Pergamon

Tetrahedron 54 (1998) 10199–10204

TETRAHEDRON

Isolation and Structure Elucidation of Tsugicolines F-H, Novel Furosesquiterpenes, and Tsugicoline I from the Fungus *Laurilia tsugicola*[1]

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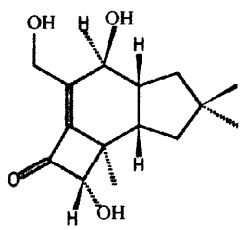
Received 6 May 1998; revised 15 June 1998; accepted 18 June 1998

Abstract: Three novel sesquiterpenes of protoilludane origin, tsugicolines F-H (**4a-6a**) , have been isolated from solid cultures of the Basidiomycetae *Laurilia tsugicola.* Their structures were elucidated by means of chemical correlation and NMR studies; a possible pathway for the formation from the protoilludane derivative tsugicoline A is reported. The furosesquiterpenes **4a** and **5a** are weakly active on bacteria but inhibited the germination of the water cress *Lepidium sativum.* A fourth metabolite, the norsesquiterpene tsugicoline I **7**, was also isolated from the same fungus.

Introduction

Basidiomycetes including the genus *Laurilia* are known to produce a series of biologically active compounds when grown in pure culture. In particular, we have recently isolated a number of norisoilludalane sesquiterpenes from *L. sulcata*[2] and of protoilludanes from liquid still cultures of *L. tsugicola*;[3] among them tsugicoline A **1** which was converted chemically into the sterpurane derivative **2**.[4] The polyoxygenated tsugicoline E **3** was isolated from the more polar chromatographic fractions.



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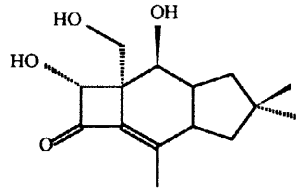

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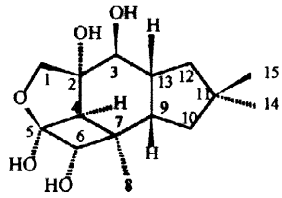

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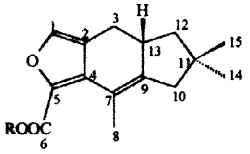

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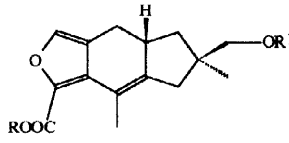
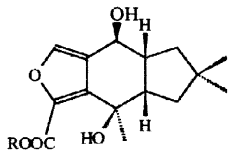
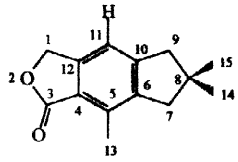
4b R = Me



5a R = R[1] = H
5b R = Me, R[1] = H
5c R = H, R[1] = Ac
5d R = Me, R[1] = Ac



6a R = H
6b R = Me



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The formation *in vivo* of the last interesting metabolite could involve a Michael addition of water on the unsaturated ketone system of compound **1**, followed by intramolecular acetalization.[1]

0040-4020/98/$ - see front matter
PII: S0040-4020(98)00613-9

In the present paper we describe the isolation of four sesquiterpenes named tsugicoline F-I **4a-7** produced by the same fungus when grown in solid culture of MPGA (malt-peptone-glucose-agar).

Results and Discussion

When a strain of *L. tsugicola* was grown on MPGA for four weeks, three main metabolites, tsugicolines **4a-6a**, were isolated by silica gel chromatography; tsugicoline F **4a** was obtained as a yellow powder, m.p. 75-77°C; $[\alpha]_D$ -26° (*c* 0.1, $CHCl_3$) and analysed for $C_{15}H_{18}O_3$ (M^+, 246); chemical ionization mass spectroscopy (CIMS; isobutane) gave a distinct peak at *m/z* 247 (MH^+) and a fragment was found at *m/z* 203 due to the ready loss of CO_2 indicating the presence in the molecule of a carboxylate function. The IR spectrum ($CHCl_3$) exhibited a large absorption at 1680 cm^{-1}, and the UV spectrum [λ_{max} 205 and 272 nm (ε 7650 and 6400)] agreed with the presence of a conjugated system. Methylation of **4a** with CH_2N_2 gave rise to the corresponding methylester **4b** which showed a peak at *m/z* 261 (CIMS)(MH^+) in agreement with a molecular formula $C_{16}H_{20}O_3$. The ^{13}C NMR spectrum of **4a** (Table 1) showed the presence of 15 resonances which were assigned to three methyl (C-8, -14 and -15), three methylene (C-3, -10 and -12), two methine (C-1 and -13) and seven quaternary (C-2, -4, -5, -6, -7, -9 and-11) carbon atoms. The one-bond ^1H - ^{13}C coupling constant of 201.5 Hz observed for C-1 together with the two- and three-bond ^1H - ^{13}C couplings of 13, 6 and 7 Hz presented by H-1 with C-2, -4 and -5 primes suggested the presence of an α', β, β' - trisubstituted furan ring[5] while the chemical shift value of 160.80 ppm for C-6 confirmed that it is part of a conjugated carboxylate moiety. First order analysis of the ^1H NMR spectrum (Table 2) revealed the presence of a -C(3)H_2-C(13)H-C(12)H_2- grouping and of a *cis*-disubstituted C(8)H_3-C(7)=C(9)-C(10)H_2- unit in which the methyl and the methylene protons, which are homoallylically coupled, exhibited mutual NOE enhancements (Experimental).

The cross-peaks observed in a COLOC spectrum, optimised for the observation of two- and three-bond ^1H - ^{13}C couplings of *ca.* 6 Hz for both 14- and 15-methyl protons with C-10, -11 and -12, not only indicated the presence of a *gem*-dimethyl group located at C-11 but also defined the mode of linking of C-11 with C-10 and -12. Additional cross-peaks observed between the 8-methyl protons and C-4, -7 and -9, together with the presence of ^1H - ^{13}C couplings of 6.5, 4.5 and 3 Hz between C-2 and H_2-3 and H-13, permitted us to join C-4 with C-7 and C-2 with C-3. Thus, to assign the structure of tsugicoline F **4a** we had only to connect C-9 with C-13 and C-5 with C-6.

Tsugicoline G **5a** was obtained as a solid, m.p. 90-95°C, $[\alpha]_D$ -36° (*c* 0.2, $CHCl_3$), and had an analysis consistent with a formulation as $C_{15}H_{18}O_4$; CIMS gave a molecular peak at *m/z* 263 (MH^+). Comparison of the ^1H and ^{13}C NMR data of **4a** and **5a** (Tables 1 and 2) revealed that both compounds share the same basic structure the only difference being the presence in **5a** of a -CH_2OH- moiety in place of a Me group. In fact, the ^1H NMR spectrum of **5a** contained an AB spin system at 3.35 and 3.30 ppm in place of the resonance at 0.99 ppm which was assigned to H_3-15; moreover, in the ^{13}C NMR spectrum of **5a** the resonances assigned to C-15 and -11 experienced downfield shifts of 41.20 and 5.31 ppm (α and β effects) and those assigned to C-10,-12 and -14 upfield shifts of 4.92-5.20 ppm (γ effects). The isolation of the monoacetate **5c** in the chromatographic separation (Experimental) with the concomitant downfield shift of *ca.* 0.5 ppm of the 15-methylene protons confirmed the presence of the hydroxy function. The NOE observed between the 15-methylene protons and H-13 (5%), assumed as β oriented in the formula, indicated that these protons are on the same side of the molecule. As expected, both compounds **5a** and **5c** gave the corresponding methyl esters **5b** and **5d**.

Tsugicoline H **6a** was isolated as a yellow oil from the more polar chromatographic fractions. Due to the difficulty in isolating the pure compound because of its instability, we have made the structural elucidation on the methyl derivative **6b**.

Comparison of the ^1H and ^{13}C NMR spectra of **6b** and **4a** (Tables 1-3) indicated that the structure of tsugicoline H differs from that of tsugicoline F in that the C(8)H_3-C(7)H=C(9) moiety and one proton at C-3 have been replaced by a C(8)H_3OH-C(7)H-C(9)H- grouping and by an hydroxy group, respectively. In fact, the COLOC spectrum of **6b** showed that the methyl protons at 1.54 ppm, assigned to H_3-8 since they couple with C-4, gave additional cross peaks with the quaternary oxygen bearing C-7 and the methine C-9 carbons while the methine proton at 4.48 ppm, assigned to H-3 since it presented a ^{13}C-^1H coupling of 3Hz with C-1, was vicinally

coupled with the hydroxy proton at 4.92 ppm; the remaining hydroxy proton which resonates as a singlet at 5.55 ppm was located at C-7.

Table 1 ^{13}C NMR data for compounds 4-7

	4a[a]				5a[b]		6b[a,c]			7[b]	
Carbon atom	δ_C		δ_C	^1J(C, H)	δ_C		δ_C		^1J(C, H)	δ_C	^1J(C,H)
1	138.93	d	(139.15)[b]	201.5	139.15	d	141.96	d	205	68.40	151.5
2	125.58	s	(125.28)		124.98	s	129.75	s			
3	25.60	t	(25.02)	130	25.24	t	66.32	d	145	171.62	
4	132.99	s	(134.83)		134.45	s	139.17	s		121.30	
5	137.71	s	(135.63)		135.87	s	139.22	s		135.27	
6	160.80	s	(163.73)		163.67	s	161.02	s		144.24	
7	149.96	s	(151.27)		150.00	s	70.65	s		45.26	129
8	17.27	q	(17.09)	127.5	17.15	q	27.38	q	127.5	40.20	
9	119.97	s	(119.21)		119.60	s	51.27	d	132	48.14	129
10	45.58	t	(45.13)	130	40.21[d]	t	44.26	t	131	150.83	
11	38.65	s	(38.26)		43.57	s	38.46	s		115.27	161.5
12	47.95	t	(47.43)	130	42.23[d]	t	46.49	t	131	146.35	
13	41.21	d	(40.47)	128	40.15	d	45.87	d	132.5	13.91	128
14	29.62	q	(29.48)	125	24.43	q	29.70	q	124	28.80	125
15	28.34	q	(28.14)	125	69.34	t	27.48	q	124	28.80	125

[a] In [$^2H_6$]acetone. [b] In $CDCl_3$. [c] The OMe carbon resonates at 52.31 ppm (^2J=147.5 Hz). [d] The assignment may be interchanged.

Table 2 ^1H NMR data for compounds 4 and 5 in [$^2H_6$]acetone

	δ_H								J / Hz		
Proton	4a		4b[a]	5a		5b	5c[b]	5d	J(H, H)	4a	5a
1	7.32	(7.25)[b]	7.06	7.37	(7.22)[b]	7.37	7.25	7.38	1, 3α	1.9	2.0
3α	1.97	(2.01)	1.98	1.99	(2.00)	1.98	2.05	1.99	3α, 3β	14.0	14.5
3β	2.83	(2.80)	2.75	2.84	(2.78)	2.84	2.81	2.85	3α, 13	13.7	14.1
8	2.10	(2.10)	2.06	2.12	(2.10)	2.08	2.12	2.09	3β, 13	6.2	6.5
10α	2.27	(2.29)	2.27	2.20	(2.26)	2.19	2.32	2.32	8, 10α	1.7	1.7
10β	2.27	(2.29)	2.27	2.45	(2.43)	2.45	2.47	2.47	8, 10β	1.7	1.7
12α	1.31	(1.30)	1.28	1.22	(1.23)	1.21	1.25	1.30	8, 13	2.5	2.5
12β	1.82	(1.82)	1.78	2.13	(2.10)	2.14	2.11	2.07	10α, 15	0.8	0.8
13	2.69	(2.72)	2.70	2.66	(2.65)	2.65	2.71	2.75	10β, 12β	1.2	1.8
14	1.15	(1.16)	1.16	1.15	(1.16)	1.14	1.17	1.17	12α, 12β	11.8	12.7
15a	0.99	(0.99)	0.99	3.35	(3.43)	3.34	3.95	3.93	12α, 13	10.8	11.0
15b				3.30	(3.37)	3.30	3.79	3.79	12α, 15	0.8	0.8
6-OR	9.50	(7.90)	3.79	9.00	(6.70)	3.81	6.10	3.80	12β, 13	7.0	7.5
15-OR				9.00	(6.70)	3.00	2.08	2.01	15a, 15b		11.0

[a] In CCl_4. [b] In $CDCl_3$.

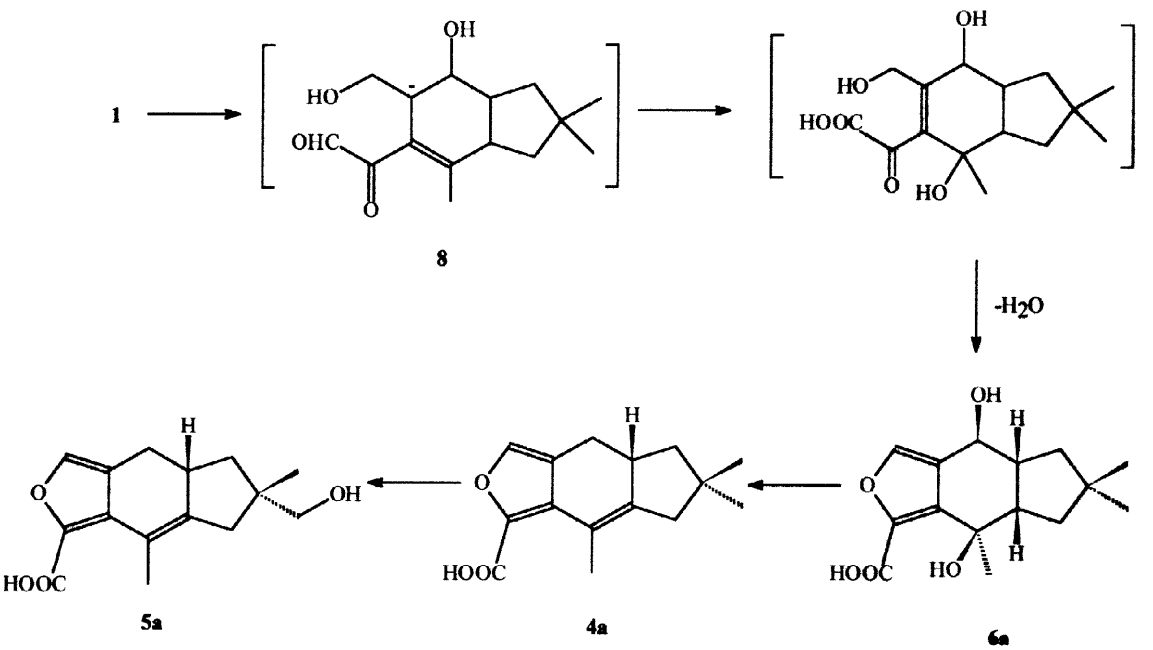
The NOE experiments reported in the Experimental gave further support to the proposed structure of tsugicoline H permitting us to assign the relative configuration. Specifically, the fact that the 15-methyl protons

at 0.99 ppm, assumed as β oriented in the formula, and the 10- and 12- methylene protons at 1.55 and 1.74 ppm, coupled each other *via* a w-type coupling of 2.2 Hz, underwent NOE enhancement (1-3.5%) by irradiation of both H-9 and H-13 indicating that all these protons are on the β side of the molecule. On the contrary, H-3 and H_3-8, which experienced mutual NOE enhancements, are α oriented since their irradiation enhanced the 10α- and the 12α-protons more than the corresponding geminal ones (4 *vs* 1%) and 7 *vs* 4%, respectively.

Table 3 ^{1}H NMR data for compound **6b** in [$^2H_6$]acetone

Proton	δ_H			δ_H		J(H, H)	Hz	J(H, H)	Hz
1	7.62	(7.54)[a]	12β	1.74	(1.74)	1, 3	0.9	10α, 15	0.8
3	4.48	(4.43)	13	2.59	(2.57)	3, 13	5.2	10β, 12β	2.2
8	1.54	(1.59)	14	1.00	(1.00)	3, 3-OH	5.4	12α, 12β	12.4
9	2.71	(2.72)	15	0.99	(0.98)	9, 10α	11.2	12α, 13	9.9
10α	1.07	(1.05)	3-OH	4.92	(3.40)	9, 10β	7.4	12α, 15	0.8
10β	1.55	(1.60)	6-OMe	3.88	(3.93)	9, 13	10.2	12β, 13	7.4
12α	1.08	(1.04)	7-OH	5.55	(4.92)	10α, 10β	12.4		

[a] In $CDCl_3$.



Scheme.- A possible mechanism of formation of tsugicolines F-H from tsugicoline A

The last metabolite, tsugicoline I 7 was isolated from the less polar fractions as optically inactive white crystals, m.p. 165-168°C; the HRMS indicated a $C_{14}H_{16}O_2$ formula like that of a norsesquiterpene derivative; the IR spectrum (KBr) exhibited a strong absorption band at 1745 cm^{-1}, suggesting the presence of a lactone moiety. The ^{1}H NMR spectrum of compound 7 (Experimental) showed the presence of 6 broadened singlets in a 1:2:2:2:3:6 ratio which were assigned to one methine (H-11), three methylene (H_2-1, -7 and -9) and three methyl groups (H_3-13, -14 and -15), two of which having the same chemical shift. The ^{13}C NMR spectrum (Table1) confirmed and extended these findings through the appearance of three methyl, three methylene, one

methine and seven quaternary carbons. The latter signals were assigned to one ester type carbonyl carbon (C-3), to a mono substituted aromatic ring (C-4, -5, -6, -10 and -12) and to one sp^3 carbon (C-8).

Finally, the NOE enhancements and the COLOC correlations reported in Experimental allowed us to assigned the structure of tsugicoline I 7.

The isolation of compounds **4a-7** confirms the important role played by the protoilludanes in the complex biosynthetic pathways of the sesquiterpenoids from Basidiomycetes.[6] In the case of *L. tsugicola* different products were obtained depending on the conditions of the culture. In liquid cultures we observed the biotransformation, also obtained by chemical conversion, of tsugicoline A **1** into the sterpurane **2**,[4] and the formation of the tsugicoline E **3** that exhibits a new skeleton having a cyclobutane ring fused to both a cyclohexane and a tetrahydrofurane ring.[1] On the contrary, in agar cultures the new furosesquiterpenes **4a-6a**, which show a fused furane ring like some marasmanes and furolactaranes,[6] were formed. It may also be observed that the recently isolated isolactarufin from *Lactarius scrobilatus*,[7] which is an isomer of tsugicoline I 7, may be also formed by rearrangement of a sterpurane.[7] In our opinion, the presence of the two oxygen functions in the four-membered ring of compound **1** is the key to the reactivity of this interesting natural intermediate.

The scheme shows a possible pathway of formation of the **4a-6a** skeleton from the protoilludane **1** *via* an oxidative process on the intermediate **8**.[4]

The tsugicoline **4a** and **5a** showed antibacterial activity against *Bacillus cereus*, *B. subtilis* and *Sarcinea lutea* (50μg/disc). The same compounds inhibited the growth of *Lepidum sativum*;[8] after 48 h the inhibition of the root elongation was *ca* 91 and 99% respectively.

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

Experimental

General.- M.p.s. were determined on a Kofler apparatus and are uncorrected; IR spectra on a Perkin-Elmer 177 spectrophotometer; mass spectra on a Finnigan-MAT-TSQ70 spectrometer; optical rotations on a JASCO-500 DIP-181 polarimeter. NMR spectra were recorded on a Bruker AC 250L spectrometer operating at 250.1 MHz for ^1H 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. Flash column chromatography was performed with Merck silica gel (0.04-0.063 mm), and TLC and preparative TLC (PLC) with Merck HF_{254} silica gel. Owing to the complexity of the purification procedure, we report the R_f value in CH_2Cl_2-MeOH (9:1) and $(Me)_2CO$-H_2O (2:1) (RP-18 plates), respectively.

Isolation and Purification of Metabolites **4a, 5a, 6a** and **7**.- A strain of *Laurilia tsugicola* (Henn and Shirai)[*Echinodontium tsugicola*](CBS 248.51) received from Centraal Bureau voor Schimmel Cultures, Baarn, was maintained on MPGA (malt, peptone, glucose, agar, 20:4:20:15 g dm^{-3}) slants and a mycelium suspension was inoculated into 30 Roux flasks containing MPGA (100 cm^3). After four weeks the cultures were extracted with EtOAc containing 1% of MeOH and the extracts were dried (Na_2SO_4) and evaporated to yield a mixture (1.6g) of sesquiterpenes. It was chromatographed on a silica gel column using CH_2Cl_2-MeOH (gradient) as eluant to give tsugicoline H **7** (90mg) and a mixture of tsugicoline F **4a** and G **5a** that was further chromatographed on a column RP_{18} silica gel with $(Me)_2CO$-H_2O (1:1) to yield the pure metabolites **4a** (115mg) and **5a** (105mg). Finally, the more polar chromatographic fractions (eluant CH_2Cl_2-MeOH 7:1) were treated with CH_2N_2 to give a mixture that was successively purified to yield the methyl ester **6b** of the tsugicoline H **6a**.

Tsugicoline F **4a**.-R_f 0.3 and 0.3. (Found: C, 73.1; H, 7.3.$C_{15}H_{18}O_3$ requires C, 73.14; H, 7.36%); ^{13}C and ^1H NMR data are reported in Tables 1 and 2. Selected NOE experiments ([$^2H_6$]acetone): {H-1} enhanced H_2-3 (1.5%), {H_3-**8**} enhanced H_2-10 (2.5%), {H_2-10} enhanced H_3-8 (3.5%), H-12α (2%), H-13 (0.5%), H_3-14 (2%), and H_3-15 (0.5%), {H-12α} enhanced H-3α (3.5%), H-10α (1%) and H-12β (16%), {H-12β} enhanced H-3β (3%), H-12α (15%), H-13 (2.5%) and H_3-15 (1%), {H_3-14} enhanced H_2-10 (3%) and H-12β (0.5%) and {H_3-15} enhanced H_2-10 (1.5%), H-12β (2.5%) and H-13(3%).

Methyl ester of tsugicoline F **4a**.- Compound **4a** (30mg) was methylated with CH_2N_2. Evapn of the solvent and PLC using hexane-EtOAc (2:1) as eluent gave ester **4b** (10mg); m.p. 58 -60°C; CIMS, *m/z* 261 (MH^+). ^1H NMR data are reported in Table 2.

Tsugicoline G **5a**.- R_f 0.12 and 0.55. UV: λ_{max} 204 and 275 nm (ε 7700 and 10.800); HR EIMS, *m/z* 262.1215 (calcd for $C_{15}H_{18}O_4$ 262.1205); ^{13}C and ^1H NMR data are reported in Tables 1 and 2. Selected NOE experiments ([$^2H_6$]acetone): {H_2-15} enhanced H-10β (4%), H-12β (3.5%), H-13 (5%) and H_3-15 (1%).

Methyl ester of tsugicoline G **5a**.- Compound **5a** (80mg) was methylated with CH_2N_2. Evapn of the solvent and PLC using CH_2Cl_2-MeOH (15:1) as eluent gave ester **5b** (26mg); m.p. 93 -95°C; CIMS, *m/z* 277 (MH^+); ^1H NMR data are reported in Table 2; acetylation of compound **5b** as usual (see below) gave compound **5d**; ^1H NMR data are reported in Table 2.

Acetylation of tsugicoline G **5a**.- Compound **5a** (50mg) was dissolved in dry pyridine (0.5cm^3) and treated with Ac_2O (1cm^3) overnight at 0°C. Standard work up followed by PLC on silica gel in hexane- EtOAc (2:1) gave the acetate derivative **5c** (40mg). M.p. 145-150°C; $[\alpha]_D$ -22° (*c* 0.1, MeOH); CIMS, *m/z* 305 (MH^+); ^1H NMR data are reported in Table 1. The same compound was isolated in poor yield during the purification of tsugicolines.

Methyl ester of tsugicoline H **6a**.- The fractions containing tsugicoline H **6a** (140mg), R_f 0.15 and 0.8, were methylated with CH_2N_2. The mixture was chromatographed on a silica gel column using EtOAc-hexane (gradient) as eluent to give ester **6b** (15mg); m.p. 135-140°C; $[\alpha]_D$ +5.6° (*c* 0.5, $CHCl_3$); (Found: C, 65.2; H, 7.4. $C_{16}H_{22}O_5$ requires C, 65.28; H, 7.53%); CIMS, *m/z* 277 (MH^+ -18); ^{13}C and ^1H NMR data are reported in Tables 1 and 3. Selected NOE experiments ([$^2H_6$]acetone): {H-3} enhanced H-1 (2.5%), H_3-8 (0.5%), H-12α (4%), H-12β (1%) and H-13 (2.5%), {H_3-8} enhanced H-3 (2%), H-10α (7%), H-10β (4%) and OMe-6 (0.5%), {H-9} enhanced H_3-8 (0.5%), H-10α (0.5%), H-10β (3%) and H_3-15 (1%), and {H-13} enhanced H-3 (2%), H-12α (0.5%), H-12β (3.5%) and H_3-15 (1%).

Tsugicoline I **7**.- R_f 0.7 and 0.3. UV: λ_{max} 214, 245, 282 and 293 nm (ε 6000, 3500, 750 and 830); HR EIMS, *m/z* 216.1134 (calcd for $C_{14}H_{16}O_2$ 216.1150); δ_H ($CDCl_3$) 7.05 (1H, br s, H-11), 5.17 (2H, br s, H_2-1), 2.80 (2H, br s, H_2-9), 2.71 (2H, br s, H_2-7), 2.57 (3H, br s, H_3-13), and 1.17 (6H, s, H_3-14 and -15); ^{13}C data are reported in Tables 2.Selected NOE experiments ($CDCl_3$): {H-1} enhanced H-11 (2%), H_2-7 enhanced H_3-13 (2%), H_3-14 and -15 (1%), {H_2-9} enhanced H-11 (2%), H_3-14 and -15 (1%), H-11 enhanced H_2-1 (1%) and H_2-9 (1%) and {H_3-13} enhanced H_2-7 (1%). Selected COLOC correlations: H_2-1 showed cross peaks with C-4, -11 and -12, H_2-7 with C-5, -6, -8, -10, -14 and -15, H_2-9 with C-6, -8, -10, -11, -14 and -15, H-11 with C-4 and -5, H_3-13 with C-4, -5 and -6, H_3-14 and -15 with C-7, -8 and -9.

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