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CHEMICAL STUDIES OF NORTH ADRIATIC SEAWEEDS—I

NEW DOLABELLANE DITERPENES FROM THE BROWN ALGA *DILOPHUS FASCIOLA*

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Abstract—From the brown alga *Dilophus fasciola* (Dictyotaceae) three new tetraoxygenated diterpenes (1–3) with a dolabellane skeleton were isolated. The structure of these was determined by spectral data and by means of chemical transformation. Two-dimensional NMR spectroscopy was proved to be a potent tool to establish the structures, the stereochemistry and to enable the assignment of all resonances in the NMR spectra. The new compounds were tested in ichthyotoxic and phytotoxic bioassays.

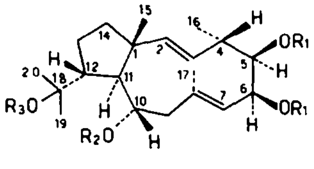
We have started to study the algae of North-Adriatic sea, because it is characterized by a peculiar ecological habitat, in the Mediterranean area, especially due to the less salinity and larger temperature variations. In the course of our studies we have isolated three new diterpenes with dolabellane skeleton (1–3) from the brown alga *Dilophus fasciola* (Roth) Howe (Dictyotaceae) collected near Rovinj, Yugoslavia. The isolation of a diterpene of mixed biogenesis (geranylgeranyl glycerol)[1] and of some sesquiterpenes from the same alga, collected in the Tyrrhenian sea, is already described in literature.[2–4]

Diterpenes with dolabellane skeleton had been originally isolated from the herbivorous sea hare *Dolabella california*.[5] Later, they were isolated also from the brown algae of family Dictyotaceae,[6–8] from the sea whip *Eunicea calyculata*[9] and recently from the mollusc *Aplysia dactylomela*.[10] In this paper the isolation and structure determinations of three new dolabellane diterpenes 1–3, from *D. fasciola*, are reported.

The alga was dried at 60° and the chloroform soluble part, of chloroform–methanol extract, was chromatographed on Si-gel, using increasing concentrations of diethyl ether in light petroleum as the eluent. Three compounds 1–3 were obtained. An initial examination of the spectral data revealed that the three compounds are very similar, only differing in the number of acetate groups. In fact, through a series of simple chemical conversions, the relations among them were determined.

Using new techniques of two-dimensional (2D) NMR, together with the classic spectroscopic techniques, we were able to establish the structure of the new compounds (1–3) and to assign all the chemical shifts for 1. In structural studies of 1, three different 2D NMR methods were used: proton–proton correlation[11] (COSY); two dimensional exchange spectroscopy[12] (NOESY); homonuclear J-resolved spectroscopy[13] (^1H J-resolved).

Diacetate 1 was converted under mild conditions (Acetic anhydride/Pyridine at room temp) to triacetate 2. At same time, the acetylation under more rigorous conditions (Acetic anhydride/Pyridine under reflux) converted both diacetate 1 and triacetate 2 to tetraacetate 3. The alkaline hydrolysis of 1–3 yielded the same tetrol 4.



1 $R_1 = Ac$; $R_2 = R_3 = H$

2 $R_1 = R_2 = Ac$; $R_3 = H$

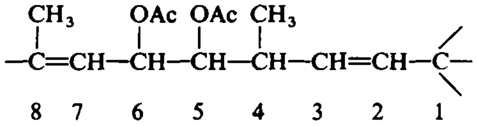
3 $R_1 = R_2 = R_3 = Ac$

4 $R_1 = R_2 = R_3 = H$

Compound 1 m.p. 201–203°; $[\alpha]_D = +11.1$ had the molecular formula $C_{24}H_{38}O_6$, from the high resolution mass measurement of the parent ion. The presence of bands at 3500, 1730 and 1230 cm^{-1} in the IR spectrum and two acetate Me signals in the ^1H-NMR at δ 2.00 and 2.16, strongly suggested that 1 was a diacetate of a tetrol. A signal in the ^1H-NMR at δ 3.48 and in the ^{13}C-NMR spectrum at 71.2 ppm (d), indicated the presence in the molecule of a secondary alcohol. The presence of two Me singlets in the ^1H-NMR at δ 1.23 and 1.29, together with a signal in the ^{13}C-NMR spectrum at 72.9 ppm (s) indicated the presence of an isopropyl alcohol moiety. The ^1H-NMR spectrum showed also: three Me signals, a singlet at δ 1.01 (Me on quaternary C), a doublet at δ 1.10 (J = 7.1 Hz) and a broad singlet at δ 1.78 (vinyl Me); two α-acetoxy proton signals at δ 5.68 (br, d, J = 10.0 Hz) and 5.17 (br, d, J = 5.8 Hz) and three vinyl protons absorbing at δ 5.08 (d, J = 16.4 Hz), 5.37 (br, d, J = 10.0 Hz) and 5.48 (dd, J = 16.4 and 5.3 Hz). The signals at 138.2 (d), 136.5 (s), 126.5 (d) and 123.6 (d) ppm in the ^{13}C-NMR spectrum also confirmed the presence of a disubstituted and a trisubstituted double bonds. The occurrence of signals at 77.6 (d) and 69.0 (d) ppm afforded an additional proof for the proposed two secondary acetates. Moreover, the ^{13}C-NMR spectrum showed three signals due to methylene groups at 46.3, 39.9 and 26.0 ppm and a quaternary C at 47.3 ppm.

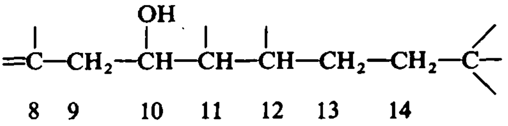
Hence, **1** having six degrees of unsaturation, must possess a carbobicyclic skeleton.

The COSY spectrum (Fig. 1) which identifies the pairs of resonances that are coupled together, allowed readily to establish the partial structure A.



A

Moreover, the COSY spectrum showed that the α-OH proton at δ 3.48 (H-10) was correlated with a methyne proton (H-11) (δ 1.46) and at the same time with the nonequivalent vinylic methylene protons (δ 2.16 and 2.45). Furthermore, the methyne proton (H-12) at δ 2.57 was correlated with H-11 (δ 1.46) and also with the nonequivalent methylene protons (δ 1.29 and 1.93). It was correlated with other nonequivalent methylene protons (δ 1.40 and 1.60). These data are in accordance with the partial structure B.



B

The spectral data quoted so far are in accordance with the bicyclic structure **1** with fused 11- and 5-membered rings. The proposed structure fits very well to the biogenetic rule. It should also be noted that the ^{13}C chemical shifts (Table 1) assigned by a series of selective decoupling in the ^{13}C spectrum are in good agreement with structure **1**.

The stereochemistry was deduced from the following evidence. The NOESY spectrum exhibited the presence of NOEs, as reported in Table 2, indicating that the bridgehead Me at C-1 and the H-3 have a β orientation, while the Me-4, Me-8, H-2, H-6 and H-11 are α. The presence of NOE between the H-11 and one of two Me's

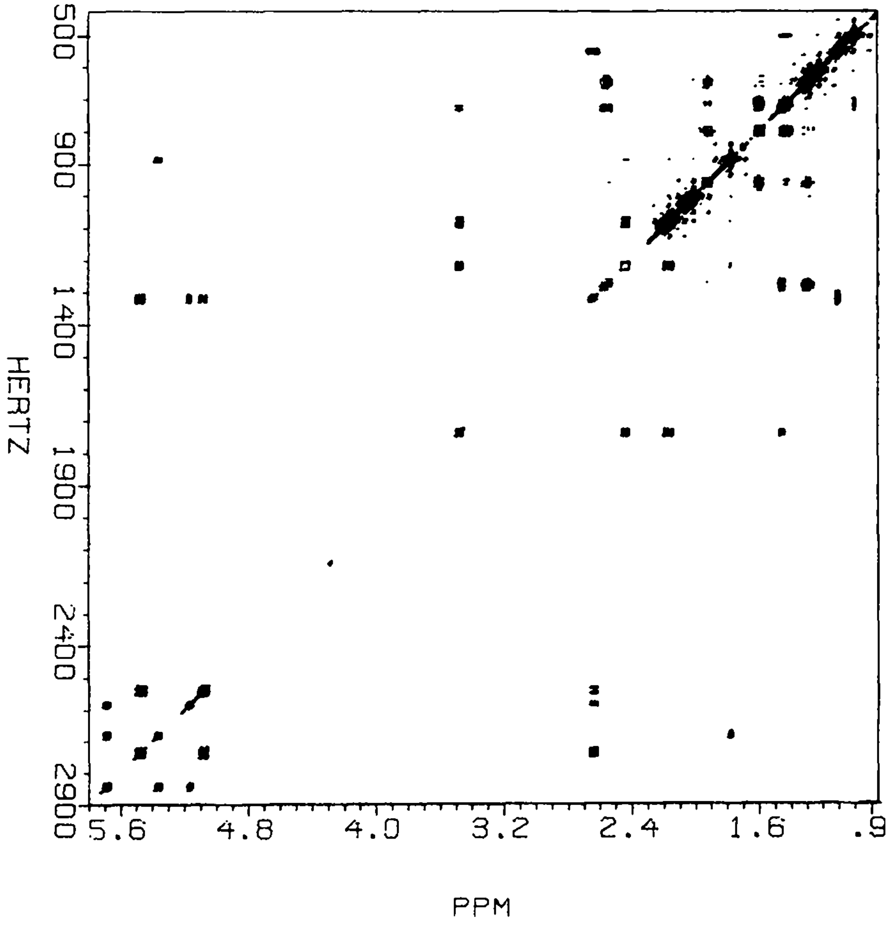


Fig. 1. Symmetrized contour plot of a 500 MHz absolute value COSY-90 spectrum of a 0.2 M solution of 1 in $CDCl_3$, T = 298°K. Sixteen FID's have been coadded for each of the 512 values with appropriate phase cycling program[14]. A 1024 × 2048 data matrix has been Fourier transformed with sine-bell filters in both domains, using DISNMR software. The digital resolution is 2.42 Hz/point in both domains.

The diagonal signals at 4.30 and 1.70 ppm originate from folded-over $CHCl_3$ and TMS, respectively.

Table 1. Carbon-13 and proton chemical shifts^ for 1

N.C	Carbon	Proton	^{1}H J-Resolved†
C-1	47.3 s	—	—
C-2	138.2 d	5.08 br d (16.4)	dd (16.4, 1.8)
C-3	126.5 d	5.48 dd (16.4, 5.3)	dd (16.4, 5.3)
C-4	35.4 d	2.64 m	m
C-5	77.6 d	5.17 br d (5.8)	dd (5.8, 2.2)
C-6	69.0 d	5.68 br d (10.0)	dd (10.0, 2.2)
C-7	123.6 d	5.37 br d (10.0)	dt (10.0, 1.8)
C-8	136.5 s	—	—
C-9	46.3 t	2.16$^+$m 2.45 br d (12.0)	m dd (12.0, 6.8)
C-10	71.2 d	3.48 m	m
C-11	54.3 d	1.46$^+$m	m
C-12	47.7 d	2.57 m	ddd (11.2, 11.2, 7.4)
C-13	26.0 t	1.29$^+$m 1.93 m	m m
C-14	39.9 t	1.40$^+$m 1.60 m	m ddd (12.8, 9.0, 2.4)
C-15	17.1 q	1.01 s	s
C-16	14.7 q	1.10 d (7.1)	d (7.4)
C-17	21.2 q	1.78 br s	d (1.8)
C-18	72.9 s	—	—
C-19	23.2 q	1.23 s	s
C-20	31.5 q	1.29$^+$s	s
2 C<u>H</u>$_3$CO	21.06 q 21.02 q	2.00 s 2.16$^+$s	s s
2 CH$_3$<u>C</u>O	170.4 s 170.2 s	— —	— —

^Referred to tetramethylsilane. Multiplicities are indicated by usual symbols. Coupling constants (hertz) are in parentheses.

$^+$ Overlapped with other signals.

† The 2D J-resolved spectrum was recorded by coaddition of 16 scans with appropriate phase cycling program[14] at each of 32 t_1 values. A 128 × 8192 data matrix is Fourier transformed in the same manner as the spectrum from Fig. 1. Only the values corresponding to the clearest cross-sections of tilted ^{1}H J-resolved spectrum are reported.

Table 2. Magnetization exchange by cross relaxation (NOE) for 1 in $CDCl_3$, as obtained from NOESY[12,14]

Cross peak co-ordinates below the diagonal $^\delta$(x)–$^\delta$(y)	Protons correlated
1.01–5.48	Me-1; H-3
1.10–5.08	Me-4; H-2
1.10–5.68	Me-4; H-6
1.78–5.08	Me-8; H-2
1.78–5.68	Me-8; H-6
1.46–1.78	H-11; Me-8
1.46–5.08	H-11; H-2
1.40–5.08	H-14; H-2
1.23–1.46	Me-19; H-11

^{1}H NOESY spectrum is recorded at 500 MHz on the same sample as on Fig. 1, with the mixing time $\tau_m = 1\ s \pm 100\ ms$ (randomly modulated[12c,14]). Only the cross-peaks not sensitive to strong filtering were reported. (Random modulation of the mixing time and the independence of the cross-peak's integral intensity on the strength of filtering function ensures cross-relaxation origin of reported cross-peaks[12c,14]).

(δ 1.23) of the isopropyl alcohol moiety suggested an α orientation for this group, moreover, it indicated a partially hindered rotation along the C-12, C-18 bond, probably due to an intramolecular H-bond between the OH groups at C-10 and C-18. If this interpretation is correct the OH group at C-10 must be α orientated.

From the NOESY experiment we could conclude the 11-membered ring of 1 to be more rigid than expected, in accordance with the previous studies[5b] concerning dolabellane skeleton. The *E* configuration of C-2 double bond was based on the magnitude of the vicinal coupling constant (16.4 Hz) between the vinyl hydrogens. The same configuration for the C-7 double bond is supported by the lack of NOE between H-7 and Me-8 and also by chemical shift of Me-8 (21.2 ppm) and C-9 (46.3 ppm) in the ^{13}C-NMR spectrum. The coupling constant $J_{5,6}$ of 2.2 Hz indicated H-5 and H-6 to be *cis*. This was also confirmed by fast lead tetraacetate cleavage on the tetrol 4. Finally, the application of the Horeau method allowed to determine the chirality at C-10 in 1 as *S*. On the whole, we determine the structure of 1 as: (1*R*, 2*E*, 4*S*, 5*R*, 6*S*, 7*E*, 10*S*, 11*S*, 12*R*) - 5,6 - diacetoxy - 10,18 - dihydroxy - 2,7 - dolabelladiene.

Compound 2, isolated as an oil, $[\alpha]_D = -25.4$, had the molecular formula $C_{26}H_{40}O_7$ (precise mass measurement). The ^{1}H-NMR spectrum of 2 was similar to that of diacetate 1, except for the presence of an additional acetate Me signal (δ 2.09) and of another α-acetoxy proton at δ 4.84, rising from the acetylation shift of the α-OH proton at δ 3.48 in the ^{1}H-NMR spectrum of 1. Thus, together with the remaining spectroscopic evidence (particularly that concerning ^{13}C-NMR data, see Experimental) as well as the quoted chemical evidence (i.e. $1 \xrightarrow{Ac_2O/Py} 2$), enabled establishment of 2 as 10-monoacethyl derivative of 1.

Compound 3, isolated as an oil, in minor amount than the above compounds, $[\alpha]_D = +1.6$, had the molecular formula $C_{28}H_{42}O_8$ (precise mass measurement). The ^{1}H- and ^{13}C-NMR data of 3 (listed in the Experimental) together with the chemical evidence

$$\text{(i.e. } 1 \xrightarrow[\text{reflux}]{Ac_2O/Py} 3 \xleftarrow[\text{reflux}]{Ac_2O/Py} 2\text{)}$$

are in accordance with the structure of 10,18-diacetyl derivative of 1 for this compound.

Compounds 1–3 when subjected to the fish toxicity bioassay (at room temp) were proved to be active at a concentration of 50 ppm, causing the death within 8 hr. Being known that some herviborous molluscs feed on brown algae of the genus *Dictyota*[6] the above ichthyotoxicity provides for molluscs a significant advantage against predators.

Further the compounds 1–3 were proved to be phytotoxic, at a concentration of 100 ppm, showing a decreasing of root grown of 60% in the Van Onckelen[15] *et al.* bioassay.

EXPERIMENTAL

General procedures. ^{1}H- and ^{13}C-NMR spectra were recorded at 500 and 125 MHz, respectively, with TMS as internal standard on a Bruker WM 500, under ASPECT 2000 control. IR spectra were recorded on a Perkin–Elmer Model 257 Infracord. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter, using a 10 cm-microcell. Low-resolution and high-resolution mass spectra were recorded on an AEI MS-30 and on an AEI MS-5 spectrometers, respectively. M.ps were measured on a Kofler apparatus and are reported uncorrected. TLC were carried out using pre-coated silica gel F_{254} plates (Merck). Kieselgel 60 (Merck) was used for chromatography. *Gambusia patruelis* (Baird Grd) (length *ca* 1 cm) and commercial *Hordeum vulgaris* seed were used for bioassays.

Extraction and isolation of compounds. The alga *Dilophus fasciola* (Roth) Howe collected in June 1983 at Lone Bay near Rovinj (YU), was dried at 60°. The dried alga (130 g after extraction) was extracted in soxhlet with $CHCl_3$: MeOH (1 : 1). The extract was evaporated *in vacuo* to obtain a brown gum (11.6 g) that was dissolved in $CHCl_3$. The $CHCl_3$ soluble part (8.2 g) after evaporation of solvent was applied on a column (5 × 100 cm) of Si-gel. The column was eluted with a solvent gradient system from light petroleum 40–70° to diethyl ether. Fractions of 40 ml were collected. The fractions 73–77 were combined to give an oil, that was rechromatographed on silica gel, using $CHCl_3$ as eluent, to obtain 3 (96 mg, 0.07% dry wt), as an oil, single spot on TLC. The fractions 90–97 contained 2 as pure oil (500 mg, 0.38% dry wt), while from the fractions 103–119, after crystallization from $CHCl_3$ was recovered 1 (210 mg, 0.16% dry wt).

(1R,2E,4S,5R,6S,7E,10S,11S,12R) - 5,6,10,18 - *Tetraacetoxy - 2,7 - dolabelladiene* (3): $[\alpha]_D + 1.6$ ($c = 2.5$ $CHCl_3$); IR ($CHCl_3$) 1730, 1230 cm^{-1}; mass spectrum *m/z* 506.2881 (4%) (M$^+$ calc for $C_{28}H_{42}O_8$, 506.2879), 446 (3%), 404 (5%), 386 (35%), 344 (15%), 326 (52%), 283 (61%), 266 (100%), 251 (55%), 225 (30%), 223 (46%), 173 (93%); ^{1}H-NMR ($CDCl_3$) δ 0.84 (s, 3H), 1.09 (d, 3H, J = 7.1 Hz), 1.24 (m, 1H), 1.38 (s, 3H, overlap m, 1H), 1.50–1.60 (m, 3H), 1.61 (s, 3H), 1.79 (br, s, 3H), 1.92 (s, 3H), 1.94 (m, 1H), 2.02 (s, 3H), 2.06 (s, 3H), 2.15 (m, 1H), 2.17 (s, 3H), 2.37 (m, 1H), 2.68 (m, 1H), 3.14 (m, 1H), 4.80 (br, d, 1H, J = 10.2 Hz), 5.07 (dd, 1H, J = 16.4, 5.3 Hz), 5.14 (br, d, 1H, J = 5.8 Hz), 5.54 (dd, 1H, J = 16.4, 5.3 Hz), 5.58 (d, 1H, J = 10.0 Hz) and 5.71 (br, d, 1H, J = 10.0 Hz); ^{13}C-NMR ($CDCl_3$) (off resonance mult.) ppm 170.6 (s), 170.3 (s), 170.2 (s), 170.1 (s), 137.4(d), 134.6 (s), 128.2 (d), 125.3 (d), 84.7 (s), 77.7 (d), 72.9 (d), 69.1 (d), 52.4 (d), 48.2 (s), 44.5 (d), 41.6 (t), 39.9 (t), 35.4 (d), 26.7 (t), 26.4 (q), 23.4 (q), 23.0 (q), 21.2 (q), 16.8 (q) and 15.2 (q).

(1R,2E,4S,5R,6S,7E,10S,11S,12R) - 5,6,10 - *Triacetoxy - 18 - hydroxy - 2,7 - dolabelladiene* (2): $[\alpha]_D - 25.4$ ($c = 1.6$, $CHCl_3$); IR ($CHCl_3$) 3500, 1730, 1230 cm^{-1}; mass spectrum *m/z* 464.2772 (1%) (M$^+$ calc for $C_{26}H_{40}O_7$, 464.2774), 418 (2%), 404 (4%), 386 (4%), 344 (8%), 326 (10%), 302 (20%), 284 (40%), 266 (44%), 251 (32%), 226 (100%), 211 (50%), 173 (60%); ^{1}H-NMR ($CDCl_3$) δ 0.87 (s, 3H), 1.10 (d, 3H, J = 7.1 Hz), 1.17 (s, 3H), 1.28 (s, 3H), 1.41 (m, 1H), 1.56 (m, 2H), 1.79 (br, s, 3H), 1.93 (m, 1H), 2.01 (s, 3H), 2.09 (s, 3H), 2.17 (s, 3H), 2.34 (m, 2H), 2.41 (m, 1H), 2.67 (m, 1H), 4.84 (br, d, 1H, J = 10.2 Hz), 5.08 (br, d, 1H, J = 16.4 Hz), 5.16 (br, d, 1H, J = 5.8 Hz), 5.54 (br, d, 1H, J = 10.0 Hz), 5.56 (dd, 1H, J = 16.4, 5.3 Hz) and 5.71 (br, d, 1H, J = 10.0 Hz); ^{13}C-NMR ($CDCl_3$) ppm 170.3 (s), 169.9 (s), 169.1 (s), 137.5 (d), 134.6 (s), 127.9 (d), 125.1 (d), 77.6 (d), 73.5 (d), 72.7 (s), 69.1 (d), 52.4 (d), 48.8 (d), 47.8 (s), 41.5 (t), 40.0 (t), 35.3 (d), 31.6 (q), 26.8 (t), 22.8 (q), 21.2 (q), 20.9 (q), 16.8 (q) and 15.1 (q).

(1R,2E,4S,5R,6S,7E,10S,11S,12R) - 5,6 - *Diacetoxy - 10,18 - dihydroxy - 2,7 - dolabelladiene* (1): m.p. 201–203°; $[\alpha]_D + 11.1$ ($c = 2.1$ $CHCl_3$); IR ($CHCl_3$) 3500, 1730, 1230 cm^{-1}; mass spectrum *m/z* 422.2670 (1%) (M$^+$ calc for $C_{24}H_{38}O_6$, 422.2668), 404 (10%), 387 (12%), 362 (16%), 345 (10%), 344 (15%), 327 (5%), 302 (100%), 287 (13%), 285 (14%), 284 (15%), 269 (22%) and 251 (9%); ^{1}H-NMR and ^{13}C-NMR (Table 1).

Partial acetylation of 1. 1 (50 mg) was dissolved in Ac_2O (5 ml) and pyridine (0.5 ml) and the mixture was kept at room temp for 24 hr. The excess reagents were removed *in vacuo* and the residue was partitioned between water and diethyl ether. The ether extracts were dried over Na_2SO_4 and the solvent was evaporated, to yield an oil which was purified on a Si-gel column, using light petroleum : diethyl ether (7 : 3) as eluent. The purified triacetate (45 mg), was identical in all respects with the natural product 2.

Peracetylation. Peracetylation of 1 (50 mg) and 2 (50 mg) was carried out with Ac_2O (5 ml) and pyridine (0.5 ml) at reflux for 2 hr. The mixtures were evaporated *in vacuo* and purified on a Si-gel column, using light petroleum : diethyl ether (7 : 3) as eluent, to obtain in both cases a tetraacetate (38 mg and 41 mg respectively), identical in all respects with the natural product 3.

Hydrolysis. Hydrolysis of 1 (50 mg), 2 (50 mg) and 3 (25 mg) was carried out with KOH 5% in MeOH/H_2O (1 : 1) (5 ml) at 50° for 4 hr. The mixtures were neutralized with HCl 1N and extracted with diethyl ether (3 × 10 ml). The ether extracts were dried over Na_2SO_4, the solvent was evaporated *in vacuo*. The crystallization from $CHCl_3$, afforded in all cases the tetrol 4 (36 mg, 32 mg and 18 mg respectively): m.p. 173–175°; $[\alpha]_D - 5.1$ ($c = 1.2$, $CHCl_3$); IR (nujol) 3500 cm^{-1}; mass spectrum *m/z* 320 (15%) (M$^+$-18), 302 (17%), 286 (12%), 235 (29%), 224 (44%), 206 (100%) and 151 (90%); ^{1}H-NMR ($CDCl_3 + CD_3OD$) δ 1.01 (s, 3H), 1.04 (d, 3H, J = 7.1 Hz), 1.21 (s, 3H, overlapping m, 1H), 1.27 (s, 3H, overlapping m, 1H), 1.40–1.44 (m, 2H), 1.58 (m, 1H), 1.70 (br, s, 3H), 1.93 (m, 1H), 2.14 (m, 1H), 2.43 (br, d, 1H, J = 12.0 Hz), 2.55 (m, 1H), 2.68 (m, 1H), 3.53 (m, 1H), 3.92 (br, d, 1H, J = 5.8 Hz), 4.43 (br, d, 1H, J = 10.0 Hz), 4.98 (br, d, 1H, J = 16.4 Hz), 5.41 (br, d, 1H, J = 10.0 Hz), 5.61 (dd, 1H, J = 16.4, 5.3 Hz); ^{13}C-NMR ($CDCl_3 + CD_3OD$) ppm 137.2 (d), 133.2 (s), 128.4 (d), 127.4 (d), 78.2 (d), 73.8 (s), 72.1 (d), 70.5 (d), 54.2 (d), 47.2 (d), 47.0 (s), 45.8 (t), 39.7 (t), 37.1 (d), 31.0 (q), 25.9 (t), 22.5 (q), 20.5 (q), 17.0 (q), 14.9 (q).

Lead tetraacetate cleavage of tetrol 4. Compound 4 (30 mg) was dissolved in MeOH (4 ml) and AcOH (0.5 ml). After addition of lead tetraacetate (50 mg) the mixture was kept at room temp for 3 hr. Upon addition of water (2 ml) the products were extracted with diethyl ether (3 × 5 ml). The ether extracts were dried over Na_2SO_4 and the solvent was evaporated *in vacuo* to yield an oil. Examination of the oil using TLC, indicated the presence of a major component, giving rise to UV visible (at 254 nm) spot, giving the positive test with dinitrophenylhydrazine reagent.[16] All attempts to separate the mixture on silica gel resulted in complete destruction of the compound. UV (MeOH) of oil λ_{max} 240 nm; IR (film liquid) of oil 3500, 1730, 1700 cm^{-1}.

Application of the Horeau method. 1 (18 mg) was treated with an excess of racemic α-phenylbutyric anhydride (40 mg) in pyridine (2 ml). Conventional workup[17] led to the isolation of ($-$)α-phenylbutyric acid (optical yield 5.1%).

Bioassays

Fish toxicity bioassay. Compounds **1–3** were dissolved in fresh water using Tween 80 (1 drop) at the concentration of 50 ppm. Two fishes, *Gambusia patruelis* (Baird Grd) were placed in each tank, at the room temp, while a tank containing only fresh water and the same concentration of Tween 80 was used as control.

Phytotoxicity bioassay.[15] In the root growth bioassay technique the samples **1–3** were dissolved in 0.5 ml EtOH and diluted with H_2O to 100 ppm. The soln was poured into 10 Petri dishes, with Whatman grade 113 paper as support and 10 *Hordeum vulgaris* seeds placed on each plate. After 4 days at 20° in the dark, the length of the main root was measured and compared with that of control plants.

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