Clavaric Acid: A Triterpenoid Inhibitor of Farnesyl-Protein Transferase from *Clavariadelphus truncatus*

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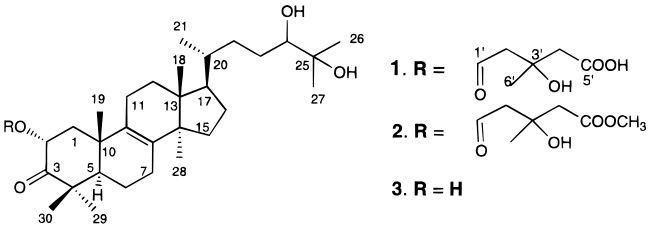
Farnesyl-protein transferase (FPTase) catalyses the specific transfer of farnesyl to Ras-peptides that is essential for oncogenic activity in oncogene-mediated tumors. Specific inhibition of FPTase activity has been shown to reduce tumor development in nude mice challenged with oncogenic forms of *ras*, thereby establishing FPTase as a viable therapeutic target. Our continued efforts to discover inhibitors of FPTase has led to the discovery of a triterpenoidal inhibitor, clavaric acid (**1**). This compound inhibits rHFPTase with an IC_{50} value of 1.3 μM. Structure elucidation, structure modifications, and biological activity of clavaric acid are herein described.

The *ras* gene is found mutated in numerous types of cancer.[1] Ras (p21) is the gene product of the *ras* oncogene and is modified posttranslationally. This posttranslational modification is required for cell transforming activity.[2] The first and critical step in the posttranslational modification is farnesylation of the carboxy terminal CaaX box by the farnesyl donor enzyme, FPTase. It has been demonstrated through animal studies that inhibitors of FPTase have the potential to be effective anticancer agents for tumors in which *ras* is found mutated and contributes to cell transformation.[3]

A number of FPTase inhibitors have been reported in the past few years. These inhibitors are derived either from rational design (CaaX mimetics) or from random screening of natural product extracts and chemical collections. A few examples of each class of the inhibitors are L-731,734[4] and benzodiazepines,[5] examples of inhibitors that are CaaX mimetics; chaetomellic acids;[6] actinoplanic acids;[7] oreganic acid;[8] cylindrols;[9] fusidienols;[10] gliotoxins;[11] CP-225,917 and CP-263,114;[12] and pepticinnamins,[13] examples of natural product inhibitors; and Sch44342 and analogues,[14] examples of inhibitors derived from the screening of chemical collections. Our continued efforts to identify nonpeptide inhibitors of FPTase led to the discovery of a novel triterpenoidal inhibitor, clavaric acid (**1**), from



Clavariadelphus truncatus, (Quélet) Donk (Clavariaceae, Aphyllophorales), a fungus collected from Cercedilla (Madrid, Spain). It is a specific inhibitor of FPTase exhibiting an IC_{50} value of 1.3 μM and does not inhibit related enzymes such as geranylgeranyl-protein transferase or squalene synthase. It inhibited FPTase activity by competing with the Ras-peptide substrate, and the inhibitory activity was reversible. More importantly, clavaric acid inhibited Ras-processing in NIH3T3 *ras*-transformed cells at 50 μM without manifesting any toxicity. To our knowledge, this is second example, cembranolide[15] being the first, of a non-nitrogenous inhibitor of FPTase that competes with Ras-peptide binding, not with FPP binding.

A solid-state fermentation of *C. truncatus* (ATCC 74314) was extracted with methyl ethyl ketone. Gel permeation chromatography of the extract resulted in the reduction of the weight by 25% and concentrated the biological activity in a medium molecular weight range. Reversed-phase high-resolution chromatography of the FPTase-active fraction gave clavaric acid (**1**, 50 mg/L) as a colorless homogeneous amorphous powder.

High-resolution FAB mass spectral analysis of clavaric acid (**1**) gave a molecular ion at *m/z* 619 $(M + H)^+$ and suggested the molecular formula $C_{36}H_{58}O_8$. The molecular formula was corroborated by ^{13}C NMR analysis that exhibited 36 resonances (Table 1). The DEPT spectrum of clavaric acid showed resonances for nine methyls, 11 methylenes, and five methines. Therefore, the remaining 11 carbons must be quaternaries. The ^1H NMR spectrum showed seven sharp singlets for tertiary methyls (δ 0.71, 0.86, 1.09, 1.13, 1.18, 1.37, 1.48) and a doublet for a secondary methyl group (δ 0.92, $J = 6.0$ Hz). The ^1H and ^{13}C NMR spectra of clavaric acid were assigned by the application of 2D $^1H-^1H$ COSY and HMQC experiments, and the assignments are summarized in Table 1. The examination of the NMR spectral data indicated that clavaric acid contained a substituted tetracyclic triterpenoidal skeletal system with an extra six-carbon substitution consisting of a 3-hydroxy-3-methylglutaric acid moiety.

The HMBC correlations of H-26 (δ 1.22) and H-27 (δ 1.17) to the quaternary carbon at δ 73.33 and to the oxymethine carbon at δ 78.74 established the hydroxy substitutions at C-25 and C-24, respectively (Figure 1). The presence of the tetrasubstituted olefin was ascertained from the HMBC correlations of the methyl protons of H-19 and H-28. Both of these methyl groups exhibited HMBC correlations to the ketone carbonyl at δ 210.16. Hence, its location at C-3 was confirmed, a conclusion that was also corroborated from the HMBC correlations of both methylene protons at C-1 (Figure 1). The remaining oxymethine group was placed on C-2 according to the HMBC correla-

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Table 1. ^{1}H and ^{13}C NMR Data of Clavaric Acid (1) in $CDCl_3$

position	^{13}C δ (mult)[a]	^{1}H δ (mult)	position	^{13}C δ (mult)	^{1}H δ (mult)
1	42.3 (t)	2.30, dd (J = 12.4, 6 Hz)	19	19.9 (q)	1.37, s
2	73.2 (d)	5.71, dd (J = 13.4, 4.2 Hz)	20	36.2 (d)	1.42, m
3	210.2 (s)		21	18.6 (q)	0.92, d (J = 6 Hz)
4	48.5 (s)		22	33.1 (t)	1.50, 1.25, m
5	52.4 (d)	1.25, m	23	28.3 (t)	1.38, m
6	18.9 (t)	1.68, m	24	78.7 (d)	3.35, brt, (J= 6 Hz)
7	28.3 (t)*	1.34, 1.97, m	25	73.3 (s)	
8	135.8 (s)		26	23.3 (q)	1.22, s
9	132.8 (s)		27	26.6 (q)	1.17, s
10	37.9 (s)		28	24.3 (q)	0.86, s
11	21.5 (t)*	2.02, m	29	21.1 (q)	1.18, s
12	26.1 (t)*	2.10, m	30	24.5 (q)	1.09, s
13	49.8 (s)		1′	170.7 (s)	
14	44.5 (s)		2′	45.0 (t)	2.70, d (J = 16 Hz)
15	30.7 (t)*	1.58, 1.2, m	3′	70.1 (s)	
16	30.8 (t)*	1.76, m	4′	45.4 (t)	2.74, d (J = 16 Hz)
17	50.5 (d)	1.48, m	5′	172.2 (s)	
18	15.8 (q)	0.72, s	6′	27.2 (q)	1.48, s

[a] Carbon shifts marked with an asterisk can be interchanged.

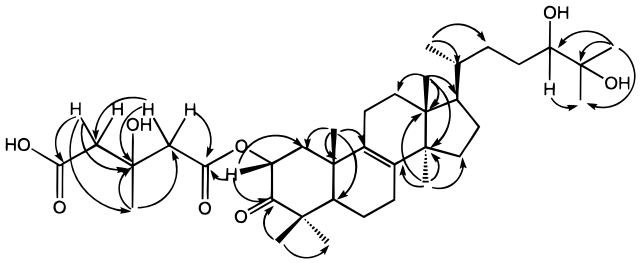

Figure 1. Selected HMBC (J_{CH} = 7 Hz) correlations of clavaric acid (**1**).

tions of the H-2 (δ 5.71) to C-1 and C-3 and COSY correlations of H-2 to H_2-1. Other significant HMBC correlations are shown in Figure 1.

The structure of the 3-hydroxy-3-methylglutaric acid moiety was deduced as follows. The tertiary methyl group H_3-6′ (δ 1.49) gave strong HMBC correlations to two methylene carbons C-2′ (δ 45.02) and C-4′ (δ 45.38) and the remaining oxygen containing tertiary carbon C-3′ (δ 70.07). In turn, methylene protons H-2′ and H-4′ gave HMBC correlations to both carboxyls C-1′(δ 170.7) and C-5′ (δ 172.15), C-3′ (δ 70.07) and C-6′(δ 27.21).

The methine proton H-2 (δ 5.71, dd, J = 13.4, 4.2 Hz) gave HMBC correlation to the C-1′ carboxyl (δ 170.7) in addition to the expected HMBC correlations to the A ring carbons of the terpenoid nucleus, hence confirming the HMG ester linkage at C2. The exhibition of large coupling (13.4 Hz) between H-2 and axial H-1 allowed the placement of the ester group in an equatorial position at C2. Methylation of clavaric acid (**1**) with diazomethane gave a monomethyl ester **2**, confirming the presence of a free carboxyl group. Mild basic hydrolysis of **1** gave 3α,24,25-trihydroxylanostan-3-one (**3**) and 3-hydroxy-3-methylglutaric acid. On the basis of these studies, the structure of clavaric acid is assigned as 2α-(3′-hydroxy-3′-methylglutaroyl)-24,25-dihydroxylanostan-3-one (**1**).

Clavaric acid is a close structural analogue of fasciculic acid, a calmodulin antagonist, isolated from a mushroom, *Naematoloma fasciculare*. The NMR assignments of clavaric acid (**1**) are in accordance with the reported NMR assignments of fasciculic acid.[16,17] Ochraceolides[18] and ganoderic acids[19] are examples of terpenoids that were recently reported as farnesyl protein transferase inhibitors.

The compounds **1**−**3** were evaluated in the Ras farnesylprotein transferase filtration assay using human recombinant FPTase enzyme as described previously.[20,21] Clavaric acid (**1**) inhibited the activity of FPTase enzyme with an IC_{50} value of 1.3 μM. The methyl ester (**2**) was completely inactive, while the hydrolysis product, clavarinone (**3**), was less active (IC_{50} 10.5 μM).

Experimental Section

General Experimental Procedures. The IR absorption spectra were obtained with a model 1750 infrared Fourier transform spectrophotometer using a multiple internal reflectance cell (MIR, ZnSe) on neat 10−20 μg samples. Mass spectral data were obtained on Finnigan-MAT TSQ700 and MAT212 instruments by electron impact at 90 eV. The FAB spectra were obtained on a MAT 731 instrument. NMR data were recorded on Varian XL-300 and Unity 400 spectrophotometers at ambient temperature in $CDCl_3$. ^{1}H NMR chemical shifts in $CDCl_3$ are given relative to the solvent peak at δ 7.26 ppm. ^{13}C NMR chemical shifts in $CDCl_3$ are given relative to the solvent peak at δ 77.05 ppm.

Organism. The producing organism MF-6001 (ATCC 74314) was isolated from the internal tissues of a fruiting body of a basidiomycetes (mushroom) collected in Cercedilla (Madrid, Spain) and was identified as *C. truncatus* (Quélet) Donk (Clavariaceae, Aphyllophorales).

Fermentation Condition. The fungus, *C. truncatus*, was inoculated into the seed culture by aseptically transferring a small amount of the preserved soil into a 250 mL Erlenmeyer flask containing 50 mL of seed medium of the following composition (in g/L); corn steep liquor, 5.0; tomato paste, 40.0; oat flour, 10.0; glucose, 10.0; and trace elements solution, 10 mL/L (consisting of, in g/L: $FeSO_4 \cdot 7H_2O$, 1.0; $MnSO_4 \cdot 4H_2O$, 1.0; $CuCl_2 \cdot 2H_2O$, 0.025; $CaCl_2 \cdot 2H_2O$, 0.1; H_3BO_3, 0.056; $(NH_4)_6MoO_{24} \cdot 4H_2O$, 0.019; $ZnSO_4 \cdot 7H_2O$, 0.2; dissolved in 0.6 N HCl). The seed culture was incubated at 25 °C, 220 rpm, for 144−150 h. Fermentations were performed on solid substrate production medium formulated as follows: millet, 15.0 g/250 mL Erlenmeyer flask to which was added 15 mL of medium of the following composition (in g/flask): 0.5 g of yeast extract, 0.1 g of sodium tartrate, 0.5 g of sucrose, 0.5 g of alfalfa, 0.1 g of corn oil; and 0.01 g of $FeSO_4 \cdot 7H_2O$. After the flasks were autoclaved once, an additional 15 mL of distilled water was added, and the flasks were autoclaved a second time. The production flasks were inoculated with 2.0 mL of vegetative seed and incubated without agitation at 25 °C for 21 days.

Isolation of Clavaric Acid (1). Solid-state fermentation (40 flasks = 2 L) was extracted with methyl ethyl ketone (3 L) for 2 h. The extract was filtered and evaporated to produce 5.0 g of residue. The residue was dissolved in 200 mL of methanol and was charged on to a 1.5 L Sephadex LH-20 column in methanol. Elution of the column with methanol afforded FPTase-active fractions in 0.6 column volume. The

active fraction was concentrated on a rotovapor to produce a brown residue (1.1 g). A portion of the residue (50 mg) was purified on a reversed-phase Zorbax RX C-8 HPLC column (22.4 × 250 mm) using 60% acetonitrile/water with 0.1% TFA at a flow rate of 7 mL/min. The activity eluted between 30 and 36 min as a broad peak. Lyophilization of the combined active fraction gave **1** (4.6 mg, 50 mg/L) as a colorless powder: $[\alpha]^{25}_D = +29°$ (*c* 0.1, MeOH); UV (MeOH) λ_{max} end absorption; IR (ZnSe) ν_{max} 3600−3100 (broad), 2972, 1716, 1681, 1388, 1203, 1149 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; FABMS (*m*/*z*) 619 $(M + H)^+$, 641 $(M + Na)^+$; HR-FABMS (*m*/*z*) 618.4019 (calcd for $C_{36}H_{58}O_8$, 618.4091).

Methylation of Clavaric Acid (1). To a solution of **1** (4.8 mg) in ether (1.0 mL) was added two drops of diazomethane in ether. The solution was stirred at room temperature until the disappearance of the starting material (3 h). Solvent was evaporated under a stream of nitrogen, and the residue was filtered through a silica plug eluting with 2% methanol in methylene chloride to produce the monomethyl ether (**2**): IR (ZnSe) ν_{max} 3600−3100 (br), 2950, 1724,1457, 1439, 1380, 1199 cm^{-1}; ^{1}HNMR δ ($CDCl_3$) 0.73 (3H, s), 0.87 (3H, s) 0.91 (3H, d, $J = 6$ Hz), 1.12 (3H, s), 1.17 (3H, s), 1.18, (3H, s), 1.22 (3H, s), 1.37 (3H, s), 1.45 (3H, s), 1.26−2.30 (m), 2.33−3.35 (1H, t, $J = 6$ Hz); EIMS (*m*/*z*) 632 (M^+).

Hydrolysis of Clavaric Acid (1). Sodium hydroxide (0.6 mL of 2% solution) was added to a cooled solution of clavaric acid (10.0 mg) in ethanol (1.0 mL) and the resulting mixture stirred overnight at room temperature. The reaction mixture was diluted with water and extracted with ether. The ethereal extract was dried (Na_2SO_4), and the solvent was evaporated under reduced pressure to produce 5.2 mg of clavarinone (**3**) as a white powder: IR (ZnSe film) ν_{max} 3600−3100 (br), 2975, 1722, 1382, 1108 cm^{-1}; ^{1}HNMR δ ($CDCl_3$) 0.72 (3H, s), 0.87 (3H, s) 0.94 (3H, d, $J = 6$ Hz), 1.12 (3H, s), 1.17 (3H, s), 1.22 (3H, s), 1.23 (3H, s), 1.26 (3H, s), 1.26−2.30 (m), 3.35 (1H, t, $J = 6$ Hz); EIMS (*m*/*z*) 474 (M^+).

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