13}C NMR spectra



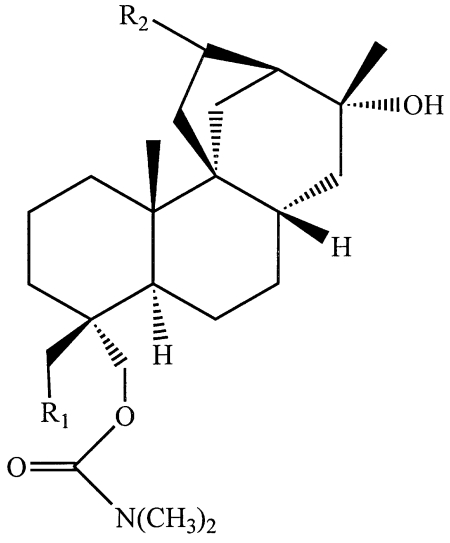
1 R_1=R_2=R_3=H, R_4=αOH, βH
2 R_1=βOH, αH, R_2=R_3=H, R_4=αOH, βH
3 R_1=R_3=H, R_2=βOH, αH, R_4= αOH, βH,
4 R_1=R_2=H, R_3=βOH, αH, R_4=αOH, βH
4a R_1=R_2=H, R_3=βOAc, αH, R_4=αOAc, βH



5 R_1=R_2=H
6 R_1=OH, R_2=H
7 R_1=H, R_2=βOH, αH



8 R_1=CH_2OH, R_2=R_3=R_4=H
9 R_1=COOH, R_2=OH, R_3=R_4=H
10 R_1=COOH, R_2=OH, R_3=H, R_4=βOH, αH
11 R_1=CH_2OH, R_2=OH, R_3=H, R_4=αOH, βH
12 R_1=COOH, R_2=R_4=H, R_3=βOH, αH



13 R_1=R_2=H
14 R_1=OH, R_2=ξOH

were recorded at 200 MHz and 50 MHz respectively using a Bruker AC200 spectrometer. NMR samples were prepared in $CDCl_3$ containing tetramethylsilane as the internal standard. ^{13}C NMR assignments are reported in Table 1. Optical rotations were measured on a Perkin Elmer 241 MC polarimeter. High resolution mass spectra (EI) were acquired on a Kratos MS50 instrument at an ionising voltage of 70 eV. Electrospray mass spectral data were obtained on Agilent Technologies 1100MSD or Micromass Zabspec-oaTOF spectrometers. Column chromatography used silica gel (230–400 mesh) for purifications. Thin layer chromatography plates were visualised by spraying with ammonium molybdate-sulfuric acid spray and heating at 120 °C for 5 min. Stemodin (**1**) and stemarin (**8**) were obtained from *Stemodia maritima* in overall yields of 0.07 and 0.02% respectively. *Aspergillus niger* ATCC 9142 was obtained from the American Type Culture Collection,

Rockville, MD, USA. Petrol refers to the petroleum fraction boiling at 60–80 °C.

3.2. Culture conditions

Aspergillus niger ATCC 9142 was grown on potato dextrose agar slants at 28 °C for 2 weeks. Five slants were used to inoculate twenty 500 ml erlenmeyer flasks each containing 125 ml liquid culture medium. This medium was comprised of glucose (20 g/l), yeast extract (5 g/l), soya meal (5 g/l), sodium chloride (5 g/l), and dipotassium hydrogen phosphate (5 g/l) (Belan et al., 1987). The flasks were shaken at 180 rpm. Substrates were pulse fed to the growing fungus (20 flasks) in portions of 10, 20, 30 and 40% at 24, 36, 48 and 60 h after inoculation.

3.3. Incubation of stemodin (*1*)

Stemodin (**1**) (500 mg) in ethanol (10 ml) was pulse fed to the culture as described above. The fermentation was allowed to proceed for 10 days after the final feed. The mycelial cells were filtered from the broth. Extraction of both with ethyl acetate afforded a brown gum (0.76 g) which was purified using column chromatography. Elution with 40% ethyl acetate in petrol afforded stemodin (200 mg). Further elution yielded 2α,3β,13-trihydroxystemodane (**2**) (97 mg) which crystallised from acetone as needles, mp 192–194 °C, $[\alpha]_D$: +7.1° (*c* 3.6, $CHCl_3$) [lit. mp 180–182 °C, $[\alpha]_D$: +17.5° (*c* 1.0, MeOH) (Hufford et al., 1992)]; IR ν_{max} cm^{-1}: 3398, 2941, 2875; ^1H NMR δ: 0.94 (3H, *s*, H-19), 1.01 (3H, *s*, H-20), 1.05 (3H, *s*, H-18), 1.12 (3H, *s*, H-17), 3.02 (1H, *d*, *J* = 9.8 Hz, H-3α), 3.72 (1H, *m*, $^w/_2$ = 20.1 Hz, H-2).

Further purification gave the 2α,7β,13-trihydroxystemodane (**3**) (3.2 mg) which did not crystallise, $[\alpha]_D$: −10.7° (*c* 1.6, $CHCl_3$) [lit. crystals, mp 241–245 °C, $[\alpha]_D$:−2.9° (*c* 1.35, pyridine) (Badria and Hufford, 1991)]; IR ν_{max} cm^{-1}: 3413, 2966, 2938; ^1H NMR δ: 0.95 (3H, *s*, H-19), 0.97 (3H, *s*, H-18), 1.01 (3H, *s*, H-20), 1.16 (3H, *s*, H-17), 3.64 (1H, *m*, $^w/_2$ = 5.1 Hz, H-7α).

The third metabolite (**4**) was characterised as its diacetate. 2α,16β-Diacetoxy-13-hydroxystemodane (**4a**) (17.5 mg) did not crystallise, $[\alpha]_D$: −22.8° (*c* 4.5, $CHCl_3$); EIMS *m*/*z* (rel. int.): 388.2614 (6) ($[M–H_2O]^+$), 346.2508 (20) ($[M–HOAc]^+$), 335.2222 (10), 328.2402 (27) ($[M–H_2O–HOAc]^+$), 286.2297 (100) ($[M–2HOAc]^+$); IR ν_{max} cm^{-1}: 3547, 2972, 2938, 1736, 1749; ^1H NMR δ: 0.89 (3H, *s*, H-19), 0.97 (3H, *s*, H-18), 1.03 (3H, *s*, H-20), 1.06 (3H, *s*, H-17), 1.92 (3H, *s*, CH_3CO_2), 1.99 (3H, *s*, CH_3CO_2), 3.31 (1H, *br d*, *J* = 13.0 Hz, H-16α), 4.95 (1H, *br s*, H-2β).

Table 1
^{13}C NMR chemical shift assignments for fed and biotransformed metabolites

	Compounds													
Carbon	**1**	**2**	**3**	**4a**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1	45.7	42.3	44.2	39.2	51.4	50.3	50.8	31.8	30.2	34.2	30.3	76.2	31.7	31.6
2	65.1	68.5	63.8	70.0	212.8	213.7	215.0	18.3	18.4	18.4	22.6	29.7	18.1	19.2
3	50.1	83.5	50.5	53.4	55.8	51.5	56.0	35.3	36.9	36.6	36.2	36.5	36.3	33.4
4	34.6	39.2	34.6	39.1	39.2	43.6	39.1	38.0	47.4	39.2	45.1	38.4	37.0	38.6
5	46.6	46.9	44.1	46.7	47.1	39.9	48.8	40.9	44.0	42.5	40.6	48.1	42.8	50.2
6	22.0	21.9	31.3	21.8	22.5	22.8	22.0	22.3	24.7	31.9	34.7	22.4	22.6	22.7
7	36.4	36.5	79.4	36.2	35.9	36.2	36.7	30.8	30.0	80.4	66.9	30.4	30.5	29.9
8	36.9	36.7	46.4	36.7	37.1	37.7	37.1	38.9	39.1	37.5	44.2	37.7	38.6[a]	37.1
9	50.6	50.1	50.4	50.2	50.1	50.4	53.1	51.6	51.0	50.9	53.8	52.7	51.6	50.5
10	40.1	39.0	40.0	40.0	44.8	44.6	43.9	38.6	42.4	39.9	37.6	38.3	38.6[b]	45.1
11	27.8	27.5	27.8	27.7	27.8	28.4	28.9	29.4	28.3	29.2	29.7	29.4	29.4	29.3
12	32.7	32.6	32.2	32.6	32.6	33.1	32.4	47.9	47.1	47.4	48.2	43.9	48.3	48.3
13	72.3	72.2	72.9	72.4	72.1	72.7	74.9	73.9	73.9	72.7	74.0	74.2	73.8	72.5
14	46.1	46.0	46.5	46.0	45.7	46.2	45.7	39.9	39.2	39.5	39.9	41.7	39.6	39.2
15	38.1	37.8	37.8	37.8	38.1	38.6	37.3	29.5	29.7	28.9	29.6	27.1	29.6	29.7
16	30.1	30.8	32.7	80.5	30.3	30.9	79.9	26.7	25.6	25.7	26.7	26.4	26.7	65.8
17	28.0	28.0	28.2	28.2	28.1	28.7	28.5	27.4	27.5	29.7	27.1	26.6	26.8	52.5
18	34.7	29.9	34.5	31.6	34.4	71.0	35.0	18.1	62.0	63.7	64.4	17.0	17.8	61.8
19	23.7	17.5	23.7	19.3	23.9	20.7	24.6	70.8	183.4	184.0	71.5	182.7	73.6	71.3
20	19.6	19.6	20.0	18.5	18.7	20.0	19.9	16.9	17.4	16.2	17.7	11.5	16.9	14.1
				170.6									156.8	162.3
				21.2									38.6[c]	36.6
				170.9									38.6[c]	36.6
				20.9										

[a] 38.61 ppm.
[b] 38.56 ppm.
[c] 38.64 ppm.

3.4. Biotransformation of stemodinone (**5**)

The diterpene (**5**) (500 mg), dissolved in ethanol (10 ml), was pulse fed over twenty flasks each containing 125 ml sterile liquid culture medium. The fermentation was allowed to proceed for 10 days after the final feed. The mycelium was filtered from the broth and both were extracted with ethyl acetate. The extracts were combined and dried with anhydrous magnesium sulfate. Removal of the solvent in vacuo afforded a combined extract (2.2 g) which was chromatographed. Elution with 20% ethyl acetate in petrol afforded stemodinone (202.4 mg).

Further elution gave 13,18-dihydroxystemodan-2-one (**6**) (84.1 mg) which crystallised from ethyl acetate as needles, mp 181–183 °C, $[\alpha]_D$: +31.3° (*c* 0.76, $CHCl_3$) [lit. gum (Hanson et al., 1994)]; IR ν_{max} cm^{-1}: 3419, 2969, 1682; ^1H NMR δ: 0.85 (3H, *s*, H-20), 1.01 (3H, *s*, H-19), 1.13 (3H, *s*, H-17), 3.06 (1H, *d*, *J* = 10.7 Hz, H-18,), 3.35 (1H, *d*, *J* = 10.7 Hz, H-18).

Further elution gave 13,16β-dihydroxystemodan-2-one (**7**) (9.4 mg), which did not crystallise, $[\alpha]_D$: −14.3° (*c* 1.4, $CHCl_3$) [lit. gum (Hanson et al., 1994)]; IR ν_{max} cm^{-1}: 3452, 2940, 1642; ^1H NMR δ: 0.92 (3H, *s*, H-19), 0.93 (3H, *s*, H-20), 1.09 (3H, *s*, H-18), 1.19 (3H, *s*, H-17), 2.91 (1H, *d*, *J* = 11.4 Hz, H-16α).

3.5. Biotransformation of stemarin (**8**)

Stemarin (**8**) (1.0 g) was dissolved in ethanol (20 ml) and pulse fed to the growing culture. The fermentation was worked up 10 days after the final feed. The broth was acidified to pH 2 using 3 M aqueous HCl before extraction with ethyl acetate. The combined extract (1.91 g) was then subjected to column chromatography. Elution with 20% ethyl acetate in petrol afforded stemarin (102.4 mg).

Further elution gave 13,18-dihydroxystemaran-19-oic acid (**9**) (47.5 mg) which did not crystallise, $[\alpha]_D$: +6.9° (*c* 19.4, $CHCl_3$); EIMS *m*/*z* (rel. int.): 318.2189 (5) ([M-H_2O]$^+$), 305.2117 (22), 287.2011 (100); ESMS *m*/*z* (rel. int.): 359.2413 [M + Na]$^+$ (100); IR ν_{max} cm^{-1}: 3452, 2933, 2833, 1705; ^1H NMR δ: 1.16 (3H, *s*, H-20), 1.22 (3H, *s*, H-15), 3.93 (1H, *d*, *J* = 25.0 Hz, H-18), 4.08 (1H, *d*, *J* = 24.0 Hz, H-18).

Elution with 25% ethyl acetate in petrol yielded 7β,13,18-trihydroxystemaran-19-oic acid (**10**) (30.4 mg) which did not crystallise, $[\alpha]_D$: +2.3° (*c* 30.5, $CHCl_3$); EIMS *m*/*z* (rel. int.): 318.2189 (5) ([M–H_2O]$^+$), 305.2117 (22), 287.2011 (100); ESMS *m*/*z* (rel. int.): 359.2413 [M + Na]$^+$ (100); IR ν_{max} cm^{-1}: 3418, 2927, 2854, 1716; ^1H NMR δ: 0.93 (3H, *s*, H-20), 1.19 (3H, *s*, H-15), 3.03 (1H, *d*, *J* = 7.0 Hz, H-18), 3.42 (1H, *d*, *J* = 7.0 Hz, H-18), 3.62 (1H, *m*, $^w/_2$ = 16 Hz, H-7α).

Further elution afforded 7α,13,18,19-tetrahydroxystemarane (**11**) (30.0 mg) which did not crystallise, $[\alpha]_D$: −9.8° (*c* 1.6, $CHCl_3$); EIMS *m*/*z* (rel. int.): 320.2351 (13) ([M-H_2O]$^+$), 305.2117 (100), 320.2246 (20); IR ν_{max} cm^{-1}: 3429, 2955, 2933, 2877; ^1H NMR δ: 0.97 (3H *s*, H-20), 1.19 (3H, *s*, H-15), 3.41 (2H, *d*, *J* = 25.6 Hz, H-19), 3.63 (2H, *d*, *J* = 20.6 Hz, H-18), 3.98 (1H, *m*, $^w/_2$ = 10.8 Hz, H-7β).

Elution with acetone gave 1β,13-dihydroxystemaran-19-oic acid (**12**) (10.3 mg) which did not crystallise, $[\alpha]_D$: −9.7° (*c* 7.5, $CHCl_3$); EIMS *m*/*z* (rel. int.) 336.2301 (11) ([M]$^+$, $C_{20}H_{32}O_4$), 318.2191 (15), 305.2117 (100), 291.2324 (30); IR ν_{max} cm^{-1}: 3418, 2927, 2877, 1705: ^1H NMR δ: 0.74 (3H, *s*, H-20), 0.93 (3H, *s*, H-15), 1.19 (3H, *s*, H-18), 3.58 (1H, *dd*, *J* = 5.1 and 11.1 Hz, H-1α).

3.6. Synthesis of 19-N,N-dimethylcarbamoxy-13-hydroxystemarane (**13**)

Stemarin (**8**) (1.0 g, 3.26 mmol) was dissolved in pyridine (5.0 ml). Dimethylcarbamyl chloride (12.0 ml, 130.64 mmol) and a catalytic amount of dimethylaminopyridine were added and the mixture was stirred at 60 °C for 2 days. The solvent was removed in vacuo and the resulting brown solution was purified by column chromatography. Elution with 1% acetone in methylene chloride afforded 19-*N,N*-dimethylcarbamoxy-13-hydroxystemarane (**13**) (164.0 mg) which crystallised from methylene chloride as amorphous crystals, mp 124–126 °C, $[\alpha]_D$: +2.9° (*c* 7.2, $CHCl_3$); EIMS *m*/*z* (rel. int.): 377.2930 ([M]$^+$, $C_{23}H_{39}NO_3$), 359.2824 (24), 288.2454 (39), 257.2269 (100); IR ν_{max} cm^{-1}: 3508, 2955, 2866, 1700, 1460; ^1H NMR δ: 0.82 (3H, *s*, H-19), 0.95 (3H, *s*, H-20), 1.13 (3H, *s*, H-15), 2.92 (6H, *s*, $N(CH_3)_2$), 3.66 (1H, *d*, *J* = 10.4 Hz, H-19), 3.77 (1H, *d*, *J* = 10.4 Hz, H-19).

3.7. Biotransformation of 19-N,N-dimethylcarbamoxy-13-hydroxystemarane (**13**)

19-*N,N*-Dimethylcarbamoxy-13-hydroxystemarane (**13**) (100 mg) was dissolved in acetone (1.0 ml) and pulse fed to two flasks containing 125 ml liquid culture medium. The fermentation was allowed to proceed for 10 days after the final feed. Extraction of the broth and mycelia afforded a brown gum (141.9 mg) which was subjected to column chromatography. Elution with 20% ethyl acetate in petrol gave the fed compound (31.4 mg).

Further elution afforded 19-*N,N*-dimethylcarbamoxy-13,17ξ,18-trihydroxystemarane (**14**) (2 mg) which did not crystallise, $[\alpha]_D$: −5.2° (*c* 3.1, $CHCl_3$); EIMS *m*/*z* (rel. int.): 345.2668 (1) ([M–CH_2OH–OH–O]$^+$), 302.2224 (2) ([M–$C_3H_7NO_2$–H_2O]$^+$), 273.2216 (5) (M–$C_3H_7NO_2$–CH_2O–OH); IR ν_{max} cm^{-1}: 3413, 2938, 1750, 1462, 1158; ^1H NMR δ: 0.96 (3H, *s*, H-20), 1.16 (3H, *s*, H-15), 2.94 (6H, *s*, $N(CH_3)_2$), 3.49 (1H, *m*, $^w/_2$ = 10 Hz, H-18), 3.64 (3H, *m*, $^w/_2$ = 14 Hz, H-17,18,19), 3.74 (1H, *m*, $^w/_2$ = 8 Hz, H-19).

Acknowledgements

This work was supported, in part, by funds secured under the University of the West Indies/Interamerican Development Bank (UWI/IDB) Programme. ARMC thanks the UWI for the granting of a Postgraduate Scholarship. The authors are grateful to Professor John C. Vederas (University of Alberta) for arranging mass spectral analyses and to Professor Herbert L. Holland (Brock University) for helpful discussions. Optical rotations were measured at the Bureau of Standards, Kingston. Fermentations were carried out in the Biotechnology Centre, UWI.

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