Journal of Natural Products
Vol. 58, No. 7, pp. 986-991, July 1995

BARCELONEIC ACID A, A NEW FARNESYL-PROTEIN TRANSFERASE INHIBITOR FROM A *PHOMA* SPECIES

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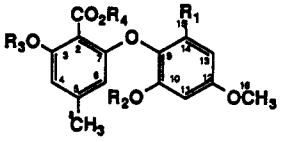
ABSTRACT.—Three new diphenyl ethers, barceloneic acids A, B, and barceloneic lactone [**1**, **2**, and **3**, respectively] were isolated from a fermentation extract of a fungus of the genus *Phoma*. The structures of compounds **1–3** were determined by a combination of spectroscopic and single-crystal X-ray diffraction methods. The effect of these compounds on the inhibition of farnesyl-protein transferase (FPTase) was evaluated and results are presented. Barceloneic acid A [**1**] is a novel and modest inhibitor of FPTase with an IC_{50} value of 40 μM.

Inhibitors of farnesyl-protein transferase (FPTase) have potential as anticancer agents for tumors in which the *Ras* gene is mutated and contributes to cell transformation (1). We have been interested for some time in finding novel and non-peptide natural product inhibitors of FPTase. From the screening of natural products from microbial sources, we have recently reported the discovery of chaetomellic acids A and B produced by *Chaetomella acutisetae* as potent and specific inhibitors of FPTase with IC_{50} values in the nanomolar range (2). More recently, we have also discovered fusidienol as a potent inhibitor of the enzyme (3). Several other groups have also reported both rationally designed compounds and natural products as inhibitors of FPTase, e.g., benzodiazepine peptidomimetics (4), gliotoxins (5), and pepticinnamins (6).

Our continuing efforts to discover additional FPTase inhibitors has resulted in the isolation and characterization of three new asteric acid analogues, barceloneic acids A [**1**] B [**2**], and barceloneic lactone [**3**] as well as the related compound, 4-methyl-2,6-dihydroxy benzoic acid from the fermentation extract of a fungus of the genus *Phoma*. Barceloneic acid A inhibited FPTase with an IC_{50} value of 40 μM.

RESULTS AND DISCUSSION

The structure of **1** was initially investigated by extensive nmr experiments and finally established by X-ray diffraction studies. The mass spectral data indicated a mol wt of 320 daltons by eims with a base peak at *m/z* 151. The molecular formula of **1** was elucidated as $C_{16}H_{16}O_7$ by hrms. The ^{1}H-nmr spectrum (Table 1) of **1** showed the

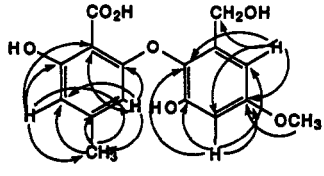


1 R_1=CH_2OH, R_2=R_3=R_4=H
2 R_1=CO_2H, R_2=R_3=R_4=H
1a R_1=CH_2OH, R_2=R_3=R_4=CH_3
1b R_1=CH_2OH, R_2=CH_3, R_3=R_4=H
1c R_1=CH_2OH, R_2=R_3=CH_3, R_4=H

TABLE 1. ^{1}H- and ^{13}C-Nmr Assignments for **1**, **2**, and **3** in Me_2CO-d_6.

Position	1		2		3	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	—	171.86	—	171.55	—	168.02
2	—	100.91	—	101.51	—	112.13
3	—	159.14	—	159.19	—	154.18
4	6.03, br s	105.31	5.94, br s	105.41	6.54, d, J=3 Hz	114.90
5	—	147.85	—	147.69	—	145.43
6	6.50, br s	112.64	6.50, br s	112.73	6.73, d, J=3 Hz	115.17
7	—	164.40	—	164.02	—	156.52
8	2.18, s	22.00	2.18, s	21.97	2.28, s	21.50
9	—	132.36	—	125.49	—	138.90
10	—	151.03	—	152.70	—	150.33
11	6.54, d, J=3 Hz	102.41	6.93, d, J=3 Hz	108.30	6.27, br s	103.50
12	—	159.25	—	158.85	—	157.16
13	6.68, d, J=3 Hz	105.51	7.13, d, J=3 Hz	108.52	6.55, br s	105.74
14	—	137.38	—	134.81	—	129.18
15	4.53, br s	60.31	—	166.28	5.14, s	69.45
16	3.80, s	55.74	3.89, s	56.12	3.74, s	55.81

presence of four aromatic protons at δ 6.03 (br s), 6.50 (br s), 6.54 (d, J=3.0 Hz), and 6.68 (d, J=3 Hz), a methoxy group at δ 3.80, an aromatic methyl group at δ 2.18, and a broad singlet for a hydroxymethyl group at δ 4.53 ppm. Methylation of compound **1**, using mild basic conditions and excess CH_3I, produced a trimethoxy derivative [**1a**] thus indicating the presence of three phenolic and/or acidic groups. The presence of a carboxylic acid group was further supported by a ^{13}C-nmr signal at δ 171.8 ppm and an ir band at 1678 cm^{-1}. The ^{13}C-nmr spectrum (Table 1) of barceloneic acid [**1**] showed sixteen carbon resonances in agreement with the assigned molecular formula. The APT spectrum of **1** revealed four aromatic methines, in addition to a methoxy, an oxymethylene, and a methyl carbon. An HMQC nmr experiment allowed the assignment of aromatic methine carbons at δ 102.4, 105.3, 105.5, and 112.6 bearing the protons at δ 6.54, 6.03, 6.68, and 6.50, respectively. The base peak at *m/z* 151 in the ms data allowed placement of CH_3, OH, and COOH in one ring and CH_2OH, OCH_3, and OH in the other. The proper positioning of these groups on their respective rings was established by an HMBC nmr experiment (see Figure 1). HMBC correlations of a CH_3 group to two methine carbons at δ 105.3 (C-4) and 112.6 (C-6) allowed its placement at C-5. Two- and three-bond proton-carbon correlations of the proton at δ 6.03 (H-4) to δ 22.0 (C-8), 100.9 (C-2), 112.6 (C-6), and 159.1 (C-3), as well as those of the proton at δ 6.50 (H-6) to 22.0 (C-8), 100.9 (C-2), 105.3 (C-4), and 164.4 (C-7), allowed the placement of OH at C-3, the COOH at C-2, and an ether linkage at C-7 on the A ring relative to the CH_3.



HMBC $^{n}J_{CH}$=7 Hz

FIGURE 1. HMBC correlations of barceloneic acid A [**1**].

The substitution pattern on the B ring, except for the ether linkage with ring A, was established by careful examination of HMBC correlations of the methoxy group to C-12 and of H-11 and H-13 to the carbons indicated in Figure 1. No HMBC correlations of H-15 to any carbons could be observed due to signal broadening. A distinction between the hydroxy-bearing carbon (C-10) and the ether carbon (C-9) was not possible on the basis of these nmr experiments. The final structure, including the ether linkage, of **1** was unambiguously established by a single crystal X-ray diffraction experiment and the final crystallographic model is shown in Figure 2.

Methylation of **1** with CH_2N_2 in anhydrous Et_2O gave the monomethyl ether **1b** (ir 1622 cm^{-1}) as sole product. The selective methylation of the phenolic group in the presence of a carboxyl group is unusual. This may be due to hydrogen bonding between the carboxyl group and the OH group at C-3. Methylation with methyl iodide under basic conditions gave the dimethyl ether monomethyl ester **1a** (ir 1733 cm^{-1}) as mentioned earlier. Hydrolysis of the trimethoxy derivative [**1a**] with aqueous K_2CO_3 in Me_2CO gave the carboxyl derivative **1c**.

The ^{1}H-nmr spectrum of compound **2** indicated the presence of four aromatic protons at δ 5.94 (br s), 6.50 (br s), 6.93 (d, J=3 Hz), 7.13 (d, J=3 Hz), one aromatic methyl at δ 2.18, and a methoxy group at δ 3.88. The ^{13}C-nmr spectrum of **2** was essentially identical to that of **1** except for the lack of an oxymethylene carbon at δ 60.3. The presence of an additional carboxyl carbon at δ 166.2 in **2** was observed as were minor carboxyl associated shifts in the signals of the ring-B carbons. This finding was corroborated by fabms and eims which gave a mol wt of 334 daltons and a formula of $C_{16}H_{14}O_8$.

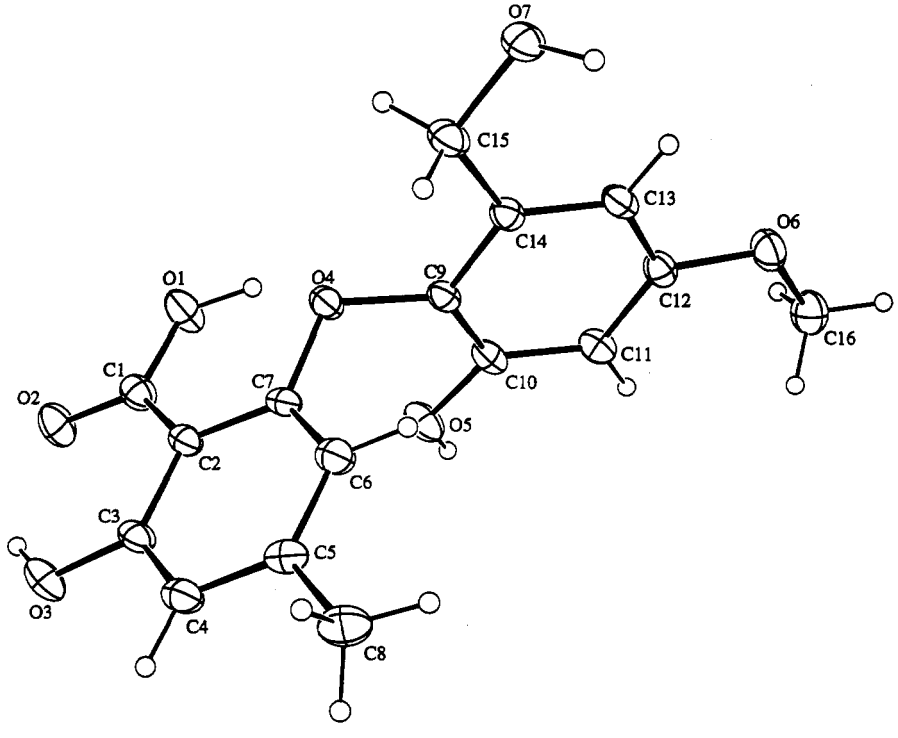


FIGURE 2. ORTEP drawing of barceloneic acid [**1**] with the crystallographic number scheme. The atom ellipsoids are drawn at the 30% probability level except for the H atoms, which are an arbitrary size.

Oxidation of barceloneic acid A [**1**] with Jones reagent produced a di-acid identical with **2**. The identities of the oxidation product and barceloneic acid B were confirmed by hplc and nmr comparisons.

Mass spectral analysis of **3** by both fabms and eims gave a mol wt of 302 daltons and the formula $C_{16}H_{14}O_6$ indicating the loss of a H_2O molecule relative to **1**. The ^{1}H-nmr spectrum (Table 1) of compound **3** displayed four aromatic protons at δ 6.54 (d, J=3 Hz), 6.55 (m), 6.73 (d, J=3 Hz), 6.27 (m), -CH_2OCO- (δ 5.14, s), -OCH_3 (δ 3.74, s), and an aromatic methyl group at δ 2.28. The structure of **3** was established on the basis of HMQC and HMBC nmr experiments similar to those described for **1**. The HMBC correlations of H-15 to the lactone carbonyl C-1 established the lactone ring. Selected HMBC correlations are illustrated in Figure 3 (only selected HMBC correlations are shown; all other correlations were identical to those observed with barceloneic acid A, **1**). Structure **3** is thus proposed for barceloneic lactone.

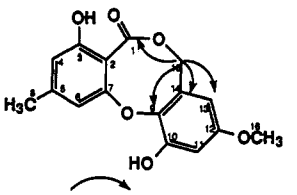


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FIGURE 3. Selected HMBC correlations of barceloneic lactone [**3**].

A monomeric compound was also isolated from the extract and determined by nmr and ms to be 4-methyl-2,6-dihydroxybenzoic acid, a constituent structural moiety of **1**.

All the compounds were evaluated in the *Ras* farnesyl-protein transferase assay using human recombinant FPTase enzyme as described earlier (7–9). Barceloneic acid A [**1**] inhibited the FPTase enzyme with an IC_{50} value of 40 μM. The other compounds [**1a–1c**, **2**, **3**, and 4-methyl-2,6-dihydroxybenzoic acid] did not show any inhibitory activity up to ca. 300 μM. The lack of activity of **2** is particularly surprising.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The ir absorption spectra were obtained with a model 1750 Ft-ir spectrophotometer using a multiple internal reflectance cell (MIR, ZnSe) on neat 10–20 μg samples. The uv absorption spectra were measured with a DU-70 spectrophotometer in MeOH solution. Mass spectral data were obtained on Finnigan-MAT TSQ700 and MAT-212 instruments by electron impact at 90 eV. The fab mass spectra were obtained on a MAT-731 instrument.

Nmr data were recorded on a Varian XL-300 or a Unity 400 spectrophotometer at ambient temperature in Me_2CO-d_6 or $CDCl_3$. ^{1}H-Nmr chemical shifts in Me_2CO-d_6 and $CDCl_3$ are given relative to the solvent peak at δ 2.03 and 7.24 ppm. ^{13}C-Nmr chemical shifts in Me_2CO-d_6 are given relative to the solvent peak at δ 28.9 ppm.

FUNGAL MATERIAL.—The producing strain was recovered from a soil sample collected at the Merck Manufacturing Division Plant, Barceloneta, Puerto Rico. In culture, the fungus produces abundant, multi-ostiolate pycnidia. These were glabrous, thin walled, and composed of pseudoparenchymatous tissue. Hyaline, elliptical, aseptate conidia were produced from subglobose to doliform, phialidic conidiogenous cells that line the pycnidial cavity. Based on these features, the strain was assigned to the form-genus *Phoma* (Deuteromycotinae, Coelomycetes). However, the strain was not identified as any of the common species of *Phoma* typically isolated from soil. The strain has been accessioned in the Merck Microbial Resources Culture Collection as *Phoma* sp., MF5789.

FERMENTATION CONDITIONS.—Fermentations were performed on solid substrate production medium

formulated as follows: brown rice, 10.0 g per 250-ml Erlenmeyer flask to which was added 20 ml of an aqueous solution containing 0.02 g yeast extract, 0.01 g sodium tartrate, and 0.01 g KH_2PO_4. Solid substrate production flasks were capped with cotton plugs and sterilized at 121° for 15 min. Immediately prior to inoculation, distilled H_2O (15.0 ml) was added to each flask, and the flasks were re-sterilized at 121° for 20 min. Each production flask was inoculated with 2.0 ml vegetative seed growth which was dispersed throughout the solid substrate. Production flasks were extracted with 50 ml MeOH.

EXTRACTION AND ISOLATION.—The methanolic extract (900 ml) was filtered through Celite and the filtrate was concentrated under reduced pressure to a brown suspension which was diluted to 500 ml with H_2O, acidified to pH 2.0, and sequentially extracted with $CHCl_3$ (2×250 ml) and EtOAc (2×250 ml). The organic extracts were combined and evaporated on a rotary evaporator to produce 1.2 g of brown residue.

The residue (1.0 g) was dissolved in MeOH (100 ml) and fractionated on a Sephadex LH-20 column eluted with MeOH. *Ras* farnesyl-protein transferase active fractions eluted after 2.0 to 2.4 liters of MeOH were concentrated on a rotary evaporator to produce a residue which was crystallized from methanol giving 80 mg of **1** as colorless needles.

Barceloneic acid B [**2**], barceloneic lactone [**3**], and a monomer, 4-methyl-2,6-dihydroxybenzoic acid, were isolated by hplc from the mother liquor of **1**. The brown residue (45 mg) was chromatographed on a Zorbax C_8 (22×250 mm) prep. column and fractions were eluted isocratically, at a flow rate of 7 ml/min, with 30% aqueous MeCN containing 0.2% trifluoroacetic acid. The separation was monitored by uv at 254 nm. Fractions were pooled on the basis of analytical hplc (Zorbax RX C_8, 4.6×250 mm, 40% aqueous MeCN containing 0.2% trifluoroacetic acid at a flow rate of 1 ml per min). Compounds **1**, **2**, **3**, and 4-methyl-2,6-dihydroxybenzoic acid were eluted after 5.64, 8.35, 8.75, and 9.03 minutes, respectively.

Barceloneic acid A [**1**].—Crystalline solid (MeOH); mp 192–193°; hreims *m/z* 320.0905 (calcd for $C_{16}H_{16}O_7$, 320.0895); ir (ZnSe film) ν max 3387 (br), 1659, 1678, 1442, 1263, 1207, 1067 cm^{-1}; uv λ max (MeOH) (log ε) 213 (4.50), 246 (3.7), 292 (3.6) nm; ^1H- and ^{13}C-nmr data, see Table 1.

Barceloneic acid B [**2**].—Crystalline solid (MeOH); mp 234–235°; hreims *m/z* 334.0046 (calcd for $C_{16}H_{14}O_8$, 334.0688); ir (ZnSe film) ν max 3287 (br), 2927, 1675, 1633, 1450, 1400, 1204, 1060 cm^{-1}; uv λ max (MeOH) (log ε) 219 (4.5), 248 (4.1), 309 (3.9) nm; ^1H- and ^{13}C-nmr data, see Table 1.

Barceloneic lactone [**3**].—Crystalline solid (MeOH); mp 195°; hreims *m/z* 302.0793 (calcd for $C_{16}H_{14}O_6$, 302.0790); ir (ZnSe film) ν max 3446 (br), 1717, 1623, 1541, 1498, 1457, 1303, 1199 cm^{-1}; uv λ max (MeOH) (log ε) 220 (4.5), 288 (4.0) nm; ^1H- and ^{13}C-nmr data, see Table 1.

4-Methyl-2,6-dihydroxybenzoic acid.—Brown gum; hreims *m/z* 168.0408 (calcd for $C_8H_8O_4$, 168.0422); ir (ZnSe film) ν max 3155 (br), 1651, 1586, 1458, 1280, 1205 cm^{-1}; uv λ max (MeOH) (log ε) 219 (4.3), 254 (3.9), 314 (3.5) nm; ^1H nmr ($CDCl_3$) δ 2.18 (3H, s, CH_3), 6.09, 6.19 (1H each, s, aromatic H), 11.8 (1H, br s, OH).

PREPARATION OF THE DIMETHYL ETHER MONOMETHYL ESTER OF BARCELONEIC ACID A [**1a**].—To a solution of **1** (20 mg) in Me_2CO (3 ml) was added anhydrous K_2CO_3 (25 mg) and methyl iodide (0.1 ml). The reaction mixture was refluxed overnight and the solvent evaporated under N_2. The residue was partitioned between H_2O and $CHCl_3$. The $CHCl_3$ extract was dried over Na_2SO_4, evaporated under reduced pressure, and purified on a small Si gel column. Elution of the column with 5% MeOH in $CHCl_3$ yielded 18 mg of the dimethyl ether monomethyl ester derivative **1a** as a wax: ir (ZnSe film) ν max 3448, 1733, 1613, 1464, 1228, 1283, 1088 cm^{-1}; ^1H nmr ($CDCl_3$) δ 2.23 (3H, s, CH_3), 3.76, 3.92 (3H each, s, OCH_3), 3.83 (6H, s, 2×OCH_3), 4.51 (2H, br s, CH_2OH), 5.93, 6.38 (1H each, br s), 6.52 (2H, br s); eims *m/z* 362.

PREPARATION OF 5′-*O*-METHYLBARCELONEIC ACID A [**1b**].—To a solution of **1** (5 mg) in Et_2O (1 ml) was added two drops of CH_2N_2 in Et_2O. The solution was stirred at room temperature until the disappearance of the starting material. Solvent was evaporated under a stream of N_2 and residue was filtered through a Si gel plug filled in a Pasteur pipette. Elution with 20% MeOH in $CHCl_3$ yielded monomethyl ether **1b** (3.8 mg) as a wax: ir (ZnSe film) ν max 3387 (br), 1622, 1442, 1263, 1207 cm^{-1}; ^1H nmr ($CDCl_3$) δ 2.17 (3H, s, CH_3), 3.82 (3H, s, OCH_3), 4.91 (3H, s, OCH_3), 4.51 (2H, br s, CH_2OH), 5.94, 6.52 (1H each, m), 6.58, 6.59 (1H each, br s); eims *m/z* 334.

PREPARATION OF THE DIMETHYL ETHER OF BARCELONEIC ACID A [**1c**].—To a solution of the ester **1a** (10 mg) in Me_2CO (2 ml) was added K_2CO_3 solution (0.5 ml of 10% solution); this was stirred at room temperature overnight until no starting material was visible on tlc. The reaction mixture was acidified to pH 2.0 by addition of 4 N HCl and extracted with EtOAc. The extract was washed with H_2O, dried over sodium sulfate, and evaporated under reduced pressure to give a residue that was purified on a short Si gel column eluting with 20% methanolic $CHCl_3$ to produce acid **1c** (6 mg) as a wax: ir (ZnSe film) ν max 3400 (br), 1713, 1613, 1465, 1227, 1204 cm^{-1}; ^1H nmr (CD_3OD) δ 2.18 (3H, s, CH_3), 3.83, 3.84, 3.92 (3H each,

s, OCH_3), 4.51 (2H, br s, CH_2OH), 5.89, 6.51 (1H each, br s), 6.58, 6.99 (1H each, d, J=3 Hz); eims *m/z* 348.

OXIDATION OF BARCELONEIC ACID A TO BARCELONEIC ACID B [**2**].—To a solution of **1** (5 mg) in Me_2CO (0.5 ml) was added Jones reagent (20 μl of 8 N solution). After stirring overnight the solution was filtered and the solvent evaporated to produce a residue which was diluted with 1 ml of H_2O and extracted with EtOAc (3×1 ml). The organic extract was removed *in vacuo* to produce 2.5 mg of a di-acid which was identical to barceloneic acid B [**2**] in all respects (hplc and nmr).

SINGLE CRYSTAL X-RAY ANALYSIS OF BARCELONEIC ACID A [**1**][1].—The crystals were grown by evaporation from a methanol solution. Crystal data: $C_{16}H_{16}O_7 \cdot CH_3OH$, M_r=352.344, triclinic, P1, a=8.419(1), b=12.915(2), c=8.1831(9) Å, α=101.08(1), β=90.11(1), γ=108.33(1)°, V=827.0(5) Å^3, Z=2, D_x=1.415 g cm^{-3}, monochromatized radiation λ (CuK_α=1.541838 Å, μ=0.92 mm^{-1}, F(000)=372, T=294° K. Data were collected on a Rigaku AFC5 diffractometer to a θ limit of 71° with 1442 observed (with $I \geq \sigma(I)$ as the criterion for being observed) reflections out of 3292 measured. The structure was solved by direct methods (SHELXS-86) (10) and refined using full-matrix least-squares on F using 227 parameters (11). The compound crystallized with a MeOH molecule in the lattice which is near and hydrogen-bonded to one OH (O-5) as well as hydrogen-bonded to a symmetry-related neighboring OH (O-7). All hydrogen atoms were located in a difference Fourier and included as 'riding' atoms with fixed thermal parameters set at 1.2 times that of the attached atom. All non-hydrogen atoms were refined with anisotropic thermal displacements. The final agreement statistics are: R=0.051, wR=0.047, S=1.81 with $(\Delta/\sigma)_{max}$ <0.01. The least-squares weights were defined as $1/\sigma^2(F)$. The maximum peak height in a final difference Fourier map is 0.20(5) eÅ^{-3} and this peak is without chemical significance.

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Received 14 September 1994

[1]Hydrogen coordinates, thermal parameters, bond distances and angles, and observed and calculated structure factors have been deposited with the Cambridge Crystallographic Data Centre and can be obtained upon request from Dr. Olga Kennard, University Chemical Laboratory, 12 Union Road, Cambridge CB2 1EZ, UK.