Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 17, pp. 2081-2084, 1996

0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00372-1

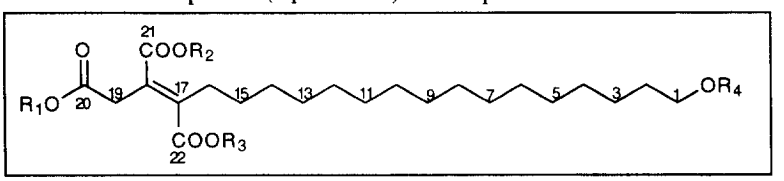
OREGANIC ACID: A POTENT NOVEL INHIBITOR OF RAS FARNESYL-PROTEIN TRANSFERASE FROM AN ENDOPHYTIC FUNGUS

Hiranthi Jayasuriya,* Gerald F. Bills, Carmen Cascales,# Deborah L. Zink, Michael A. Goetz, Rosalind G. Jenkins, Keith C. Silverman, Russell B. Lingham and Sheo B. Singh

Merck Research Laboratories, P. O. Box 2000, Rahway, New Jersey 07065
#Centro de Investigación Básica, Merck Sharp & Dohme de España S.A., Josefa Valcárcel 38, 28027, Madrid, Spain

Abstract: Inhibitors of farnesyl-protein transferase (FPTase) have the potential of being anticancer agents for tumors in which *ras* is found mutated and contributes to cell transformation . From the screening of the extracts of an endophytic fungus isolated from living leaves of *Berberis oregana*, we have discovered the tricarboxylated alkylsulfate, oreganic acid (**1**), as a potent (IC_{50} = 14 nM) and specific inhibitor of FPTase. Its desulfated analog (**4**) was less active (IC_{50} = 3.3μM). The trimethylester (**2**) and its desulfated analog (**3**) were inactive.

Ras (p^{21}) proteins are post-translationally farnesylated on a carboxy-terminal cysteine. Farnesylation is required for Ras membrane localization which is essential for cell transformation. Inhibition of farnesylation would, therefore, alter the membrane localization and potentially oncogenic effect of Ras. Therefore, FPTase inhibitors have potential utility as anticancer agents for tumors in which Ras is found mutated and contributes to cell transformation.[1] We have reported a number of inhibitors of FPTase - e.g. chaetomellic acids,[2] fusidienol,[3] actinoplanic acids,[4] cylindrol A,[5] preussomerins [6] and barceloneic acid[7] - from microbial sources. Our continued screening for novel non-peptide natural product inhibitors of FPTase resulted in the discovery of a tricarboxylated alkylsulfate, oreganic acid (**1**), from the extracts of an endophytic fungus isolated from living leaves of *Berberis oregana*. Oreganic acid is a novel, highly potent (IC_{50} = 14 nM) and specific inhibitor of FPTase. The mode of inhibition is competitive (K_i = 4.5 nM) with respect to FPP.[8]



1: $R_1 = R_2 = R_3 = H$, $R_4 = SO_3H$
2: $R_1 = R_2 = R_3 = CH_3$, $R_4 = SO_3H$
3: $R_1 = R_2 = R_3 = CH_3$, $R_4 = H$
4: $R_1 = R_2 = R_3 = R_4 = H$

Isolation

Size exclusion chromatography of the methyl ethyl ketone extract of a 1.6 L solid state fermentation of the unidentified endophytic fungus (MF 6046) resulted in the elution of FPTase activity in a broad band. The active fraction was chromatographed on a reverse phase C-8 column to give oreganic acid (**1**) as a gum (399 mg/L).

Structure elucidation

Oreganic acid failed to produce useful data by any of the mass spectrometric methods (+ and - ion FAB, ESI, CI, and EI). Reaction of oreganic acid with diazomethane gave a trimethyl ester (**2**) which was used for structure elucidation. Positive and negative ion FABMS of **2** gave ions at *m/z* 575 (M+K) and *m/z* 535 (M-H) respectively. Negative ion ESI gave an ion at *m/z* 535 (M-H). High resolution analysis of the M+K ion in the positive ion FAB spectrum gave a molecular formula of $C_{25}H_{44}O_{10}S$ for the trimethyl ester; the molecular formula of oreganic acid is therefore $C_{22}H_{38}O_{10}S$. The EI spectrum of **2** did not give a molecular ion but showed a parent ion at *m/z* 438 resulting from a facile loss of H_2SO_4. The ^{13}C NMR was in consistent with the mass spectral derived molecular formula. From the examination of the ^1H NMR spectra of **1** and **2**, it was obvious that this compound had an unbranched long fatty chain, substituted at both ends. The ^1H NMR signal at δ 1.24 integrated for twelve methylene groups. Lack of a terminal methyl group and the presence of a triplet at δ 4.08 resulted in the placement of the hydroxy methyl group at the terminal position bearing the sulfate group. The ^{13}C NMR indicated the presence of three carbonyl groups (δ 166.76, 169.82, and 170.43) and a single tetra-substituted double bond (δ 125.31 and 146.34).

The C-19 methylene protons (δ 3.41) gave HMBC correlations (Figure 1) to two carbonyl carbons (δ 166.76 and 170.43) and to both olefinic carbons (δ 125.31 and 146.34) and allowed the placement of the C-20 and C-21 ester groups and the C17-C18 double bond relative to this methylene. The placement of the third ester group (C22) on the olefinic carbon (C17) was established from the HMBC correlations from the C-16 vinylic proton (16-H_2; δ 2.34) to the C-22 ester carbonyl at δ 169.82. The methyl ester groups were assigned based on their HMBC correlations to the respective carbonyls.

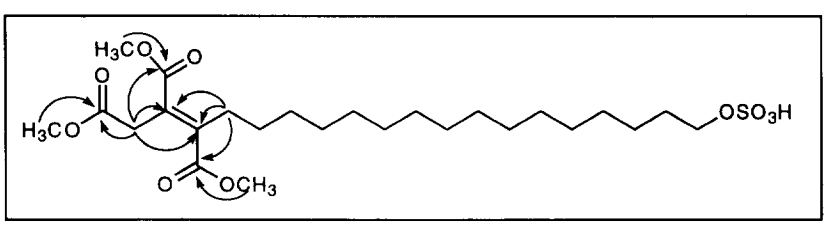


Figure 1: HMBC correlations (J_{CH} = 7 Hz) of 2.

The structure was further supported by the removal of the sulfate ester as follows. Refluxing of **2** in dioxane for 3 hr afforded exclusively the desulfated compound **3** (similar treatment of free oreganic acid resulted in a complex mixture). As expected, the terminal methylene group was shifted upfield by ~0.4 ppm in the ^1H NMR spectrum of **3** compared to **2**. Based on all the evidence discussed, structure **1** was assigned to oreganic acid.

Biological Activity

All four compounds were evaluated in the human recombinant FPTase *in vitro* assay[9,10] and the result is shown below. The free acid (**1**) was most potent (IC_{50} = 14 nM), removal of the sulfate (**4**; IC_{50} = 3.3 μM) or methylation (**2**; IC_{50} = 10.6 μM) diminished the biological activity. The desulfated trimethylester compound (**3**) was completely inactive at the highest level tested (>20 μM).

Experimental Procedure

For general experimental procedures see refs 6-7.

Producing Fungus

The producing fungus was an endophyte, isolated from living, surface-sterilized leaves of *Berberis oregana* (Berberidaceae), collected from near Lord Ellis Summit, Humboldt Co., California. The fungus could not be identified because of its nonsporulation under laboratory conditions. The fungus (MF6046) is maintained in the Merck Microbial Resources Culture Collection, Rahway, NJ.

Fermentation Conditions

The production medium was formulated as follows (g/L): sucrose, 75.0; tomato paste, 10.0; malt extract, 5.0; $(NH_4)_2SO_4$, 1.0; soy flour, 1.0; and KH_2PO_4, 9.0. This medium was prepared using distilled water, the pH was adjusted to 7.0 by adding NaOH; 50 mL of the media was dispensed into 250 mL Erlenmeyer flasks plugged with cotton before being autoclaved at 121^{0}C for 20 min. Production flasks were inoculated with 2.0 mL of vegetative seed growth and were incubated at 25^{0}C, on a gyratory shaker (220 rpm, 5.1 cm throw) for 8 days. At the time of the harvest, contents of fermentation flasks were homogenized with 40.0 mL of methyl ethyl ketone (MEK), shaken for 30 min and the extracts pooled.

Isolation

The methyl ethyl ketone extract (1.6 L) was fractionated on a Sephadex LH 20 column (1.5 L) and eluted with methanol. The FPTase activity eluted in a broad band. The active fraction was further purified on a HPLC column [Zorbax RX C-8 (22.4 X 250 mm), acetonitrile-water (0.1% TFA) 40:60]. Three major peaks (t_R = 9.64, 12.99, 30.32 min) were collected separately to produce three active fractions. Despite collection of a single peak during the preparative run, the three active fractions all contained varying ratios of a set of 3 peaks when examined by analytical HPLC under identical conditions. This could be attributed to the existence of multiple charged species under the chromatographic conditions due to different pKa values of the carboxylic acids and the sulfate group. ^{1}H NMR study of the three active fractions in CD_3OD indicated the gradual appearance of a triplet at δ 3.5 ppm with concomitant decrease in the intensity of the triplet at δ 3.99 ppm due to solvolysis. The compound was stabilized by esterification (*vide infra*) and the ester was used for the structural elucidation.

Oreganic acid (**1**). Light brown gum, IR (ZnSe) ν_{max}:, 2920, 2852, 1772, 1719, 1620, 1468, 1219, 1072 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.28 (24H, brs), 1.58 (2H, m, CH_2), 1.66 (2H, m, CH_2), 2.50 (2H, t, J = 7.6, 16-CH_2), 3.73 (2H, brs, 19-CH_2), 3.99 (2H, t, J = 6.6, 1-CH_2).

Preparation of Oreganic Acid Trimethyl Ester(**2**). To a solution of **1** (5 mg) in Et_2O (1 mL) was added three drops of CH_2N_2 in Et_2O. The solution was stirred at room temperature until disappearance of the starting material. Solvent was evaporated under a stream of nitrogen and and residue was filtered through a silica gel plug eluting with 25% MeOH in $CHCl_3$. Oreganic acid triemethyl ester (**2**, 4.0 mg) was obtained as a white wax. IR (ZnSe) ν_{max}: 2921, 2852, 1732, 1436, 1242, 1217, 1109, 964 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.24 (24H, brs), 1.43 (2H, m, CH_2), 1.63 (2H, m, CH_2), 2.34 (2H, t, J = 7.6, 16-CH_2), 3.41 (2H, brs, 19-CH_2), 3.69 and 3.74 (3H each, s, 20 & 21-OCH_3), 3.79 (3H, s, 22-OCH_3), 4.08 (2H, brt, 1-CH_2); ^{13}C NMR ($CDCl_3$) δ 25.80, 27.54, 29.27, 29.29, 29.38, 29.43, 29.50, 29.56, 29.61, 29.66, 29.73, 29.76, 29.81, 29.88 (C2 to 15); 31.41 (C16); 33.74 (C19); 52.37 (20 C-OCH_3); 52.56 (21 &22 C-OCH_3); 69.91 (C1); 125.31 and 146.34 (C17 and

18); 166.76 and 170.43 (C-20 and 21) 169.82 (C22); HRFABMS *m/z* 575.2102 (M+K, calcd. for $C_{25}H_{44}O_{10}SK$: 575.2292).

Preparation of Desulfated Compound (3). A solution of **2** (5 mg) in dioxane (1 mL) was refluxed at 100 ^{0}C for 3 hrs. Evaporation of dioxane under reduced pressure and filtration of the residue through a pipette filled with silica gel and elution with 25% MeOH in $CHCl_3$ produced **3** (3.4 mg) as a colorless wax; IR(ZnSe) ν_{max}: 2918, 2851, 1725, 1473, 1293, 1229 cm^{-1}, 1040; ^{1}H NMR ($CDCl_3$) δ 1.28 (24H, brs), 1.43 (2H, m, CH_2), 1.56 (2H, m, CH_2), 2.34 (2H, t, J = 7.6, 6-CH_2), 3.41 (2H, brs, 19-CH_2), 3.64 (2H, t, J = 7.6, 1-CH_2), 3.69, 3.74, 3.79 (3H, s, -$COOCH_3$); ESI *m/z* 479 (M+Na), 495 (M+K).

Preparation of Desulfated Oreganic Acid (4). A solution of **3** (2 mg) in acetone (1 mL) was stirred at room temperature with LiOH (6.0mg) overnight. After evaporation of the solvent under reduced pressure, water was added. Citric acid (10%) was added after cooling to adjust the pH (pH = 5). The product **4** (1mg) was extracted into ethyl acetate. IR(ZnSe) ν_{max}: 2923, 2853, 1733, 1437, 1268, 1201, 1075 cm^{-1}; ^{1}H NMR (CD_3OD) δ 1.29 (24H, brs), 1.52 (2H, m, CH_2), 1.57 (2H, m, CH_2), 2.63 (2H, brt, 6-CH_2), 3.22 (2H, brs, 19-CH_2), 3.53(2H, t, J = 7.6, 1-CH_2); HR-EI *m/z* 414.2551.

References

1. (a) Barbacid, M. *Ann. Rev. Biochem.* **1987**, *56*, 779. (b) Rodenhuis, S. *Semin. Cancer Biol.* **1992**, *3*, 241. (c) Casey, P.; Solski, P. A.; Der, C. J.; Buss, J. E. *Proc. Natl. Acad. Sci. U. S. A.* **1989**, *86*, 8323.(d) Gibbs, J. B. *Semin. Cancer Biol.* **1992**, *3*, 383. (e) Gibbs, J. B. *Cell* **1991**, *65*, 1 and references cited therein. (f) Der, C. J.; Cox, A. D. *Cancer Cells* **1991**, *3*, 331.(g) Reiss, Y.; Goldstein, J. L.; Seabra, M. C.; Casey, P. J.; Brown, M. S. *Cell* **1990**, *62*, 81. (h) Schaber, M. D.; O'Hara, M. B.; Garsky, V. M.; Mosser, S. D., Bergstrom, J. D.; Moores, S. L.; Marshall, M. S.; Friedman, P. A.; Dixon, R. A. F.; Gibbs, J. B. *J. Biol. Chem.* **1990**, *265*, 14701. (i) Gibbs, J. B.; Pompliano, D. L.; Mosser, S. D.; Rands, E.; Lingham, R. B.; Singh, S. B.; Scolnick, E. M.; Kohl, N. E.; Oliff, A. *J. Biol. Chem.* **1993**, *268*, 7617. (j) Kohl, N. E.; Mosser, S. D.; S. J. deSolms, Giuliani, E. A.; Pompliano, D. L.; Graham, S. L.; Smith, R. L.; Scolnick, E. M.; Oliff, A.; Gibbs, J. B. *Science* **1993**, *260*, 1934.
2. Singh, S. B.; Zink, D. L.; Liesch, J. M.; Goetz, M. A.; Jenkins, R. G.; Nallin-Omstead, M.; Silverman, K. C.; Bills, G. F.; Mosley, R. T.; Gibbs, J. B.; Albers-Schonberg, G.; Lingham, R .B. *Tetrahedron* **1993**, *49*, 5917.
3. Singh, S.B.; Jones, E.T.; Goetz, M.A.; Bills, G.F.; Nallin-Omstead, M.; Jenkins, R.G.; Lingham, R.B.; Silverman, K.C.; Gibbs, J.B.*Tetrahedron Lett.* **1994**, *35*, 4693.
4. (a) Singh, S. B.; Liesch, J. M.; Lingham, R .B; Goetz, M. A.; Gibbs, J. B. *J. Am. Chem. Soc.* **1994**, *116*, 11606. (b) Singh, S. B.; Liesch, J. M.; Lingham, R .B; Silverman, K. C.; Sigmund, J. M.; Goetz, M. A. *J. Org. Chem.* **1995**, *60*, 7896.
5. Singh, S. B.; Zink, D. L.; Bills, G. F.; Jenkins, R. G.; Silverman, K. C.; Lingham, R. B. *Tetrahedron Lett.* **1995**, *36*, 4935.
6. Singh, S. B.; Zink, D. L.; Liesch, J. M.; Ball, R. G.; Goetz, M. A.; Bolessa, E. A.; Giacobbe, R. A.; Silverman, K. C.; Bills, G. F.; Pelaez, F.; Cascales, C.; Gibbs, J. B.; and Lingham, R. B. *J. Org. Chem.* **1994**, *59*, 6296.
7. Jayasuriya, H.; Ball, R. G.; Zink, D. L.; Smith, J. L.; Goetz, M. A.; Jenkins, R. G.; Nallin-Omstead, M.; Silverman, K. C.; Bills, G. F.; Lingham, R. B.; Pelaez, F.; Cascales, C. and Singh, S. B. *J. Nat. Prod.*, **1995**, *58*, 986.
8. Silverman, K. C.; Jayasuriya, H.; Cascales, C.; Jenkins, R. G.; Singh, S. B.; Lingham, R. B. in preparation.
9. Lingham, R. B.; Silverman, K. C.; Bills, G. F.; Cascales, C.; Sanchez, M.; Jenkins, R. G.; Gartner, S. E.; Martin, I.; Diez, M. T.; Pelaez, F.; Mochales, S.; Kong, L.; Burg, R. W.; Meinz, M.; Huang, L.; Nallin-Omstead, M.; Mosser, S. D.; Shaber, M. D.; Omer, C. A.; Pompliano, D. L.; Gibbs, J. B.; Singh, S. B. *Appl. Microbiol. Biotechnol.* **1993**, *40*, 370.
10. Omer, C. A.; Kral, A. M.; Diel, R. E.; Prendergast, G. C.; Powers, S .; Allen, C. M.; Gibbs, J. B.; Kohl, N. E. *Biochemistry* **1993**, *32*, 5167.

(*Received in USA* 15 *March* 1996; *accepted* 1 *August* 1996)