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Stemodane skeletal rearrangement: chemistry and microbial transformation

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

Abstract

Solvolytic rearrangement of the C/D ring system of the tetracyclic diterpenoid stemodinone (**2**) afforded the compounds 15(13→12)*abeo*-13β-hydroxystemaran-2-one (**5**) and 15(8→9)*abeo*-8β(H)-12β-hydroxystachan-2-one (**10**). Terpene **5** possesses a novel diterpene skeleton. Oxidation of these compounds yielded their respective diketones. Bioconversion of **5** by *Rhizopus oryzae* yielded 15(13→12)*abeo*-7β,13β-dihydroxystemaran-2-one (**18**) while microbial transformation of **10** provided 15(8→9)*abeo*-8β(H)-6α,12β-dihydroxystachan-2-one (**19**), 15(8→9)*abeo*-8β(H)-7β,12β-dihydroxystachan-2-one (**20**) and 15(8→9)*abeo*-8β(H)-6α,12β,14β-trihydroxystachan-2-one (**21**). A rationale for the formation of the rearranged compounds is proposed.
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Keywords: *Rhizopus oryzae* ATCC 11145; *Stemodia maritima*; Stemodane; Stachane; Stemarane; Diterpene; Biotransformation; Hydroxylation

1. Introduction

Microbial transformation of terpenes has gained increasing popularity as new metabolites are produced with enhanced biological activity (Abraham et al., 2000; Aranda et al., 1991; Maurs et al., 1999). In addition the insertion of a hydroxyl group in one step with high regio- and stereo-selectivity at an unactivated carbon remains unmatched by chemical means (Holland, 1982). These terpenes and their derivatives are used as hemisynthesis intermediates, chiral auxiliaries, and chiral synthons for asymmetric synthesis (Azerad, 2000).

The fungal conversion of the cytotoxic and antiviral diterpene stemodin (**1**) and its derivatives in our group has led to the production of a number of metabolites (Buchanan and Reese, 2001; Chen and Reese, 2002). In this present work stemodinone (**2**), an analogue of stemodin (**1**), was solvolysed to yield 2 new rearranged diterpenes: 15(13→12)*abeo*-13β-hydroxystemaran-2-one (**5**) and 15(8→9)*abeo*-8β(H)-12β-hydroxystachan-2-one (**10**). The skeleton of **5** is novel; however, it resembles those of the stemarane **6** (Manchand and Blount, 1976) and the biologically active scopadulane compounds, scopadulcic acids A (**7**) and B (**8**) (Hayashi et al., 1987) as well as scopadulciol (**9**) (Hayashi et al., 1991a; Ahmed and Jakupovic, 1990). Diterpenoid **8** possesses anti-ulcer (Hayashi et al., 1990b), anti-viral (Hayashi et al., 1988, 1990a) and anti-tumour (Hayashi et al., 1991b, 1992) activities. It also exhibits inhibitory effects on bone resorption and osteoclast formation in vitro (Miyahara et al., 1999). The skeleton of **10** is known; examples include the 15(8→9)*abeo*-8β(H)-stachane class of compounds **11** (Lupi et al., 1984) and **12** (Hanson et al., 1992a). Compound **12** was formed from the solvolysis of an analogue of the potent cytotoxic, antiviral fungal metabolite aphidicolin (**13**).

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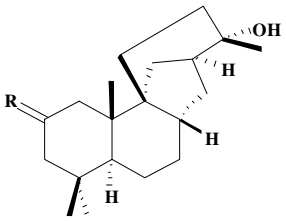
The transformation of these rearranged terpenoids is part of a larger investigation of the mono-oxygenase enzymes of *Rhizopus oryzae* ATCC 11145 (formerly *Rhizopus arrhizus* ATCC 11145). The aim is to garner useful information about the relationship between the functional groups of substrates and the site of hydroxylation (Martin et al., 2004a,b).

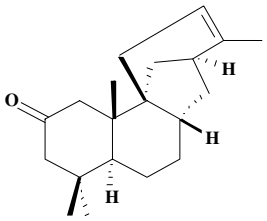
2. Results and discussion

Stemodin (**1**) (Hufford et al., 1992), obtained in large quantities from the plant *Stemodia maritima* (Scrophulariaceae), was oxidised to stemodinone (**2**) (Manchand et al., 1973). The acid catalysed solvolysis of **2** was expected to yield stemod-12-en-2-one (**3**) (Manchand et al., 1973) and 13(*R*)-hydroxystemodan-2-one (**4**) (Martin et al., 2004b). These compounds were required as substrates for investigation of the biotransformation of stemodin analogues by *R. oryzae*. Along with **4** two new unexpected rearranged products, **5** and **10**, were obtained. As the ^{13}C NMR profiles of **5** and **10** were similar it was first thought that they shared the same skeleton and were epimers of each other at a CHOH carbon. This was disproved, however, when small samples of each were oxidised to yield different ketones, **14** and **15**, respectively.



1 R= αOH, βH

2 R=O



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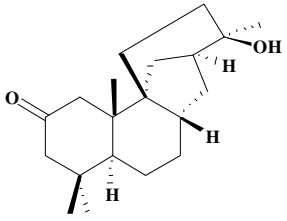

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Data from the HRMS(EI) (M^+ = 304.2407) of compound **5** gave a molecular formula of $C_{20}H_{32}O_2$. Absorption in the FTIR spectrum showed hydroxyl and carbonyl stretches at 3358 and 1710 cm^{-1}, respectively. The structure was confirmed from HSQC, HMBC, ^1H–^1H COSY and T-ROESY experiments (Table 1).

Table 1
^1H, ^{13}C and 2D NMR data of 15(13→12)*abeo*-13β-hydroxystemaran-2-one (**5**)

Carbon	δ_C	δ_H	HMBC	^1H–^1H COSY	T-ROESY
1	48.8	2.45α_{ax} (*d*, 12.0)	C-2, C-9, C-20	H-1β, *H-3α*, H-20	H-11β
		2.20β_{eq} (*dd*, 12.0, 2.5)	C-2, C-3, C-5, C-10, C-20	H-1α, *H-3β*	H-17
2	212.9				
3	56.5	2.28α_{ax} (*d*, 12.8)	C-2, C-4, C-18, C-19	*H-1α*, H-3β, *H-19*	
		2.10β_{eq} (*dd*, 12.8, 2.5)	C-2, C-4, C-5, C-18, C-19	*H-1β*, H-3α	
4	39.3				
5	48.6	1.65α_{ax} (*m*)			
6	22.7	1.73α_{eq} (*m*)			
		1.43β_{ax} (*m*)	C-5, C-7, C-10	H-5α, H-6α	
7	29.5	1.62α_{ax} (*m*)			
		1.62β_{eq} (*m*)			
8	38.8	1.85β_{ax} (*m*)	C-9, C-11, C-13, C-14	H-7, *H-11β*, H-14β	H-11β
9	52.5				
10	44.9				
11	39.1	1.65α_{ax} (*m*)		H-11β	H-13α
		1.32β_{eq} (*m*)	C-8, C-9, C-12, C-13, C-15	*H-8β*, H-11α	H-1, H-8β
12	46.1				
13	74.1	3.56α_{ax} (*dd*, 11.0, 5.9)	C-11, C-14, C-15, C-16	H-14α, H-14β	H-11α, H-14α
14	36.1	1.63α_{eq} (*m*)		H-13α	
		1.50β_{ax} (*m*)	C-8, C-7, C-13	H-8β, H-13α	
15	23.6	1.05 (*s*)	C-11, C-12, C-13, C-16		H-11β, H-13α, H-16
16	28.6	1.70 (*m*)			
		1.07 (*m*)		H-16, H-17	
17	31.7	1.68 (*m*)			H-1β, H-20
		1.24 (*m*)	C-9, C-11, C-12, C-16	H-16	
18	33.5	1.03 (*s*)	C-3, C-4, C-5, C-19		H-3α, H-3β, H-5α, H-6α, H-19
19	23.1	0.84 (*s*)	C-3, C-4, C-5, C-18		H-3β, H-6β, H-18
20	17.3	0.89 (*s*)	C-5, C-9, C-10		H-1β, H-6β, H-8, H-17

Determined in $CDCl_3$.
Long range (4 bond) COSY correlations are italicised.

T-ROE couplings for H-20 (δ 0.89) with both H-17 protons (δ 1.68, 1.24) as well as that of H-1β with H-17 (δ 1.68) implied that the C-16, C-17 bridgehead had β-stereochemistry. The T-ROESY correlation of H-1α (δ 2.45) to H-11β (δ 1.32) suggested that the stereochemistry at C-11 was α. With ring C (that is, for carbons 8, 9, 11, 12, 13 and 14) in the chair form, H-13α was designated as being pseudo-axial due to a large diaxial coupling (J = 11.0 Hz) between H-13α (δ 3.54) and H-14β (1.50). T-ROE coupling was noted between H-13α and H-11α (δ 1.65) and this suggested a β-stereochemistry for the C-13 hydroxyl group. ^{1}H–^{1}H COSY data showed "w" coupling between H-11β (δ 1.32) and H-8β (δ 1.85) (Table 1). ^{13}C NMR data affirmed the presence of the C-13 hydroxyl functional group with a resonance at 74.1 ppm.

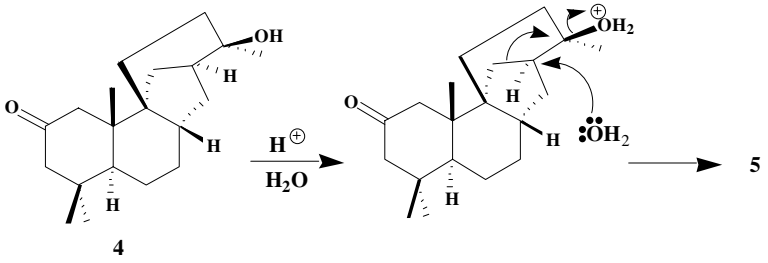
The second rearranged analogue (**10**) had a molecular formula of $C_{20}H_{32}O_2$ (*m/z* 304.2408). The FTIR spectrum showed absorptions for hydroxyl and carbonyl functions at 3454 and 1703 cm^{-1}, respectively. ^{1}H NMR data contained the four methyl singlets at δ 0.80 (H-17), 0.87 (H-20), 0.89 (H-19) and 1.06 (H-18) as well as a signal for H-12 at 3.60 ppm. HSQC, HMBC, ^{1}H–^{1}H COSY and T-ROESY experiments were performed to determine the structure of compound **10** (Table 2). The data showed that rings C and D were converted from the bicyclo[3.2.1]octane of stemodinone (**2**) to the bicyclo[2.2.2]octane ring system. T-ROE correlations between H-11α (δ 2.54) and H-5α_{ax} (δ 1.90) as well as H-1α_{ax} (δ 2.32) and H-11β (δ 1.11) fixed the stereochemistries of H-11α and H-11β. The stereochemistry of the hydroxyl group at C-12 was determined from the correlations of H-11α and H-11β with H-12α (δ 3.60). The dihedral angle of H-11α and H-12α was 0° as deduced from a coupling constant of 10 Hz, whereas that of H-11β, H-12α was 5 Hz corresponding to a dihedral angle of ca. 120°. The stereochemistry of the hydroxyl group was therefore determined as β. The ^{13}C NMR spectrum supported the presence of hydroxyl and carbonyl groups with signals at 73.8 and 212.9 ppm, respectively.

The rationale for the formation of these compounds is as follows. Compound **5** was obtained directly from 13(*R*)-hydroxystemodan-2-one (**4**) (Scheme 1) while **10** was obtained from stemodinone (**2**) (Scheme 2). The

Table 2
^{1}H, ^{13}C and 2D NMR data of 15(8→9)*abeo*-8β(H)-12β-hydroxystachan-2-one (**10**)

Carbon	δ_C	δ_H	HMBC	^{1}H–^{1}H COSY	T-ROESY
1	48.8	2.32α_{ax} (*d*, 11.8)	C-2, C-9, C-10, C-20	H-1β, *H-3α*, H-20	H-5α, H-11β
		2.06β_{eq} (*dd*, 11.8, 2.5)	C-2, C-3, C-5, C-10, C-20	H-1α, *H-3β*	H-15α
2	212.9				
3	56.4	2.26α_{ax} (*d*, 13.1)	C-2, C-4, C-18, C-19	*H-1α*, H-3, *H-19*	
		2.11β_{eq} (*dd*, 13.1, 2.5)	C-1, C-2, C-4, C-19	*H-1β*, H-3α	
4	39.1				
5	46.8	1.90α_{ax} (*dd*, 12.2, 2.9)	C-4, C-9, C-10, C-19, C-20	H-6α, H-6β	H-1α, H-11α
6	23.0	1.66α_{eq} (*m*)			
		1.31β_{ax} (m)	C-5, C-8, C-7, C-10	H-6α, H-7α	
7	33.1	1.64β_{eq} (*m*)	C-5, C-8, C-14		
		1.19α_{ax} (*m*)	C-6, C-8, C-14	H-8β	
8	33.9	1.75β_{ax} (*m*)		H-7α, H-14α	
9	39.9				
10	44.8				
11	34.2	2.54 (*m*)	C-12, C-15	H-11β, H-12α, *H-15*	H-5α, H-12α
		1.11β (*m*)	C-8, C-9, C-12, C-15		H-1α
12	73.8	3.60α (*ddd*, 9.4, 5.6, 1.8)		H-11α, H-11β	H-11α, H-14α
13	32.8				
14	40.8	1.71β (*m*)			
		0.74α (*m*)	C-8, C-9, C-12, C-13, C-16	H-8	
15	26.8	1.34β_{ax} (*m*)	C-9, C-10, C-11, C-16	*H-11α*, *H-11β*, H-16β	
		1.34α_{eq} (*m*)	C-9, C-10, C-11, C-16	*H-11α*, *H-11β*, H-16β	H-1β
16	25.5	1.70β (*m*)			
		1.11α (*m*)	C-9, C-12, C-15		
17	22.8	0.80β_{eq} (*s*)	C-12, C-13, C-14, C-16		H-12α, H-14β
18	34.3	1.06α_{eq} (*s*)	C-3, C-4, C-5, C-19		H-3α, H-3β, H-5α, H-6α, H-19
19	23.6	0.89β_{ax} (*s*)	C-3, C-4, C-5, C-18		H-3β, H-6β, H-18
20	16.1	0.87β_{ax} (*s*)	C-1, C-5, C-9, C-10		H-1β, H-6β, H-8β, H-15

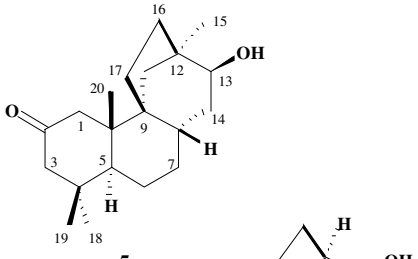
Determined in $CDCl_3$.
Long range (4 bond) COSY correlations are italicised.

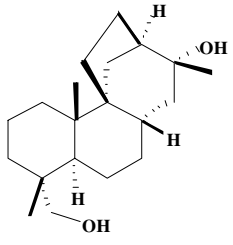


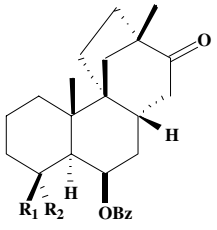
Scheme 1.

departure of the leaving group resulted in the migration of the alkyl group that was *anti* to it. Attack by water afforded products **5** and **10**. Both reactions appear to be concerted. The formation of the corresponding olefins (**16** and **17**), along with stemod-12-en-2-one (**3**), were likely since the reaction was performed at reflux temperature.

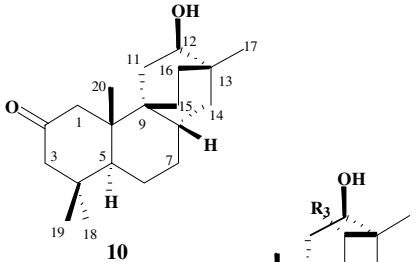
The aforementioned skeletal rearrangement of the C/D ring system of diterpenoids **4** is novel, however, that of **2** is not unprecedented. The incubation of a tetracyclic diterpenoid possessing the bicyclo[3.2.1]octane moiety with the fungus *Cephalosporium aphidicola* was reported to yield a compound with the bicyclo[2.2.2]octane ring system, albeit in less than 4% yield (Hanson et al., 1992b). Interestingly the reverse rearrangement, that is the conversion of the bicyclo[2.2.2]octane to the bicyclo[3.2.1]octane ring system has been employed in the total synthesis of aphidicolane and stemodane diterpenes (Toyota and Ihara, 1999).

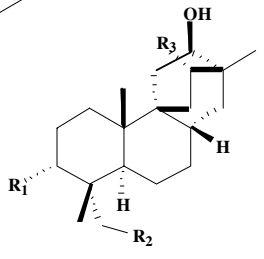


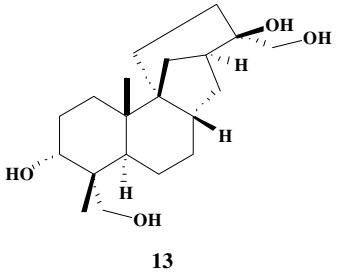




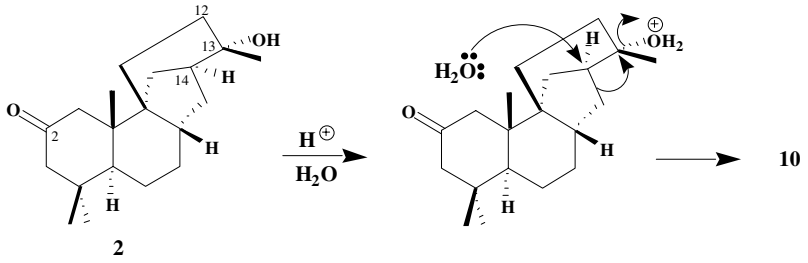
7 $R_1 = CO_2H$, $R_2 = CH_2OH$
8 $R_1 = CH_3$, $R_2 = CO_2H$
9 $R_1 = CH_3$, $R_2 = CH_2OH$







11 $R_1 = R_2 = R_3 = H$
12 $R_1 = R_2 = R_3 = OH$

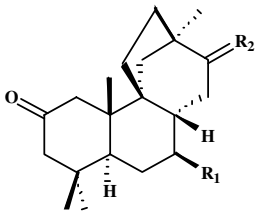


Scheme 2.

Oxidation of **5** gave 15(13→12)*abeo*-stemarane-2,13-dione (**14**). Data from HRMS(EI) (*m*/*z* 302.2246) suggested a molecular formula of $C_{20}H_{30}O_2$. A carbonyl stretch at 1707 cm^{-1} was observed in the FTIR spectrum. ^{13}C NMR data verified the absence of the hydroxyl group with the disappearance of the C-13 methine group at 74.1 ppm and the appearance of a new carbonyl peak at 214.3 ppm. Oxidation of **10** gave the 15(8→9)*abeo*-8β (H)-stachane-2,12-dione (**15**). Data from the HRMS(EI) (*m*/*z* 302.2241) suggested a molecular formula of $C_{20}H_{30}O_2$. The FTIR spectrum showed an absorption at 1718 cm^{-1} (>C=O). This was supported by ^{13}C NMR data in which the C-12 carbonyl signal was noted at δ 216.6.

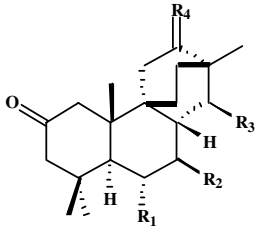
Incubation of 15(13→12)*abeo*-13β-hydroxystemaran-2-one (**5**) with *R. oryzae* yielded 15(13→12)*abeo*-7β,13β-dihydroxystemaran-2-one (**18**). The HRMS data (*m*/*z* 320.2363) of **18** indicated that the compound possessed the molecular formula $C_{20}H_{32}O_3$. In the FTIR spectrum hydroxyl stretches at 3568 and 3370 cm^{-1} as well as carbonyl absorptions at 1726 cm^{-1} were observed. ^1H NMR data showed a new resonance at δ 3.69 for H-7. ROE cross-peaks confirmed the stereochemistry of the C-7 hydroxyl group as β from correlations of H-7α with H-5α (δ 1.78), H-6α (δ 2.05), H-11α (δ 1.48) and H-14α (δ 2.27). The presence of a C-7 hydroxyl moiety was also supported in the ^{13}C NMR spectrum with a signal at 69.3 ppm as well as accompanying β-downfield (+7.9, +9.8 ppm) and γ-gauche (−6.5 to +0.7 ppm) shifts.

Bioconversion of 15(8→9)*abeo*-8β(H)-12β-hydroxystachan-2-one (**10**) by *Rhizopus* afforded 15(8→9)*abeo*-8β(H)-6α,12β-dihydroxystachan-2-one (**19**), 15(8→9)*abeo*-8β (H)-7β,12β-dihydroxy-stachan-2-one (**20**) and 15(8→9)*abeo*-8β(H)-6α,12β,14β-trihydroxystachan-2-one (**21**). Compound **19** was assigned a molecular formula of $C_{20}H_{32}O_3$ from the HRMS data (*m*/*z* 320.2356). Carbonyl and hydroxyl stretches were observed in the FTIR spectrum at 1710 and 3420 cm^{-1}, respectively. A signal at 3.84 ppm was noted for H-6β in the ^1H NMR spectrum. T-ROESY correlations were seen for H-6β with H-7β (δ 1.90), H-8β (δ 1.88), H-19 (δ 1.17) and H-20 (δ 0.90). These cross-peaks suggested that the stereochemistry of the C-6 hydroxyl group was α. A C-6 resonance at 69.7 ppm along with C-5 (+5.0 ppm) and C-7 (+11.0 ppm) downfield shifts in the ^{13}C NMR spectrum also confirmed that hydroxylation had occurred. γ-Gauche shifts ranged between −5.5 and −0.6 ppm. A molecular formula of $C_{20}H_{32}O_3$ was determined for the metabolite **20** based on HRMS data (M^+ = 320.2346). The FTIR spectrum displayed absorptions at 1702 and 3392 cm^{-1} indicative of the presence of carbonyl and hydroxyl functionalities, respectively. A new signal at δ 3.40 for H-7 was noticed in the ^1H NMR spectrum. Cross-peaks between H-7α and H-5α (δ 1.97), H-6α (δ 1.97), H-11α (δ 2.37), H-12α (δ 3.62), and H-14α (δ 1.28) in the T-ROESY data proved that the stereochemistry of the C-7 hydroxyl group was β. The C-7 hydroxylation was also detected in the ^{13}C NMR spectrum with a new resonance at 74.3 ppm and accompanying shifts at C-6 (+9.4 ppm) and C-8 (+7.9 ppm). HRMS data (*m*/*z* 336.2299) of metabolite **21** confirmed the presence of a dihydroxylated metabolite ($C_{20}H_{32}O_4$). Absorptions in the FTIR spectrum at 1674 and 3404 cm^{-1} revealed carbonyl and hydroxyl moieties. The ^1H NMR data showed new resonances at 2.93 and 3.80 ppm for H-14 and H-6, respectively. The stereochemistry of the C-6 hydroxyl group was designated α due to ROE couplings between H-6β and H-7β (δ 2.08), H-8β (δ 1.62), H-19 (δ 1.17) and H-20 (δ 0.92). The stereochemistry of the other hydroxyl group at C-14 was determined as β from ROE correlations of H-14α with H-7β (δ 2.08), H-7α (δ 1.29), H-8β (δ 1.62), H-12α (δ 3.45) and H-17 (δ 0.89). ^{13}C NMR data supported the hydroxylations with signals at δ 69.2 and 79.5 for C-6 and C-14, respectively. β-Downfield shifts at C-8 (+12.3 ppm) and C-13 (+6.0 ppm) were observed when the data for **21** was compared with that obtained for **19**. It is probable that **21** was produced from hydroxylation of that compound.



14 $R_1 = H, R_2 = O$

18 $R_1 = OH, R_2 = \beta OH, \alpha H$

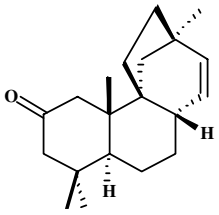


15 $R_1 = R_2 = R_3 = H, R_4 = O$

19 $R_1 = OH, R_2 = R_3 = H, R_4 = \beta OH, \alpha H$

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

21 $R_1 = R_3 = OH, R_2 = H, R_4 = \beta OH, \alpha H$



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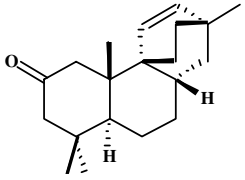

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In summary, 8 new diterpenes were prepared by chemical and microbial means. Solvolysis of stemodinone (**2**) yielded 2 hitherto unreported rearranged analogues, one of which possessed a unique diterpene skeleton. Bioconversion of these substrates afforded 4 new products. The position and stereochemistry of hydroxylation on compounds **5** and **10** are in accord with previous results (Martin et al., 2004a,b); that is, diterpenoids possessing two potential binding groups are usually functionalised by *R. oryzae* at the C-6 or -7 equatorial position. It is hoped that, while an X-ray crystal structure of the mono-oxygenase of *Rhizopus* is unavailable, the results of such experiments can aid in the development of a model of the enzyme active site.

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 NaCl disks on a Perkin–Elmer FTIR Paragon 1000 spectrophotometer. ^1H 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 chloroform ($CDCl_3$) was used as solvent with tetramethylsilane (TMS) as internal standard. The complete NMR data for the rearranged compounds are shown in Tables 1, 2. ^{13}C NMR assignments for the diterpenoids **14**, **15**, **18**–**21** are listed in Table 3. 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 dia.). Detection of 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%. Stemodinone was prepared by oxidation of stemodin using Jones reagent (Manchand et al., 1973). *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 for *R. oryzae* consisted of (per litre) K_2HPO_4 (5 g), NaCl (5 g), peptone (5 g), yeast extract (5 g) and glucose (20 g). 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. 24, 36, 48 and 60 h after inoculation 10%, 20%, 30% and 40% of the substrate, respectively; dissolved in ethanol, was

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

C	14	15	18	19	20	21
1	48.6	49.0	49.0	48.6	48.9	48.5
2	212.0	211.7	215.0	212.3	212.0	214.7
3	56.3	56.1	56.6	56.9	56.0	57.0
4	39.1	39.1	39.6	38.5	38.6	38.8
5	48.4	46.9	47.7	51.8	45.0	51.5
6	22.4	22.4	32.5	69.7	32.4	69.2
7	29.4	31.8	69.3	44.1	74.3	41.5
8	40.2	33.1	46.7	31.9	41.8	44.2
9	52.8	42.0	53.2	45.0	44.2	45.1
10	44.7	44.5	45.1	39.3	40.3	39.2
11	40.2	39.9	40.9	34.0	35.1	33.6
12	53.3	216.6	46.2	73.5	73.7	71.0
13	214.3	43.2	73.6	32.6	32.6	38.6
14	42.4	40.4	29.6	40.3	36.5	79.5
15	20.0	29.8	23.7	26.5	27.0	26.4
16	35.0	25.5	29.1	25.5	25.2	20.0
17	31.2	19.5	32.4	23.0	22.9	18.8
18	33.4	34.1	33.5	37.9	34.2	37.8
19	22.9	23.4	23.2	25.0	23.6	25.0
20	17.3	15.9	17.6	17.3	16.2	17.3

Determined in $CDCl_3$.

fed to the growing fungus. Controls consisted of flasks to which only solvent was administered. The medium was shaken for a further 5 d. 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.

3.3. Solvolysis of stemodinone (2)

To stemodinone (**2**) (3 g, 9.889 mmol) in acetone (300 ml) was added 6 M sulfuric acid (300 ml). The reaction mixture was heated under reflux for 2 h at 70–80 °C. The mixture was neutralised with 20% KOH solution (ca. 1100 ml) and the acetone was removed in vacuo. The aqueous solution was extracted with ethyl acetate (2 × 400 ml). The organic solution was dried, filtered and concentrated to yield a dark brown solid (2.89 g).

3.3.1. 15(13→12)abeo-13β-Hydroxystemaran-2-one (5)

The solvolysed extract (2.89 g) was chromatographed on silica gel. Elution with 3% acetone in petrol afforded a mixture of elimination products (400 mg), the purification of which proved impossible. Elution in 4–5% acetone in petrol gave 15(13→12)*abeo*-13β-hydroxystemaran-2-one (**5**) (862 mg) which crystallised from acetone as needles, m.p. 245–247 °C, $[\alpha]_D^{27}$ +15.2° (*c* 0.8, Me_2CO); FTIR: ν_{max} cm^{-1}: 3358 (OH), 1710 (>C=O); HRMS(EI): *m/z* (rel. int.): 304.2407 $[M]^+[C_{20}H_{32}O_2$ requires 304.2402], 289.2286 $[M - CH_3]^+$ (100); ^1H and ^{13}C NMR data of **5** are listed in Table 1.

3.3.2. 13(R)-Hydroxystemodan-2-one (4)

Elution with 6% acetone in petrol yielded 13(*R*)-hydroxystemodan-2-one (**4**) (60 mg) the data of which was the same as that previously reported (Martin et al., 2004b).

3.3.3. 15(8→9)abeo-8β(H)-12β-Hydroxystachan-2-one (10)

Elution in 6–8% acetone in petrol provided 15(8→9)*abeo*-8β (H)-12β-hydroxystachan-2-one (**10**) (810 mg) which crystallised from acetone as prisms, m.p. 167–170 °C, $[\alpha]_D^{27}$ −11.8 ° (*c* 2.07, Me_2CO); FTIR: ν_{max} cm^{-1}: 3454 (OH), 1703 (>C=O); HRMS(EI): *m/z* (rel. int.): 304.2408 $[M]^+$(100) $[C_{20}H_{32}O_2$ requires 304.2402], 289.1994 $[M - CH_3]^+$ (58.2), 247.1893 (51.1); ^1H and ^{13}C NMR data of **10** are listed in Table 2.

Elution in 18% acetone in petrol provided unchanged stemodinone (**2**) (40 mg).

3.3.4. 15(13→12)abeo-Stemarane-2,13-dione (14)

To 15(13→12)*abeo*-13β-hydroxystemaran-2-one (**5**) (30.4 mg, 0.10 mmol) in acetone (20 ml) at −15 °C (ice salt) was added Jones reagent (2.5 M, 0.25 ml, 0.625 mmol) dropwise and with stirring. After 10 min EtOH (0.5 ml) was added and the mixture was neutralised with 20% aqueous NaOH (0.25 ml). The solvent was removed in vacuo. The crude residue was diluted with water (50 ml) and was extracted with ethyl acetate (2 × 30 ml). The organic solution was dried, filtered and the solvent was concentrated to provide 15(13→12)*abeo*-stemarane-2,13-dione (**14**) (28.3 mg) which crystallised from acetone as cubes, m.p. 139–140 °C, $[\alpha]_D^{27}$ −83.1° (*c* 0.95, Me_2CO); FTIR: ν_{max} cm^{-1}: 1707 (>C=O);

HRMS(EI): *m/z* (rel. int.): 302.2246 $[M]^+$ (61.7) [$C_{20}H_{30}O_2$ requires 302.2246]; ^1H NMR (200 MHz, $CDCl_3$): δ 0.86 (3H, *s*, H-19), 0.99 (3H, *s*, H-20), 1.04 (3H, *s*, H-18), 1.10 (3H, *s*, H-15), 2.53 (1H, *d*, J = 12.0 Hz, H-1α), 2.70 (1H, *dd*, J = 16.1, 8.4 Hz, H-14β).

3.3.5. 15(8→9)abeo-8β(H)-Stachane-2,12-dione (15)

To 15(8→9)*abeo*-8β(H)-12β-hydroxystachan-2-one (**10**) (30.2 mg, 0.0993 mmol) in acetone (20 ml) at −15 °C (ice salt) was added Jones reagent (2.5 M, 0.25 ml, 0.625 mmol) dropwise and with stirring. After 10 min EtOH (0.5 ml) was added and the mixture was neutralised with 20% aqueous NaOH (0.25 ml). The solvent was concentrated in vacuo. The crude residue was diluted with water (50 ml) and was extracted with ethyl acetate (2 × 30 ml). The organic solution was dried, filtered and concentrated to provide 15(8→9)*abeo*-8β(H)-stachane-2,12-dione (**15**) (29.1 mg) which crystallised from acetone as amorphous crystals, m.p. 167–169 °C, $[\alpha]_D^{27}$ +13.7° (*c* 0.73, Me_2CO); FTIR: ν_{max} cm^{-1}: 1718 (>C=O); HRMS(EI): *m/z* (rel. int.): 302.2241 $[M]^+$ (100) [$C_{20}H_{30}O_2$ requires 302.2246]; ^1H NMR (200 MHz, $CDCl_3$): δ 0.91 (3H, *s*, H-19), 0.93 (3H, *s*, H-18), 0.98 (3H, *s*, H-17), 1.07 (3H, *s*, H-20), 2.79 (1H, *dd*, J = 6.7, 2.2 Hz, H-11α).

3.4. Bioconversion of 15(13→12)abeo-13β-Hydroxystemaran-2-one (5)

The bioconversion of 15(13→12)*abeo*-13β-hydroxystemaran-2-one (**5**) (500 mg) provided mycelial (1.63 g) and broth (0.71 g) extracts. The fed material was present only in the mycelial extract while the transformed metabolite was seen in the broth extract. Trituration of the mycelial extract with petrol provided the fed material (133 mg). Washing the broth extract with cold acetone:petrol (1:2) mixture yielded 15(13→12)*abeo*-7β,13β-dihydroxystemaran-2-one (**18**) (12 mg) which crystallised from acetone as needles, m.p. 192–195 °C, $[\alpha]_D^{27}$ +35.0° (*c* 0.77, Me_2CO); FTIR: ν_{max} cm^{-1}: 3568 (OH), 3370, 1726 (>C=O); HRMS(EI): *m/z* (rel. int.): 320.2363 $[M]^+$(14) [$C_{20}H_{32}O_3$ requires 320.2351]; ^1H NMR (500 MHz, $CDCl_3$): δ 0.88 (3H, *s*, H-19), 0.93 (3H, *s*, H-20), 1.06 (3H, *s*, H-15), 1.09 (3H, *s*, H-18), 3.52 (1H, *dd*, J = 11.1, 5.5 Hz, H-13), 3.69 (1H, *m*, *w*/2 = 16.1Hz, H-7).

3.5. Bioconversion of 15(8→9)abeo-8β(H)-12β-hydroxystachan-2-one (10)

The incubation of 15(8→9)*abeo*-8β(H)-12β-hydroxystachan-2-one (**10**) (500 mg) with the fungus gave mycelial (2.06 g) and broth (0.91 g) extracts. The broth extract, which contained only products of transformation, was purified on silica gel. Elution with 50% ethyl acetate in petrol afforded 15(8→9)*abeo*-8β(H)-6α,12β-dihydroxystachan-2-one (**19**) (183 mg) which crystallised from acetone as amorphous crystals, m.p. 153–155 °C, $[\alpha]_D^{27}$ −7.09° (*c* 1.10, Me_2CO); FTIR: ν_{max} cm^{-1}: 3420 (OH), 1710 (>C=O); HRMS(EI): *m/z* (rel. int.): 320.2356 $[M]^+$ (100) [$C_{20}H_{32}O_3$ requires 320.2351]; ^1H NMR (500 MHz, $CDCl_3$): δ 0.82 (3H, *s*, H-17), 0.90 (3H, *s*, H-20), 1.17 (3H, *s*, H-19), 1.32 (3H, *s*, H-18), 3.61 (1H, *m*, *w*/2 = 10.3 Hz, H-12), 3.84 (1H, *m*, *w*/2 = 18.8 Hz, H-6).

Further elution gave 15(8→9)*abeo*-8β(H)-7β,12β-dihydroxystachan-2-one (**20**) (60 mg) which crystallised from acetone as prisms, m.p. 237–240 °C, $[\alpha]_D^{27}$ +10.9° (*c* 1.00, Me_2CO); FTIR: ν_{max} cm^{-1}: 3392 (OH), 1702 (>C=O); HRMS(EI): *m/z* (rel. int.): 320.2346 $[M]^+$ (77) [$C_{20}H_{32}O_3$ requires 320.2351]; ^1H NMR (500 MHz, $CDCl_3$): δ 0.86 (3H, *s*, H-17), 0.91 (3H, *s*, H-20), 0.93 (3H, *s*, H-19), 1.09 (3H, *s*, H-18), 3.40 (1H, *dt*, J = 10.8, 4.8 Hz, H-7), 3.62 (1H, *ddd*, J = 9.2, 5.7, 1.6 Hz, H-12).

Elution with 80% ethyl acetate in petrol yielded 15(8→9)*abeo*-8β(H)-6α,12β,14β-trihydroxystachan-2-one (**21**) (41 mg) as a gum, $[\alpha]_D^{27}$ +46.0° (*c* 1.98, MeOH); FTIR: ν_{max} cm^{-1}: 3404 (OH), 1674 (>C=O); HRMS(EI): *m/z* (rel. int.): 336.2299 $[M]^+$ (87) [$C_{20}H_{32}O_4$ requires 336.2301]; ^1H NMR (500 MHz, $CDCl_3$): δ 0.89 (3H, *s*, H-17), 0.92 (3H, *s*, H-20), 1.17 (3H, *s*, H-19), 1.32 (3H, *s*, H-18), 2.93 (1H, *bs*, H-14), 3.45 (1H, m, *w*/2 = 13.8 Hz, H-12), 3.80 (1H, *td*, J = 10.9, 4.6 Hz, H-6).

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