Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytochemistry 67 (2006) 1088–1093
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Bioconversion of *Stemodia maritima* diterpenes and derivatives by *Cunninghamella echinulata* var. *elegans* and *Phanerochaete chrysosporium*

Andrew S. Lamm [a], William F. Reynolds [b], Paul B. Reese [a,*]

[a] *Department of Chemistry, University of the West Indies, Mona, Kingston 7, Jamaica*
[b] *Department of Chemistry, University of Toronto, Toronto, Ont., Canada M5S 3H6*

Received 19 February 2006; accepted 3 April 2006
Available online 24 May 2006

Abstract

Stemodane and stemarane diterpenes isolated from the plant *Stemodia maritima* and their dimethylcarbamate derivatives were fed to growing cultures of the fungi *Cunninghamella echinulata* var. *elegans* ATCC 8688a and *Phanerochaete chrysosporium* ATCC 24725.

C. echinulata transformed stemodin (**1**) to its 7α-hydroxy- (**2**), 7β-hydroxy- (**3**) and 3β-hydroxy- (**4**) analogues. 2α-(*N*,*N*-Dimethylcarbamoxy)-13-hydroxystemodane (**6**) gave 2α-(*N*,*N*-dimethylcarbamoxy)-6α,13-dihydroxystemodane (**7**) and 2α-(*N*,*N*-dimethylcarbamoxy)-7α,13-dihydroxystemodane (**8**). Stemodinone (**9**) yielded 14-hydroxy-(**10**) and 7β-hydroxy- (**11**) congeners along with **1**, **2** and **3**. Stemarin (**13**) was converted to the hitherto unreported 6α,13-dihydroxystemaran-19-oic acid (**18**). 19-(*N*,*N*-Dimethylcarbamoxy)-13-hydroxystemarane (**14**) yielded 13-hydroxystemaran-19-oic acid (**17**) along with the two metabolites: 19-(*N*,*N*-dimethylcarbamoxy)-2β,13-dihydroxystemarane (**15**) and 19-(*N*,*N*-dimethylcarbamoxy)-2β,8,13-trihydroxystemarane (**16**).

P. chrysosporium converted **1** into **3**, **4** and 2α,11β,13-trihydroxystemodane (**5**). The dimethylcarbamate (**6**) was not transformed by this microorganism. Stemodinone (**9**) was hydroxylated at C-19 to give **12**. Both stemarin (**13**) and its dimethylcarbamate (**14**) were recovered unchanged after incubation with *Phanerochaete*.

Keywords: Stemodane; Stemarane; *Cunninghamella echinulata* var. *elegans* ATCC 8688a; *Phanerochaete chrysosporium* ATCC 24725; Diterpene; Hydroxylation; Biotransformation; *Stemodia maritima*

1. Introduction

The shrub *Stemodia maritima* produces diterpenoids possessing mild antiviral and cytotoxic properties (Hufford et al., 1991). Some of these compounds are stemodin (**1**), stemodinone (**9**) and stemarin (**13**) among others (Manchand et al., 1973; Manchand and Blount, 1975, 1976; Hufford et al., 1992; Hufford, 1988). Compounds **1** and **9** are structurally related to aphidicolin, a potent cytotoxic and antiviral agent (Dalziel et al., 1973). Structural modifications of these substrates and their derivatives via microbial transformation would yield unique analogues with potential biological activity.

Cunninghamella echinulata var. *elegans* ATCC 8688a is recognised for its potential for steroid hydroxylation (Yang and Davis, 1991) and has been noted for its ability to mimic mammalian hepatic metabolism with other substrates (Hezari and Davis, 1992). The white rot fungus *Phanerochaete chrysosporium* ATCC 24725, previously known as *Sporotrichum pulverulentum*, degrades lignin (Moore-Landecker, 1996) and environmental toxins such as aromatic and halogenated hydrocarbons, pesticides, among others (Sandermann, 1997; Sheremata and Hawari, 2000). Examples of terpene transformation with this basidiomycete are extremely rare.

* Corresponding author. Tel.: +1 (876) 935 8460; fax: +1 (876) 977 1835.
E-mail address: paul.reese@uwimona.edu.jm (P.B. Reese).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.04.001

In this paper we report the action of *C. echinulata* var. *elegans* and *P. chrysosporium* on stemodin (**1**), stemodinone (**9**), stemarin (**13**), and the dimethylcarbamate derivatives of **1** and **13**.

2. Results and discussion

2.1. C. echinulata var. elegans

Incubation of *C. echinulata* with stemodin (**1**) gave 2α,7α,13-trihydroxy- (**2**), 2α,7β,13-trihydroxy- (**3**), and 2α,3β,13-trihydroxy-stemodane (**4**). Compounds **2** and **3** were converted to their novel 2,7-diacetates **2a** and **3a**, respectively, in order to facilitate purification. Previously Hufford reported that *C. echinulata* var. *echinulata* (ATCC 9244) transformed **1** to **2** and **3** as well as the 2α,13,14-triol (Badria and Hufford, 1991). The molecular formulae for **2a** and **3a** were shown to be $C_{24}H_{38}O_5$ by CIMS and this indicated that monohydroxylation of **1** had taken place. The ^{13}C NMR data for **2a** established the position of functionalisation based on HMBC data and the large shift of the C-7 signal and smaller ones for resonances for C-5, -6 and -8. The stereochemistry of the 7-acetoxy group was deduced to be α from the value of the H-7, H-8 diaxial coupling constant (J 18.3 Hz). For **3a** the ^{13}C NMR data was similar, however, in the ^1H NMR spectrum the resonance for H-7α showed an axial–equatorial coupling (J 10.1 Hz) and this indicated that a β-acetoxy substituent was present. The spectral data for **4** showed that it was identical with maristeminol, a natural product previously isolated from *S. maritima* (Hufford et al., 1992).

The protection of alcohols as their carbamates has been shown to improve the docking potential of some substrates, thus increasing the yield of metabolites (Buchanan and Reese, 2001). Fermentation of 2α-(*N*,*N*-dimethylcarbamoxy)-13-hydroxystemodane (**6**) yielded two metabolites. The ESMS spectra suggested a molecular formula of $C_{23}H_{39}NO_4$ for both products **7** and **8**, and this indicated that monohydroxylation had occurred. Analysis of the HMBC data for **7** showed that C-6 hydroxylation of **6** had occurred through correlations of H-6 with C-5, 7 and 8. T-ROESY data was used to deduce that the alcohol had α stereochemistry; H-6β showed correlations with H-7β, -19 and -20. Therefore the first metabolite was the novel 2α-(*N*,*N*-dimethylcarbamoxy)-6α,13-dihydroxystemodane (**7**). The HMBC data of the second, more polar, metabolite showed the presence of a strong correlation between H-7 and C-8 while the T-ROESY experiment correlated H-7β with H-8. In the T-ROESY experiment H-7β was seen to be coupled with H6α, -6β and -8. These results confirmed that the second compound was the previously unreported 2α-(*N*,*N*-dimethylcarbamoxy)-7α,13-dihydroxystemodane (**8**).

Stemodinone (**9**) afforded the novel 13,14-dihydroxystemodan-2-one (**10**) and 7β,13-dihydroxystemodan-2-one (**11**). Terpene **10** had a molecular formula of $C_{20}H_{32}O_3$ (EIHRMS), and this implied that monohydroxylation of **9** had occurred. In the ^{13}C NMR spectrum the C-14 methine signal had disappeared and was replaced by a peak at δ 79.6 for a non-protonated, oxygen-bearing carbon. The spectral data for metabolite **11** was identical with that reported in the literature (Chen et al., 2005). Stemodin (**1**) was also produced in this fermentation along with trace amounts of **2** and **3**.

The incubation of stemarin (**13**) yielded the novel 6α,13-dihydroxystemaran-19-oic acid (**18**) as the sole metabolite. HRMS data indicated that the compound had a molecular formula of $C_{20}H_{32}O_4$. The ^{13}C NMR spectrum showed the loss of the methylene at C-19 and the appearance of a new carbonyl group at 183.0 ppm. Diaxial coupling of H-6β (δ 4.29) with H-5 (J 13.1 Hz) in the ^1H NMR spectrum revealed that 6α-hydroxylation had occurred.

Bioconversion of 19-(*N*,*N*-dimethylcarbamoxy)-13-hydroxystemarane (**14**) gave three metabolites. Comparison of the spectral data of the least polar metabolite (**17**) with that in the literature indicated that it was 13-hydroxystemaran-19-oic acid (Buchanan and Reese, 2001). Monohydroxylation at C-19 was believed to result in loss of the dimethylcarbamoxy group to give the corresponding aldehyde which was further oxidised to an acid functionality. HRMS analysis of the second metabolite, **15**, showed that its molecular formula was $C_{23}H_{39}NO_4$, i.e. it was the product of monohydroxylation. Analysis of the ^{13}C NMR spectra showed that the C-2 signal (18.1 ppm) of **14** had disappeared and was replaced by a methine resonance (δ 75.0). HMBC correlations were used to establish that hydroxylation had indeed occurred at C-2. Assignment of the β-stereochemistry of the alcohol was determined from T-ROESY interactions of H-2 (δ 3.81) with H-19 (δ 3.64) and H-5 (δ 1.50). The most polar of the transformation products (**16**) possessed a molecular formula of $C_{23}H_{39}NO_5$ (HRMS). The HMBC data confirmed that C-2 and -8 hydroxylation had been effected. The NMR signal for H-2 (δ 3.89) showed nOe cross peaks with H-19 (δ 3.73) and H-5 (δ 1.55), thus fixing the stereochemistry of the 2-hydroxyl as β. The very strong T-ROESY correlation between H-2α and H-5 in **15** and **16** indicated that ring A was forced into the boat conformation in an effort to minimise interactions of the 2β-hydroxyl with the axial 18- and 20-methyls. Hence **15** and **16** were 19-(*N*,*N*-dimethylcarbamoxy)-2β,13-dihydroxystemarane and 19-(*N*,*N*-dimethylcarbamoxy)-2β,8,13-trihydroxystemarane, respectively; neither compound has been previously described in the literature.

2.2. P. chrysosporium

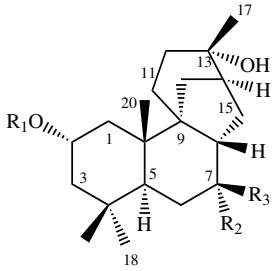
Biotransformation of stemodin (**1**) with *P. chrysosporium* produced three metabolites. The least polar of the products, 2α,7β,13-trihydroxystemodane (**3**), was identified via its 2,7-diacetate (**3a**). Spectral data indicated that the second metabolite was 2α,3β,13-trihydroxystemodane (maristeminol) (**4**). The third product formed in this

fermentation was identified as 2α,11,13-trihydroxystemodane (**5**) from its spectral and physical data (Chen et al., 2005).

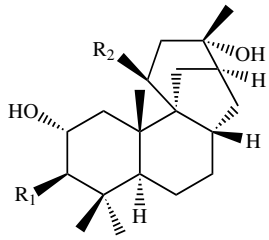
Stemodin dimethylcarbamate (**6**) was recovered unchanged after incubation with the microorganism. Fermentation of stemodinone (**9**) yielded 13,18-dihydroxystemodan-2-one (**12**) (Hanson et al., 1994).

No products of transformation were obtained from the fermentation of stemarin (**13**) or its dimethylcarbamate derivative (**14**) with this fungus.

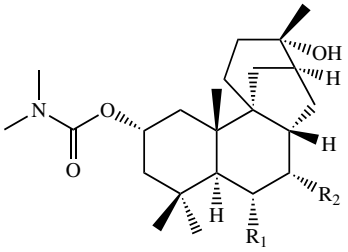
In summary, five substrates were fed to *Cunninghamella* and *Phanerochaete* to yield 13 metabolites, six of which had not been previously described. Furthermore, in an effort to purify two known compounds, their novel diacetates were prepared.



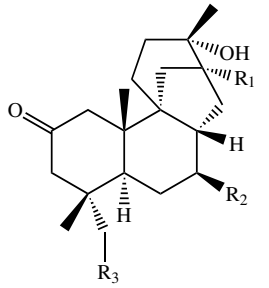
1 R_1=R_2=R_3=H
2 R_1=R_3=H, R_2=OH
2a R_1=Ac, R_2=OAc, R_3=H
3 R_1=R_2=H, R_3=OH
3a R_1-Ac, R_2=H, R_3=OAc



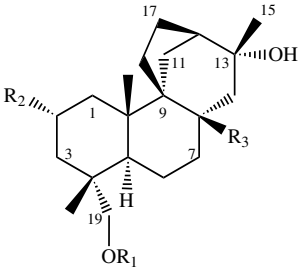
4 R_1=OH, R_2=H
5 R_1=H, R_2=OH



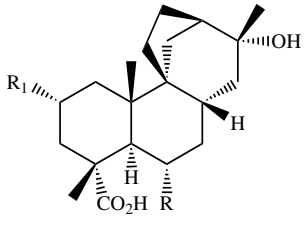
6 R_1=R_2=H
7 R_1=OH, R_2=H
8 R_1=H, R_2=OH



9 R_1= R_2=R_3=H
10 R_1=OH, R_2=R_3=H
11 R_1=R_3=H, R_2=OH
12 R_1= R_2=H, R_3=OH



13 R_1= R_2=R_3=H
14 R_1=C(O)N(CH_3)$_2$, R_2=R_3=H
15 R_1=C(O)N(CH_3)$_2$, R_2=OH, R_3=H
16 R_1=C(O)N(CH_3)$_2$, R_2=R_3=OH



17 R=H
18 R=OH

3. Experimental

3.1. General experimental

NMR spectra were obtained using Bruker AC200 and Varian 500 MHz spectrometers. Samples were analysed in deuterated chloroform with tetramethlysilane as the internal standard. Column chromatography utilised basic alumina and silica gel (230–400 mesh). TLC plates were sprayed with ammonium molybdate/sulfuric acid spray and heated. Infrared data was obtained on a Perkin–Elmer FTIR spectrophotometer 1000 using KBr discs. Optical rotations were conducted on a Perkin–Elmer 241 mc polarimeter and are for chloroform solutions unless otherwise stated. Melting points were done on a Reichert melting point apparatus. HRMS (EI) analyses were determined on a Kratos MS50 direct probe instrument with an ionising energy of 70 eV. Fungi were obtained from the American Type Culture Collection, Rockville, MD, USA. *S. maritima* was collected from Hellshire, St. Catherine from which stemodin (**1**) and stemarin (**13**) were obtained. A voucher specimen was deposited in the Botany Herbarium, UWI (Accession No. 34649). 2α-(*N*,*N*-Dimethylcarbamoxy)-13-hydroxystemodane (**6**) was prepared as previously described (Buchanan and Reese, 2001). Jones oxidation of **1** gave stemodinone (**9**). The synthesis of 19-(*N*,*N*-dimethylcarbamoxy)-13-hydroxystemarane (**14**) has already been reported (Chen and Reese, 2002). ^{13}C NMR data of new compounds is listed in Table 1.

3.2. C. echinulata var. elegans

3.2.1. Fermentation procedure

Slants of the fungus were maintained on media composed of (per litre) peptone (10 g), maltose (40 g), agar (20 g). Five 2 week old slants were used to inoculate twenty 500 ml conical flasks each containing 125 ml of growth medium. The liquid growth medium consisted of (per litre) glucose (20 g), soya bean meal (5 g), yeast extract (5 g), NaCl (5 g) and K_2HPO_4 (5 g). The substrate was fed 60 h after inoculation. 10 d after feeding the mycelium was filtered from the broth and both were extracted with ethyl acetate. These extracts were dried with magnesium sulfate and the solvents were evaporated in vacuo.

3.2.2. Fermentation of stemodin (*1*)

Stemodin (**1**) (1 g), dissolved in ethanol (46 ml), was fed to the fungus. The mycelial and broth extracts were combined (1.1 g) and subjected to chromatography. Elution with 25% acetone in petrol gave untransformed substrate (**1**) (422 mg). Increasing the polarity of the eluant to 35% acetone in petrol yielded 2α,7α,13-trihydroxystemodane (**2**) (210 mg). The metabolite was converted to 2α,7α-diacetoxy-13-hydroxystemodane (**2a**) (30 mg) which resisted crystallisation, $R_f = 0.62$, 40% ethyl acetate in petrol, $[\alpha]_D$ −2.6° ($c = 5.8$, $CHCl_3$); IR: ν_{max} 2965, 2930, 2360, 1733, 1261 cm^{-1}; CIMS: *m*/*z* (rel. int.): 406 (81) M^+; HRMS (EI): *m*/*z* (rel. int.): 388.2615 (42) $[M - H_2O]^+$

Table 1
^{13}C NMR data for all novel terpenoids

C	2a	3a	7	8	10	15	16	18
1	41.4	41.6	42.9	42.5	51.7	39.6	41.6	32.2
2	68.8	68.8	69.8	70.6	212.1	75.0	66.3	18.0
3	46.3	46.2	49.1	47.1	55.8	51.0	43.9	36.9
4	34.5	34.5	35.5	34.7	38.8	37.4	41.9	71.8
5	43.0	42.8	46.53	44.5	47.6	43.1	39.6	43.5
6	27.3	27.3	69.4	31.2	19.8	22.8	19.8	72.4
7	81.2	81.3	46.3	79.0	32.4	35.8	35.7	39.2
8	43.9	43.9	51.8	46.5	43.6	40.6	76.3	31.7
9	51.7	51.9	50.1	52.0	51.3	51.7	56.4	53.3
10	39.8	39.7	42.3	40.2	44.3	44.0	37.3	38.5
11	27.9	27.8	38.5	28.1	23.6	34.1	27.7	29.8
12	31.8	32.6	32.8	32.6	43.0	47.9	47.1	45.7
13	83.9	72.2	72.2	72.2	72.4	73.6	73.6	73.9
14	43.1	46.0	46.45	46.3	79.6	42.1	50.2	39.8
15	34.9	35.6	37.3	36.5	47.5	29.0	28.6	27.4
16	30.6	31.4	30.3	31.7	33.4	32.2	32.5	25.3
17	23.7	28.1	27.85	27.8	28.1	27.1	24.5	29.3
18	34.4	34.4	33.7	34.5	34.6	17.9	27.5	17.0
19	22.5	21.5	23.9	23.7	24.0	72.8	73.5	183.0
20	19.6	19.5	20.5	19.8	17.3	21.0	21.1	16.9
CH_3	21.4	21.4	–	–	–	–	–	–
CH_3	21.6	23.5	–	–	–	–	–	–
CO	170.5	170.7	–	–	–	–	–	–
CO	170.6	171.1	–	–	–	–	–	–
NCO	–	–	157.5	157.1	–	154.2	157.7	–
NCH_3	–	–	36.2	36.1	–	31.0	36.0	–

($C_{24}H_{36}O_4$ requires 388.2614), 328.2405 (74) $[M - CH_3\text{-}CO_2H - H_2O]^+$, 268.2195 (100) $[M - 2(CH_3CO_2H) - H_2O]^+$; ^1H NMR: δ 0.98 (3H, *s*, H-18), 1.08 (3H, *s*, H-19), 1.25 (3H, *s*, H-20), 1.37 (3H, *s*, H-17), 2.02 (3H, *s*, CH_3CO_2-2), 2.05 (3H, *s*, CH_3CO_2-7), 2.78 (1H, *t*, $J = 12.5$ Hz, H-8), 4.61 (1H, *m*, $w/_2 = 18.3$ Hz, H-2), 4.91 (1H, *m*, $w/_2 = 21.8$ Hz, H-7).

Continued elution gave 2α,7β,13-trihydroxystemodane (**3**) (223 mg) which was acetylated. 2α,7β-Diacetoxy-13-hydroxystemodane (**3a**) (100 mg) resisted crystallisation, $R_f = 0.60$, 40% ethyl acetate in petrol, $[\alpha]_D$ +2.5° ($c = 3.55$, $CHCl_3$); IR: ν_{max} 3509, 3020, 2963, 2877, 1733, 1465 cm^{-1}; CIMS: *m/z* (rel. int.): 424 (44) $[M + NH_3]^+$, 406 (25) M^+; HRMS (EI): *m/z* (rel. int.): 391.2465 (1) $[M - CH_3]^+$ ($C_{23}H_{35}O_5$ requires 391.2484), 328.2404 (8) $[M - CH_3CO_2H - H_2O]^+$, 286.2300 (57) $[M - 2(CH_3CO_2\text{-}H)]^+$; ^1H NMR: δ 0.97 (3H, *s*, H-18), 0.98 (3H, *s*, H-19), 1.09 (3H, *s*, H-20), 1.13 (3H, *s*, H-17), 2.02 (3H, *s*, CH_3CO_2-2), 2.06 (3H, *s*, CH_3CO_2-7), 4.63 (1H, *t*, $J = 10.1$ Hz, H-7), 4.91 (1H, *m*, $w/2 = 18.0$ Hz, H-2).

Further elution gave 2α,3β,13-trihydoxystemodane (**4**) (88 mg) which crystallised from ethyl acetate as cubes, $R_f = 0.32$, ethyl acetate, the physical and spectral data of which was identical to that of an authentic sample (Hufford et al., 1992).

3.2.3. Fermentation of 2α-(*N*,*N*-dimethylcarbamoxy)-13-hydroxystemodane (**6**)

2α-(*N*,*N*-Dimethylcarbamoxy)-13-hydroxystemodane (**6**) (0.10 g) in acetone (2 ml) was fed to the fungus in nine 500 ml flasks. The combined broth and mycelial extract (1.10 g) was purified using column chromatography. Elution with 10% acetone in dichloromethane gave recovered **6** (0.04 g). Elution with 40% ethyl acetate in petrol yielded 2α-(*N*,*N*-dimethylcarbamoxy)-6α,13-dihydroxystemodane (**7**) (4 mg) which resisted crystallisation, $R_f = 0.20$, 20% ethyl acetate in petrol, $[\alpha]_D$ −2.6° ($c = 0.39$, $CHCl_3$); IR: ν_{max} 3402, 1701, 1368, 1269 cm^{-1}; ESMS: *m/z* (rel. int.): 416.2776 (100) $[M + Na]^+$ ($C_{23}H_{39}NNaO_4$ requires 416.2777); ^1H NMR: δ 1.05 (3H, *s*, H-18), 1.08 (3H, *s*, H-20), 1.12 (3H, *s*, H-17), 1.17 (3H, *s*, H-19), 2.91 (6H, *s*, $N[CH_3]_2$), 3.75 (1H, *tt*, $J = 4.4$, 10.7 Hz, H-6), 4.74 (1H, *m*, $w/2 = 20.7$ Hz, H-2).

Further elution gave 2α-(*N*,*N*-dimethylcarbamoxy)-7α,13-dihydroxystemodane (**8**) (38 mg) which crystallised as needles from acetone, $R_f = 0.14$, 20% ethyl acetate in petrol, m.p. 108–110 °C, $[\alpha]_D$ +11.2° ($c = 0.20$, $CHCl_3$); IR: ν_{max} 3399, 1710, 1336, 1266 cm^{-1}; ESMS: *m/z* (rel. int.): 416.2766 (100) $[M + Na]^+$ ($C_{23}H_{39}NNaO_4$ requires 416.2777); ^1H NMR: δ 0.99 (3H, *s*, H-18), 1.01 (3H, *s*, H-19), 1.08 (3H, *s*, H-20), 1.14 (3H, *s*, H-17), 2.91 (6H, *s*, $N[CH_3]_2$), 3.32 (1H, *m*, $J = 10.3$ Hz, H-7), 4.78 (1H, *tt*, $J = 3.8$, 15.6 Hz, H-2).

3.2.4. Fermentation of stemodinone (**9**)

Stemodinone (**9**) (480 mg), dissolved in ethanol, was fed to ten 500 ml conical flasks. The broth extract (0.24 g) was chromatographed on silica. Elution with 15% ethyl acetate in petrol yielded the fed compound (**9**) (98 mg). Elution with 20% ethyl acetate in petrol afforded 13,

14-dihydroxystemodan-2-one (**10**) (56 mg) which crystallised from acetone as cubes, $R_f = 0.23$, 20% ethyl acetate in petrol, m.p. 218–219 °C (sublimed to needles at 182–184 °C), $[\alpha]_D$ +18.5° ($c = 0.96$, acetone); IR: ν_{max} 3432, 2944, 2906, 1684, 1289 cm^{-1}; HRMS (EI): *m/z* (rel. int.): 320.2357 (22) M^+ ($C_{20}H_{32}O_3$ requires 320.2351), 316.2041 (8) $[M - 2H_2]^+$, 302.2239 (19) $[M - H_2O]^+$, 284.2139 (22) $[M - 2H_2O]^+$; ^{1}H NMR: δ 0.95 (3H, *s*, H-19), 1.08 (3H, *s*, H-18), 1.11 (3H, *s*, H-20), 1.16 (3H, *s*, H-17), 2.34 (2H, *s*, H-16).

Further elution gave 7β,13-dihydroxystemodan-2-one (**11**) (38 mg) which crystallised from acetone as needles, $R_f = 0.29$, 15% acetone in CH_2Cl_2, the physical and spectral data of which was identical to that of an authentic sample (Chen et al., 2005).

Elution with 50% ethyl acetate in petrol yielded stemodin (**1**) (13 mg), followed by 2α,7α,13-trihydroxystemodane (**2**) (8 mg) and 2α,7β,13-trihydroxystemodane (**3**) (8 mg) the spectral data of which were identical to those of authentic samples (Badria and Hufford, 1991). Chromatography of the mycelial extract (200 mg) gave untransformed stemodinone (**9**) (190 mg).

3.2.5. Fermentation of stemarin (7)

Stemarin (**13**) (100 mg) in ethanol (8 ml) was fed to two flasks. The broth and mycelial extracts were combined (130 mg) and chromatographed. Elution with acetone gave 6α-hydroxystemaran-19-oic acid (**18**) (5 mg) which resisted crystallisation, $R_f = 0.33$, 15% acetone in dichloromethane, $[\alpha]_D$ +34.4° ($c = 0.10$, MeOH); IR: ν_{max} 3428, 2927, 2869, 1694, 1254 cm^{-1}; HRMS (EI): *m/z* (rel. int.): 336.2282 (4) M^+ ($C_{20}H_{32}O_4$ requires 336.2301), 334.2143 (12) $[M - H_2]^+$, 318.2192 (100) $[M - H_2O]^+$, 316.2029 (4) $[M - H_2O - H_2]^+$, 303.1968 (19) $[M - H_2O - CH_3]^+$, 300.2088 (15) $[M - 2H_2O]^+$, 291.2311 (8) $[M - CH_2O]^+$; ^{1}H NMR: δ 1.13 (3H, *s*, H-20), 1.16 (3H, *s*, H-18), 1.19 (3H, *s*, H-17), 4.29 (1H, *m*, $w/2 = 13.1$ Hz, H-6).

3.2.6. Fermentation of 19-(N,N-dimethylcarbamoxy)-13-hydroxystemarane (14)

19-(*N*,*N*-Dimethylcarbamoxy)-13-hydroxystemarane (**14**) (0.38 g) in acetone (10 ml) was fed to the fungus growing in nine flasks. Column chromatography of the combined extracts (0.28 g) with 20% acetone in chloroform eluted 13-hydroxystemaran-19-oic acid (**17**) (6 mg) which crystallised as needles from acetone, $R_f = 0.16$, 10% acetone in dichloromethane, the physical and spectral data of which was identical to that of an authentic sample (Buchanan and Reese, 2001).

The second metabolite, 19-(*N*,*N*-dimethylcarbamoxy)-2β,13-dihydroxystemarane (**15**) (7 mg), crystallised as needles from acetone, $R_f = 0.30$, 15% acetone in dichloromethane, m.p. 187–190 °C, [α] +19.2° ($c = 0.59$, $CHCl_3$); IR: ν_{max} 3438, 1704, 1470, 1408 cm^{-1}; HRMS (EI): *m/z* (rel. int.): 393.2870 (20) M^+, ($C_{23}H_{39}NO_4$ requires 393.2879); ^{1}H NMR: δ 0.81 (3H, *s*, H-18), 1.05 (3H, *s*, H-20), 1.15 (3H, *s*, H-15), 2.928 (3H, *s*, NCH_3), 2.934 (3H, *s*, NCH_3), 3.64 (1H, *d*, $J = 10.4$ Hz, H-19), 3.74 (1H, *d*, $J = 9.0$ Hz, H-19), 3.81 (1H, *m*, $w/2 = 15.7$ Hz, H-2).

Finally 19-(*N*,*N*-dimethylcarbamoxy)-2β,8,13-trihydroxystemarane (**16**) (15 mg) was eluted and this crystallised as needles from acetone, $R_f = 0.21$, 15% acetone in dichloromethane, m.p. 198–200 °C, $[\alpha]_D$ +4.34° ($c = 4.60$, Me_2CO); IR: ν_{max} 3410, 2963, 2949, 1704, 1689, 1408, 1204 cm^{-1}; HRMS (EI): *m/z* (rel. int.): 409.2818 (2) M^+ ($C_{23}H_{39}NO_5$ requires 409.2828), 391.27202 (48) $[M - H_2O]$+, 373.2026 (6) $[M - 2H_2O]^+$, 365.2319 (2) $[M - (CH_3)_2N]^+$, 347.2207 (4) $[M - (CH_3)_2N - H_2O]^+$, 329.2129 (2) $[M - (CH_3)_2N - 2H_2O]^+$, 88.0395 (5) $[C_3H_6O_2N]^+$, 72.04495 (97) $[C_3H_6ON]^+$; ^{1}H NMR: δ 0.99 (3H, *s*, H-20), 1.15 (3H, *s*, H-15), 1.37 (3H, *s*, H-18), 2.95 (3H, *s*, NCH_3), 2.94 (3H, *s*, NCH_3), 3.73 (1H, *d*, $J = 10.4$ Hz, H-19), 3.86 (1H, *d*, $J = 10.4$ Hz, H-19), 3.86 (1H, *m*, $w/2 = 16.2$ Hz, H-2).

3.3. P. chrysosporium

3.3.1. Fermentation procedures

Cultures were maintained on slants of potato dextrose agar. For 1 g of substrate, five 2 week old slants were used to inoculate twenty 500 ml Erlenmeyer flasks each containing 125 ml liquid culture medium. A modified Richard's medium which consisted of (per litre) glucose (40 g), yeast extract (2 g), KNO_3 (10 g), $MgSO_4 \cdot 7H_2O$ (1.5 g), and KH_2PO_4 (2.5 g) was used. The flasks were shaken at 230 rpm. Incubations were conducted using the single phase, pulse feed technique. A solution of the substrate (10% of the total mass to be fed) was fed 24 h after inoculation. Thereafter 20%, 30% and 40% of the substrate was fed at 36, 48 and 60 h after inoculation, respectively. The mycelium was filtered from the fermentation broth 10 d after the last feed. The cells were homogenised with hot ethyl acetate while the broth was extracted with the same solvent. The organic extracts were then dried and concentrated.

3.3.2. Fermentation of stemodin (1)

Stemodin (**1**) (1 g) in ethanol (46 ml) was fed to twenty flasks. The mycelium extract was recrystallised to provide untransformed substrate (**1**) (300 mg); whereas the broth extract was chromatographed with 40% ethyl acetate in petrol to afford more **1** (116 mg). Elution with 50% ethyl acetate in petrol yielded 2α,7β,13-trihydroxystemodane (**3**) (85 mg) which was acetylated to give 2α,7β-diacetoxy-13-hydroxystemodane (**3a**) which resisted crystallisation, $R_f = 0.60$, 40% ethyl acetate in petrol, the spectral data of which was described earlier in this paper.

Increasing the polarity of the eluant mixture to 60% ethyl acetate in petrol afforded 2α,3β,13-trihydroxystemodane (**4**) (7 mg) which crystallised as cubes from acetone, $R_f = 0.32$, ethyl acetate, the physical and spectral data of which was described earlier in this paper.

Further elution yielded 2α,11β,13-trihydroxystemodane (**5**) (5 mg) as a gum, $R_f = 0.18$, ethyl acetate, the spectral

data of which was identical to that of an authentic sample (Chen et al., 2005).

3.3.3. Fermentation of 2α-(N,N-dimethylcarbamoxy)-13-hydroxystemodane (6)

2α-(*N*,*N*-Dimethylcarbamoxy)-13-hydroxystemodane (**6**) (0.1 g), dissolved in acetone (4 ml) was incubated with *P. chrysosporium*. Extraction of the broth and mycelium yielded a brown gum (0.2 g). Chromatography with mixtures of ethyl acetate in petrol gave untransformed **6**.

3.3.4. Fermentation of stemodinone (9)

Stemodinone (**9**) (0.5 g) in ethanol (20 ml) was fed to ten 500 ml conical flasks. The mycelial extract (391 mg) was recrystallised to afford the fed material (**9**) (380 mg) while the broth extract (146 mg) was subjected to column chromatography. Elution with 20% ethyl acetate in petrol yielded untransformed **9** (21 mg). Continued elution gave 13,18-dihydroxystemodan-2-one (**12**) (52 mg) as a gum, $R_f = 0.18$, 30% acetone in petrol, the spectral data of which was identical to that of an authentic sample (Hanson et al., 1994).

3.3.5. Fermentation of stemarin (13)

Stemarin (**13**) (0.5 g) was dissolved in ethanol (8 ml) and fed to ten conical flasks containing *P. chrysosporium*. The broth and mycelial extracts gave no products of transformation, and only **13** was recovered.

3.3.6. Fermentation of 19-(N,N-dimethylcarbamoxy)-13-hydroxystemarane (14)

19-(*N*,*N*-Dimethylcarbamoxy)-13-hydroxystemarane (**14**) (0.4 g), dissolved in acetone (8 ml) was fed to the fungus in eight 500 ml flasks. The substrate (**14**) was recovered untransformed.

Acknowledgements

The authors thank the University of the West Indies/Inter-American Development Bank (UWI/IDB) Programme for partial support. We are grateful to Professor John C. Vederas (University of Alberta) for mass spectral analysis, the Biotechnology Centre (UWI) for accommodation of our fermentations, and the Jamaica Bureau of Standards for the use of their polarimeter. A.S. Lamm thanks the Board for Graduate Studies (UWI) for the granting of a postgraduate scholarship.

References

Badria, F.A., Hufford, C.D., 1991. Microbial transformations of stemodin, a *Stemodia* diterpene. Phytochemistry 30, 2265–2268.

Buchanan, G.O., Reese, P.B., 2001. Biotransformation of diterpenes and diterpenes derivatives by *Beauveria bassiana* ATCC 7159. Phytochemistry 56, 141–151.

Chen, A.R.M., Reese, P.B., 2002. Biotransformation of terpenes from *Stemodia maritima* by *Aspergillus niger* ATCC 9142. Phytochemistry 59, 57–62.

Chen, A.R.M., Ruddock, P.L.D., Lamm, A.S., Reynolds, W.F., Reese, P.B., 2005. Stemodane and stemarane diterpenoid hydroxylation by *Mucor plumbeus* and *Whetzelinia sclerotiorum*. Phytochemistry 66, 1898–1902.

Dalziel, W., Hesp, B., Stevenson, K.M., Jarvis, J.A.J., 1973. The structure and absolute configuration of the antibiotic aphidicolin: a tetracyclic diterpenoid containing a new ring system. J. Chem. Soc. Perkin Trans. I, 2841–2851.

Hanson, J.R., Reese, P.B., Takahashi, J.A., Wilson, M.R., 1994. Biotransformation of some stemodane diterpenoids by *Cephalosporium aphidicola*. Phytochemistry 36, 1391–1393.

Hezari, M., Davis, P., 1992. Microbial models of mammalian metabolism: N-dealkylation of furosemide to yield the mammaliam metabolite CSA using *Cunninghamella elegans*. Drug metab. Dispos. 20, 882–888.

Hufford, C.D., 1988. ^{1}H NMR and ^{13}C NMR assignments for the *Stemodia* diterpenes, stemodin, stemodinone, and maritimol. J. Nat. Prod. 51, 367–369.

Hufford, C.D., Badria, F.A., Abou-Karam, M., Shier, W.T., Rogers, R.D., 1991. Preparation, characterization, and antiviral activity of microbial metabolites of stemodin. J. Nat. Prod. 54, 1543–1552.

Hufford, C.D., Oguntimien, B.O., Muhammad, I., 1992. New stemodane diterpenes from *Stemodia maritima*. J. Nat. Prod. 55, 48–52.

Manchand, P., Blount, J., 1975. X-ray structure and absolute stereochemistry of stemarin, a diterpene with a new skeleton. J. Chem. Soc. Chem. Commun., 894–895.

Manchand, P., Blount, J., 1976. X-ray structure and absolute stereochemistry of stemolide, a novel diterpene bisepoxide. Tetrahedron Lett. 29, 2489–2492.

Manchand, P., White, J., Wright, H., Clardy, J., 1973. Structures of stemodin and stemodinone. J. Am. Chem. Soc. 95, 2705.

Moore-Landecker, E., 1996. Fundamentals of the Fungi, fourth ed. Prentice Hall, New Jersey, p. 1, 16, 391.

Sandermann, H., 1997. Ex situ process for treating PAH-contaminated soil with *Phanerochaete chrysosporium*. Environ. Sci. Technol. 31, 2626–2633.

Sheremata, T., Hawari, J., 2000. Mineralization of RDX by the white rot fungus *Phanerochaete chrysosporium* to carbon dioxide and nitrous oxide. Environ. Sci. Technol. 34, 3384–3388.

Yang, W., Davis, P., 1991. Microbial models of mammalian metabolism: biotransformation of *N*-methylcarbazole using the fungus *Cunninghamella echinulata*. Drug metab. Dispos. 20, 38–41.