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Microbial transformation of cadina-4,10(15)-dien-3-one, aromadendr-1(10)-en-9-one and methyl ursolate by *Mucor plumbeus* ATCC 4740

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

Abstract

The sesquiterpenes cadina-4,10(15)-dien-3-one (**1**) and aromadendr-1(10)-en-9-one (squamulosone) (**14**) along with the triterpenoid methyl ursolate (**21**) were incubated with the fungus *Mucor plumbeus* ATCC 4740. Substrates **1**, **14** and ursolic acid (**20**) were isolated from the plant *Hyptis verticillata* in large quantities. *M. plumbeus* hydroxylated **1** at C-12 and C-14. When the iron content of the medium was reduced, however, hydroxylation at these positions was also accompanied by epoxidation of the exocyclic double bond. In total nine new oxygenated cadinanes have been obtained. Sesquiterpene **14** was converted to the novel 2α,13-dihydroxy derivative along with four other metabolites. Methyl ursolate (**21**) was transformed to a new compound, methyl 3β,7β,21β-trihydroxyursa-9(11),12-dien-28-oate (**22**). Two other triterpenoids, 3β,28-dihydroxyurs-12-ene (uvaol) (**23**) and 3β,28-bis(dimethylcarbamoxy)urs-12-ene (**24**) were not transformed by the micro-organism, however.

Keywords: *Mucor plumbeus* ATCC 4740; Cadinane; Aromadendrane; Ursane; Biotransformation; Hydroxylation; *Hyptis verticillata*; Sesquiterpene; Triterpene

1. Introduction

Hyptis verticillata Jacq. (Labiatae) has been used traditionally in the treatment of eczema, psoriasis, scabies, athlete's foot, rheumatoid arthritis and a host of cold-related problems (Morton, 1981; Ayensu, 1981; Stanley, 1928). A number of compounds have been isolated and characterised from the plant (German, 1971; Novelo et al., 1993; Kuhnt et al., 1994, 1995), including the terpenes cadina-4,10(15)-dien-3-one (**1**) (Porter et al., 1995), aromadendr-1(10)-en-9-one (squamulosone) (**14**) (Collins et al., 2001) and ursolic acid (**20**) (Porter et al., 1995). A previous incubation of **1** with the fungus *Beauveria bassiana* ATCC 7159 resulted in the isolation of nine metabolites (Buchanan and Reese, 2000), three of which were more potent against the sweet potato weevil *Cylas formicarius elegantulus* than the naturally occurring compound. The fungal metabolism of **14** has also been investigated using *Curvularia lunata* ATCC 12017, producing seven new aromadendranes (Collins et al., 2001). In our ongoing programme to examine the metabolism of plant derived terpenes and their derivatives by fungi (Hanson et al., 1994; Buchanan and Reese, 2000), compounds **1**, **14** and methyl ursolate (**21**) were incubated with *Mucor plumbeus* (formerly *M. spinosus*) ATCC 4740. Additionally, the incubation of substrate **1** in two media, a low iron (LIM) and a high iron medium (HIM) (Aranda et al., 1991a), highlighted the effect of varying the iron content of the medium upon the outcome of bioconversion. Seven metabolites emerged with the use of HIM and nine with LIM. Four of the compounds were common to both fermentations. Interestingly epoxidation of the exocyclic double bond only occurred with the use of LIM. Transformations of substrates **14** and **21** were performed only in HIM. Five congeners were obtained when **14** was fed to the fungus, only one

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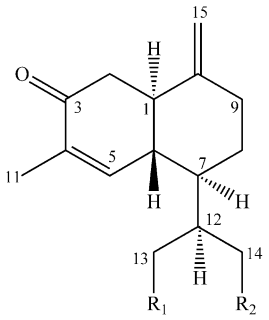

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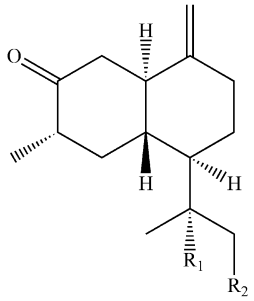

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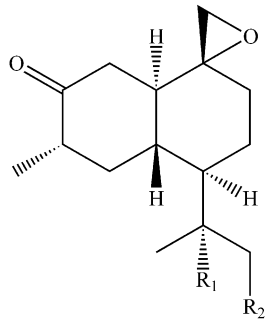

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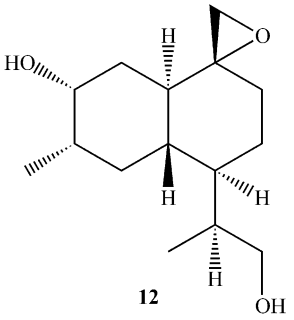

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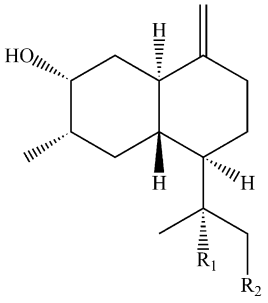

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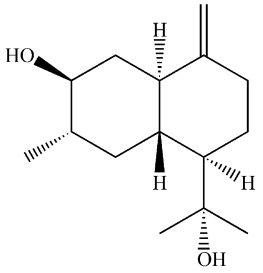
6: $R_1 = OH$, $R_2 = H$
7: $R_1 = H$, $R_2 = OH$



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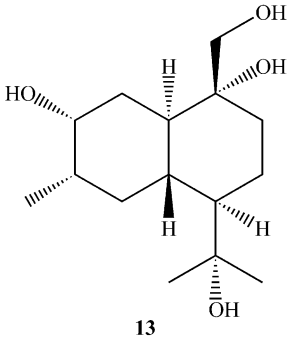

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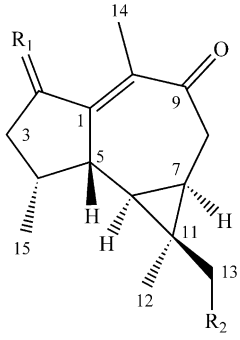


14: $R_1 = H_2$, $R_2 = H$
15: $R_1 = \beta OH,\alpha H$, $R_2 = H$
16: $R_1 = \alpha OH,\beta H$, $R_2 = H$
17: $R_1 = H_2$, $R_3 = OH$
18: $R_1 = \beta OH,\alpha H$, $R_3 = OH$
19: $R_1 = \alpha OH,\beta H$, $R_3 = OH$

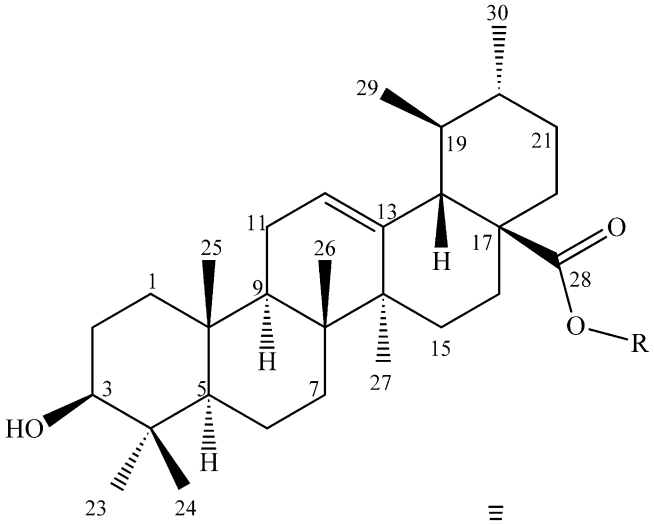
of which was new. Bioconversion of the triterpenoid methyl ursolate (**21**) gave a novel compound. Attempts at the transformation of two other triterpenoids: 3β,28-dihydroxyurs-12-ene (uvaol) (**23**) and 3β,28-bis(dimethylcarbamoxy)urs-12-ene (**24**) were unsuccessful.

M. plumbeus has been employed in the metabolism of a number of terpene substrates (Aranda et al., 1991a,b, 1992; Azerad, 2000; Boaventura et al., 1995; Guillermo et al., 1997; Fraga et al., 1998, 2001; Arantes et al., 1999a,b,c,d; Arantes and Hanson, 1999; Maurs et al., 1999). This list, while not extensive, includes the transformation of three aromadendrane derivatives (Guillermo et al., 1997). Fungal transformations of triterpenes are extremely rare and none have been reported on ursane substrates or by *M. plumbeus*.

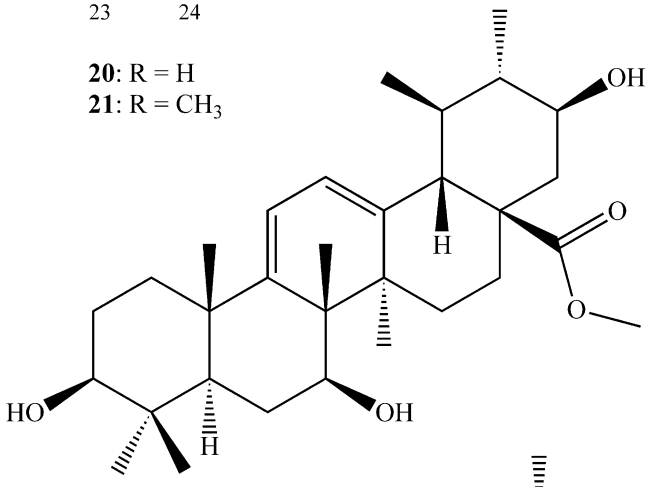
2. Results and discussion

The incubation of **1** with *M. plumbeus* in HIM produced seven metabolites that were identified as 14-hydroxycadina-4,10(15)-dien-3-one (**2**), (4*S*)-12-hydroxycadin-10(15)-en-3-one (**3**), (4*S*)-14-hydroxycadin-10(15)-en-3-one (**1**), (4*S*)-3α-hydroxycadin-10(15)-ene (**2**), (4*S*)-3α,12-dihydroxycadin-10(15)-ene (**2**), (4*S*)-3α,14-dihydroxycadin-10(15)-ene (**1**) and (4*S*)-3β,12-dihydroxycadin-10(15)-ene (**8**). Compounds **3**, **4** and **5** were identified by comparison of their spectral data with those in the literature. Previously, however, metabolite **4** had been mistakenly identified as the 13-hydroxy derivative (Buchanan and Reese, 2000). This correction was based on the accepted principle that hydroxyl insertion at C-1 will cause upfield shifts of up to 6 ppm for C-3 (Silverstein and Webster, 1998), and as such the chemical shifts of **4** are consistent with hydroxylation at C-14 and not C-13. The assignments were aided by the fact that the signals for the C-13 and-14 methyls were distinct, having a difference of ~6 ppm. A repeat fermentation of **1**, this time using LIM, yielded nine cadinanes only four of which (congeners **4**, **5**, **6** and **7**) were obtained in common with the initial fermentation. The new metabolites were identified as 13-hydroxycadina-4,10(15)-dien-3-one (**9**), (4*S*)-10α,15-epoxy-12-hydroxycadinan-3-one (**10**), (4*S*)-10α,15-epoxy-14-hydroxycadinan-3-one (**11**), (4*S*)-10α,15-epoxy-3α,14-dihydroxycadinane (**21**) and (4*S*)-3α,10α,12,15-tetrahydroxycadinane (**13**).

The IR absorption band at 3429 cm^{-1} for **1** revealed the presence of a hydroxyl group. The new functionality was placed at C-14 based on a comparison of the chemical shifts of **2** with those of **1**.



20: R = H
21: R = CH_3



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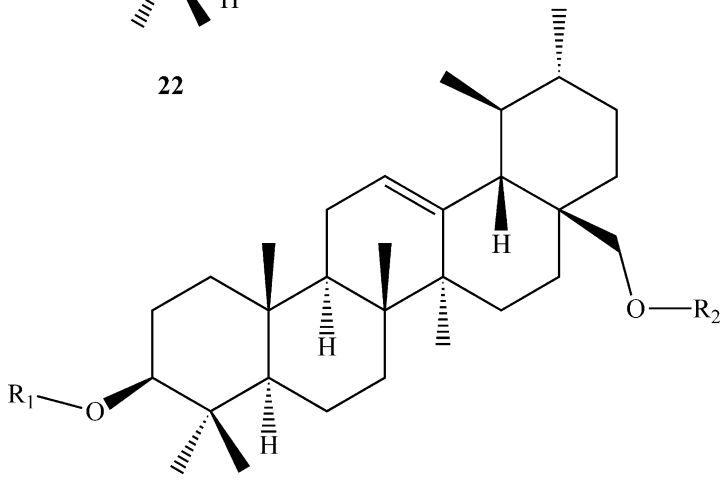

23: $R_1 = R_2 = H$
24: $R_1 = R_2 = CON(CH_3)_2$

The reduction of the α,β-unsaturated system in **6** to form the saturated alcohol was proposed by the absence of the olefinic resonances from its ^{13}C NMR spectrum. The latter contained two resonances corresponding to hydroxyl bearing carbons, one of which was assigned to C-3. The stereochemistry of this hydroxyl at C-3 was determined to be α based on the small coupling constant of H-3 ($J = 2.9$ Hz). The (*S*)-configuration of the C-11 methyl followed from its relatively large resonance value (Buchanan and Reese, 2000). The other hydroxyl, attached to a tertiary carbon, was assigned to C-12.The chemical shifts of the geminal dimethyls are consistent with this placement.

Congener **7**, having a molecular formula of $C_{15}H_{26}O_2$ ($[M]^+ = 238.1939$) as suggested by HR(EI)MS data, differed from **6** in that the reduction of the enone to the

3α-alcohol was accompanied by C-14 hydroxylation. Compound **8** was found only to be the C-3 epimer of **6**. In compound **1** the substrate had undergone a previously unreported hydroxylation at C-13 of the isopropyl group.

Data from the HR(EI)MS of **10** suggested a molecular formula of $C_{15}H_{24}O_3$ ($[M]^+ = 252.1726$). The increase in the chemical shift of the carbonyl to 211.9 ppm as compared with that of **1** indicated a loss of conjugation. Notable was the absence of the olefinic carbons representing an exocyclic double bond and the appearance of a sharp band at 1186 cm^{-1} in the IR spectrum which corresponded to an epoxide function. The presence of the epoxy moiety was suggested by two new C–O resonances (49.3 and 60.5 ppm) in the ^{13}C NMR spectrum. An IR band at 3462 cm^{-1} suggested that the molecule also contained a hydroxy function. This tertiary hydroxyl (δ_C 73.9) was accommodated by C-12. The stereochemical assignments of the epoxide as well as the C-11 methyl were aided significantly by T-ROESY data. For example the enhancements of H_{ax}-2 (δ 1.84) or H_{ax}-9 (δ 1.42) when either H_S-15 (δ 2.80) or H_R-15 (δ 2.48) was irradiated firmly fixed the stereochemistry of the epoxide as α.

Compound **11**, when analysed by HR(EI)MS, also furnished an m/z of 252.1726 ($[M]^+$) that translated into a molecular formula of $C_{15}H_{24}O_3$. In this case, however, the ^1H NMR spectrum revealed the presence of only two methyl groups within the structure. Like **10**, three different functionalities were apparent: a carbonyl (1713 cm^{-1}), an epoxide (1230 cm^{-1}), and a hydroxyl (3421 cm^{-1}). This hydroxyl group (δ_C 66.5) was found to reside on C-14.

Metabolite **12** had comparable chemical shifts to **11** for ring B, but the absence of a carbonyl in ring A was quite evident from its ^{13}C NMR and IR spectra. With a molecular formula of $C_{15}H_{26}O_3$ ($[M]^+ = 254.1882$), a hydroxyl function indubitably existed at C-3. Its α-stereochemistry was again suggested by the low value of the H-3 coupling constant ($J = 2.5$ Hz), while the adjacent C-11 methyl possessed an (*S*)-configuration as in the case of **6**. HMQC, HMBC and ^1H–^1H COSY data were used to confirm the proposed structure. The stereochemistry of the epoxide, determined from T-ROESY data, was the same as that in **10**.

Absent from the IR spectrum of congener **13** were bands characteristic of carbonyl or epoxide functions. This information along with that gleaned from the ^{13}C NMR data indicated that **13** contained four hydroxyl groups. HMQC, HMBC and ^1H–^1H COSY data were quite useful in establishing the connectivities within the molecule as the chemical shifts varied considerably from other metabolites. The stereochemistry of the groups was again established by T-ROESY data. Metabolite **13** is perceived to have transited through an initial 10,15-epoxide that opened to form the vicinal diol.

The incubation of aromadendr-1(10)-en-9-one (squamulosone) (**14**) with *M. plumbeus* yielded five metabolites that were identified as 2β-hydroxyaromadendr-1(10)-en-9-one (**15**), 2α-hydroxyaromadendr-1(10)-en-9-one (**16**), 13-hydroxyaromadendr-1(10)-en-9-one (**17**), 2β,13-dihydroxyaromadendr-1(10)-en-9-one (**18**), and 2α,13-dihydroxyaromadendr-1(10)-en-9-one (**19**). Compounds **15**–**18** were all identified by comparison of their spectral data with those of known metabolites (Collins et al., 2001). HR(EI)MS data of **19** suggested a molecular formula of $C_{15}H_{22}O_3$ ($[M]^+ = 250.1569$) and this indicated the presence of two additional hydroxyl groups. The two new oxygen-bearing carbons observed in the ^{13}C NMR were present as a methine (δ_C 74.2) and a methylene (δ_C 71.9) and were assigned to C-2 and C-13, respectively. The stereochemistry of the new C-2 function followed from the large coupling constant (14.6 Hz) for H-2 (Collins et al., 2001).

Table 1
Carbon-13 NMR resonances for cadina-4,10(15)-dien-3-one (**1**), squamulosone (**14**) and metabolites of both

	Compounds											
	1	**2**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**19**
C-1	45.31	45.1	46.2	45.0	45.9	45.21[a]	43.6	41.9	37.1	39.9	166.4	163.7
C-2	41.4	41.3	36.6	36.5	38.3	41.2	40.4	40.2	32.5	34.9	34.1	74.7
C-3	199.9	199.9	70.1	70.4	75.9	199.9	211.9	212.0	69.6	71.6	32.2	42.3
C-4	135.5	135.7	35.9	35.9	40.0	135.4	44.5	44.2	35.8	38.4	37.2	32.4
C-5	148.4	146.0	34.3	31.9	39.9	146.3	42.1	39.8	31.9	33.7	45.2	44.2
C-6	45.29	44.6	38.5	39.1	45.0	45.18[a]	46.4	46.0	42.6	41.5	31.3	28.0
C-7	45.1	40.1	53.1	42.8	53.0	45.0	52.4	42.2	42.3	39.3	22.8	19.6
C-8	26.3	26.6	31.0	26.8	31.0	26.2	27.9	23.4	23.7	16.9	41.9	41.5
C-9	35.5	35.2	36.4	36.4	36.3	35.3	34.5	34.4	35.0	30.6	201.0	201.2
C-10	149.8	149.6	152.6	152.8	151.7	149.7	60.5	60.6	61.2	75.2	130.3	133.9
C-11	16.0	15.9	18.6	18.6	18.5	15.9	14.4	14.3	18.5	18.1	25.7	31.9
C-12	26.4	34.6	74.2	34.6	74.2	38.6	73.9	34.9	34.4	76.1	16.0	11.7
C-13	15.2	10.2	24.2	10.2	24.2	68.0	24.1	10.2	10.2	28.33[a]	28.2	71.9
C-14	21.5	66.4	31.7	67.4	32.0	15.1	32.4	66.5	67.0	28.29[a]	14.9	14.4
C-15	105.3	105.5	103.6	103.8	104.2	105.2	49.3	49.3	49.9	66.1	15.4	16.4

[a] Assignments are interchangeable within a column.

Three triterpenoids: methyl ursolate (**21**), 3β,28-dihydroxyurs-12-ene (uvaol) (**23**) and 3β,28-bis(dimethylcarbamoxy)urs-12-ene (**24**) were also incubated with *M. plumbeus*. While substrates **23** and **24** were recovered untransformed from the fermentation broth, **21** underwent hydroxylation to give methyl 3β,7β,21β-trihydroxyursa-9(11),12-dien-28-oate (**22**) as the sole product. Pivotal in the structural elucidation of **22** was the complete resolution of all methyl groups in the ^{1}H NMR spectrum, a rarity for triterpenes. As such HMBC correlations were used to unambiguously assign the resonances. The presence of two additional hydroxyl groups and a second double bond was substantiated by a suggested molecular formula of $C_{31}H_{48}O_5$ ($[M]^+ = 500.3502$). Again T-ROESY data aided stereochemical propositions and indicated that ring E was slightly distorted from its normal chair conformation. The two new hydroxyls were equatorial. The formation of **21** is thought to have proceeded through an 11-hydroxy intermediate that readily eliminated to form the conjugated diene. It is not sure, however, if this reaction occurred at an early or late stage in the metabolism of the substrate. The failure of the fungus to effect reaction on either substrate **23** or **24** may be a direct result of polarity of the former and size of the latter.

In summary sesquiterpenes **1** and **14** and triterpenoids **21**, **23** and **24** were incubated with the fungus *M. plumbeus*. Substrate **1** yielded seven metabolites in a high iron medium (HIM) and nine metabolites in a low iron medium (LIM). Epoxidation of the 10(15)-exocyclic double bond occurred exclusively in the latter. Nine new cadinanes were thus produced. Substrate **14** was transformed to five metabolites, only one of which was new. Here reactions were centred on C-2 and C-13 with little stereoselectivity. Of the three triterpenoids, only one, **21**, was transformed by the fungus and this resulted in the isolation of a single, but novel metabolite.

Table 2
Carbon resonances for ursane triterpenoids

	21	22	23	24
C-1	38.5	37.5	38.7	38.4
C-2	27.0	27.8	26.1	27.2
C-3	78.7	78.5	81.8	79.0
C-4	38.5	38.7	38.0	38.0
C-5	55.1	47.9	55.2	55.1
C-6	18.1	29.3	18.2	18.3
C-7	32.8	71.6	32.7	32.8
C-8	39.3	41.8	40.0	40.0
C-9	47.4	154.9	47.5	47.6
C-10	36.7	39.1	36.7	36.8
C-11	23.1	116.7	23.5[a]	23.33[a]
C-12	125.4	123.9	125.4	125.0
C-13	137.9	139.0	138.1	138.7
C-14	41.8	48.8	41.9	42.0
C-15	27.8	30.2	24.1[a]	26.0
C-16	24.0	25.8	23.4	23.27[a]
C-17	47.9	48.1	37.2	36.8
C-18	52.7	51.0	54.4	54.0
C-19	38.8	37.6	39.4	39.4
C-20	38.7	46.6	39.2	39.3
C-21	30.5	71.1	30.6	30.6
C-22	36.4	44.5	35.8	35.1
C-23	28.0	28.2	28.1	28.1
C-24	15.5	15.6	17.3	15.7
C-25	15.3	24.9	15.7	15.6
C-26	16.7	13.8	16.7	16.7
C-27	23.4	18.3	23.3	23.2
C-28	177.9	176.5	72.1	69.9
C-29	16.9	17.1	16.9	17.3
C-30	21.0	15.8	21.3	21.3
OCH_3	51.3	51.9		
OCO-N($\underline{C}H_3$)$_2$-28			35.8	
OCO-N($\underline{C}H_3$)$_2$-3			36.3	
O$\underline{C}$O-N(CH_3)$_2$-28			156.7	
O$\underline{C}$O-N(CH_3)$_2$-3			156.9	

[a] Assignments are interchangeable within a column.

3. Experimental

^{1}H and ^{13}C NMR spectra were generated in deuterated chloroform at 200 and 50 MHz respectively using a Bruker AC200 instrument. 2D NMR data were generated on a Varian Unity 500 spectrometer. Tetramethylsilane (TMS) was used as the internal standard. ^{13}C NMR assignments for the cadinanes and aromadendranes are listed in Table 1 while those for the ursanes are reported in Table 2. Melting points were measured on a Thomas–Hoover capillary melting point apparatus. Infrared data were obtained on a Perkin Elmer FTIR Paragon 1000 instrument using KBr disks. The UV spectra were determined on a Hewlett Packard HP 8452A spectrophotometer. Optical rotations were acquired on a Perkin Elmer 241 polarimeter. High-resolution electron impact mass spectrometry [HR(EI)MS] was performed on a VG 70-250S or a Kratos MS50 instrument at an ionising voltage of 70 eV. Column chromatography was performed with Kieselgel silica (40–63 μm). Compounds on TLC plates were visualised by the use of the ammonium molybdate/sulfuric acid spray reagent followed by heating at 120 °C. *Mucor plumbeus* ATCC 4740 was obtained from the American Type Culture Collection, Rockville, MD, USA. Petrol refers to the petroleum fraction boiling between 60 and 80 °C.

3.1. Generation of substrates

Hyptis verticillata plant material was collected in St Andrew, Jamaica. A voucher specimen was deposited in the Botany Herbarium, UWI (accession number 33483). Two separate collections of the plant material were necessary to isolate the different compounds. In the first, the green leaves and stems (17.61 kg) were collected in the first quarter of the year, chopped and extracted twice with CH_2Cl_2 (47 l) at room temperature.

The extracts were pooled and concentrated in vacuo to produce a dark green gum (145.5 g). The crude extract was chromatographed on silica gel using 3% EtOAc in petrol to give eight major fractions (A–H). The moderately nonpolar fraction B (42.3 g) was subjected to further purification by column chromatography using 0.5% EtOAc in petrol. This led to the isolation of a cadina-4,10(15)-dien-3-one (**1**) (4.7 g).

3.1.1. Cadina-4,10(15)-dien-3-one (*1*)

Needles from acetone, mp 74–75 °C; $[\alpha]_D^{25}$: +134° ($c=10.3$, $CHCl_3$) [lit. mp 79–80 °C, $[\alpha]_D^{25}$: +127° ($c=1.6$, $CHCl_3$) (Porter et al., 1995)]; IR ν_{max} cm^{-1} 2950, 1680, 890; ^1H NMR: δ 0.80 (3H, *d*, $J=12$ Hz, H-14), 1.00 (3H, *d*, $J=12$ Hz, H-13), 1.80 (3H, *m*, w/2 = 17 Hz, H-11), 2.25 (1H, *m*, w/2 = 38 Hz, H-12), 4.47 (1H, *s*, H-15), 4.73 (1H, *s*, H-15), 6.82 (1H, *s*, H-5).

Further elution in 1% EtOAc in petrol led to the isolation of aromadendr-1(10)-en-9-one (**14**) (10.3 g).

3.1.2. Aromadendr-1(10)-en-9-one (*14*)

Needles from acetone, mp 50–51 °C; $[\alpha]_D^{25}$: −202° ($c=1.43$, $CHCl_3$) [lit. mp 45–46 °C, $[\alpha]_D$: −234° ($c=1.2$, $CHCl_3$) (Batey et al., 1971)]; IR ν_{max} cm^{-1} 2954, 2871, 1639 (C=O), 1468, 1315; UV (MeOH) λ_{max} (log ε) 254 (3.81); ^1H NMR: δ 0.71 (1H, *ddd*, $J=5.2$, 9.5, 11.8 Hz, H-7), 0.82 (1H, *dd*, $J=9.5$, 11.1 Hz, H-6), 1.04 (3H, *d*, $J=6.5$ Hz, H-15), 1.07 (3H, *s*, H-13), 1.18 (3H, *s*, H-12), 1.42 (1H, *m*, w/2 = 26.7 Hz, H-3), 1.78 (3H, *bs*, H-14), 1.87 (1H, *m*, w/2 = 18.7 Hz, H-3), 2.21 (1H, *m*, w/2 = 24.0 Hz, H-4), 2.34 (1H, *m*, w/2 = 22.3 Hz, H-2), 2.35 (1H, *dd*, $J=11.8$, 14.8 Hz, H-8), 2.62 (1H, *bt*, $J=11.0$ Hz, H-5), 2.67 (1H, *bdd*, $J=8.2$, 18.0 Hz, H-2), 2.81 (1H, *dd*, $J=5.2$, 14.8 Hz, H-8).

The green leaves and stems (12.1 kg) of the second collection, done in the second quarter, were used to produce the concentrated dichloromethane (97 g from 44.5 l). The plant residue was dried at 40 °C for 4 days, milled, and then percolated with acetone (19.5 l). Removal of the solvent gave a dark green gum (33.7 g). The dichloromethane extract contained only very small quantities of sesquiterpenes **1** and **14**. The acetone extract was chromatographed on silica gel using progressively increasing proportions of acetone in petrol to give five main fractions (I–M). Fraction K (11.6 g) was subjected to further purification by column chromatography using 60% EtOAc in petrol. Ursolic acid (**20**) (5.6 g), thus isolated, was characterised as its methyl ester (**21**).

3.2. Methyl 3β-hydroxyurs-12-en-28-oate (*21*)

To a suspension of crude ursolic acid (**20**) (2.18 g, 4.8 mmol) in CH_2Cl_2 (30 ml) was added excess ethereal diazomethane. When the reaction was complete (TLC) the excess diazomethane was removed by passing nitrogen through the solution. The solution was concentrated in vacuo, chromatographed using 10% ethyl acetate in petrol, and the product was recrystallised from acetone to yield methyl 3β-hydroxyurs-12-en-28-oate (methyl ursolate) (**21**) (2.14 g, 4.6 mmol).

3.2.1. Methyl 3β-hydroxyurs-12-en-28-oate (*21*)

Amorphous crystals from acetone, mp 166–168 °C; $[\alpha]_D^{25}$: +61° ($c=19.8$, $CHCl_3$) [lit. mp 168–170 °C, $[\alpha]_D^{31}$: +56.0° (Gupta and Mahadevan, 1968); IR ν_{max} cm^{-1} 3453, 1726, 1459; ^1H NMR: δ 0.74 (3H, *s*, H-26), 0.78 (3H, *s*, H-24), 0.86 (3H, *d*, $J=6.2$ Hz, H-29), 0.92 (3H, *s*, H-25), 0.93 (3H, *d*, $J=6.6$ Hz, H-30), 0.98 (3H, *s*, H-23), 1.08 (3H, *s*, H-27), 3.20 (1H, *t*, $J=6$ Hz, H-3), 3.60 (3H, *s*, CO_2Me), 5.24 (1H, *t*, $J=3.7$ Hz, H-12).

3.2.2. 3β,28-Dihydroxyurs-12-ene (uvaol) (*23*)

Methyl 3β-hydroxyurs-12-en-28-oate (**21**) (1.4 g, 3.0 mmol) was dissolved in THF (40 ml) and lithium aluminium hydride (0.5 g, 13.5 mmol) was added. The reaction was refluxed for 3 h. The addition of water to the reaction mixture was followed by extraction with EtOAc (3×50 ml) and concentration in vacuo to give 3β,28-dihydroxyurs-12-ene (**23**) (1.25 g, 2.8 mmol): Needles from acetone, mp 223–224 °C; $[\alpha]_D^{25}$: +67.0° ($c=8.1$, $CHCl_3$) [lit. mp 226–227 °C, $[\alpha]_D$: +70° ($c=2.6$, $CHCl_3$) (Orzalesi et al., 1969)]; IR ν_{max} cm^{-1} 3358, 2927, 1457, 1043; ^1H NMR: δ 0.79 (3H, *s*, H-24), 0.81 (3H, *d*, $J=6.0$ Hz, H-29), 0.93 (3H, *d*, $J=3.8$ Hz, H-30), 0.94 (3H, *s*, H-25), 0.99 (6H, *s*, H-23, H-26), 1.10 (3H, *s*, H-27), 3.18 (1H, *d*, $J=11.1$ Hz, H-28), 3.20 (1H, *m*, w/2 = 12.0 Hz, H-3), 5.13 (1H, *t*, $J=3.8$ Hz, H-12).

3.2.3. 3β,28-Bis(dimethylcarbamoxy)urs-12-ene (*24*)

3β,28-Dihydroxyurs-12-ene (**23**) (1.0 g, 2.3 mmol) was dissolved in pyridine (2.3 ml, 2.3 g, 29.1 mmol) and dimethylcarbamyl chloride (19 ml, 16.3 g, 151.3 mmol) was added. The reaction mixture was refluxed for 24 h, cooled and then $CuSO_4$ (aq) was added. The mixture was extracted with EtOAc (3×50 ml) and concentrated in vacuo to yield 3β,28-bis(dimethylcarbamoxy)urs-12-ene (**24**) (900 mg, 2 mmol). Plates from acetone, mp 175–177 °C; $[\alpha]_D^{25}$: +61.9 ($c=8.4$, $CHCl_3$); IR ν_{max} cm^{-1} 2927, 2870, 1704, 1492, 1397, 1190; EIMS *m/z* 584.4553 (1) $[M]^+$, 569.4444 (1), 495.40494 (100), 406.3600 (8), 217.1916 (11), 216.1878 (40.27), 204.1878 (13), 203.1800 (24); ^1H NMR: δ 0.81 (3H, *d*, $J=4.7$ Hz, H-29), 0.87 (3H, *s*, H-24), 0.91 (3H, *s*, H-25), 0.94 (3H, *d*, $J=6.6$ Hz, H-30), 0.96 (3H, *s*, H-26)*, 1.01 (3H, *s*, H-23)*, 1.10 (3H, *s*, H-27), 2.90 (12H, *bs*, (2 x $(CH_3)_2N$), 4.34 (1H, *dd*, $J=5.1$, 11.1 Hz, H-3), 5.13 (1H, *t*, $J=3.2$ Hz, H-12). *Assignments may be interchanged.

3.3. Feeding protocol

The fungus was maintained on PDA slants. The high iron (HIM) liquid growth medium contained per litre:

glucose (30 g), corn steep solids (5 g), sodium nitrate (2 g), potassium chloride (0.5 g), magnesium sulfate (0.5 g) and iron(II) sulfate (0.02 g) (Aranda et al., 1991a). The only difference with the low iron (LIM) medium was a change in the concentration of iron sulfate (0.002 g/l). One 14 day old slant was used to inoculate three 500 ml conical flasks each containing 125 ml of liquid medium. The flasks were incubated at 200 rpm at 27 °C. The substrates **1** (1 g), **14** (1 g) and **21** (0.5 g), were each dissolved in ethyl acetate (3 ml) and fed to the fungus 72 h after inoculation. The fermentation was allowed to proceed for 10 days. The pH was measured and the mycelium was filtered from the broth. The mycelium was filtered and the broth was extracted with EtOAc (3×500 ml). The mycelium was homogenised in EtOAc. The extracts were dried with sodium sulfate, concentrated in vacuo, and analysed by thin layer chromatography.

3.4. Incubation of cadina-4,10(15)-dien-3-one (**2**)

Cadina-4,10(15)-dien-3-one (**1**) (1 g) was fed to *M. plumbeus* in 20 flasks of each medium as outlined above. After 10 days the fungus was harvested to give broth extracts (0.532 and 0.613 g) and mycelial extracts (0.464 and 0.453 g) from HIM and LIM respectively. Analysis of both extracts by TLC indicated the presence of biotransformed compounds. The broth and mycelial extracts of each fermentation were combined and subjected to column chromatography using increasing concentrations of EtOAc in petrol.

3.4.1. 14-Hydroxycadina-4,10(15)-dien-3-one (**3**) (HIM, 34.4 mg)

Oil; $[\alpha]_D^{25}$:−30.8° ($c=1.6$, $CHCl_3$); IR ν_{max} cm^{-1} 3429, 2924, 1659; EIMS *m/z* (rel. int.) 234.1622 (3) $[M]^+$, 216.1514 (19), 175.1121 (100), 174.1038 (26), 173.0965 (18), 149.0242 (16), 147.1175 (18), 133.1018 (16); ^{1}H NMR: δ 0.81 (3H, *d*, $J=7.0$ Hz, H-13), 1.80 (3H, *q*, $J=1.6$, Hz, H-11), 3.58 (2H, *d*, $J=7.3$ Hz, H-14), 4.54 (1H, *bs*, H-15), 4.75 (1H, *m*, w/2 = 4.4 Hz, H-15), 6.85 (1H, *s*, H-5);

3.4.2. (4S)-12-Hydroxycadin-10(15)-en-3-one (**3**) (HIM, 58.7 mg)

Needles from acetone, mp 61–62 °C; $[\alpha]_D^{25}$:−96.3° ($c=23.6$, $CHCl_3$) which was identified by comparison of its physical and spectral data with those in the literature (Buchanan and Reese, 2000).

3.4.3. (4S)-14-Hydroxycadin-10(15)-en-3-one (**4**) (HIM, 23.0 mg; LIM, 13.8 mg)

Oil; $[\alpha]_D^{25}$:−15.3° ($c=5.1$, $CHCl_3$) which was identified by comparison of its physical and spectral data with those in the literature (Buchanan and Reese, 2000).

3.4.4. (4S)-3α-Hydroxycadin-10(15)-ene (**5**) (HIM, 48.6 mg; LIM, 15.9 mg)

Cubes from acetone, mp 132–134 °C; $[\alpha]_D^{25}$: +14.7° ($c=1.9$, $CHCl_3$) which was identified by comparison of its physical and spectral data with those in the literature (Buchanan and Reese, 2000).

3.4.5. (4S)-3α,12-Dihydroxycadin-10(15)-ene (**6**) (HIM, 88.6 mg; LIM, 41.8 mg)

Cubes from acetone, mp 91–92 °C; $[\alpha]_D^{25}$:−0.2° ($c=5.2$, $CHCl_3$); IR ν_{max} cm^{-1} 3385, 2924, 1710; EIMS *m/z* (rel. int.) 238.1932 (1) $[M]^+$, 220.1828 (26), 202.1728 (29), 180.1517 (16), 162.1413 (76), 159.1178 (40), 147.1177 (66), 133.1019 (32); ^{1}H NMR: δ 0.97 (3H, *d*, $J=7.0$ Hz, H-11), 1.15 (3H, *s*, H-14), 1.21 (3H, *s*, H-13), 3.90 (1H, *d*, $J=2.9$ Hz, H-3), 4.51 (1H, *bs*, H-15), 4.64 (1H, *d*, $J=1.3$ Hz, H-15).

3.4.6. (4S)-3α,14-Dihydroxycadin-10(15)-ene (**7**) (HIM, 70.8 mg; LIM, 44.5 mg)

Cubes from acetone, mp 99–101 °C; $[\alpha]_D^{25}$: +0.6° ($c=1.68$, $CHCl_3$); IR ν_{max} cm^{-1} 3410, 2924, 1646, 1450 cm^{-1}; EIMS *m/z* (rel. int.) 238.1939 (1) $[M]^+$, 220.1829 (32), 189.1646 (40), 162.1066 (73), 161.1333 (100), 147.1175 (18), 133.1018 (55); ^{1}H NMR: δ 0.73 (3H, *d*, $J=7.6$ Hz, H-13), 0.99 (3H, *d*, $J=7.0$ Hz, H-11), 3.50 (2H, *dd*, $J=3.2$, 7.3 Hz, H-14), 3.93 (1H, *m*, w/2 = 6.2 Hz, H-3), 4.50 (1H, *bs*, H-15), 4.64 (1H, *d*, $J=1.6$ Hz, H-15).

3.4.7. (4S)-3β,12-Dihydroxycadin-10(15)-ene (**8**) (HIM, 19.5 mg)

Oil; $[\alpha]_D^{25}$:−0.91° ($c=1.1$, $CHCl_3$); IR ν_{max} cm^{-1} 3426, 3304, 2929, 1716 cm^{-1}; EIMS *m/z* (rel. int.) 238.1928 (1) $[M]^+$, 220.1830 (31), 180.1515 (11), 162.1411 (70), 159.1175 (37), 149.0239 (100), 147.1175 (58), 133.1019 (41); ^{1}H NMR: δ 1.03 (3H, *d*, $J=6.3$ Hz, H-11), 1.16 (3H, *s*, H-13), 1.20 (3H, *s*, H-14), 3.22 (1H, *m*, w/2 = 15.0 Hz, H-3), 4.58 (1H, *bs*, H-15), 4.66 (1H, *dd*, $J=1.6$, 3.5 Hz, H-15).

3.4.8. 13-Hydroxycadina-4,10(15)-dien-3-one (**9**) (LIM, 62.4 mg)

Oil; $[\alpha]_D^{25}$:−76.8° ($c=62.4$, $CHCl_3$); IR ν_{max} cm^{-1} 3320, 1728, 1679, 1451; EIMS *m/z* (rel. int.) 234.1620 (3.4) $[M]^+$, 218.1671 (86), 175.1123 (32) 147.1174 (100); ^{1}H NMR: δ 0.80 (3H, *d*, $J=7.0$ Hz, H-13), 1.81 (3H, *s*, H-11), 3.58 (2H, *d*, $J=7.3$ Hz, H-14), 4.54 (1H, *s*, H-15), 4.75 (1H, *s*, H-15), 6.89 (1H, 2, H-5).

3.4.9. (4S)-10α,15-Epoxy-12-hydroxycadinan-3-one (**10**) (LIM, 21.0 mg)

Oil; $[\alpha]_D^{25}$:−2.3° ($c=19$, $CHCl_3$); IR ν_{max} cm^{-1} 3462, 1713, 1375, 1186, 1136; EIMS *m/z* (rel. int.) 252.1726 (9) $[M]^+$, 194.1307 (15), 163.1123 (6), 161.0966 (14), 147.1174 (15), 145.1017 (6), 120.0939 (7), 119.0861 (6),

111.0810 (21), 105.0704 (9), 91.0548 (20); ^{1}H NMR: δ 1.03 (3H, *d*, J=6.5 Hz, H-11), 1.22 (1H, *m*, w/2=13.0 Hz, H-5), 1.24 (1H, *m*, w/2=7.5 Hz, H-8), 1.25 (3H, *s*, H-13), 1.30 (3H, *s*, H-14), 1.32 (1H, *m*, w/2=10.0 Hz, H-7), 1.42 (1H, *dt*, J=3.5, 12.9 Hz, H-9), 1.78 (1H, *dd*, J=2.1, 3.5 Hz, H-1), 1.84 (1H, *dt*, J=1.2, 13.1 Hz, H-2), 1.92 (1H, *tq*, J=1.8, 3.0 Hz, H-9), 2.02 (1H, *d*, J=3.6 Hz, H-8), 2.05 (1H, *t*, J=2.8 Hz, H-6), 2.37 (1H, *d*, J=3.6 Hz, H-4), 2.38 (1H, *dd*, J=3.7, 13.1 Hz, H-2), 2.48 (1H, *d*, J=4.0 Hz, H-15), 2.80 (1H, *dd*, J=2.0, 4.0 Hz, H-15), 2.98 (1H, *ddd*, J=3.5, 4.5, 14.0 Hz, H-3).

3.4.10. (4S)-10α,15-Epoxy-14-hydroxycadinan-3-one (**11**) (LIM, 9.8 mg)

Oil; $[\alpha]_D^{25}$: +6.9° (c=4.9, $CHCl_3$); IR ν_{max} cm^{-1} 3421, 1713, 1230, 1028; EIMS *m/z* (rel. int.) 252.1726 (100) $[M]^+$, 234.1620 (7), 221.1542 (17), 220.1463 (24), 205.1229 (15), 194.1307 (67), 193.1229 (41), 176.1201 (46); ^{1}H NMR: δ 0.83 (3H, *d*, J=7.0 Hz, H-13), 1.02 (3H, *d*, J=6.3 Hz, H-11), 2.49 (1H, *d*, J=3.8 Hz, H-15), 2.79 (1H, *dd*, J=2.0, 4.1 Hz, H-15), 3.56 (1H, *d*, J=7.3 Hz, H-14), 3.56 (1H, *d*, J=7.3 Hz, H-14).

3.4.11. (4S)-10α,15-Epoxy-3α,14-dihydroxycadinane (**12**) (LIM, 31.8 mg)

Oil; $[\alpha]_D^{25}$: +45.7° (c=12.7, $CHCl_3$); IR ν_{max} cm^{-1} 3404, 1029, 991; EIMS *m/z* (rel. int.) 254.1882 (20) $[M]^+$, 236.1776 (15), 205.1592 (10), 178.1358 (28), 149.0239 (100); ^{1}H NMR: δ 0.77 (3H, *d*, J=7.0 Hz, H-13), 0.98 (3H, *d*, J=7.0 Hz, H-11), 0.98 (1H, *m*, w/2=18.0 Hz, H-2), 1.09 (1H, *t*, J=12 Hz, H-5), 1.20 (1H, *m*, w/2=15.0 Hz, H-6), 1.25 (1H, *m*, w/2=15.0 Hz, H-8), 1.36 (1H, *dt*, J=4.0, 12.5 Hz, H-9), 1.44 (1H, *dt*, J=3.5, 10.5 Hz, H-7), 1.49 (1H, *m*, w/2=20.0 Hz, H-4), 1.63 (1H, *m*, w/2=11.5 Hz, H-5), 1.66 (1H, *m*, w/2=20.0 Hz, H-8), 1.84 (1H, *dt*, J=3.0, 13.5 Hz, H-2), 1.94 (1H, *tq*, J=2.0, 12.5 Hz, H-9), 2.01 (1H, *ddd*, J=3.3, 10.1, 10.3 Hz, H-1), 2.08 (1H, w/2=13.4 Hz, H-12), 2.43 (1H, *d*, J=4.6 Hz, H-15), 2.76 (1H, *dd*, J=2.0, 2.6 Hz, H-15), 3.51 (1H, *dd*, J=3.2, 9.7 Hz, H-14), 3.54 (1H, *dd*, J=3.2, 9.7 Hz, H-14), 3.85 (1H, *d*, J=2.5 Hz, H-3).

3.4.12. (4S)-3α,10α,12,15-Tetrahydroxycadinane (**13**) (LIM, 23.2 mg)

Oil; $[\alpha]_D^{25}$: +70.2° (c=9.9, $CHCl_3$); IR ν_{max} cm^{-1} 3420, 1379; EIMS *m/z* (rel. int.) 254.18820 (30) $[M-H_2O]^+$, 236.1776 (4), 223.1698 (6), 168.1514 (10), 153.0916 (11), 142.0994 (43), 111.0810 (100); ^{1}H NMR: δ 1.02 (3H, *d*, J=7.0 Hz, H-11), 1.25 (3H, *s*, H-13), 1.27 (1H, *d*, J=4.0 Hz, H-7), 1.31 (3H, *s*, H-14), 1.35 (1H, *dq*, J=2.0, 7.0 Hz, H-5), 1.37 (1H, *ddd*, J=0.5, 10.5, 13.0 Hz, H-9), 1.49 (1H, *dd*, J=12.0, 23.5 Hz, H-5), 1.54 (1H, *dd*, J=2.5, 12.0 Hz, H-2), 1.61 (1H, *tt*, J=1.5, 12.5 Hz, H-1), 1.69 (1H, *m*, w/2=18 Hz, H-8), 1.72 (1H, *m*, w/2=12 Hz, H-6), 1.77 (1H, *m*, w/2=8 Hz, H-4), 1.79 (1H, *m*, w/2=10 Hz, H-2), 1.84 (1H, *m*, w/2=18 Hz, H-8), 1.98 (1H, *m*, w/2=18 Hz, H-9), 3.31 (1H, *d*, J=11.0 Hz, H-15), 3.50 (1H, *d*, J=11.5 Hz, H-15), 3.96 (1H, *dd*, J=3.0, 5.5 Hz, H-12).

3.5. Incubation of aromadendr-1(10)-en-9-one (**14**)

Aromadendr-1(10)-en-9-one (**14**) (1 g) was fed to *M. plumbeus* in 20 flasks of HIM as outlined above. After 10 days the fungus was harvested to give broth (0.405 g) and mycelial (0.832 g) extracts. Analysis of both extracts by TLC indicated the presence of biotransformed compounds. The extracts were combined and subjected to column chromatography using increasing concentrations of EtOAc in petrol.

3.5.1. 2β-Hydroxyaromadendr-1(10)-en-9-one (**15**) (57.7 mg)

Cubes from acetone, mp 85–86 °C; $[\alpha]_D^{25}$:−186° (c=6.3, $CHCl_3$) which was identified by comparison of its physical and spectral data with those in the literature (Collins et al., 2001).

3.5.2. 2α-Hydroxyaromadendr-1(10)-en-9-one (**16**) (53.5 mg)

Cubes from acetone, mp 72–74 °C; $[\alpha]_D^{25}$:−91° (c=18.6, $CHCl_3$) which was identified by comparison of its physical and spectral data with those in the literature (Collins et al., 2001).

3.5.3. 13-Hydroxyaromadendr-1(10)-en-9-one (**17**) (52.6 mg)

Oil; $[\alpha]_D^{25}$: −118° (c=4.4, $CHCl_3$) which was identified by comparison of its physical and spectral data with those in the literature (Collins et al., 2001).

3.5.4. 2β,13-Dihydroxyaromadendr-1(10)-en-9-one (**18**) (48.1 mg)

Needles from acetone, mp 145–146 °C; $[\alpha]_D^{25}$:−128° (c=0.6, $CHCl_3$) which was identified by comparison of its physical and spectral data with those in the literature (Collins et al., 2001).

3.5.5. 2α,13-Dihydroxyaromadendr-1(10)-en-9-one (**19**) (33.5 mg)

Oil; $[\alpha]_D^{25}$: −101.0° (c=0.5, $CHCl_3$); IR: ν_{max} cm^{-1} 3457, 2928, 1632; UV ($CHCl_3$) λ_{max} nm (log ε) 231 (2.69); EIMS *m/z* (rel. int.) 250.1569 (22) $[M]^+$, 204.1511 (29), 162.1042 (40), 149.0964 (41), 119.0860 (45), 107.0861 (100); ^{1}H NMR: δ 0.99 (1H, *m*, w/2=9.2 Hz, H-6), 1.13 (3H, *d*, J=7.0 Hz, H-15), 1.29 (3H, *s*, H-12), 1.65 (1H, *dd*, J=3.3, 7.0 Hz, H-3), 1.96 (3H, *bs*, H-14), 2.22 (1H, *m*, w/2=15.8 Hz, H-8), 2.89 (1H, *dd*, J=5.1, 15.2 Hz, H-8), 3.39 (2H, *d*, J=3.2 Hz, H-13), 4.82 (1H, *t*, J=13.3 Hz, H-2).

3.6. Incubation of methyl 3β-hydroxyurs-12-en-28-oate (**21**)

Methyl 3β-hydroxyurs-12-en-28-oate (**21**) (500 mg) was fed to *M. plumbeus* in 20 flasks of HIM as outlined above. After 10 days the fungus was harvested to give broth (0.155 g) and mycelial (0.550 g) extracts. Analysis of both extracts by TLC indicated the presence of biotransformed compounds. The extracts were separately subjected to column chromatography using increasing concentrations of EtOAc in petrol.

The mycelial extract contained, almost exclusively, untransformed methyl ursolate (**21**). When chromatographed, the broth was found to contain methyl 3β,7β,21β-trihydroxyursa-9(11),12-dien-28-oate (**22**) (6.4 mg): Needles from acetone, mp 123–125 °C, $[\alpha]_D^{25}$: +164.2° ($c=6.4$, $CHCl_3$); IR: ν_{max} 3406, 1715, 1456, 1199 cm^{-1}; EIMS m/z 500.3502 (52) $[M]^+$, 482.3396 (100), 468.3240 (15), 440.3290 (25), 427.2848 (88); ^1H NMR: δ 0.81 (3H, *s*, H-24), 0.86 (1H, *m*, w/2 = 11.5 Hz, H-5), 0.92 (3H, *d*, *J* = 6.5 Hz, H-29), 0.96 (3H, *s*, H-27), 0.98 (1H, *d*, *J* = 2 Hz, H-20), 1.00 (3H, *s*, H-26), 1.03 (3H, *s*, H-23), 1.08 (3H, *d*, *J* = 6.3 Hz, H-30), 1.18 (3H, *s*, H-25), 1.30, *m*, w/2 = 13.3 Hz, H-15), 1.32 (1H, *m*, w/2 = 12.5 Hz, H-1), 1.43 (1H, *m*, w/2 = 12 Hz, H-19), 1.50 (1H, *dd*, *J* = 3.8, 14.9 Hz, H-22), 1.52 (1H, *dd*, *J* = 11.3 14.1 Hz, H-6), 1.64 (1H, *m*, w/2 = 30 Hz, H-2), 1.73 (1H, *m*, w/2 = 20 Hz, H-2), 1.80 (1H, *dd*, *J* = 5, 12.5 Hz, H-6), 1.87 (1H, *dd*, *J* = 4.3, 9.3 Hz, H-16), 1.91 (1H, *m*, w/2 = 7.5 Hz, H-16), 1.98 (1H, *dt*, *J* = 4.8, 10.9 Hz, H-1), 2.09 (1H, *dd*, *J* = 4.5, 12.5 Hz, H-22), 2.17 (1H, *dt*, *J* = 5.8, 13.8 Hz, H-15), 2.41 (1H, *d*, *J* = 10.1 Hz, H-18), 3.23 (1H, *dd*, *J* = 4.6, 11.7 Hz, H-2), 3.45 (1H, *m*, w/2 = 25 Hz, H-21), 3.66 (3H, *bs*, CO_2Me), 4.05 (1H, *dd*, *J* = 5.4, 11.4 Hz, H-7), 5.58 (1H, *d*, *J* = 6.0 Hz, H-12), 5.63 (1H, *d*, *J* = 6.0 Hz, H-11).

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