Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytochemistry 65 (2004) 701–710
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Investigation of the importance of the C-2 oxygen function 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, Ontario, Canada M5S 3H6*

Received 30 July 2003; received in revised form 6 January 2004; accepted 8 January 2004

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

Abstract

A new stemodinoside, stemodin-α-L-arabinofuranoside (**5**), was isolated from the plant *Stemodia maritima*. Incubation of stemodin (**2**) with *Rhizopus oryzae* ATCC 11145 gave 2α,7β,13(*S*)-trihydroxystemodane (**17**) and 2α,3β,13(*S*),16α-tetrahydroxystemodane (**18**) whilst stemodinone (**8**) afforded 6α,13(*S*)-dihydroxystemodan-2-one (**19**). The bioconversion of 2β,13(*S*)-dihydroxystemodane (**10**) by the fungus yielded 2β,7β,13(*S*)-trihydroxystemodane (**20**) whereas stemod-12-en-2-one (**9**) provided 7β,17-dihydroxystemod-12-en-2-one (**21**). The results provide useful information about the relationship between the functional groups of the substrates and their potential for bioconversion.

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

1. Introduction

Stemodia maritima (Scrophulariaceae), known in the Northern Caribbean as "poor man's strength," has been used as a home remedy to treat various types of body ailments such as stomach ache, dropsy and swelling (Ayensu, 1981). Previous phytochemical analyses of the plant (Manchand et al., 1973; Manchand and Blount, 1976a,b; Hufford et al., 1976, 1992) list the isolation of a number of diterpenoids and their glycosides. Herein we report the characterisation of a new stemodinoside.

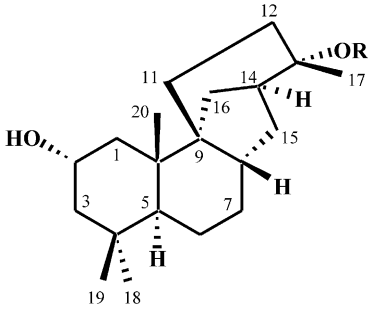
Previous work in this laboratory on the fungal transformation of the antiviral and cytotoxic diterpene stemodin (**2**) from *S. maritima* (Hufford et al., 1991) and its analogues has yielded a number of interesting compounds (Buchanan and Reese, 2001; Chen and Reese, 2002). In this study stemodin (**2**) was chemically converted to 2α-hydroxystemod-12-ene (**7**), stemodinone (**8**), stemod-12-en-2-one (**9**), 2β,13(*S*)-dihydroxystemodane (**10**), 2β-hydroxystemod-12-ene (**11**), 13(*S*)-hydroxystemodane (**12**), and stemod-12-ene (**13**). Incubation of stemodin (**2**) and its analogues with *R. oryzae* were studied to determine the effect of the C-2 functional group on the site of hydroxylation. The ability of the fungus to metabolise analogues of the triterpene betulinic acid (**14**) was also investigated.

2. Results and discussion

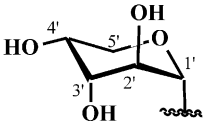
Phytochemical investigation of the acetone extract of the plant afforded the previously reported stemarin (**1**), stemodin (**2**), stemodinosides A (stemodin-α-L-arabinopyranoside) (**3**) and B (stemodin-β-D-glucopyranoside) (**4**) and betulinic acid (**14**) (Manchand et al., 1973; Manchand and Blount, 1976a,b; Hufford et al., 1992). Purification of stemodin (**2**) from betulinic acid (**14**) aided by the use of a basic adsorbent. A new stemodinoside, stemodin-α-L-arabinofuranoside (**5**), was also obtained.

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

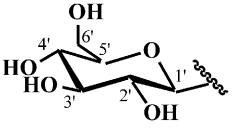
0031-9422/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2004.01.011



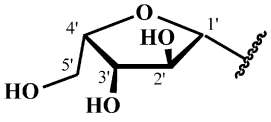
2 R = H



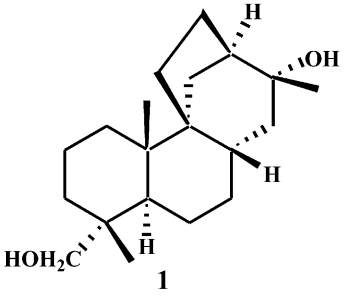
3 R =



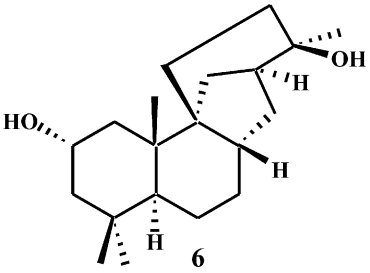
4 R =



5 R =



1

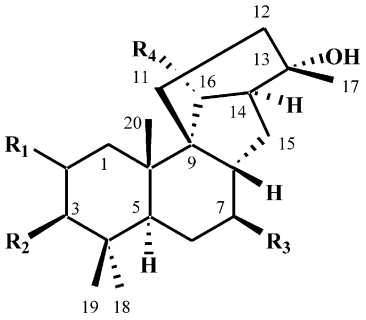


6

HRMS(EI) analysis of stemodin-α-L-arabinofuranoside (**5**) ($M^+ = 438.2981$) suggested a molecular formula of $C_{25}H_{42}O_6$. Hydroxyl and C–O–C absorptions were noted in the infrared spectrum at 3394 and 1124 cm^{-1} respectively. A comparison of the ^1H and ^{13}C NMR data of compound **5** with that of stemodin (**2**) confirmed the presence of the stemodane skeleton. The ^{13}C NMR data showed noticeable upfield shifts of the C-17 methyl signal to δ 24.2 as well as the expected shift to lower field of the C-13 quaternary carbon peak to δ 79.4. The stereochemistry of the C-17 methyl group was assigned as β due to T-ROE couplings of H-17 (δ 1.16) with H-15β (δ 1.73) and H-12β (δ 1.42). With stemodin (**2**) confirmed as the aglycone of stemodinoside **5**, the pentose sugar moiety was left to be resolved. The low value of the signal at 62.3 ppm in the ^{13}C NMR spectrum implied that the pentose was in the furanose form. This was supported from ^1H to ^1H COSY and HMBC data (Table 1). Determination of the stereochemistry of the protons within the sugar ring was difficult due to very small vicinal couplings (ca. 1–3 Hz) which were observed. Fortunately the pentose was confirmed as α-L-arabinofuranose based on extensive comparison with data from other sugars (Gorin and Mazurek, 1975). This compound which has not been reported previously is being designated as stemodinoside D.

Stemodin (**2**) was solvolysed to give the novel 2α,13(*R*)-dihydroxystemodane (**6**) and 2α-hydroxystemod-12-ene (**7**). The rationale for their formation under acidic conditions may be explained accordingly. Protonation of C-13 hydroxyl resulted in the loss of water in an S_N1 fashion to generate the tertiary carbocation. Elimination of the carbocation produced the olefin **7** while hydration from either face afforded the starting material (**2**) or its 13-epimer (**6**). Trans elimination across the C-12,13 bond of stemodin (**2**) was a possible pathway to the elimination product (**7**) (Scheme 1). HRMS(EI) data of compound **6** ($M^+ = 306.2533$) gave the molecular formula $C_{20}H_{34}O_2$. Hydroxyl absorption in the FTIR spectrum was seen at 3260 cm^{-1}. The major difference between stemodin (**2**) and its C-13 epimer (**6**) was observed in ^{13}C NMR data with a noticeable upfield shift of the C-17 resonance by ca. 2 ppm. Compound **7** was identical in all respects to that previously reported by Buchanan and Reese (2001).

Both compounds **2** and **7** were oxidised under Jones conditions to yield their corresponding ketones stemodinone (**8**) and stemod-12-en-2-one (**9**), respectively (Manchand et al., 1973). IR spectra showed the expected carbonyl stretches at 1690 and 1714 cm^{-1} for substrates **8** and **9**, respectively. The absence of the C-2 hydroxyl groups in the products was made evident in the ^1H NMR spectra by the disappearance of H-2 signals. ^{13}C NMR data also verified this with the emergence of carbonyl resonances at 212.5 ppm for both compounds.



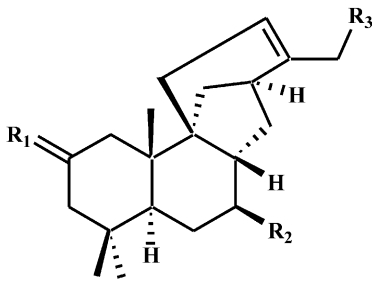
10 $R_1 = \beta OH, R_2 = R_3 = R_4 = H$

12 $R_1 = R_2 = R_3 = R_4 = H$

17 $R_1 = \alpha OH, R_2 = R_4 = H, R_3 = OH$

18 $R_1 = \alpha OH, R_2 = R_4 = OH, R_3 = H$

20 $R_1 = \beta OH, R_2 = R_4 = H, R_3 = OH$



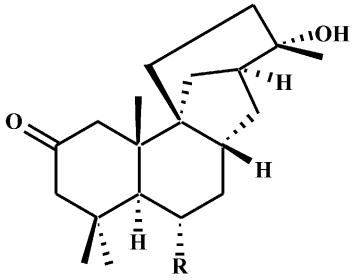
7 $R_1 = \alpha OH, \beta H, R_2 = R_3 = H$

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

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

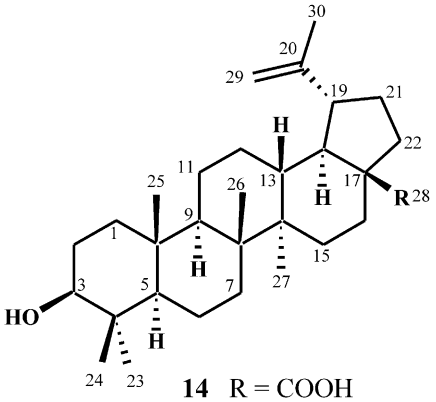
13 $R_1 = H_2, R_2 = R_3 = H$

21 $R_1 = O, R_2 = OH, R_3 = OH$



8 R = H

19 R = OH



14 R = COOH

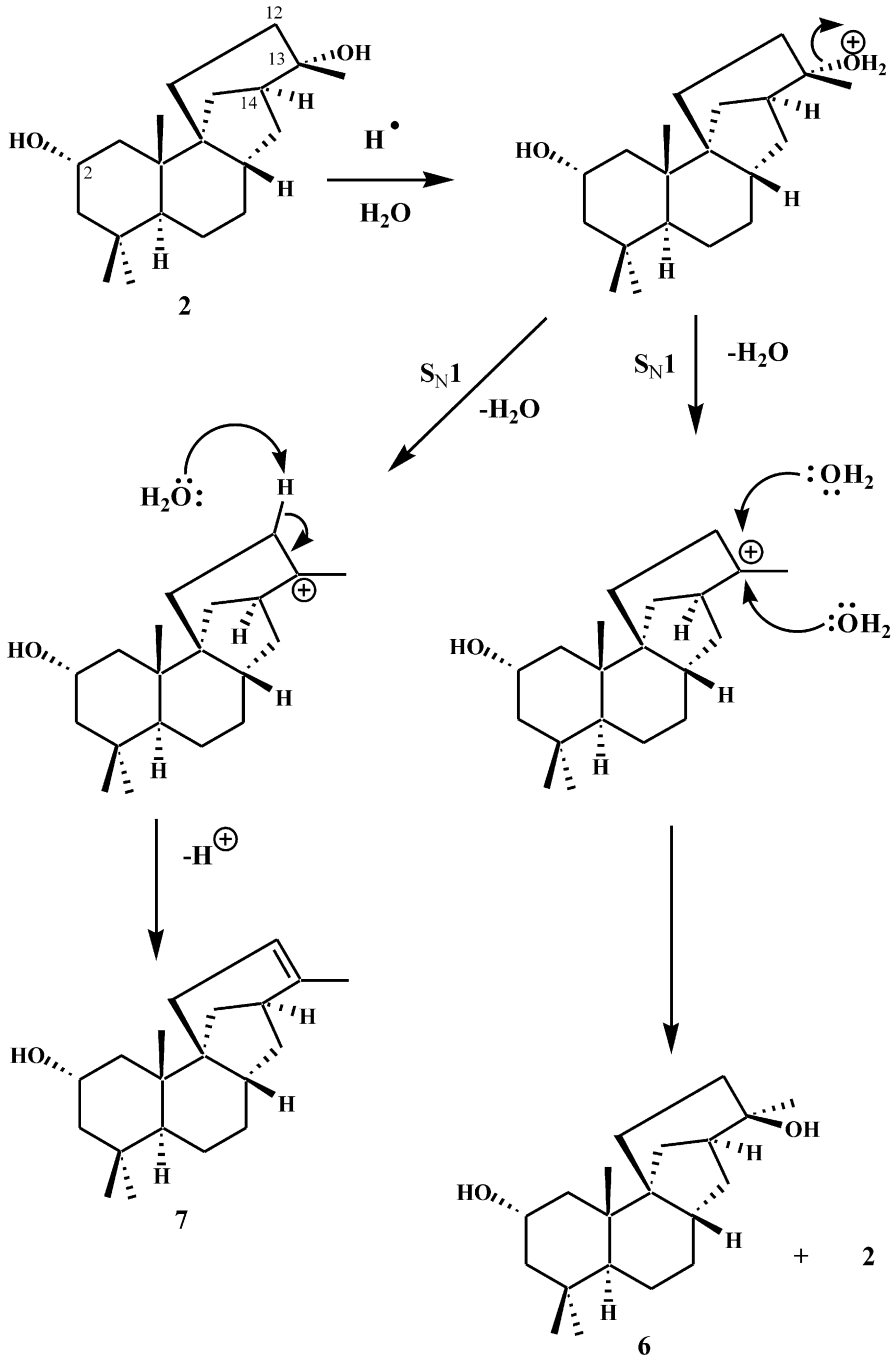
15 R = COOMe

16 $R = CH_2OH$

Stereospecific reduction of products **8** and **9** using sodium borohydride in methanol afforded the novel 2β,13(*S*)-dihydroxystemodane (**10**) and 2β-hydroxystemod-12-ene (**11**), respectively. HRMS(EI) data of **10** suggested a molecular formula of $C_{20}H_{34}O_2$ (*m*/*z* 306.2559). Absorption at 3352 cm^{-1} in the IR spectrum indicated the presence of a hydroxyl functional group. 2β,13(*S*)-Dihydroxystemodane (**10**), the C-2 epimer of stemodin (**2**), showed noticeable downfield shifts of 4.02 (H-2) and 67.8 (C-2) ppm in ^1H and ^{13}C NMR spectra, respectively when compared with **2**. Compound **11** was determined to have a molecular formula of $C_{20}H_{32}O$ from HRMS(EI) ($M^+ = 288.2454$). The IR spectrum suggested the presence of hydroxyl and olefin functionalities at 3390 and 1644 cm^{-1}, respectively. ^1H NMR data supported these findings with the appearance of an H-2 resonance at δ 4.03 and an olefin resonance at 5.01 ppm for H-12. In addition ^{13}C NMR analysis revealed the absence of the carbonyl resonance and the appearance of a new hydroxyl signal at 67.8 ppm.

Treatment of compounds **8** and **9** under the modified Wolff Kishner conditions (Huang-Minlon, 1949) gave the 2-deoxy compounds 13(*S*)-hydroxystemodane (**12**) (Manchand et al., 1973; Kelly et al., 1983; Lupi et al., 1984) and stemod-12-ene (**13**) (Manchand et al., 1973), respectively in high yields. The IR spectrum of **12** indicated the presence of a hydroxyl stretch at 3382 cm^{-1}. ^{13}C NMR data confirmed the absence of the C-2 carbonyl signal with the presence of a new methylene carbon peak at 18.8 ppm. IR data for compound **13** indicated an olefin stretch at 1678 cm^{-1}. ^1H NMR spectrum showed only an olefin resonance at 5.00 ppm for H-12. ^{13}C NMR data verified this with signals at 117.4 and 145.8 ppm for C-12 and C-13, respectively.

Methylation of betulinic acid (**14**) utilising diazomethane afforded methyl betulinate (**15**) (Monaco and Previtera, 1984). Subsequent reduction of **15** (LAH, THF, reflux) yielded betulin (**16**) (Monaco and Previtera, 1984, Tinto et al., 1992, Akihisa et al., 2001).



Scheme 1.

Incubation of stemodin (**2**) with *R. oryzae* gave two products. The least polar metabolite **17** showed absorption in the IR spectrum at 3304 and 3358 cm^{-1} corresponding to hydroxyl groups. ^{13}C NMR resonance for the C-7 methylene carbon had disappeared and a new methine carbon at δ 79.4 was observed. This agreed well with literature (Hufford et al., 1991; Badria and Hufford, 1991) and the metabolite was, therefore, determined to be 2α,7β,13(*S*)-trihydroxystemodane (**17**).

The more polar metabolite **18** had a molecular ion at *m/z* 361.2347 in the ESMS which was assigned to $[C_{20}H_{34}O_4+Na]^+$. Absorption at 3404 cm^{-1} in the FTIR spectrum was indicative of the presence of a hydroxyl group. T-ROESY data exhibited ROE of H-3α (δ 3.07) with H-1α (δ 1.72), H-5α (δ 2.36) and H-18 (δ 1.03). This suggested that the stereochemistry of the hydroxyl group at C-3 was β. The size of the coupling constant of H-3α ($J=10$ Hz) also verified the diaxial coupling between H-3α and H-2β. The stereochemistry of the hydroxyl group at C-16 was confirmed as α because of the ROE correlations of H-16β (δ 4.32) with H-1α (δ 1.98), H-1β (δ 1.72), H-11α (δ 1.47), H-11β

(δ 1.40) and H-14α (δ 1.82). ^{13}C NMR data supported these findings with the emergence of new methine signals at ca. 79 and 84 ppm for C-16 and C-3, respectively. The metabolite was affirmed as the hitherto unreported 2α,3β,13(*S*),16α-tetrahydroxystemodane (**18**).

The results from the abovementioned fermentation differed somewhat from those performed previously by Hufford et al. (1991) where the production of both the 7α- and 7β-hydroxy products as well as the 18-hydroxy- and 16,18-dihydroxy metabolites were reported. Hufford, however, employed a double phase fermentation method whereas a single phase protocol was used in all our experiments.

Incubation of stemodinone (**8**) with the fungus afforded the novel 6α,13(*S*)-dihydroxystemodan-2-one (**19**) as the sole metabolite. HRMS(EI) conferred a molecular formula of $C_{20}H_{32}O_3$ (*m*/*z* 320.2351) for 6α,13(*S*)-dihydroxystemodan-2-one (**19**). The FTIR spectrum showed the presence of hydroxyl and carbonyl functionalities at 3388 and 1696 cm^{-1}, respectively. ROE correlations of H-6β (δ 3.86) with H-19 (δ 1.20), H-20 (δ 0.99) and H-7β (δ 2.17) were noted in the T-ROESY data and this indicated that the stereochemistry of C-6 hydroxyl group was α. The size of the H-6 coupling (*dt*, $J = 11.0$, 4.4 Hz) was consistent with these findings. ^{13}C NMR spectra supported the presence of a new methine peak at 69.1 ppm for C-6 with accompanying shifts for C-5 and C-7.

Incubation of 2β,13(*S*)-dihydroxystemodane (**10**) for 10 days with *R. oryzae* gave seven metabolites in poor yield and their purification proved difficult. However, reduction of the incubation period to 5 days yielded metabolite **20** in sufficient quantity for characterisation. Compound **20** was the product of monohydroxylation with a molecular formula of $C_{20}H_{34}O_3$ as indicated from the HRMS (EI) data $[M-1]^+$ (*m*/*z* 321.2657). Absorption in the FTIR spectrum suggested the presence of a hydroxyl group (3380 cm^{-1}). In the ^{1}H NMR

Table 1
^{1}H, ^{13}C and 2D NMR data of stemodin-α-L-arabinofuranoside (**5**)

Carbon	δ_C	δ_H	HMBC	^{1}H–^{1}H COSY	T-ROESY
1	45.6	1.97β	65.5, 50.8, 46.7, 40.3	1.75, 1.13, 3.74, 0.99	1.66
		1.15α			
2	65.5	3.75		1.97, 1.75, 1.15, 1.08	
3	50.9	1.75β	66.5, 46.7, 45.6		
		1.08α	65.5, 34.8, 23.8	3.74, 1.76, 0.92	
4	34.8				
5	46.7	1.21α	22.1	1.08	
6	22.1	1.41α	40.3, 37.3		
		1.18β		1.92, 1.41	
7	36.5	1.92β	49.2, 46.7, 37.3	1.73, 1.41, 1.18, 1.13	
		1.13α			
8	37.3	1.73β	49.8, 43.9, 28.0	1.28	
9	49.9				
10	40.3				
11	27.9	1.66	79.7, 40.3, 37.3		1.97β
		1.38		1.73, 1.65, 1.42	
12	31.2	1.65α	79.7, 40.3, 37.3		
		1.42β			1.16
13	79.7				
14	43.9	2.15	79.7, 49.8, 37.3, 31.2, 24.2	1.73, 1.65, 1.28	
15	37.7	1.73	79.7, 49.8, 43.9, 37.3		1.16
		1.28	79.7, 36.4, 24.2	2.15, 1.93, 1.65	
16	30.3	1.73β		37.3	
		1.65α			
17	24.2	1.16	79.7, 43.9, 31.2		1.42, 1.73
18	34.7	0.96	50.8, 48.7, 34.8, 23.7		
19	23.8	0.92	50.8, 34.8, 34.7, 46.7	1.08	
20	19.6	0.99	49.8, 46.7, 45.6, 40.3	1.97	
1′	103.5	5.28	87.2, 79.5, 78.6	3.99	
2′	79.5	3.99	103.5, 87.2, 79.5, 78.6, 62.3	5.28, 4.23, 4.01	4.22
3′	78.6	4.01	103.5, 87.2, 79.5, 78.6, 62.3	4.23, 3.45, 3.99	3.45
4′	87.2	4.23	78.6, 62.3	4.01, 3.87, 3.83	
5′	62.3	3.87	87.2, 78.6	4.23	
OH_2'		4.22		3.99	
OH_3'		3.45	78.6	4.01	
OH_5'		2.88			
OH_2		1.81			

Determined in $CDCl_3$.

data a new signal was observed at δ 3.34 (H-7α). T-ROESY data confirmed the stereochemistry of the C-7 hydroxyl group as being β due to ROE correlations between H-7α and H-15α (δ 1.85) and H-16α (δ 1.53). The presence of a new methine signal at 79 ppm and absence of the methylene resonance for C-7 were noted in the ^{13}C NMR spectrum. 2β,7β,13(*S*)-Trihydroxystemodane (**20**) has not been reported previously.

The bioconversion of stemod-12-en-2-one (**9**) afforded a single metabolite. HRMS (EI) data (*m*/*z* 318.2195) of compound **21** suggested the presence of a dihydroxylated product ($C_{20}H_{30}O_3$). Absorptions in the FTIR spectrum at 3441, 1652 and 1636 cm^{-1} corresponded to hydroxyl, carbonyl and olefin stretches, respectively. Allylic hydroxylation at C-17 was noted in the ^{1}H NMR data with the absence of the expected H-17 methyl singlet (1.64 ppm) and the presence of hydroxymethylene protons at 4.03 ppm. T-ROESY data showed ROE cross-peaks between H-7β (δ 3.45), H-15α (δ 1.98) and H-5α (δ 1.93) which implied that the C-7 hydroxyl moiety was of β stereochemistry. The ^{13}C NMR spectrum also verified hydroxylations at carbons 7 and 17 with resonances at 77.2 and 64.7 ppm, respectively. The metabolite was therefore established as the new 7β,17-dihydroxystemod-12-en-2-one (**21**).

2α-Hydroxystemod-12-ene (**7**), 2β-hydroxystemod-12-ene (**11**), 13(*S*)-hydroxystemodane (**12**), stemod-12-ene (**13**), methyl betulinate (**15**) and betulin (**16**) were not transformed by the fungus.

Table 2
Results from the incubation of the stemodanes with *R. oryzae*

	13(*S*)-OH	12-ene
2α-OH	**17** 7β-OH (8%) **18** 3β,16α-diOH (4%)	–
2-one	**19** 6α-OH (1%)	**21** 7β,17-diOH (4%)
2β-OH	**20** 7β-OH (10%)	–
2-H	–	–

2.1. Substrate properties vs. potential for biotransformation

The results from the incubation of the aforementioned compounds have provided some useful information about the types of substrates that would be accepted for bioconversion. A change in the stereochemistry of the hydroxyl functional group at C-2 from α to β resulted in multiple hydroxylations. Conversion of the functional group at C-2 to a ketone produced a shift in the site of hydroxylation. Complete removal of the functionality at C-2 yielded a product which did not undergo transformation. Similar alterations of the C-2 functional group of the olefins **7**, **9**, **11** and **13** were effected, however, transformation was only observed for substrate **9** (Table 2).

The results for the substrates would suggest that two docking groups (whether carbonyl or hydroxyl) are preferable for bioconversion to occur. Variation of the C-2 functional group seems to affect the way in which the substrate is orientated in the enzyme active site and hence the site of hydroxylation. No transformation was observed for the triterpene derivatives methyl betulinate (**15**) and betulin (**16**) although they had the required

Table 3
^{13}C NMR resonances of the stemodanes

C	2[a]	6[a]	7[b]	8[a]	9[a]	10[a]	11[a]	12[a]	13[a]	17[b]	18[b]	19[a]	20[b]	21[a]
1	45.7	45.5	44.9	51.5	50.7	42.8	42.4	36.3	36.1	46.5	43.0	51.3	42.2	50.7
2	65.1	65.3	65.2	212.5	212.5	67.8	67.8	18.8	18.8	69.5	69.6	212.3	67.8	211.6
3	50.6	50.8	50.9	55.9	56.1	46.2	46.6	41.9	42.1	51.3	84.3	56.6	46.1	55.9
4	34.6	34.9	34.8	39.2	39.1	32.6	33.0	33.2	33.3	35.3	40.6	39.0	32.6	38.8
5	46.6	47.0	47.1	47.2	47.7	45.8	45.4	47.2	47.8	45.4	49.1	52.2	43.4	45.7
6	22.0	22.0	22.0	22.5	22.4	22.3	22.3	22.3	22.2	32.2	22.7	69.1	31.5	31.7
7	36.4	36.4	36.8	36.1	36.8	36.2	37.1	36.7	36.9	80.1	38.2	45.8	79.0	77.2
8	36.9	37.2	39.2	37.3	38.9	36.6	38.8	37.2	39.2	47.5	37.5	36.5	46.3	52.3
9	50.1	50.5	50.0	50.1	49.9	50.7	50.7	50.0	50.0	52.7	54.5	49.7	52.5	52.1
10	40.1	40.0	40.5	44.8	45.0	38.4	38.9	38.4	38.8	41.0	41.0	45.3	40.1	44.9
11	27.8	29.1	33.1	27.9	33.4	27.8	32.8	27.6	33.0	29.1	29.5	27.8	27.9	36.9
12	32.7	33.7	117.5	32.7	117.4	32.6	117.7	32.8	117.9	33.3	32.8	32.7	32.5	118.1
13	72.3	72.3	145.7	72.1	145.8	72.6	146.0	72.5	145.8	72.9	75.5	72.1	72.4	149.5
14	46.1	46.7	42.6	45.9	42.9	45.2	42.4	46.2	43.0	47.4	57.0	46.1	46.1	35.2
15	38.1	35.7	43.9	38.1	44.0	38.2	44.0	38.1	44.0	37.4	37.8	38.2	36.3	42.8
16	30.1	32.8	35.9	30.4	35.4	29.9	35.6	29.9	35.4	32.5	79.7	30.5	31.5	35.2
17	28.0	26.2	17.7	28.2	17.1	28.1	20.7	28.1	21.7	28.2	28.3	28.2	28.1	64.7
18	34.7	34.8	34.5	34.4	34.3	34.3	34.0	34.5	34.5	35.0	30.8	37.3	34.4	34.3
19	23.6	23.7	23.4	23.9	23.7	25.3	25.4	22.9	22.8	24.2	21.3	25.0	25.1	24.1
20	19.6	19.8	21.7	18.8	21.6	22.2	21.7	18.9	16.9	20.5	18.9	19.6	22.2	17.5

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

Table 4
^{13}C NMR resonances of the lupane triterpenoids

C	14[a]	15[b]	16[b]
1	38.5	38.6	38.7
2	26.5	27.3	27.0
3	80.6	78.9	80.0
4	38.7	38.8	38.8
5	55.2	55.3	55.2
6	18.2	18.2	18.3
7	34.2	34.2	34.2
8	40.7	40.6	40.9
9	50.4	50.5	50.4
10	37.1	37.1	37.1
11	20.8	20.8	20.8
12	25.4	25.4	25.2
13	38.4	38.2	37.3
14	42.4	42.3	42.7
15	30.4	30.5	29.7
16	32.0	32.1	33.9
17	56.6	56.5	56.5
18	46.9	46.9	47.8
19	49.2	49.4	48.7
20	150.1	150.5	150.5
21	29.6	29.6	29.1
22	37.1	36.9	27.3
23	27.8	27.9	28.0
24	15.3	15.3	15.4
25	15.9	15.9	15.9
26	16.0	16.0	16.1
27	14.6	14.6	14.7
28	183.5	176.8	60.5
29	109.9	109.5	109.7
30	19.3	19.3	19.1

[a] Determined in $CDCl_3$ + TFA (trace).
[b] Determined in $CDCl_3$.

docking groups at C-3 and C-28. It is possible that these substrates were too large to be accepted by the enzyme.

In summary, a new stemodinoside **5** was isolated from *S. maritima* and seven novel analogues of stemodin (**2**) were prepared by chemical and microbial means. Although the compounds **9**, **12**, **13** and **15** were not new their full spectral analyses are reported for the first time. Preliminary results from the incubation of the stemodanes (**2**, **7**–**13**) and lupanes (**15**–**16**) with *R. oryzae* have yielded some insights of the substrate requirements of the mono-oxygenase enzyme system of the fungus (Table 2). Future work will explore this area further.

3. Experimental

3.1. General

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 FTIR Paragon 1000 spectrophotometer. ^{1}H and ^{13}C NMR data were obtained on Brüker 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 and lupane terpenoids are listed in Tables 3 and 4. HRMS(EI) was carried out on a Kratos MS50 instrument at an ionising voltage of 70 eV. Electrospray mass spectral data were carried out on Aligent Technologies 1100MSD or Micromass Zabspec-oa TOF spectrometers. Column chromatography was performed on silica gel (37–63 μm dia.) or alumina (basic, activity I). Detection of compounds on thin layer chromatography (TLC) was achieved by spraying the plates with phosphomolybdic acid/cerium(IV) sulfate or ammonium molybdate/sulfuric acid solutions followed by heating until the colour developed. Stemarin (**1**), stemodin (**2**), stemodin-α-L-arabinopyranoside (**3**), stemodin-β-D-glucopyranoside (**4**) and betulinic acid (**14**), were obtained from the acetone extract of *Stemodia maritima* in overall yields of 1.2, 12, 13, 10 and 15%, respectively. *Rhizopus 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 among twenty 500-ml Erlenmeyer flasks and was sterilised. Spore suspensions of the fungus were used to inoculate the flasks. After 24, 36, 48 and 60 h, 10, 20, 30 and 40%, respectively of the substrate in ethanol 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 no substrate was fed to the growing fungus. The medium was shaken for 5 days after the final feed. 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. The extracts were analysed by TLC.

3.3. Stemodin-α-L-arabinofuranoside (5)

Initial purification of the acetone extract (53 g) of *S. maritima* (220 g) afforded stemarin (**1**) (0.7 g), stemodin (**2**) (6.4 g) and the stemodinoside mixture (Hufford et al., 1992). The stemodinoside extract (17.3 g) was

further purified on silica gel. Elution in ethyl acetate produced stemodin-α-L-arabinofuranoside (**5**) (1.51 g) which crystallised from acetone as needles, mp 215–217 °C; $[\alpha]_D^{27}$ −40.8° (MeOH, *c* 0.91); FTIR: ν_{max} cm^{-1} 3382 (OH), 1383, 1124; HRMS (EI) *m/z* (rel. int.): 438.2981 $[M]^+$ [$C_{25}H_{42}O_6$ requires 438.2981], 420.2876 $[M–H_2O]^+$ (0.15), 271.3003 $[M–H_2O–C_5H_9O_5]^+$ (100); ^1H and ^{13}C NMR data of **5** are listed in Table 1.

Elution in 2–10% methanol in ethyl acetate yielded the previously isolated stemodinosides A (**3**) (6.9 g) and B (**4**) (5.3 g) (Hufford et al., 1992).

3.4. 2α,13(R)-dihydroxystemodane (6) and 2α-hydroxystemod-12-ene (7)

To stemodin (**2**) (8.01 g, 26.1 mmol) in acetone (400 ml) at 0 °C (ice salt) was slowly added 3 M sulfuric acid (400 ml). The reaction mixture was allowed to warm to room temperature and was stirred for 68 h. The mixture was neutralised with 20% aqueous potassium hydroxide solution (ca. 980 ml), acetone was removed in vacuo, and the aqueous solution was extracted with ethyl acetate (2 × 1000 ml). The organic solution was dried, filtered and the solvent was evaporated to yield an off-white solid (6.4 g) which was chromatographed on silica gel. Elution with dichloromethane afforded 2α-hydroxystemod-12-ene (**7**) (2.35 g) which crystallised from ethyl acetate as prisms, mp 132–133 °C; $[\alpha]_D^{27}$ +18.0° (MeOH, *c* 0.75); lit. mp 128–130 °C; $[\alpha]_D$ +19.9° ($CHCl_3$, *c* 1.08) (Buchanan and Reese, 2001).

Elution with 20% acetone in dichloromethane gave 2α,13(*R*)-dihydroxystemodane (**6**) (2.28 g) which crystallised from ethyl acetate as plates, mp 235–237 °C; $[\alpha]_D^{27}$ +16.5° (MeOH, *c* 0.38); FTIR ν_{max} cm^{-1} 3260 (OH); HRMS (EI) *m/z* (rel. int.): 306.2533 $[M]^+$ (0.4) [$C_{20}H_{34}O_2$ requires 306.2559], 288.2449 $[M–H_2O]^+$ (7.9), 273.2218 $[M–H_2O–CH_3]^+$ (13.9), 217.1956 (100), 94.0781 (64.3); ^1H NMR (200 MHz, $CDCl_3$): δ 0.92 (3H, s, H-19), 0.95 (3H, s, H-18), 0.99 (3H, s, H-20), 1.23 (3H, s, H-17), 3.80 (1H, m, $^w/_2$ = 37.0 Hz, H-2).

Elution with acetone produced stemodin (**2**) (0.58 g).

3.5. Stemodinone (8)

Stemodinone (**8**) was prepared by the previously reported method (Buchanan and Reese, 2001). The product (**8**) (1.69 g) crystallised from ethyl acetate as needles, mp 209–210 °C; $[\alpha]_D^{27}$ +10.2° (Me_2CO, *c* 1.25); lit. mp 215–216 °C; $[\alpha]_D$ +14.3 ($CHCl_3$, *c* 1.00) (Manchand et al., 1973).

3.6. 2β,13(S)-dihydroxystemodane (10)

To stemodinone (**8**) (0.21 g, 0.704 mmol) in methanol (10 ml) at −5 °C (ice salt) was slowly added sodium borohydride (133 mg, 3.51 mmol) with stirring. The reaction was allowed to warm to room temperature for 15 min, poured into water (100 ml), and then was neutralised with 6 M sulfuric acid. 2β,13(*S*)-Dihydroxystemodane (**10**) (0.20 g) was filtered and recrystallised from acetone to give needles: mp 157–159 °C; $[\alpha]_D^{27}$ +11.9° (MeOH, *c* 0.78); FTIR ν_{max} cm^{-1} 3352 (OH); HRMS (EI) *m/z* (rel. int.): 306.2559 $[M]^+$ (3.8) [$C_{20}H_{34}O_2$ requires 306.2559], 288.2448 $[M–H_2O]^+$ (26), 273.2215 $[M–H_2O–CH_3]^+$ (22.4), 217.1952 (78.5), 94.0778 (100); ^1H NMR (200 MHz, $CDCl_3$): δ 0.87 (3H, s, H-18), 1.02 (3H, s, H-19), 1.07 (3H, s, H-20), 1.12 (3H, s, H-17), 4.02 (1H, m, $^w/_2$ = 15.5 Hz, H-2).

3.7. 13(S)-hydroxystemodane (12)

Stemodinone (**8**) (2.0 g, 6.58 mmol) was reduced under modified Wolff Kishner conditions (Huang-Minlon, 1949; Manchand et al., 1973) to yield 13(*S*)-hydroxystemodane (**12**) (1.9 g) which crystallised from methanol as amorphous crystals, mp 126–130 °C; $[\alpha]_D^{27}$ +2.8° (MeOH, *c* 0.56); lit. mp 143–144 °C; $[\alpha]_D$ +6.2° ($CHCl_3$, *c* 1.02) (Manchand et al., 1973); FTIR: ν_{max} cm^{-1} 3382 (OH); ^1H NMR (200 MHz,$CDCl_3$): δ 0.87 (3H, s, H-19), 0.88 (3H, s, H-18), 0.95 (3H, s, H-20), 1.11 (3H, s, H-17).

3.8. Stemod-12-en-2-one (9)

2α-Hydroxystemod-12-ene (**7**) (2.5 g, 8.68 mmol) was used to synthesise stemod-12-en-2-one (**9**) by the same method as described above for stemodinone (**8**). Stemod-12-en-2-one (**9**) (2.48 g) crystallised from acetone as amorphous crystals, mp 73–75 °C; $[\alpha]_D^{27}$ +34.2° (Me_2CO, *c* 1.90); lit. mp 93–95 °C, (Manchand et al., 1973); FTIR: ν_{max} cm^{-1} 1714 (>C=O); ^1H NMR (200 MHz, $CDCl_3$): δ 0.93 (3H, s, H-19), 0.97 (3H, s, H-20), 1.10 (3H, s, H-18), 1.65 (3H, s, H-17), 5.0 (1H, bs, H-12).

3.9. 2β-Hydroxystemod-12-ene (11)

To stemod-12-en-2-one (**9**) (1.09 g, 3.81 mmol) in methanol (30 ml) at −15 °C (ice salt) was slowly added sodium borohydride (0.55 g, 14.55 mmol). The reaction was stirred for 15 min and was poured into water (80 ml). The solution was neutralised with 3 M sulfuric acid and was extracted with diethyl ether (2 × 50 ml). The solvent was dried, filtered and concentrated in vacuo to afford 2β-hydroxystemod-12-ene (**11**) (0.78 g) which crystallised from methanol as amorphous crystals, mp 82–85 °C; $[\alpha]_D^{27}$ +20.9° (MeOH, *c* 1.63); FTIR: ν_{max} cm^{-1} 3390 (OH), 1644 (C=C); HRMS (EI): *m/z* (rel. int.): 288.2454 $[M]^+$ (100) [$C_{20}H_{32}O$ requires 288.2453], 273.2216 $[M–CH_3]^+$ (22.9), 270.2351 $[M–H_2O]^+$ (16.9), 255.2119 $[M–H_2O–CH_3]^+$ (18.6), 189.1643 (16.6); ^1H NMR (200 MHz, $CDCl_3$): δ 0.93 (3H, s, H-19), 1.05 (3H, s, H-20), 1.17 (3H, s, H-18), 1.65 (3H, s, H-17), 4.03 (1H, m, $^w/_2$ = 14.2 Hz, H-2), 5.01 (1H, br s, H-12).

3.10. Stemod-12-ene (13)

Stemod-12-ene (**13**) was prepared from stemod-12-en-2-one (**9**) (2.0 g, 6.99 mmol) in accordance with the modified Wolff Kishner conditions (Huang-Minlon, 1949; Manchand et al., 1973). Stemod-12-ene (**13**) (0.9 g) crystallised from chloroform as an amorphous solid, mp 43–45 °C; $[\alpha]_D^{27}$ +33.6° (*c* 5.77, $CHCl_3$); lit. mp 52–53 °C; $[\alpha]_D$ +36.6° ($CHCl_3$, *c* 1.00) (Manchand et al., 1973); FTIR: ν_{max} cm^{-1} 1678 (>C=O), 1447, 1378; ^1H NMR (200 MHz, $CDCl_3$): δ 0.87 (3H, s, H-19), 0.89 (3H, s, H-20), 0.95 (3H, s, H-18), 1.64 (3H, s, H-17), 5.00 (1H, bs, H-12).

3.11. Methyl betulinate (15)

To a solution of potassium hydroxide (1.0 g, 17.82 mmol) dissolved in ethanol (16.5 ml) and water (5 ml) at 65 °C was slowly added a solution of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (diazald) (6.9 g, 32.20 mmol) dissolved in diethyl ether (52 ml). The ethereal diazomethane distillate was added to a solution of betulinic acid (**14**) (1.0 g, 2.19 mmol) in dichloromethane (170 ml) at 0 °C (ice bath). The organic solution was removed in vacuo to afford methyl betulinate (**15**) (1 g) which crystallised from dichloromethane as needles, mp 220–221 °C; $[\alpha]_D^{27}$ +10.3° (CH_2Cl_2, *c* 1.55); lit. mp 221–223 °C; $[\alpha]_D$ +5° (Monaco and Previtera, 1984); FTIR: ν_{max} cm^{-1} 3568 (OH), 3370 (OH), 1726 (>C=O), 1635 (C=C), 1452, 1378, 1154, 1046; ^1H NMR (200 MHz, $CDCl_3$): δ 0.75 (3H, s, H-24), 0.82 (3H, s, H-25), 0.91 (3H, s, H-23), 0.96 (6H, s, H-26, H-27), 1.68 (3H, s, H-30), 2.99 (1H, m, $^w/_2$ = 14.9 Hz, H-19), 3.19 (1H, dd, *J* = 9.5, 5.5 Hz, H-3), 3.67 (3H, s, H-31), 4.60 (1H, m, $^w/_2$ = 4.7 Hz, H_a-29), 4.74 (1H, d, *J* = 1.6 Hz, H_b-29).

3.12. Betulin (16)

To a solution of the methyl betulinate (**15**) (0.70 g, 1.489 mmol) in tetrahydrofuran (35.0 ml) was added lithium aluminium hydride (0.23 g, 6.06 mmol). The reaction mixture was refluxed for 3 h and ethyl acetate (35 ml) was added. Water (100 ml) was added and the mixture was extracted with ethyl acetate (3 × 35 ml). The organic solution was dried, filtered and removed in vacuo to afford betulin (**16**) (0.65 g) which crystallised from acetone as needles, mp 245–246 °C; $[\alpha]_D^{27}$ +17.9° (Me_2CO, *c* 0.64); lit. mp 236–238 °C; $[\alpha]_D$ +24° (*c* 0.075, py) (Tinto et al., 1992).

3.13. Incubation of stemodin (2)

Stemodin (**2**) (1.0 g) was incubated with *R. oryzae*. The fermentation was harvested to provide mycelial (0.36 g) and broth (0.38 g) extracts. The broth extract was purified on silica gel. Elution with 50–70% ethyl acetate in petrol afforded 2α,7β,13(*S*)-trihydroxystemodane (**17**) (76 mg) which crystallised from methanol as cubes, mp 240–241 °C; $[\alpha]_D^{27}$ +22.6° (MeOH, *c* 0.44); lit. mp 241–245 °C, $[\alpha]_D$ −2.9° (*c* 1.35, py) (Badria and Hufford, 1991).

Elution with ethyl acetate yielded 2α,3β,13(*S*),16α-tetrahydroxystemodane (**18**) (38 mg) which crystallised from methanol as prisms, mp 239–240 °C; $[\alpha]_D^{27}$ −17.9° (MeOH, *c* 0.47); FTIR: ν_{max} cm^{-1} 3404 (OH); ESMS (EI) *m/z* (rel. int.): 361.2347 $[M+Na]^+$ (100) [($C_{20}H_{34}O_2$ + Na) requires 361.2354]; ^1H NMR (200 MHz, CD_3OD): δ 0.87 (3H, s, H-19), 0.97 (3H, s, H-20), 1.03 (3H, s, H-18), 1.14 (3H, s, H-17), 3.07 (1H, br d, *J* = 10.0 Hz, H-3), 3.58 (1H, dt, *J* = 10.3, 4.3 Hz, H-2), 4.32 (1H, s, H-16).

3.14. Incubation of stemodinone (8)

Stemodinone (**8**) (1 g) was fed to the growing fungus. The mycelial (0.57 g) and broth (0.54 g) extracts were combined and chromatographed. Elution with 40% ethyl acetate in petrol provided 6α,13(*S*)-dihydroxystemodan-2-one (**19**) (14.3 mg) which crystallised from acetone as needles, mp 191–193 °C; $[\alpha]_D^{27}$ +55.6° ($CHCl_3$, *c* 4.5); FTIR ν_{max} cm^{-1} 3388 (OH), 1696 (C=C); HRMS (EI): *m/z* (rel. int.): 320.2351 $[M]^+$ (41) [$C_{20}H_{32}O_3$ requires 320.2351], 302.2246 $[M-H_2O]^+$ (30), 233.1905 (100); ^1H NMR (200 MHz, $CDCl_3$): δ 0.99 (3H, s, H-20), 1.15 (3H, s, H-17), 1.20 (3H, s, H-19), 1.31 (3H, s, H-18), 3.86 (1H, dt, *J* = 11.0, 4.4 Hz, H-6).

3.15. Incubation of 2β,13(S)-dihydroxystemodane (10)

Incubation of 2β,13(*S*)-dihydroxystemodane (**10**) (1 g) with the fungus gave mycelial (0.89 g) and broth (0.34 g) extracts after work up. Thin layer chromatography (TLC) showed that five metabolites were formed. The combined extract (1.23 g) was purified on silica gel. Elution in 10% acetone in dichloromethane gave 2β,7β,13(*S*)-trihydroxystemodane (**20**) (53 mg) as a gum, $[\alpha]_D^{27}$ +24.6° (MeOH, *c* 3.6); FTIR ν_{max} cm^{-1} 3380 (OH); HRMS (EI) *m/z* (rel. int.): 321.2657 $[M-1]^+$ (8.6) [$C_{20}H_{34}O_3$ requires 322.2508], 289.2530 $[M-CH_3-H_2O]^+$ (22.2), 250.2190 (19.8), 233.2173 (100); ^1H NMR (200 MHz, $CDCl_3$): δ 0.93 (3H, s, H-18), 1.11 (3H, s, H-19), 1.14 (3H, s, H-17), 1.19 (3H, s, H-20), 3.34 (1H, ddd, *J* = 14.6, 9.3, 5.3 Hz, H-7), 4.08 (1H, q, *J* = 3.8 Hz, H-2).

Purification of the other metabolites proved impractical.

3.16. Incubation of stemod-12-en-2-one (9)

Stemod-12-en-2-one (**9**) (500 mg) was fed to *R. oryzae*. Workup yielded mycelial (0.73 g) and broth (0.44 g) extracts which were pooled and purified on silica gel.

Elution in 5% ethyl acetate in petrol afforded 7β,17-dihydroxystemod-12-en-2-one (**21**) (13 mg) which crystallised from acetone as needles, mp 220–225 °C; $[\alpha]_D^{27}$ −10.9° (Me_2CO, *c* 2.8); FTIR: ν_{max} cm^{-1} 3441 (OH), 1652 (>C=O), 1636 (C=C); HRMS (EI): *m*/*z* (rel. int.): 318.2195 $[M]^+$ (96.8) [$C_{20}H_{30}O_3$ requires 318.2195], 282.1984 $[M\text{-}2H_2O]^+$ (86.6), 267.1750 $[M–CH_3\text{-}2H_2O]^+$ (20.5), 201.1278 (32.2); ^1H NMR (500 MHz, $CDCl_3$): δ 0.97 (3H, s, H-19), 1.02 (3H, s, H-20), 1.14 (3H, s, H-18), 3.45 (1H, m, $^w/_2 = 17.9$ Hz, H-7), 4.05 (2H, d, $J = 1.5$ Hz, H_a-17, H_b-17), 5.32 (1H, bs, H-12).

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. Optical rotations were carried out at the Jamaica Bureau of Standards. GDAM thanks the Chemistry Department for the granting of a Teaching Assistantship.

References

Akihisa, T., Takamine, Y., Yoshizumi, K., Tokuda, H., Kimura, Y., Ukiya, M., Nakahara, K., Yokochi, T., Ichiishi, E., Nishin, H., 2002. Microbial transformation of two lupane-type triterpenes and anti-tumor-promoting effects of the transformation products. J. Nat. Prod. 65, 278–282.

Ayensu, E.S., 1981. Medicinal Plants of the West Indies. Reference Publications, Algonác, MI, USA.

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

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

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

Gorin, P.A.J., Mazurek, M., 1975. Further studies on the assignment of signals in ^{13}C magnetic resonance spectra of aldoses and derived methyl glycosides. Can. J. Chem. 53, 1212–1223.

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, characterisation, and antiviral activity of microbial metabolites of stemodin. J. Nat. Prod. 54, 1543–1552.

Hufford, C.D., Guerrero, R.O., Doorenbos, N.J., 1976. Two new diterpenes from *Stemodia maritima* L. J. Pharm. Sci. 65, 778–780.

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

Kelly, R.B., Harley, M.L., Alward, S.J., Rej, R.N., Gowda, G., Mukhopadhyay, A., Manchand, P.S., 1983. Total synthesis of stemodane type-diterpenoids, (±)-2-desoxystemodinone, (+)-2-desoxystemodinone and (±)-stemodinol. Can. J. Chem. 61, 269–275.

Lupi, A., Patamia, M., Grgurina, I., Bettolo, R.M., Leo, O.D., Gioia, P., Antonaroli, S., 1984. Biogenetic-type total synthesis of (±)-2-desoxystemodinone. 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.

Manchand, P.S., Blount, J.F., 1976a. X-ray structure and absolute stereochemistry of stemarin, a diterpene with a new skeleton. J. Chem. Soc. Chem. Commun. 894–895.

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

Monaco, P., Previtera, L., 1984. Isoprenoids from the leaves of *Quercus suber*. J. Nat. Prod. 47, 673–676.

Tinto, W.F., Blair, L.C., Alli, A., Reynolds, W.F., McLean, S., 1992. Lupane triterpenoids of *Salacia cordata*. J. Nat. Prod. 55, 395–398.