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FUNGAL MACROLIDES: STRUCTURE DETERMINATION AND BIOSYNTHESIS OF ACHAETOLIDE, A LACTONE FROM *ACHAETOMIUM CRISTALLIFERUM*

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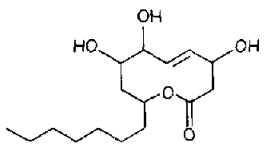
Key Word Index—*Achaetomium cristalliferum*: fungus; new macrocyclic lactone; ^1H and ^{13}C NMR spectra; biosynthesis.

Abstract—The structure of a new ten-membered lactone, achaetolide, isolated from cultures of *Achaetomium cristalliferum* is deduced from its mass and NMR spectra and from the study of some derivatives. The ^{13}C NMR spectra of achaetolide enriched with [1-^{13}C], [2-^{13}C] and [1, 2-^{13}C]acetate established its formation from eight intact acetate units via a precursor octaketide chain.

INTRODUCTION

Several compounds of biological interest have been isolated from fungi belonging to the genus *Chaetomium* [1]. But no chemical studies have been carried out on the related *Achaetomium* genus.

We now report on an investigation of an *Achaetomium* species [2], *A. cristalliferum*, originating from an arid soil in Egypt. From the filtrate of the culture broth of this fungus [3] and of *A. strumarium* Rai, Tewari and Mukerjii (IMI 82624) [unpublished results] a new macrocyclic lactone, achaetolide, was isolated. The spectral and chemical data described below led to structure **1** for this new lactone. The Bittner test [4] showed achaetolide to increase transpiration of cut barley leaves.



1

RESULTS AND DISCUSSION

Structure of achaetolide

The peak of highest mass found in the mass spectrum (HREI) of achaetolide is at *m/z* 282.1831 and corresponds to $C_{16}H_{26}O_4$ (calc. 282.1833). Chemical ionization mass spectrometry showed a pseudomolecular ion at *m/z* 301 $[M+H]^+$ to be present, thus showing the molecular ion, not observed in EIMS, to be 18 amu above *m/z* 282. If the 18 amu represent loss of water, the formula $C_{16}H_{28}O_5$ can be assigned to **1**, which implies three unsaturations. The IR spectrum suggests an ester carbonyl group (1718 cm^{-1}), a double bond (1630 cm^{-1}) and alcohol groups (3450 cm^{-1}).

The proton decoupled ^{13}C NMR spectra of achaetolide (Table 1) can be used to assign all of the 16 carbon atoms in the molecule. Analysis of the off resonance spectrum and of the chemical shifts shows one ester carbonyl group, two ethylenic carbons (–CH = CH–), four trisubstituted carbons bearing an oxygen atom (> CH–O–), eight $-CH_2-$ groups and one methyl group [5]. Hence, of the 28 hydrogen atoms of the molecule, 25 are bound to carbons. The ^1H NMR displays 28 protons, three of which undergo isotopic exchange with D_2O and must be bound to heteroatoms. The formation of a triacetate (**2**) confirms this point and shows there are three alcohol groups present. The five oxygen atoms are allotted as follows: three in secondary alcohol groups (> CHOH) and two in an ester of a secondary alcohol group (> CH–O–CO–). Two of the hydroxyl groups are vicinal as established by a positive reaction with periodic acid. Among the three unsaturations deduced from the molecular formula two are accounted for by the carbonyl group and the double bond: since no other sp^2 carbons are found, a ring system must be present. A short saturated linear chain is indicated from the ^1H NMR and IR spectra.

The molecular architecture can be defined from the analysis of reciprocal fine splittings between proton signals in the 250 MHz NMR spectrum. Coupling values and chemical shifts have been obtained by homonuclear spin decoupling experiments and results have been controlled by a calculation of the major spectrum systems. Some vicinal coupling constants, however, could not be accurately determined ($^3J < 0.2$ Hz). The results are shown in Table 2.

We have mentioned already the presence of four > CH–O– groups in **1**. The four protons H_c, H_d, H_e and H_f of these four groups are easily connected to the four signals found between δ 3.70 and 4.90 (Fig. 1). H_d is the X part of an ABX system, the AB part of which is constituted by H_g and H_h. Their chemical shift ($\delta \simeq 2.60$) indicates H_g and H_h to be vicinal to the lone carbonyl group of the molecule. H_d is further split with the olefinic protons H_a and H_b: $^3J_{H_dH_a} = 3.0$ Hz and $^4J_{H_dH_b} = 1.2$ Hz [6]. The adjacent oxygen atom and its conjugation with a double bond account for the deshielding of H_d. Similar consider-

Table 1. ^{13}C NMR spectral data for compound **1**

Carbon No.	δ_C^*	Fine structure $^1J_{C-H}$ (Hz)	Incorporation CH_3–$^{13}COONa$ 90%§	Incorporation $^{13}CH_3$–$COONa$ 90%§	Incorporation $^{13}CH_3$–$^{13}COONa$ $^1J_{C-C}$ (Hz) ‖	¶	T_1** (sec)
1	171.1	*s*	+	0	58.7	—	6.0
4	131.0	*d* 159	0	+	46.1	73	1.3
5	125.3	*d* 153	+	0	47.2	73	1.0
7	75.5	*d* 148	+	0	39	—	1.0
9	73.4†	*d* 144	+	0	38	37	0.9
6	73.4†	*d* 144	0	+	47.2	—	1.0
3	67.3	*d* 145	+	0	46.1	30	0.8
2	43.9	*t* 132	0	+	58.7	30	1.0
8	37.1‡	*t* 128	0	+	39	37	0.9
10	37.0‡	*t* 128	0	+	38	—	1.2
14	31.8	*t* 128	0	+	34	—	2.7
12	29.4	*t* 128	0	+	34	—	1.8
13	29.2	*t* 126	+	0	34	—	2.0
11	25.1	*t* 125	+	0	34	—	1.8
15	22.6	*t* 128	+	0	34.6	33.4	3.3
16	14.1	*q* 126	0	+	34.7	—	4.3

*Spectrum recorded in $CDCl_3$ at 20.1 MHz with TMS as int. reference.
†Split into two signals when D_2O added.
‡Assignment may be reversed.
§+, Enhanced signal, 0, non-enhanced signal.
‖Coupling constants of intact acetate units.
¶Interacetate coupling constants of adjacent carbons.
**T_1, relaxation time.

Table 2. ^1H NMR spectral data of **1** (250 MHz, $CDCl_3$, internal standard δ 7.27)

δ	No. of hydrogens	Band structure	(J)	Assignment of hydrogen	Coupled bands	Group
6.02	1	*ddd*	(15.9 – 3.0 – 1.8)	a	b, d, e	>CH=
5.68	1	*ddd*	(15.9 – 2.4 – 1.2)	b	a, e, d	>CH=
4.82	1	*dt*	(8.1 – 7.0)	c	i, jk	>CH–O–
4.76	1	*m*(*u*)		d	g, h, a, b	>CH–O–
4.57	1	*m*(*u*)		e	b, a	>CH–O–
3.77	1	*d*	(10.0)	f	i	>CH–O–
2.62	1	*dd*	(11.6 – 3.7)	g	h, d	$-CH_2-$
2.58	1	*dd*	(11.6 – 3.7)	h	g, d	$-CH_2-$
2.34	1	*ddd*	(15.7 – 10.0 – 8.1)	i	l, f, c	$-CH_2-$
2.25	3	*br s*		—	—	–OH × 3
1.54	2	*m*		jk	c	$-CH_2-$
1.48	1	*d*	(15.7)	l	i	$-CH_2-$
1.25	10	*m*(*u*)		m	jk	$-(CH_2)_5-$
0.88	3	*t*	(6.4)	n	m	–Me

Coupling constants are in order of decreasing magnitude from left to right and coupled band codes are in the same order (column 5).

ations may be made for H_e which is also split with the olefinic protons H_b and H_a. Thus, H_e must be vicinal to H_b. The large value of the coupling constant between H_a and H_b ($^3J_{H_aH_b}$ = 15.9 Hz) shows them to be in a *trans* configuration. All these data allowed structural unit **B** (Fig. 1) to be determined.

H_f though found at higher fields is also connected to an oxygen-bearing carbon atom. The spin coupling constant

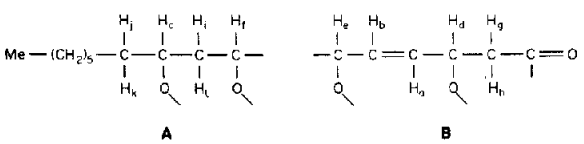


Fig. 1. Structural units.

with H_i can be determined ($^3J_{H_fH_i} = 10.0$ Hz), but those with H_e or H_l are very small (< 0.2 Hz). H_i and H_l, strongly non-equivalent are nevertheless situated on the same carbon atom giving rise to a characteristic geminal splitting ($^2J_{H_iH_l} = 15.7$ Hz). On the other hand, H_i is coupled with H_c: the value of the constant $J = 8.1$ Hz indicates a vicinal coupling. H_c, strongly shifted downfield is involved in a $>CH-O-\overset{O}{\overset{\|}{C}}-$ system and split with the $>C<^{H_j}_{H_k}$ group. This last methylene group is again linked to a short chain $Me-(CH_2)_5-$. We thus assign structure **A** (Fig. 1) to the second pattern.

There is only one possible way to connect structural units **A** and **B** (Fig. 1) taking into account all the data presented above: the carbon bearing H_e must be linked to that bearing H_f as shown in Fig. 2.

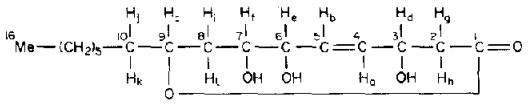
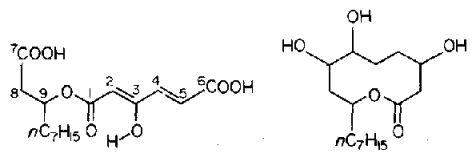


Fig. 2. Distribution of hydrogen and oxygen atoms on C-2–C-10 of **1**.

We now have to fix the three mobile protons upon three of the four oxygen atoms and close the cycle on the fourth. These four oxygen atoms are bonded onto C-3, C-6, C-7 and C-9, respectively. Cyclization at position 3 would produce a propiolactone which is not in agreement with the carbonyl absorption in the IR spectrum. Cyclization on position 6 is not possible since it introduces a *trans* double bond in a seven-membered ring. The positions left are 7 and 9. The chemical shifts of H_f and H_c linked to C-7 and C-9, respectively, differ by more than δ1: H_f must be assigned to the >CH–OH group, because of its upfield shift, H_c being involved in the >CH–O–CO group is shifted downfield. Thus the lactone ring must be closed at C-9.

Analysis of the ^{1}H NMR spectrum leads to structure **1**. Achaetolide is thus a novel unsaturated 10-membered hydroxylated lactone, related to diplodiolide A [7] and pyrenolide [8], but bearing in addition an apolar side chain.

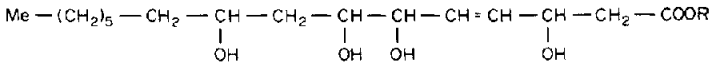
Further support for this structure is provided by the following chemical transformations. (1) Hydrolysis under basic conditions produces only one acid-alcohol compound (**3**), $C_{16}H_{30}O_6$, resulting from the expected opening of the lactone ring. This acid, **3**, via diazomethane, is transformed into the corresponding methyl ester **4**, $C_{17}H_{32}O_6$. (2) Periodic oxidation of achaetolide gives a mixture of aldehydes which on TLC as their hydrazone derivatives are separated into a major compound (**5**) and a minor one (**6**). (3) Oxidation of achaetolide with Jones' reagent gives a compound, $C_{16}H_{24}O_7$, with structure **7**. Only one >CH–O– group is found in its ^{13}C NMR spectrum giving rise to a signal at δ71.0 which is assigned to C-9. The ^{1}H NMR spectrum shows the proton linked to this carbon (δ5.37) to be split by two $-CH_2-$ groups: one on the side chain and the other (δ2.67) adjacent to a carbonyl. The ketone group at position 3 is in an enolic form. This point is supported by the presence of an ethylenic proton (on C-2) at δ5.70, a chelated hydroxyl at 11.54 and also a positive reaction with ferric chloride. Compound **7** arises from achaetolide after oxidation of the lone alcohol group into a ketone group, as expected, and oxidative cleavage of the α-glycol into two acid functions. (4) Catalytic hydrogenation of **1** produces a major compound, $C_{16}H_{30}O_5$ (**8**), resulting from the saturation of the achaetolide carbon carbon double bond.



7 **8**

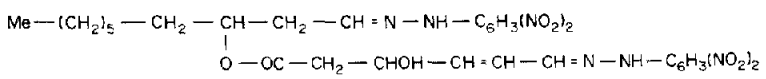
In the mass spectrum the *m/z* 282 ion **a** is produced by loss of a water molecule from the molecular ion, which as noted earlier, is not observed. The main fragmentation of ion **a** is the breaking of the bond between C-6 and C-7 (α-diol), the lactone ring being opened (Fig. 3). In this way the abundant ions **b** (*m/z* 155.1436, $C_{10}H_{19}O$, calc. 155.1440) and **d** (*m/z* 128.0473, $C_6H_8O_3$, calc. 128.0477) are produced, the latter resulting from a hydrogen transfer. Each is able to lose a water molecule to give, respectively ions **c** and **e**.

The longitudinal relaxation times (T_1) of the side chain carbon atoms (Table 1) are consistent with an isotopic intramolecular dipolar relaxation mechanism. The greater correlation time τ_c of the ring carbon explains their smaller T_1 values [9]. The relaxation time T_1 observed for the doubly protonated carbons in the macrocyclic ring is not twice as weak as the monoprotonated one: this may result from an anisotropic reorientation of the ring.



3 R = H

4 R = Me



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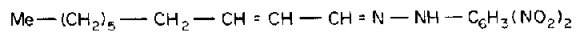

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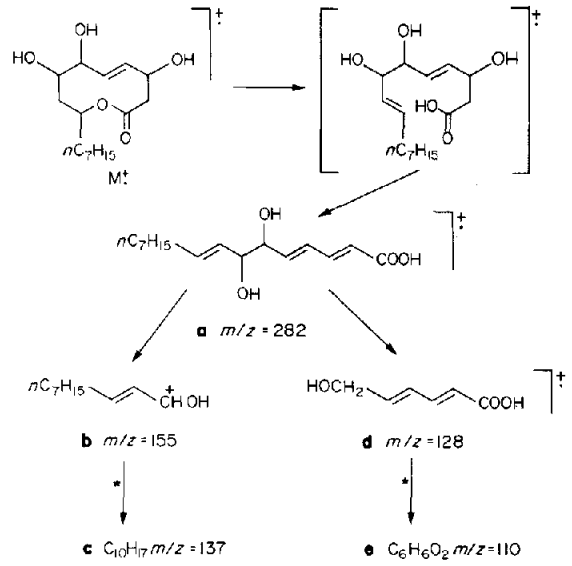

Fig. 3. Main fragmentations observed in the mass spectrum of achaetolide.

Biosynthesis

The ^{13}C NMR spectra of achaetolide enriched with [1-^{13}C], [2-^{13}C] and [1, 2-^{13}C]acetate (Table 1) are consistent with its formation, as expected by inspection of the structure, from a linear octaketide (Fig. 4).

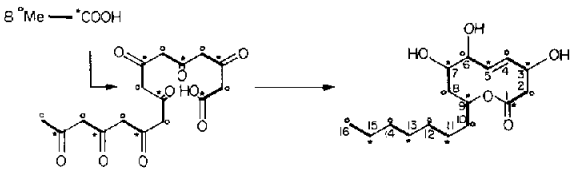

Fig. 4. Incorporation of acetate units into achaetolide.

EXPERIMENTAL

^1H NMR spectra were recorded at 250 and 80 MHz. and the ^{13}C NMR spectra at 20.115 MHz. T_1 was measured by inversion recovery sequence (180-τ-90-$5T_1$) on enriched achaetolides.

Isolation of achaetolide. Achaetomium cristalliferum Faurel & Locquin–Linard (strain PC 3252) was cultivated in Roux flasks each containing 180 ml Czapek–Dox medium, for 12 days at 29°. The mycelium was filtered off, and the filtered fermentation broth (2 l.) extracted (× 5) with Et_2O. The Et_2O layer was dried (Na_2SO_4) and the solvent removed under red. pres. The crude product (60 mg) was chromatographed over Si gel (hexane with increasing amounts of Me_2CO). Hexane–Me_2CO (5:3) eluted a mixture (45 mg) of achaetolide and another macrocyclic lactone, achaetolidone (**3**). Both compounds were further separated by prep. TLC, Si gel ($CHCl_3$–MeOH–Me_2CO, 45:3:2). The ratio achaetolide–achaetolidone was not constant over several runs.

Incorporation experiments. A. cristalliferum was grown on Czapek–Dox medium distributed in five Roux flasks (180 ml/flask) for 12 days at 29°. The different precursors (see Table 1) (0.5 g) were dissolved in sterile H_2O (25 ml) and introduced 3 days after inoculation.

Achaetolide (**1**). Mp 122° (Me_2CO); $[\alpha]_D^{21}$ − 19.3° (EtOH; *c* 1.46); IR ν_{max}^{KBr} cm^{-1}: 3450, 2960, 2925, 2860, 1718, 1470, 1410, 1260, 1170, 1125, 1050, 1030, 985, 965, 950; MS (70 eV, 150°) *m/z* (rel. int.): 283 (6), 282 [M − H_2O]$^+$ (19), 215 (8), 199 (2), 194 (2), 185 (5), 173 (2), 171 (4), 155 (65), 154 (8), 153 (9), 139 (4), 138 (11), 137 (72), 136 (2), 135 (4), 131 (13), 130 (4), 129 (28), 128 (94), 127 (35), 126 (6), 123 (7), 115 (6), 113 (6), 112 (9), 111 (35), 110 (100), 109 (12), 99 (19), 97 (20), 96 (19), 95 (84), 87 (25), 86 (87), 85 (43), 84 (23), 83 (77), 82 (61).

Triacetyl achaetolide (**2**). Achaetolide (64.4 mg) was dissolved in pyridine–Ac_2O (1:1; 1.6 ml) and kept 30 min at 40°. H_2O was added to the mixture and the crude product was chromatographed (Si gel–$CHCl_3$) to give **2** (71 mg) as an oil. IR ν_{max}^{KBr} cm^{-1}: 1740 (*br*); ^1H NMR($CDCl_3$): δ5.69 (4H, *m*, –CH = CH + 2 >CH–OCO), 4.85 (2H, *m*, 2 >CH–OCO), 2.61 (2H, *m*, –CH_2–CO), 2.16 (3H, *s*), 2.10 (3H, *s*), 2.00 (3H, *s*), (3 Me–COO–), 1.50–1.00 (14H, *m*, 7 –(CH_2)–), 0.87 (3H, *t*, *J* = 6.5 Hz, CH_3–CH_2); MS (70 eV, 150°) *m/z*: 426 [M]$^+$.

Alkaline hydrolysis of achaetolide. KOH (5 ml; 1 N) was added to **1** (30 mg) dissolved in MeOH (1.5 ml). The mixture was stirred at room temp. for 5 hr, then acidified with 1 M HCl and poured into a satd NaCl soln and extracted with Et_2O several times. The crude product (26 mg) from the Et_2O extracts was recrystallized (Et_2O) to give 3,6,7,9-tetrahydroxy-4-hexadecenoic acid **3**, $C_{16}H_{30}O_6$, mp 123–124°. IR ν_{max}^{KBr} cm^{-1}: 1700.

The acid **3** was converted into its methyl ester, **4**, by treatment with Et_2O–CH_2N_2. Compound **4**, $C_{17}H_{32}O_6$, mp 75–76° (Et_2O); IR ν_{max}^{KBr} cm^{-1}: 1735; ^1H NMR ($CDCl_3$): δ5.83 (2H, *m*, –CH = CH–), 4.58–3.70 (4H, *m*, 4 > CH–OH), 3.71 (3H, *s*, OMe), 3.50 (4H, *br s*, –OH), 2.57 (2H, *d*, *J* = 6.6 Hz, –CH_2–COO), 1.26 (14H, *m*, 7 CH_2), 0.86 (3H, *t*, *J* = 6 Hz, Me).

Periodic oxidation of **1**. KIO_4 (35 mg) in 0.5 M H_2SO_4 (1.5 ml) was added to a stirred soln of achaetolide (30 mg) in MeOH (12 ml). After 24 hr, H_2O was added and the soln extracted with Et_2O. The product recovered from the extract was dissolved in EtOH (5 ml). A soln (EtOH) of 2,4-dinitrophenylhydrazone and a drop of HCl were added. The mixture was heated for a few min then cooled, poured onto H_2O and extracted with Et_2O. The gummy product obtained was separated by prep. TLC on Si gel with toluene–HOAc (17:3). The more polar product **5** (R_f 0.33) was crystallized from EtOH–H_2O, mp 79–80°, $C_{28}H_{34}N_8O_{11}$. ^1H NMR ($CDCl_3$): δ11.10 (1H, *s*, NH), 11.04 (1H, *s*, NH), 9.10 (2H, *m*), 8.31 (2H, *m*) and 7.91 (2H, *m*) (6H arom.), 7.74 (1H, *d*, *J* = 8.5 Hz, –NH = CH–), 7.50 (1H, *t*, *J* = 5.7 Hz, –NH = CH–), 6.57 (1H, *ddd*, *J* = 15.7, 8.5, 1.5 Hz) and 6.20 (1H, *dd*, *J* = 15.7, 4.5 Hz) (–CH = CH–), 5.29 (1H, *tt*, *J* = 5.7, 6.0 Hz, >CH–O–), 4.76 (1H, *ddt*, *J* = 6.2, 4.5, 1.5 Hz, >CH–O), 2.66 (2H, *d*, *J* = 6.2 Hz, CH_2), 2.66 (2H, *t*, *J* = 5.7 Hz, CH_2), 1.26 (12H, *m*, –(CH_2)$_6$–), 0.87 (3H, *t*, *J* = 6.5 Hz, Me).

The less polar product, **6** (R_f 0.87) had mp 118–120° ($CHCl_3$). ^1H NMR ($CDCl_3$): δ11.05 (1H, *br s*, NH), 9.11 (1H, *d*, *J* = 2.5 Hz, Ar), 8.32 (1H, *dd*, J_1 = 2.5 Hz, J_2 = 10 Hz, Ar), 7.93 (1H, *d*, *J* = 10 Hz, Ar), 7.75 (1H, *m*, –N = CH–), 6.33 (2H, *m*, –CH = CH–), 2.22 (2H, *m*, –CH_2–C =), 1.25 (10H, *br* signal, 5 CH_2), 0.86 (3H, *t*, *J* = 6 Hz, Me).

Oxidation of **1** *with Jones' reagent.* Achaetolide (49 mg) in Me_2CO (10 ml) was treated with Jones' reagent at room temp. for 20 min. After the addition of H_2O the product was recovered in Et_2O, and chromatographed on Si gel ($CHCl_3$–HOAc, 49:1) to give **7**: crystallized from Me_2CO–H_2O (mp 107.5–108.5°), $C_{16}H_{24}O_7$. ^1H NMR ($CDCl_3$): δ11.54 (1H, *s*, OH), 6.97 (1H, *d*, *J* = 15 Hz), 6.63 (1H, *d*, *J* = 15 Hz) (–CH = CH–), 5.37 (1H, *tt*, J_1 = J_2 = 6.5 Hz, >CH–O–CO), 5.30 [1H, *s*, –CH = C(OH)–],

2.67 (2H, *d*, $J = 6.5$ Hz, $-CH_2-CO$), 1.61–1.00 [12H, *br* signal, $-(CH_2)_6-$], 0.86 (3H, *t*, $J = 6$ Hz, Me).

Catalytic hydrogenation of **1**. Achaetolide (58 mg) in EtOH (20 ml) was hydrogenated over 10% Pd–C for 10 hr to give a mixture which on prep. TLC (toluene–HOAc, 17:3) (R_f 0.07) gave **8**: crystallized ($CHCl_3$) (mp 89–91°), $C_{16}H_{30}O_5$. ^1H NMR ($CDCl_3$): δ 4.97 (1H, *m*, >CH–O–CO), 4.08 (1H, *m*, >CH–OH at C-3), 3.85 (1H, *m*) 3.46 (1H, *m*) (2 >CH–OH), 3.00 (3H, *br s*, OH), 2.83 (1H, *dd*, $J_1 = 4.1$ Hz, $J_2 = 14.9$ Hz) 2.49 (1H, *dd*, $J_1 = 7.8$ Hz, $J_2 = 14.9$ Hz) (CH_2 at C-2), 2.10–1.00 (18H, *br*, 9 CH_2), 0.86 (3H, *t*, $J = 6$ Hz, Me).

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