Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytochemistry 65 (2004) 2211–2217
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Investigation of the importance of the C-2 and C-13 oxygen functions in the transformation of stemodin analogues by *Rhizopus oryzae* ATCC 11145

Glenroy D.A. Martin [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 14 December 2003; received in revised form 8 May 2004
Available online 19 June 2004

Dedicated to the memory of Professor Herbert L. Holland (Brock University) for his contribution to Bio-organic Chemistry for over 30 years

Abstract

Incubation of 2α,13(*R*)-dihydroxystemodane (**3**) with *Rhizopus oryzae* ATCC 11145 gave 2α,7β,13(*R*)-trihydroxystemodane (**11**) while biotransformation of 13(*R*)-hydroxystemodan-2-one (**5**) yielded 6α,13(*R*)-dihydroxystemodan-2-one (**12**) and 7β,13(*R*)-dihydroxystemodan-2-one (**13**). Bioconversion of 2β,13(*R*)-dihydroxystemodane (**7**) with *Rhizopus* afforded 2β,7,13(*R*)-trihydroxystemodane (**14**). The results complement data from our previous work and provide more information about the effect of functional groups of stemodane substrates on the site of hydroxylation.

Keywords: *Rhizopus oryzae* ATCC 11145; *Stemodia maritima*; Scrophulariaceae; Stemodane; Diterpene; Biotransformation; Hydroxylation; *Rhizopus arrhizus*

1. Introduction

Rhizopus oryzae ATCC 11145 (synonyms: IMI 90340, CBS 381.52, IFO 5780, IFO 6155, Upjohn Co. RH 176, CECT 2339 and DSM 906), formerly known as *Rhizopus arrhizus*, is commonly found in the soil, vegetables, animal faeces, old bread and decaying fruits (Moore-Landecker, 1982). The fungus is efficient and versatile in the transformation of a wide range of xenobiotes. A few examples include the microbial conversion of steroids (Peterson et al., 1952; Holland et al., 1998), terpenes (El Sayed et al., 1998; De Oliveira et al., 1999), prostaglandins (Holland et al., 1988, 1990), thioethers (Auret et al., 1974), aromatic compounds (Hufford et al., 1981; Orabi et al., 1999), esters (Cabon et al., 1992; Abalain et al., 1996), octalin and hydrindenone derivatives (Arséniyadis et al., 1991, Ouazzani et al., 1991) amongst others. It is widely known as the C-6β hydroxylating fungus of Δ^4-3-ketosteroids (Holland, 1984).

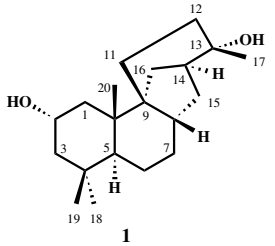
The cytochrome P450 enzyme system within *Rhizopus* is believed to be responsible for these reactions. To date, the X-ray crystal structure of this enzyme in its natural conformation has not been determined (Holland, 1984). This is due to the hydrophobic nature of the catalyst, hence, formation of single crystals is difficult since the proteins tend to aggregate (Feiters et al., 2000). By probing the enzyme active site indirectly information about its binding and hydroxylating sites as well as the type of substrates which will be accepted for bioconversion can be generated.

It is to this end that analogues of the mild antiviral and cytotoxic diterpene stemodin (**1**), obtained from *Stemodia maritima* (Manchand et al., 1973; Hufford et al., 1992), were incubated with *R. oryzae* (Hufford et al., 1991; Badria and Hufford, 1991; Hanson et al., 1994). These compounds include 2α,13(*R*)-dihydroxystemodane (**3**),

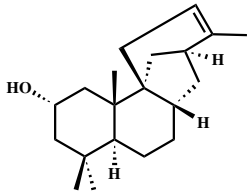
* Corresponding author. Tel.: +1-876-935-8460; fax: +1-876-977-1835.
E-mail address: paul.reese@uwimona.edu.jm (P.B. Reese).

0031-9422/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2004.05.016

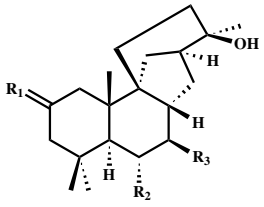
2α-hydroxystemodane (**4**), 13(*R*)-hydroxystemodan-2-one (**5**), stemodan-2-one (**6**), 2β,13(*R*)-dihydroxystemodane (**7**), 2β-hydroxystemodane (**8**), 13(*R*)-hydroxystemodane (**9**) and stemodane (**10**). The results from the fermentations show the relationship between the functional groups of the stemodane substrates and their products of bioconversion. Furthermore, this work is part of a larger study which examines the potential of fungi to effect transformation of bioactive terpenes (Buchanan and Reese, 2001; Chen and Reese, 2002).

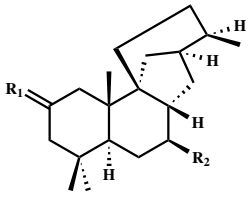


1



2





3 $R_1 = \alpha OH, \beta H, R_2 = R_3 = H$

4 $R_1 = \alpha OH, \beta H, R_2 = H$

5 $R_1 = O, R_2 = R_3 = H$

6 $R_1 = O, R_2 = H$

7 $R_1 = \beta OH, \alpha H, R_2 = R_3 = H$

8 $R_1 = \beta OH, \alpha H, R_2 = H$

9 $R_1 = H_2, R_2 = R_3 = H$

10 $R_1 = H_2, R_2 = H$

11 $R_1 = \alpha OH, \beta H, R_2 = H, R_3 = OH$

12 $R_1 = O, R_2 = OH, R_3 = H$

13 $R_1 = O, R_2 = H, R_3 = OH$

14 $R_1 = \beta OH, \alpha H, R_2 = H, R_3 = OH$

2. Results and discussion

Stemodin (**1**), isolated from the plant *S. maritima*, served as the starting material in the synthesis of eight analogues. The solvolysis of stemodin (**1**) yielded 2α-hydroxystemod-12-ene (**2**) (Buchanan and Reese, 2001) and 2α,13(*R*)-dihydroxystemodane (**3**) (Martin et al., 2004).

Catalytic hydrogenation of 2α-hydroxystemod-12-ene (**2**) gave 2α-hydroxystemodane (**4**), a new product. HRMS(EI) data of **4** ($M^+ = 290.2610$) suggested a molecular formula of $C_{20}H_{34}O$. ^1H NMR data affirmed the absence of the olefin moiety with the methyl doublet for H-17 resonating at δ 0.73. The C-17 methyl group was assigned β stereochemistry based on the T-ROE correlations for H-17 with H-15β (δ 1.79), H-14α (δ 1.90) and H-13α (δ 1.43). The disappearance of olefinic resonances in the ^{13}C NMR spectrum was also confirmed with the emergence of new methylene (δ 28.2, C-12) and methine (δ 36.6, C-13) signals.

Oxidation of 2α,13(*R*)-dihydroxystemodane (**3**) and 2α-hydroxystemodane (**4**) under Jones conditions gave 13(*R*)-hydroxystemodan-2-one (**5**) and stemodan-2-one (**6**), respectively, both previously unreported. HR MS(EI) analysis of **5** ($M^+ = 304.2402$) suggested a molecular formula of $C_{20}H_{32}O_2$. Absorption in the infrared spectrum at 3408 and 1695 cm^{-1} corresponded to hydroxyl and carbonyl stretches, respectively. A new resonance at 212.2 ppm accounted for the carbonyl carbon and accompanying α shifts were noted in the ^{13}C NMR data. HRMS(EI) data ($M^+ = 288.2453$) for **6** supported a molecular formula of $C_{20}H_{32}O$. Absorption at 1711 cm^{-1} in the FT-IR spectrum was indicative of a carbonyl group. ^{13}CNMR data also confirmed the presence of the carbonyl functionality at C-2 with a signal at 212.6 ppm.

Treatment of 13(*R*)-hydroxystemodan-2-one (**5**) and stemodan-2-one (**6**) with sodium borohydride in methanol afforded the novel 2β,13(*R*)-dihydroxystemodane (**7**) and 2β-hydroxystemodane (**8**), respectively. HRMS(EI) data of **7** ($M^+ = 306.2540$) suggested a molecular formula of $C_{20}H_{34}O_2$. The FT-IR spectrum indicated the presence of a hydroxyl stretch at 3355 cm^{-1}. The appearance of a new resonance at δ 4.08 in the ^1H NMR data was correlated with H-2, and a new signal at 68 ppm in the ^{13}C NMR spectrum confirmed that reduction had taken place. Compound **8** was assigned a molecular formula of $C_{20}H_{34}O$ based on HRMS(EI) data ($M^+ = 290.2610$). Absorption in the FT-IR spectrum at 3364 cm^{-1} signified the presence of a hydroxyl moiety. In the ^1H NMR data a signal at δ 4.07 for H-2 was observed. The presence of a hydroxyl group at C-2 was also verified in ^{13}C NMR spectrum with a resonance at 68.1 ppm.

Reduction of 13(*R*)-hydroxystemodan-2-one (**5**) and stemodan-2-one (**6**) under modified Wolff Kishner conditions (Huang-Minlon, 1949) afforded 13(*R*)-hydroxystemodane (**9**) (Lupi et al., 1984) and the saturated parent hydrocarbon (**10**), respectively (Manchand et al., 1973). An absorption at 3318 cm^{-1} for **9** in the FT-IR spectrum suggested the presence of a hydroxyl moiety. ^{13}C NMR data confirmed the absence of the C-2 carbonyl functional group and the presence of a new methylene resonance at 18.8 ppm. Analogue **10** showed the typical C–H stretch at 2930 cm^{-1} in the FT-IR spectrum. The loss of the carbonyl group was verified in the ^{13}C NMR data by the presence of a new methylene signal at δ 18.9 for C-2.

Bioconversion of 2α,13(*R*)-dihydroxystemodane (**3**) by *R. oryzae* produced only one metabolite **11** in high yield. Evidence that monohydroxylation had occurred was seen in the HRMS(EI) data ($m/z = 322.2480$, $C_{20}H_{34}O_3$). ^1H NMR data exhibited a new resonance at δ 3.33 (H-7α). The stereochemistry of C-7 hydroxyl group was resolved as β based on ROE couplings between H-7α and H-6α (δ 1.70), H-15α (δ 1.52) and H-5α

(δ 1.22). ^{13}C NMR data verified C-7 hydroxylation with a signal at 79.4 ppm. The metabolite was therefore determined to be 2α,7β,13(*R*)-trihydroxystemodane (**11**).

Bioconversion of 13(*R*)-hydroxystemodan-2-one (**5**) yielded two new compounds: 6α,13(*R*)-dihydroxy stemodan-2-one (**12**) and 7β,13(*R*)-dihydroxystemodan-2-one (**13**). Compound **12** was shown to be a product of monohydroxylation based on the HRMS(EI) data ($m/z = 320.2348$, $C_{20}H_{32}O_3$). Absorptions were noted in the FT-IR spectrum for hydroxyl and carbonyl stretches at 3332 and 1704 cm^{-1}. ^{1}H NMR data revealed a new proton resonance at δ 3.78 for H-6. T-ROESY data confirmed the stereochemistry of the hydroxyl group at C-6 as α based on ROE couplings between H-6β and H-8β (δ 1.90), H-11β (δ 1.46), H-16β (δ 1.33), H-19 (δ 1.19) and H-20 (δ 1.00). The new resonance at 68.7 ppm in the ^{13}C NMR spectrum was assigned to C-6.

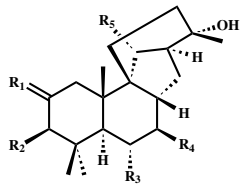
The second metabolite **13** was deduced to have a molecular formula of $C_{20}H_{32}O_3$ as supported by HRMS(EI) data ($M^+ = 320.2351$). Carbonyl and hydroxyl stretches were seen in the FT-IR spectrum at 1700 and 3380 cm^{-1}, respectively. A new signal at H-7α was observed at δ 3.42 in the ^{1}H NMR data. The hydroxyl group was assigned as β due to T-ROE correlations between H-7α and H-16α (δ 1.89), H-5α (δ 1.85) and H-15α (δ 1.60). The evidence of a new hydroxyl group was also noted in the ^{13}C NMR spectrum at δ 78.8. The metabolite was confirmed to be 7β,13(*R*)-dihydroxystemodan-2-one (**13**).

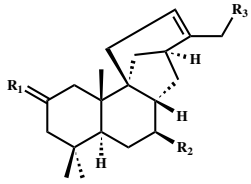
Incubation of 2β,13(*R*)-dihydroxystemodane (**7**) with *R. oryzae* yielded seven metabolites. Only the new 2β,7β,13(*R*)-trihydroxystemodane (**14**) was present in sufficient yield to be characterised. A molecular formula of $C_{20}H_{34}O_3$ was assigned to **14** based on the HRMS(EI) data ($m/z = 322.2480$). The FT-IR spectrum showed an absorption at 3272 cm^{-1}, indicative of a hydroxyl moiety. A signal at δ 3.33 (1H, *m*, $w/2 = 12.1$ Hz) was seen for H-7 in the ^{1}H NMR data. The stereochemistry of the hydroxyl group at C-7 was designated as β due to the ROE cross-peaks between H-7α and H-6α (δ 1.70), H-1α (δ 1.70), H-15α (δ 1.54) and H-5α (δ 1.33). A new resonance at 79.2 ppm in the ^{13}C NMR spectrum supported the fact that hydroxylation had occurred at C-7.

There was no evidence of transformation of the other substrates, 2α-hydroxystemodane (**4**), stemodan-2-one (**6**), 2β-hydroxystemodane (**8**), 13(*R*)-hydroxystemodane (**9**) and stemodane (**10**), and these were recovered from the fermentations.

2.1. Substrate properties vs. potential for biotransformation

In our previous work, we reported on the incubation of the stemodin analogues **15**–**18** (*S*-stereochemistry at C-13) and the olefins **2**, **19**–**21** with *R. oryzae* (Martin et al., 2004). Substrates **15**–**17** yielded mainly monohydroxylated products while the olefins showed bioconversion for only compound **19**. These results compared well with our present data as similar sites of hydroxylation were observed for xenobiotes **3**, **5** and **7** (C-13 with *R*-stereochemistry). These metabolites, however, were obtained in much higher yields. No transformation was seen for substrates **4**, **6**, **8** and **10** in which the functionality at C-13 was removed. The results are summarised in Table 1.





15 R_1 = αOH, βH, $R_2 = R_3 = R_4 = R_5$ = H

16 R_1 = O, $R_2 = R_3 = R_4 = R_5$ = H

17 R_1 = βOH, αH, $R_2 = R_3 = R_4 = R_5$ = H

18 $R_1 = H_2$, $R_2 = R_3 = R_4 = R_5$ = H

22 R_1 = αOH, βH, $R_2 = R_3 = R_5$ = H, R_4 = OH

23 R_1 = αOH, βH, $R_2 = R_5$ = OH, $R_3 = R_4$ = H

24 R_1 = O, $R_2 = R_4 = R_5$ = H, R_3 = OH

25 R_1 = βOH, αH, $R_2 = R_3 = R_5$ = H, R_4 = OH

19 R_1 = O, $R_2 = R_3$ = H

20 R_1 = βOH, αH, $R_2 = R_3$ = H

21 $R_1 = H_2$, $R_2 = R_3$ = H

26 R_1 = O, $R_2 = R_3$ = OH

The above data confirms the necessity for the stemodane xenobiotes to possess two docking groups in order to effect their biotransformation. Variation of the C-2 functional group (that is, from an alcohol to a ketone) while keeping that at C-13 unchanged (*R*-stereochemistry) seems to alter the orientation of the substrate in the enzyme active site and hence the site of

Table 1
Overall results of the incubation of the stemodane substrates with *R. oryzae*

	13-OH (*S*)	13-epi-OH (*R*)	12-ene	13-H (*R*)
2α-OH	**15** → **22** 7β-OH (8%) **15** → **23** 3β,16α-diOH (4%)	**3** → **11** 7β-OH (83%)	NP	NP
2-one	**16** → **24** 6α-OH (1%)	**5** → **12** 6α-OH (17%) **5** → **13** 7β-OH (13%)	**19** → **26** 7β,17-diOH (4%)	NP
2β-OH	**17** → **25** 7β-OH (10%)	**7** → **14** 7β-OH (10%)	NP	NP
2-H	NP	NP	NP	NP

NP, no products.

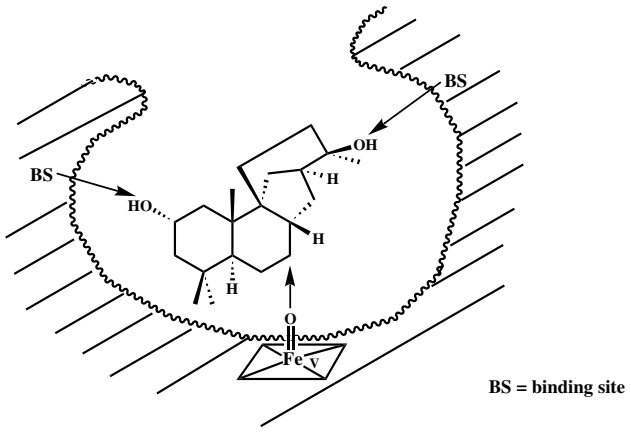


Fig. 1. Possible three-dimensional relationship between the substrate binding sites and the site of hydroxylation.

hydroxylation. It is possible that the same cytochrome P450 enzyme system was responsible for the transformations since hydroxylations occurred mainly at C-6 and C-7. Likewise, lateral insertion of the activated haem-bound oxygen along the carbon skeleton was apparent since the hydroxylations were observed at equatorial positions (Feiters et al., 2000). As with steroids (Holland, 1982), a three-dimensional relationship between the substrate binding sites and the position of hydroxylation can be applied to the stemodane substrates (Fig. 1).

In summary, we now report the preparation of nine new analogues of stemodin (**1**) by chemical and microbial means. In addition, the full spectral data of the known substrates **9**–**10** are reported for the first time. The results from the incubation of compounds **3**–**10** with *R. oryzae* indicate the types of stemodane xenobiotes that will be accepted and transformed by the enzyme systems of the fungus. It is our hope that, in the absence of an X-ray crystal structure of the mono-oxygenase of *Rhizopus*, a model of the enzyme active site can be generated to assist in predicting the outcome of these biological reactions.

3. Experimental

3.1. General experimental

Melting points were determined on a Thomas Hoover capillary melting point apparatus and are uncorrected. Optical rotations were performed on a Perkin–Elmer 241 MC polarimeter. Infrared spectra were recorded using KBr pellets or NaCl disks on a Perkin–Elmer FT-IR Paragon 1000 spectrophotometer. ^{1}H and ^{13}C NMR data were obtained on Bruker AC200 and Varian Unity 500 NMR spectrometers. 1D experiments were carried out on the former while 2D experiments were done on the latter. Deuterated methanol (CD_3OD) and chloroform ($CDCl_3$) were used as solvents with tetramethylsilane (TMS) as internal standard. ^{13}C NMR assignments for the stemodane terpenoids are listed in Table 2. HRMS(EI) was carried out on a Kratos MS50 instrument at an ionising voltage of 70 eV. Column chromatography was performed on silica gel (37–63 μm diameter). Detection of

Table 2
^{13}C NMR resonances of the stemodanes

C	1[a]	3[a]	4[a]	5[a]	6[a]	7[a]	8[a]	9[a]	10[a]	11[b]	12[b]	13[b]	14[b]
1	45.7	45.5	45.5	51.3	51.4	42.5	42.5	36.2	36.2	45.6	51.5	51.9	42.4
2	65.1	65.3	65.2	212.2	212.6	68.0	68.1	18.8	18.9	64.9	214.6	214.5	67.8
3	50.6	50.8	50.6	55.8	55.9	46.6	46.5	41.8	42.0	50.6	57.2	56.1	46.5
4	34.6	34.9	34.7	39.1	39.1	32.6	32.6	33.2	33.2	34.8	39.6	39.5	33.0
5	46.6	47.0	46.8	47.4	47.2	45.6	45.4	47.3	47.2	44.8	52.4	45.6	43.9
6	22.0	22.0	22.4	22.5	22.5	22.3	22.3	22.2	22.4	31.3	68.7	31.9	31.5
7	36.4	36.4	36.9	36.0	36.2	36.2	36.4	36.6	36.9	79.4	45.8	78.8	79.2
8	36.9	37.2	38.5	37.5	38.5	37.1	38.1	37.6	38.6	46.8	37.2	47.3	46.7
9	50.1	50.5	50.8	50.5	50.5	51.2	51.2	50.5	50.5	52.7	50.6	52.6	53.5
10	40.1	40.0	40.3	44.5	44.8	38.1	38.3	38.2	38.4	40.1	45.7	45.1	38.4
11	27.8	29.1	31.4	29.3	31.2	29.1	31.1	29.0	31.1	29.7	29.4	30.1	29.7
12	32.7	33.7	28.2	33.2	27.7	33.9	27.9	33.8	28.1	33.6	33.3	33.5	33.7
13	72.3	72.3	36.6	72.4	36.1	72.7	36.3	72.8	36.4	72.7	72.3	72.4	72.9
14	46.1	46.7	40.9	46.7	40.2	46.7	40.2	47.0	40.6	47.2	46.9	47.1	47.0
15	38.1	35.7	34.2	35.8	34.1	35.8	34.0	35.7	34.1	34.4	36.2	34.8	34.4
16	30.1	32.8	38.0	33.5	38.1	32.8	37.7	32.7	37.7	34.6	33.4	35.2	34.6
17	28.0	26.2	19.6	26.2	18.7	26.2	19.6	26.1	18.9	26.0	26.0	26.1	26.1
18	34.7	34.8	34.9	34.5	34.4	34.4	34.4	34.6	34.6	34.7	37.5	34.5	34.5
19	23.6	23.7	23.8	23.9	23.9	25.2	25.2	22.8	22.8	23.9	25.1	24.3	25.4
20	19.6	19.8	19.8	19.0	19.4	22.3	22.1	19.1	19.7	20.4	20.0	19.6	22.7

[a] Determined in $CDCl_3$.
[b] Determined in CD_3OD.

compounds on thin layer chromatography (TLC) was achieved by spraying the plates with ammonium molybdate/sulfuric acid solution followed by heating until the colour developed. Stemodin (**1**) was obtained from the acetone extract of *S. maritima* in an overall yield of 12%. *R. oryzae* ATCC 11145 was obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA. Petrol refers to the petroleum fraction boiling at 60–80 °C.

3.2. Culture conditions

The fungus was maintained on 4.5% malt agar slants. The liquid medium (2.5 l) for *R. oryzae* consisted of dipotassium hydrogen phosphate (5 g/l), sodium chloride (5 g/l), peptone (5 g/l), yeast extract (5 g/l) and glucose (20 g/l). The fermentation medium (2.5 l) was distributed equally amongst twenty 500 ml Erlenmeyer flasks and was sterilised. Spore suspensions of the fungus were used to inoculate the flasks. The substrate was fed over a period of 3 days. 24, 36, 48 and 60 h after inoculation 10%, 20%, 30% and 40%, respectively, of the substrate was fed (pulse feed protocol) to the growing fungus. The total substrate concentration was 0.20–0.40 mg ml^{-1} of the culture medium. Controls consisted of flasks to which only solvent was fed to the growing fungus. The medium was shaken for 5 days after the last feeding. The fermentation beer was pooled and extracted with ethyl acetate (4×700 ml). The fungal cells were homogenised and extracted in warm ethyl acetate (400 ml). The organic extracts were dried, filtered, and the solvent was removed in vacuo.

4. Preparation of the stemodane substrates

4.1. 13(R)-Hydroxystemodan-2-one (5)

To 2α,13(*R*)-dihydroxystemodane (**3**) (560 mg, 1.628 mmol) in acetone (60 ml) at −10 °C (ice salt) was added Jones reagent (2.5 M, 1.0 ml, 2.5 mmol). The reaction was stirred for 0.5 h. Ethanol (3.0 ml) was added and the reaction mixture was neutralised with saturated aqueous sodium hydrogen carbonate. The solvent was removed in vacuo and the residue was diluted with water (ca. 20 ml) and extracted with ethyl acetate (2×30 ml). The organic solution was dried, filtered, and the solvent was evaporated to yield 13(*R*)-hydroxystemodan-2-one (**5**) (488 mg) which crystallised from acetone as needles, m.p. 183–184 °C; $[\alpha]_D^{27}$: +21.4° (MeOH; $c = 0.98$); FT-IR: ν_{max} cm^{-1} 3408 (OH), 1695 (>C=O); HRMS(EI): *m/z* (rel. int.): 304.2402 $[M]^+$ (30.4) [$C_{20}H_{32}O_2$ requires 304.2402], 286.2294 $[M–H_2O]^+$ (18.1), 271.2062 $[M–CH_3–H_2O]^+$ (11.6), 233.1904 (100); ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.92 (3H, *s*, H-19), 0.97 (3H, *s*, H-20), 1.09 (3H, *s*, H-18), 1.23 (3H, *s*, H-17).

4.2. 13(R)-Hydroxystemodane (9)

13(*R*)-Hydroxystemodane (**9**) was prepared from 13(*R*)-hydroxystemodan-2-one (**5**) (2.0 g, 6.579 mmol) under Wolff Kishner conditions (Huang-Minlon, 1949). 13(*R*)-Hydroxystemodane (**9**) (1.81 g) crystallised from methanol as prisms, m.p. 151–153 °C; $[\alpha]_D^{27}$: +16.7° (MeOH; $c = 0.56$); FT-IR: ν_{max} cm^{-1} 3318 (OH); ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.87 (3H, *s*, H-18), 0.87 (3H, *s*, H-19), 0.94 (3H, *s*, H-20), 1.23 (3H, *s*, H-17).

4.3. 2β,13(R)-Dihydroxystemodane (7)

To 13(*R*)-hydroxystemodan-2-one (**5**) (25 mg, 0.0822 mmol) in methanol (2 ml) at 0 °C (ice salt) was slowly added sodium borohydride (15.6 mg, 0.412 mmol) over 3 min. The reaction was allowed to warm to room temperature and then was stirred for 15 min. The mixture was poured into water (20 ml) and was neutralised with 6 M sulfuric acid (ca. 1 drop). The white precipitate was filtered off under vacuum to afford 2β,13(*R*)-dihydroxystemodane (**7**) (30 mg) which crystallised from acetone as amorphous crystals, m.p. 176–178 °C; $[\alpha]_D^{27}$: +18.0° (MeOH; $c = 0.39$); FT-IR: ν_{max} cm^{-1} 3355 (OH); HRMS(EI): *m/z* (rel. int.): 306.2540 $[M]^+$ (1.1) [$C_{20}H_{34}O_2$ requires 306.2559], 288.2450 $[M–H_2O]^+$ (13.1), 273.2218 $[M–H_2O–CH_3]^+$ (18.9), 217.1949 (100); ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.91 (3H, *s*, H-19), 1.02 (3H, *s*, H-18), 1.07 (3H, *s*, H-20), 1.17 (3H, *s*, H-17), 4.08 (1H, *m*, $w/2 = 15.5$ Hz, H-2).

4.4. 2α-Hydroxystemodane (4)

To 2α-hydroxystemod-12-ene (**2**) (4.0 g, 13.88 mmol) in ethyl acetate (200 ml) was added 10% palladium on carbon (0.4 g). The reaction mixture was hydrogenated at 15 psi for 6 h. The mixture was filtered under vacuum and the filtrate was concentrated in vacuo to provide 2α-hydroxystemodane (**4**) (3.91 g) which crystallised from acetone as needles, m.p. 106–108 °C; $[\alpha]_D^{27}$: +1.46° (MeOH; $c = 1.05$); FT-IR: ν_{max} cm^{-1} 3325 (OH); HRMS(EI): *m/z* (rel. int.): 290.2610 $[M]^+$ (3.7) [$C_{20}H_{34}O$ requires 290.2610], 275.2374 $[M–CH_3]^+$ (7.2), 272.2496 $[M–H_2O]^+$ (7.7), 257.2271 $[M–CH_3–H_2O]^+$ (28.0), 233.1902 (21.6), 215.1799 (100); ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.73 (3H, d, $J = 6.3$ Hz, H-17), 0.92 (3H, *s*, H-19), 0.95 (3H, *s*, H-18), 0.97 (3H, *s*, H-20), 3.72 (1H, *tt*, $J = 11.4$, 7.6 Hz, H-2).

4.5. Stemodan-2-one (6)

To 2α-hydroxystemodane (**4**) (3.3 g, 11.37 mmol) in acetone (300 ml) at −18 °C (ice salt) was added Jones reagent (4.5 ml, 11.25 mmol). The reaction was stirred

for 1 h and ethanol (5 ml) was added. The reaction was neutralised with saturated sodium hydrogen carbonate (50 ml) and the acetone was removed in vacuo. The mixture was diluted with water (100 ml) and extracted with ethyl acetate (2×150 ml). The organic solution was dried, filtered, and the solvent was removed in vacuo to provide stemodan-2-one (**6**) (3.2 g) which crystallised from methanol as cubes, m.p. 68–71 °C; $[\alpha]_D^{27}$: +19.9° ($CHCl_3$; $c = 5.7$); FT-IR: ν_{max} cm^{-1} 1711 (>C=O); HRMS(EI): *m/z* (rel. int.): 288.2453 $[M]^+$ (67.8) [$C_{20}H_{32}O$ requires 288.2453], 273.2215 $[M–CH_3]^+$ (31.0), 231.1747 (100); ^1H NMR (200 MHz, $CDCl_3$): δ 0.73 (1H, d, $J = 6.3$ Hz, H-17), 0.92 (3H, *s*, H-19), 0.95 (3H, *s*, H-18), 1.08 (3H, *s*, H-20), 2.29 (1H, *bs*, H-1), 2.35 (1H, *d*, $J = 1.6$ Hz, H-3).

4.6. Stemodane (*10*)

Stemodan-2-one (**6**) (1.1 g, 3.82 mmol) was reduced under modified Wolff Kishner conditions (Huang-Minlon, 1949; Manchand et al., 1973) to afford stemodane (**10**) (0.96 g) as a gum, $[\alpha]_D^{27}$: +8.2° ($CHCl_3$; $c = 3.64$); literature $[\alpha]_D^{27}$: +10.1 ($CHCl_3$; $c = 1.13$) (Manchand et al., 1973); FT-IR: ν_{max} cm^{-1} 2930 (CH); ^1H NMR (200 MHz, $CDCl_3$): δ 0.72 (1H, *d*, $J = 6.3$ Hz, H-17), 0.86 (3H, *s*, H-19), 0.87 (3H, *s*, H-18), 0.92 (3H, *s*, H-20).

4.7. 2β-Hydroxystemodane (*8*)

To stemodan-2-one (**6**) (1.0 g, 3.47 mmol) in methanol (30 ml) at −20 °C was slowly added sodium borohydride (0.39 g, 10.41 mmol). The reaction was stirred for 15 min and was poured into water (80 ml). The mixture was extracted with dichloromethane (2×50 ml). The organic solution was dried, filtered, and the solvent was removed in vacuo to provide 2β-hydroxystemodane (**8**) (1.0 g) which crystallised from methanol as cubes, m.p. 96–97 °C; $[\alpha]_D^{27}$: +11.3° (MeOH; $c = 1.66$); FT-IR: ν_{max} cm^{-1} 3364 (OH); HRMS(EI): *m/z* (rel. int.): 290.2610 $[M]^+$ (44.9) [$C_{20}H_{34}O$ requires 290.2610], 275.2373 $[M–CH_3]^+$ (15.2), 257.2266 $[M–CH_3–H_2O]^+$ (19.1), 233.1902 (69.1), 215.1798 (100); ^1H NMR (200 MHz, $CDCl_3$): δ 0.72 (1H, *d*, $J = 6.3$ Hz, H-17), 0.91 (3H, *s*, H-19), 1.06 (3H, *s*, H-18), 1.14 (3H, *s*, H-20), 4.07 (1H, *q*, $J = 4.43$ Hz, H-2).

5. Biotransformation of the stemodane analogues

5.1. Incubation of 2α,13(R)-dihydroxystemodane (*3*)

2α,13(*R*)-Dihydroxystemodane (**3**) (500 mg) was fed and the fermentation was harvested to yield broth (0.44 g) and mycelial (0.83 g) extracts. The transformed metabolite was present only in the broth. The broth extract was washed with a cold acetone:petrol (1:2) mixture to afford 2α,7β,13(*R*)-trihydroxystemodane (**11**) (440 mg) which crystallised from methanol as amorphous crystals, m.p. 259–261 °C; $[\alpha]_D^{27}$: +8.3° (MeOH; $c = 0.02$); FT-IR: ν_{max} cm^{-1} 3328 (OH); HRMS(EI): *m/z* (rel. int.): 322.2480 $[M]^+$ (0.14) [$C_{20}H_{34}O_3$ requires 322.2508], 289.2161 $[M–CH_3–H_2O]^+$ (5.4), 250.1928 (14.8), 233.1896 (100); ^1H NMR (500 MHz, CD_3OD): δ 0.96 (3H, *s*, H-19), 0.98 (3H, *s*, H-18), 1.03 (3H, *s*, H-20), 1.22 (3H, *s*, H-17), 3.33 (1H, *m*, $w/2 = 6.63$ Hz, H-7), 3.74 (1H, *tt*, $J = 11.5$, 3.8 Hz, H-2).

5.2. Incubation of 13(R)-hydroxystemodan-2-one (*5*)

13(*R*)-Hydroxystemodan-2-one (**5**) (500 mg) was incubated with *Rhizopus* to produce mycelial (0.52 g) and broth (0.63 g) extracts. The broth extract which contained only the transformed metabolites was purified on silica gel. Elution with 10% acetone in dichloromethane provided 6α,13(*R*)-dihydroxystemodan-2-one (**12**) (64 mg) which crystallised from methanol as prisms, m.p. 140–142 °C; $[\alpha]_D^{27}$: +62.1° (MeOH; $c = 1.2$); FT-IR: ν_{max} cm^{-1} 3332 (OH), 1704 (>C=O); HRMS(EI): *m/z* (rel. int.): 320.2348 $[M]^+$ (28.9) [$C_{20}H_{32}O_3$ requires 320.2351], 305.2117 $[M–CH_3]^+$ (28.4), 302.2243 $[M–H_2O]^+$ (68.5), 249.1852 (100), 231.1749 (48.6); ^1H NMR (500 MHz, $CDCl_3$): δ 1.00 (3H, *s*, H-20), 1.19 (3H, *s*, H-19), 1.23 (3H, *s*, H-17), 1.32 (3H, *s*, H-18), 3.78 (1H, *m*, $w/2 = 18.5$ Hz, H-6).

Elution with 15% acetone in dichloromethane yielded 7β,13(*R*)-dihydroxystemodan-2-one (**13**) (57 mg) which crystallised from methanol as prisms, m.p. 189–192 °C; $[\alpha]_D^{27}$: +42.0° (MeOH; $c = 0.5$); FT-IR: ν_{max} cm^{-1} 3380 (OH), 1700 (>C=O); HRMS(EI): *m/z* (rel. int.): 320.2351 $[M]^+$ (28.9) [$C_{20}H_{32}O_3$ requires 320.2351], 305.2112 $[M–CH_3]^+$ (11.0), 249.1854 (100), 231.1748 (88.8); ^1H NMR (500 MHz, $CDCl_3$): δ 0.97 (3H, *s*, H-19), 1.00 (3H, *s*, H-20), 1.14 (3H, *s*, H-18), 1.23 (3H, *s*, H-17), 3.42 (1H, *m*, $w/2 = 16.0$ Hz, H-7).

5.3. Incubation of 2β,13(R)-dihydroxystemodane (*7*)

The fermentation of 2β,13(*R*)-dihydroxystemodane (**7**) (500 mg) with the fungus yielded mycelial (0.81 g) and broth (0.34 g) extracts. The combined extracts were chromatographed with 25% acetone in petrol to afford 2β,7β,13(*R*)-trihydroxystemodane (**14**) (40 mg) which crystallised from methanol as amorphous crystals, m.p. 250–254 °C; $[\alpha]_D^{27}$: +56.4° (MeOH; $c = 0.48$); FT-IR: ν_{max} cm^{-1} 3272 (OH); HRMS(EI): *m/z* (rel. int.): 322.2480 $[M]^+$ (0.4) [$C_{20}H_{34}O_3$ requires 322.2508], 289.2167 $[M–CH_3–H_2O]^+$ (8.1), 250.1928 (17.6), 233.1903 (100); ^1H NMR (500 MHz, CD_3OD): δ 0.94 (3H, *s*, H-18), 1.11 (3H, *s*, H-19), 1.19 (3H, *s*, H-20), 1.23 (3H, *s*, H-17), 3.33 (1H, *m*, $w/2 = 12.1$ Hz, H-7), 4.04 (1H, *m*, $w/2 = 11.1$ Hz, H-2).

Acknowledgements

This work was supported in part by the University of the West Indies/Inter-American Development Bank (UWI/IDB) Programme. The authors are grateful to Professor John C. Vederas (University of Alberta) for mass spectral data and the late Professor Herbert L. Holland (Brock University) for helpful discussions. Patrice Peart, Kamau Francis, Katrina Gordon and Melanie Camacho are thanked for technical assistance. Fermentations were performed in the Biotechnology Centre, UWI. G.D.A.M. thanks the Chemistry Department for the granting of a Teaching Assistantship.

References

Abalain, C., Buisson, D., Azerad, R., 1996. Selective synthesis of 1-, and 3-carbomethoxy 2-tetralol stereoisomers by microbial reduction of the corresponding tetralones. Tetrahedron: Asymmetry 7, 2983–2996.

Arséniyadis, S., Ouazzani, J., Rodriguez, R., Rumbero, A., Ourisson, G., 1991. Biohydroxylations of hydrindenone derivatives. Tetrahedron Lett. 32, 3573–3576.

Auret, B.J., Boyd, D.R., Henbest, H.B., Watson, C.G., 1974. Species-strain dependence of stereoselectivity in microbial oxidation of thioethers. Phytochemistry 13, 65–68.

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 diterpene and diterpene derivatives by *Beauveria bassiana* ATCC 7159. Phytochemistry 56, 141–151.

Cabon, O., Larchevêque, M., Buisson, D., Azerad, R., 1992. Microbial reduction of 2-chloro-3-aryl-3-oxopropionic acid esters. Tetrahedron Lett. 33, 7337–7340.

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

De Oliveira, B.H., Dos Santos, M.C., Leal, P.C., 1999. Biotransformation of the diterpenoid, isosteviol, by *Aspergillus niger*, *Penicillium chrysogenum* and *Rhizopus arrhizus*. Phytochemistry 51, 737–741.

El Sayed, K.A., Hamann, M.T., Waddling, C.A., Jensen, C., Lee, S.K., Dunstan, C.A., Pezzuto, J.M., 1998. Structurally novel bioconversion products of the marine natural product sarcophine effectively inhibit JB6 cell transformation. J. Org. Chem. 63, 7449–7455.

Feiters, M.C., Rowan, A.E., Nolte, R.J.M., 2000. From simple to supramolecular cytochrome P450 mimics. Chem. Rev. Soc. 29, 375–384.

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.

Holland, H.L., 1982. The mechanism of the microbial hydroxylation of steroids. Chem. Soc. Rev. 11, 371–395.

Holland, H.L., 1984. Biotransformation of Δ^4-3-ketosteroids by the fungus *Rhizopus arrhizus*. Acc. Chem. Res. 17, 398–402.

Holland, H.L., Brown, F.M., Chenchaiah, P.C., Rao, J.A., 1990. Hydroxylation of prostanoids by fungi. Synthesis of (−)-15-deoxy-19-(*R*)-hydroxy-PGE_1 and (−)-15-deoxy-18-(*S*)-hydroxy-PGE_1. Can. J. Chem. 68, 282–293.

Holland, H.L., Brown, F.M., Rao, J.A., Chenchaiah, P.C., 1988. Synthetic approaches to the prostaglandins using microbial biotransformation. Dev. Ind. Microbiol. 29, 191–195.

Holland, H.L., Lakshmaiah, G., Ruddock, P.L., 1998. Microbial hydroxylation of acetylaminosteroids. Steroids 63, 484–495.

Huang-Minlon, 1949. Reduction of steroid ketones and other carbonyl compounds by modified Wolff Kishner method. J. Am. Chem. Soc. 71, 3301–3303.

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., Capiton, G.A., Clark, A.M., Baker, J.K., 1981. Metabolism of impramine by microorganisms. J. Pharm. Sci. 70, 151–155.

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

Lupi, A., Patamia, M., Grgurina, I., Bettolo, R.M., Leo, O.D., Gioia, P., Antonaroli, S., 1984. Biogenetic-type total synthesis of (±)-2-deoxystemodinone. Helv. Chim. Acta 67, 2261–2263.

Manchand, P.S., White, J.D., Wright, H., Clardy, J., 1973. Structures of stemodin and stemodinone. J. Am. Chem. Soc. 93, 2705–2706.

Martin, G.D.A., Reynolds, W.F., Reese, P.B., 2004. Investigation of the importance of the C-2 oxygen function in the transformation of stemodin analogues by *Rhizopus oryzae* ATCC 11145. Phytochemistry 65, 701–710.

Moore-Landecker, E., 1982. Fundamentals of the Fungi. Prentice-Hall, Englewood Cliffs, NJ, p. 84.

Orabi, K.Y., Li, E., Clark, A.M., Hufford, C.D., 1999. Microbial transformation of sampangine. J. Nat. Prod. 62, 988–992.

Ouazzani, J., Arséniyadis, S., Alvarez-Manzaneda, R., Rumbero, A., Ourisson, G., 1991. Microbial hydroxylations of further octalin derivatives. Tetrahedron Lett. 32, 1983–1986.

Peterson, D.H., Murray, H.C., Eppstein, S.H., Reineke, L.M., Weintraub, A., Meister, P.D., Leigh, H.M., 1952. Microbiological transformation of steroids. I. Introduction of oxygen at carbon-11 of progesterone. J. Am. Chem. Soc. 74, 5933–5936.