Secondary mould metabolites: Part 53.[1] Transformation of the protoilludane sesquiterpene tsugicoline A into a sterpurane derivative and its microbiological reduction

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Tsugicoline A 1 is transformed into the sterpurane derivative 3a at pH 7–8; its structure and stereochemistry are elucidated by means of NMR studies. The isolation of compound 3a is the first example of the conversion of a protoilludane into a sterpurane sesquiterpene; biotransformation of compounds 1 and 3a gives the dihydro derivatives 2 and 4c, respectively. A compound similar to clavicoronic acid has also been isolated starting from compound 1.

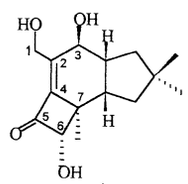
In the course of a programme aimed at identifying new bioactive metabolites produced by specific strains of microorganisms, we have studied *Laurilia* spp. (Basidiomycetae). From *Laurilia sulcata* we have isolated sulcatine B, a Δ^5-protoilludene-2,3-diol sesquiterpene, and sulcatines C and D, new norisoilludalane derivatives.[2] The growth of a strain of *L. tsugicola*, in liquid culture, gave tsugicoline A **1** in high yield (0.8 g l^{-1}). We have carried out chemical reactions on this metabolite in the presence of different nucleophiles, since the α,β-unsaturated carbonyl moiety present was expected to behave as a Michael acceptor.[3] Subsequently, we have submitted tsugicoline A **1** to biotransformation.

The treatment of tsugicoline A with baker's yeast or *Aspergillus niger* to reduce the carbonyl group was unsuccessful. In contrast tsugicoline A **1**, in liquid cultures with *Bacillus megaterium* as a biocatalyst (Table 2), gave after 4 days products **2** and **3a** in a 1:2.5 ratio (yield 50%).

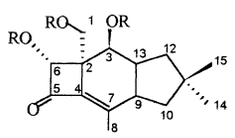
The structure of compound **2** was readily assigned since it was identical with the major product obtained by reduction with $NaBH_4$ of **1**[3] while compound **3a** was identified as a sterpurane derivative on the basis of chemical and NMR evidence. It was isolated as a white powder, mp 95–98 °C (CH_2Cl_2–hexane); $[\alpha]_D$ +42 (*c* 0.1 in MeOH) and gave an analysis consistent with the molecular formula $C_{15}H_{22}O_4$; chemical ionization mass spectrometry (isobutane) gave a distinct peak at *m/z* 267 (MH^+). The IR spectrum (liquid film) revealed a large absorption at 1730 cm^{-1} (CO), and the UV spectrum [λ_{max} (EtOH) 258 nm (ε 7900)] was consistent with the presence of a conjugated system.

^{13}C NMR experiments carried out on **3a** indicated that the carbons of metabolites **1** and **3a** have the same multiplicities but different chemical-shifts, especially pronounced for the resonances of the α,β-unsaturated ketonic group and for those of the carbons in close proximity. The ^{1}H NMR analysis (Table 1) of **3a** confirmed the presence of a cyclopentane moiety bearing two methyl groups at C-11 and a CHOH unit at C-13; moreover, the downfield shift exhibited by the 8-methyl protons, when compared with their chemical shift values in compound **1**, suggested that they are situated on a double bond while the concomitant upfield shift of the 1-methylene protons with the variation of the ^2J from 16.0 to 11.3 Hz indicated that they are no longer allylic. The formation of the triacetate **3b** caused a downfield shift for the 1-H_2, 3-H and 6-H protons, and thus supported the presence in **3a** of three OH groups.

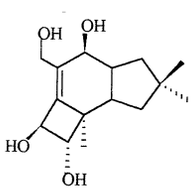
The NOEs found for **3b** allowed us to assign the absolute stereochemistry of the newly formed stereocentres C-2 and C-6.



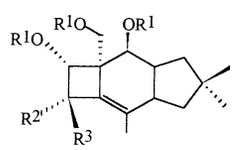
1



3a R = H
3b R = Ac



2



4a R^1 = R^2 = H, R^3 = OH
4b R^1 = Ac, R^2 = H, R^3 = OAc
4c R^1 = R^3 = H, R^2 = OH
4d R^1 = Ac, R^2 = OAc, R^3 = H

In fact, the mutual NOEs observed between 9-H and 13-H (6.5 and 7%), in a β disposition in the formula, confirmed their *cis* relationship while the NOEs observed for 3-H (7%) as well as for 10α-H (2.5%) and 12α-H (11%), which presented *trans* diaxial couplings with 9β-H and 13β-H, upon irradiation of 1-H_2 indicated that these protons are on the same α-face of the molecule. Finally, the small mutual NOEs observed between 1-H_2 and 6-H (1.5 and 0.5%) suggested that these protons are in a *trans* disposition (see Experimental section).

$NaBH_4$ reduction of **3a** afforded **4a** and **4c** in a 9:1 ratio. On acetylation these gave the corresponding tetraacetates **4b** and **4d**. The mutual NOEs observed in **4b** between 1a-H and 5-H (3 and 4%) indicated their *cis* relationship while smaller NOEs (0.5%) were observed between the *trans* disposed 1-H_2 and 6-H protons.

A screening of several fungi and bacteria was performed successively on tsugicoline A **1** (see Table 2); in one case (*Diplodia gossypina*) the substrate afforded **4c**, together with **3a**, through the stereospecific reduction of the carbonyl function.

Compound **3a** was easily obtained in high yields from **1** with a wide range of bioagents and in cultures which, during fermentation, become weakly basic; this is not the case when the medium was slightly acidic (*i.e. A. niger* and baker's yeast) suggesting that the process was not a biological but a chemical reaction whose course is pH dependent (obviously this is not the case for the formation of compounds **2** and **4c**). In fact, tsugicoline A **1** dissolved in a buffer solution at pH 8 at room temperature and was completely converted after 4 days (the

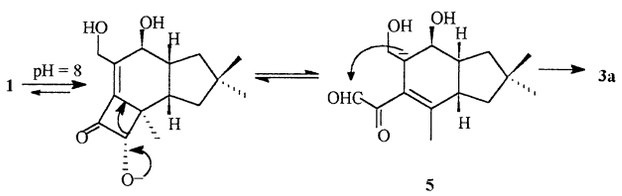
Table 1 ^{1}H NMR data for compounds **3a**, **3b**, **4a**, **4b**, **4c** and **4d**

	δ_H							J/Hz[f]
Proton	**3a**[a]	**3b**[a]	**4a**[b]	**4b**[c]	**4c**[a]	**4d**[c]	$J_{H,H}$	**3a**
1a	3.94	4.29	3.97	4.27	4.08	4.75	1a, 1b	11.3
1b	3.78	4.23	3.54	4.13	3.63	4.16	1a, 1-OH	6.5
3	4.17	5.32	4.13	5.13	3.89	5.23	1b, 1-OH	6.5
5			4.70	5.97	4.63	5.78	1b, 6	0.6
6	4.96	5.69	4.38	5.17	4.47	5.24	3, 13	1.3
8	1.94	2.07	1.74	1.71	1.67	1.81	3, 3-OH	5.0
9	2.85	2.99	2.57	2.64	2.60	2.66	6, 6-OH	7.5
10α	1.05	1.15	0.95	1.02	0.96	1.00	8, 9	0.6
10β	2.04	2.11	1.91	1.95	1.91	1.98	9, 10α	10.9
12α	1.51	1.55	1.47	1.36	1.50	1.35	9, 10β	8.0
12β	1.75	1.85	1.70	1.83	1.68	1.82	9, 13	9.7
13	2.58	2.58	2.50	2.43	2.56	2.51	10α, 10β	12.5
14	1.05	1.09	1.02	1.04	1.03	1.05	10α, 15	0.8
15	1.01	1.02	0.97	0.94	0.94	0.94	10β, 12β	2.0
1-OR	3.76	2.05[d]	*e*	2.13[d]	3.51	2.13[d]	12α, 12β	12.7
3-OR	3.95	2.02[d]	*e*	2.11[d]	3.93	2.08[d]	12α, 13	12.3
5-OR			*e*	2.08[d]	4.53	2.07[d]	12α, 15	0.8
6-OR	4.92	2.00[d]	*e*	2.02[d]	3.93	2.00[d]	12β, 13	8.4

[a] In [$^2H_6$]acetone. [b] In [$^2H_6$]acetone + D_2O. [c] In $CDCl_3$. [d] Assignments may be interchanged. [e] Not assigned. [f] Compounds **4a**, **4b**, **4c** and **4d** exhibited $J_{5,6}$ 5.5, 5.8, 5.5 and 5.8 Hz respectively.

Table 2 Microbial transformations of tsugicoline A **1**

Run	Microorganism	Conversion (%)	Metabolites (%)	
1	*Bacillus megaterium* DSM 32	50	**3a** (70)	**2** (30)
2	*Rhodococcus rhodocrous* ATCC 990	100	**3a** (60)	—
3	*Beauveria bassiana* ATCC 7159	100	**3a** (45)	—
4	*Diploidia gossypina* ATCC 10936	70	**3a** (65)	**4c**(25)
5	*Chaetomium cochliodes* ATCC 10195	100	**3a** (25)	—



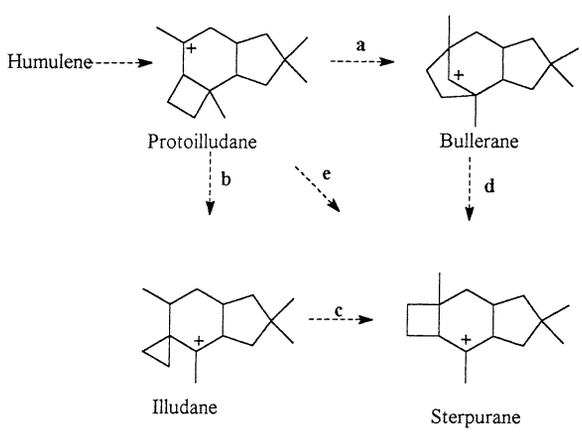
Scheme 1 A possible mechanism for the conversion of compound **1** into **3a**

time of incubation) into compound **3a**, probably *via* the intermediate **5** (see Scheme 1).

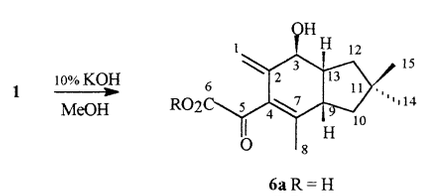
The isolation of compound **3a** is, to the best of our knowledge, the first example of the conversion of a protoilludane into a sterpurane and this confirms the important role played by the protoilludanes in the complex biosynthetic pathways of the sesquiterpenoids from Basidiomycetes.[4] The presence of sterpuranes is restricted to the fungi of the genus *Stereum purpureum*[4] and *Merullius tremellosus*[5] and they are considered the causative agents of the so called 'silver leaf disease' in fruit trees. From a biogenetic point of view, the occurrence of compound **3a** suggests that the sterpuranes may arise directly from protoilludanes (path e) rather than by the proposed pathways (a + d) and (b + c) (Scheme 2).[4,6]

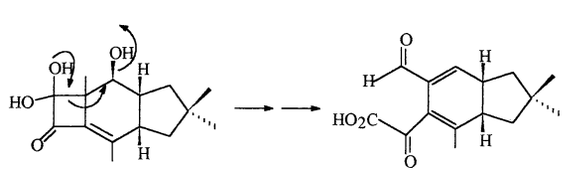
Treatment of tsugicoline A **1** with 10% KOH in MeOH gave, after acidification, compound **6a**, isolated as methyl ester **6b**. The ^{1}H and ^{13}C NMR spectra of compound **6b** (see Experimental section) agreed with the proposed structure, presenting signals attributable to a $-\underset{|}{C}=CH_2$ unit and to a $Me-\underset{|}{C}=\underset{|}{C}-CO-CO_2Me$ grouping; the remaining resonances being assigned to the cyclopentane ring moiety having, as in the starting compound **1**, two methyl groups at C-11 and a CHOH moiety at C-13.

Compound **6a** possesses a structure similar to that exhibited by clavacoronic acid **8**; the latter compound, which acts as an



Scheme 2





Scheme 3

inhibitor of reverse transcriptases, was isolated from the Basidiomycete *Clavicorona pyxidata*, and its formation was ascribed to the fragmentative opening of the cyclobutane ring

and subsequent oxidation of the hypothetical intermediate **7** (Scheme 3).[7] Since the sterpurane **3a** gave an intractable mixture with 10% KOH–CH_2N_2 (see Experimental section as for compound **6b**), we believe that acid **6a** arises from the protoilludane **1** *via* an oxidative process on the intermediate **5**, followed by elimination of water, and not from **3a**. Finally, it must also be noted that the clavacoronic acid **8** may derive from **1a** through an analogous mechanism.

Work is in progress to identify some minor compounds obtained from the reaction of compound **1** under more controlled basic conditions.

Experimental

Mps were determined on a Kofler apparatus and are uncorrected. IR and UV spectra were recorded with a Perkin-Elmer 177 instrument and a JASCO Uvidec-510 spectrophotometer, respectively; optical rotations were obtained on a JASCO Dip-181 polarimeter and values are given in 10^{-1} deg cm^2 g^{-1}; mass spectra were obtained with a Finnigan-MATT TSQ70 spectrometer. NMR spectra were acquired on a Bruker AC 250L spectrometer operating at 250.1 MHz for ^{1}H and 62.9 MHz for ^{13}C. Chemical shifts are in ppm (δ) from $SiMe_4$ as internal standard, and *J* values are given in Hz. TLC and PLC were performed with Merck HF_{254} silica gel.

Isolation and purification of compounds 2, 3a and 4c

Each microorganism (see Table 2) was grown for 2 days at 28 °C in shaken Erlenmeyer flasks (250 cm^3) at 140 rev min^{-1} containing the YMP medium [yeast extract (3 g l^{-1}), malt extract (2 g l^{-1}) and peptone (10 g l^{-1}); 50 cm^3]. Metabolite **1**[3] (according to a standard procedure; 20 mg per flask) dissolved in DMSO (100 μl) was added to the grown culture and the incubation was continued for 4 days. Each resulting mixture was extracted with ethyl acetate, and the combined organic phases were dried and evaporated under reduced pressure; the composition of each crude residue was determined by TLC; PLC in CH_2Cl_2–MeOH (9:1) was carried out for each extract. The fractions containing compound **3a** were collected. Alternatively, the metabolite **1** (10 mg), dissolved in DMSO (50 μl) was added to phosphate buffer (5 cm^3), at pH 7, 7.5 and 8, respectively, at room temperature; after 4 days the % of conversion into compound **3a** was: pH 7 (40%), 7.5 (70) and 8 (95). Compound **3a** (Found: C, 67.4; H, 8.3. $C_{15}H_{22}O_4$ requires C, 67.64; H, 8.33%); *m/z* (CI, isobutane), 267 (MH^+, 20%), 249 (100), 231 (58) and 203 (32); δ_C($CDCl_3$): 64.21 (t, C-1), 51.80 (s, C-2), 68.88 (d, C-3), 113.81 (s, C-4), 200.21 (s, C-5), 82.71 (d, C-6), 147.95 (s, C-7), 21.08 (q, C-8), 42.83 (d, C-9), 49.50 (t, C-10), 38.80 (s, C-11), 46.10 (t, C-12), 46.68 (d, C-13), 29.26 (q, C-14) and 26.54 (q, C-15). ^{1}H NMR spectroscopic data are reported in Table 1.

Compounds **2** and **4c** obtained from biotransformations with *B. megaterium* and *D. gossypina* were identical (TLC and ^{1}H NMR) with samples isolated by $NaBH_4$ reduction of tsugicoline A **1**[3] and sterpurane **3a**, respectively (see below).

Acetylation of compound 3a

Compound **3a** (15 mg) was dissolved in dry pyridine (0.3 cm^3) containing Ac_2O (0.6 cm^3) and the solution was kept at 0 °C for 6 h. The mixture was then poured into ice–water and extracted with CH_2Cl_2. Evaporation of the extract followed by PLC in hexane–EtOAc (2:1) of the residue gave the triacetate **3b** as an oil: *m/z* (FAB, thioglycerine), 393 (MH^+, 40%), 351 (25), 322 (40), 307 (48), 273 (37) and 231 (33); ^{1}H NMR data are in Table 1; NOE experiments: {1-H_2} enhanced 3-H (7%), 6-H (1.5%), 10α-H (2.5%), 12α-H (11%); {3-H} enhanced 1-H_2 (1.5%), 6-H (2.5%), 12α-H (2.5%), 12β-H (1.5%), 13-H (5%); {6-H} enhanced 1-H_2 (0.5%), 3-H (1.5%); {9-H} enhanced 8-H_3 (1.5%), 10β-H (4%), 13-H (6.5%), 15-H_3 (1%); {13-H} enhanced 3-H (4.5%), 9-H (7%), 12β-H (3.5%) and 15-H_3 (1%).

Reduction of compound 3a

Sterpurane **3a** (100 mg) was treated with $NaBH_4$ (20 mg) in MeOH (5 cm^3); work-up gave a 90:10 mixture of two compounds, which were purified by PLC in EtOAc (2 runs) and identified as compound **4a** (70 mg), R_f 0.15, and its C-5 epimer **4c** (8 mg), R_f 0.2. The compounds were acetylated as above to yield after PLC purification the tetraacetyl derivatives **4b** and **4d**, respectively.

Compounds 4a–d

Compound **4a** was isolated as a solid, mp >300 °C (decomp.) (Found: C, 67.0; H, 8.9. $C_{15}H_{24}O_4$ requires C, 67.13; H, 9.02%); *m/z* (CI-isobutane) 267 ($M^+ - 1$, 35%), 251 (13), 249 (27), 233 (100) and 203 (20). Compound **4b** as an oil; *m/z* (CI) 437 (MH^+, 4%), 436 (M^+, 8), 377 ($MH^+ - 60$, 100), 334 (10), 317 (18) and 257 (10). Compound **4c**, solid, mp 280 °C (decomp.); *m/z* (CI) 251 ($MH^+ - 18$, 22%), 249 (35), 233 ($MH^+ - 32$, 100), 203 (23) and 187 (10); *m/z* (EI), 233, 221, 203 (base peak), 189, 175 and 161; *m/z* (FAB), 269 (MH^+). Compound **4d** as an oil; *m/z* (CI) 437 (MH^+, 377). ^{1}H NMR data for the compounds **4a,b,c,d** are listed in Table 1.

Compound 6b

Tsugicoline A **1** (100 mg) was dissolved in MeOH (5 cm^3) and treated with 10% KOH (5 cm^3) for 3 h at room temp.; the mixture was concentrated by solvent evaporation after which it was acidified and extracted with EtOAc; to the residue a solution of CH_2N_2 in diethyl ether was added. PLC of the mixture on hexane–EtOAc (2:1) gave compound **6b** (16 mg) as an oil [Found: *m/z* (HREI) 278.1572. $C_{16}H_{22}O_4$ requires 278.1518]; δ_H($CDCl_3$): 5.13 and 4.79 (2 H, br s, 1-H_2), 4.14 (1 H, d, *J* 4.3, 3-H), 3.85 (3 H, s, OMe), 2.91 (1 H, br ddd, *J* 10.0, 8.8 and 8.3, 9-H), 2.54 (1 H, dddd, *J* 10.5, 8.8, 7.5 and 4.3, 13-H), 2.00 (1 H, br, OH), 1.97 (1 H, ddd, *J* 12.4, 8.3 and 2.0, 10β-H), 1.95 (3 H, br s, 8-H_3), 1.62 (1 H, ddd, *J* 12.5, 7.5 and 2.0, 12β-H), 1.16 (1 H, br dd, *J* 12.5 and 10.5, 12α-H), 1.15 (1 H, br dd, *J* 12.4 and 10.0, 10α-H), 1.04 (3 H, s, 14-H_3) and 1.02 (3 H, br s, 15-H_3); δ_C($CDCl_3$) 187.26 (s, C-5), 163.85 (s, C-6), 154.72 and 127.78 (s, C-4 and C-7), 141.85 (s, C-2), 112.45 (t, C-1), 73.69 (d, C-3), 52.93 (q, OMe), 47.22 and 44.03 (t, C-10 and C-12), 44.03 and 43.61 (d, C-9 and C-13), 37.91 (s, C-11), 29.19 and 27.58 (q, C-14 and C-1) and 19.91 (q, C-8).

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Paper 6/08174F
Received 3rd *December* 1996
Accepted 14*th January* 1997