Isolation and Structure of Leukotriene-A_4 Hydrolase Inhibitor: 8(*S*)-Amino-2(*R*)-methyl-7-oxononanoic Acid Produced by *Streptomyces diastaticus*

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Received April 18, 1996[⊗]

The novel amino acid 8(*S*)-amino-2(*R*)-methyl-7-oxononanoic acid (**1**) was isolated from the soil-borne microorganism *Streptomyces diastaticus* during our screening for inhibitors of leukotriene-A_4 hydrolase (LTA_4H), a requisite enzyme in the biosynthesis of the potent inflammatory mediator leukotriene-B_4 (LTB_4). The structure of **1** was determined by detailed spectroscopic analyses and is related to 7-keto-8-aminopelargonic acid (**2**), a biosynthetic precursor of biotin. The relative potency of **1** (LTA_4H IC_{50} = 0.6 μM) warranted further biological studies.

Leukotriene-B_4 (LTB_4) is a potent inflammatory mediator that is chemotactic for neutrophils and eosinophils and activates neutrophil aggregation and degranulation.[1] Blockage of LTB_4 action or production is likely to have therapeutic utility in a number of diseases, as elevated LTB_4 production has been shown to occur in human diseases including psoriasis, inflammatory bowel disease, contact dermatitis, asthma, rheumatoid arthritis, and gout.[1,2]

Leukotriene-A_4 hydrolase (LTA_4H) is a requisite enzyme in the biosynthetic pathway of LTB_4 production.[3−5] Screening natural product extracts for inhibitors of LTA_4H led to the discovery of the soil-borne microorganism *Streptomyces diastaticus* (Streptomycetaceae) producing the novel amino acid 8(*S*)-amino-2(*R*)-methyl-7-oxononanoic acid (**1**). This paper describes the isolation, structure elucidation, and biological testing of **1**.

Bioassay-guided fractionation of two 10-L fermentations using cation exchange chromatography and reversed-phase HPLC yielded 16 mg of the active compound as a colorless oil. The HRFABMS of **1** yielded the molecular formula $C_{10}H_{19}NO_3$ indicating two degrees of unsaturation. The ^{1}H-NMR spectrum of **1** in DMSO-d_6 showed two methyl doublets, several deshielded protons between δ 2.0 and 4.5, and a broad downfield signal at δ 8.1 (brs, 3H), which disappeared in D_2O, indicating the presence of three exchangeable protons. The ^{1}H-NMR spectrum of **1** in D_2O (Table 1) showed an isolated methine quartet at δ 4.25 (J = 7.4 Hz), two multiplets at δ 2.58 (m, 1H) and 2.66 (m, 1H) in an ABX_2 system, a methine multiplet at δ 2.37, six aliphatic multiplets between δ 1.2 and 1.7, and two methyl doublets at δ 1.48 (J = 7.4 Hz) and 1.05 (J = 6.9 Hz).

COSY showed two distinct spin systems (Table 1). A proton absorbing at δ 4.25 (q, J = 7.4 Hz, 1H) was coupled to the methyl protons at δ 1.48. This connectivity group was not further extended with COSY, which suggested the methine was bracketed by a quaternary carbon and a heteroatom. The second connectivity pattern evaluated in conjunction with the HMQC experiment showed the methyl doublet at δ 1.05 coupled with the methine multiplet at δ 2.37, which was further extended through four contiguous aliphatic methylenes terminating with geminal protons at δ 2.58 and 2.66, appropriate for adjacency to an acyl group. The combination of HMQC and HMBC experiments allowed complete assignment of the carbon spectrum (Table 1) with the placement of the C-7 ketone by HMBC correlation with the δ 1.48 methyl, and the C-1 acid by HMBC correlation with the δ 1.05 methyl.

Table 1. δ Carbon, δ Proton (J in Hz), COSY, and HMBC NMR Data for **1** (D_2O)

position	C-13	H-1	COSY	HMBC
1	182.2			1.05, 1.38, 2.37
2	39.1	2.37 m	1.55, 1.38, 1.05	1.05
3	33.5	1.55 m	1.25, 1.38, 2.37	1.05, 1.25
		1.38 m	2.37, 1.25, 1.55	
4	25.7	1.25 m	1.38, 1.5−1.6	1.38, 2.58
5	22.4	1.55 m	1.25, 2.58, 2.66	1.25, 2.58
6	37.8	2.58 m	2.66, 1.55	
		2.66 m	2.58, 1.55	
7	209.2			1.48, 2.58, 2.66
8	54.7	4.25 q (7.4)	1.48	1.48
9	14.6	1.48 d (7.4)	4.25	4.25
10	16.3	1.05 d (6.9)	2.37	

Table 2. Determination of Absolute Stereochemistry of **1** Using Molecular Rotation Additivities

model compound	$[\alpha]^{24}_D$	$[M]^{24}_D$
3(*R*)	−18	−18
4(*S*)	−3.5	−10
3(*S*) + **4**(*S*)		8
3(*S*) + **4**(*R*)		28
3(*R*) + **4**(*S*)		−28
3(*R*) + **4**(*R*)		−8
1	−13	−24

The absolute stereochemistry of **1** was based on the molecular additivity method using 2(*R*)-methylbutyric acid (**3**)[6] and 2(*S*)-aminooctadecan-3-one (**4**)[7] as models. As Table 2 indicates, the 2(*R*) and 8(*S*) configurations for **1** give the best fit.

LTA_4H is a zinc-dependent enzyme with both hydrolase (LTA_4 to LTB_4 conversion) and arginine aminopeptidase activity.[5] The potent hydrolase (IC_{50} = 0.6 μM) and aminopeptidase activity of **1** (IC_{50} = 0.3 μM) raised

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[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1996.

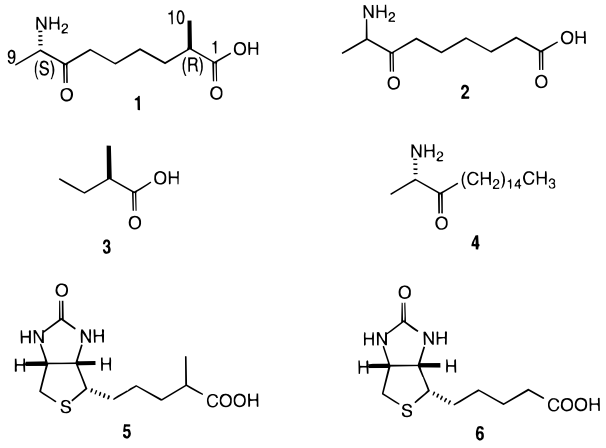
S0163-3864(96)00398-9 CCC: $12.00

Table 3. Bioactivity and Selectivity of **1**

bioassay	IC_{50} μM (n)
rhLTA$_4$H: LTB$_4$ production	0.57 ± 0.17 (n = 3)
rhLTA4: aminopeptidase	0.3
human whole blood: LTB$_4$ production	7
human whole blood: TxB$_2$ production	>30
rabbit lung ACE	>100

the question of selectivity and possible coordination of **1** with the zinc cofactor. Testing of **1** with another zinc-containing system such as angiotensin-converting enzyme (ACE) indicated that **1** was functioning in a selective manner (Table 3). The LTA$_4$H activity of **1** in human whole blood was approximately 12-fold less than in the isolated enzyme assay, indicating that **1** maintained activity in a biologically relevant matrix. The activity of **1** was selective for inhibition of LTB$_4$ vs the cyclooxygenase product thromboxane (TxB$_2$) (Table 3).

Compound **1** is structurally similar to the known biotin precursor, 7-keto-8-aminopelargonic acid (**2**), except for the methyl group at the 2-position. This similarity is also found between α-methylbiotin (**5**) and biotin (**6**), suggesting that **1** could be a precursor to α-methylbiotin in microorganisms, based upon the biotin biosynthesis scheme outlined by Rolfe and Eisenberg.[8] Biotin is a coenzyme to carboxylases[9] and α-methylbiotin and α-methyldethiobiotin have been reported to be antimetabolites of biotin and antimicrobial agents.[10]



Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Varian XR-500 spectrometer at 500 MHz (^{1}H) or 125 MHz (^{13}C) in D_2O using external referencing. LRESMS were obtained on a Sciex API-III, and the HRFABMS was obtained on a Finnigan MAT 90 using Electro Calibration with PEG. The optical rotation was obtained on a Perkin-Elmer 241 polarimeter using a 1.0 mL microcell.

Culture Conditions. A prepared seed vial of A12285 *Streptomyces diastaticus* was inoculated into a 2.8-L shake flask containing 500 mL of growth medium and placed on a rotary shaker at 200 rpm at 30 °C for 48 h. The shake culture was inoculated into a 15-L fermenter containing 9.5 L of medium, consisting of maltrin (10 g/L), MOPS (5.25 g/L), NH_4NO_3 [1.0 g/L, $MgSO_4$ (1.0 g/L), NaCl (0.5 g/L), and 1.0 mL/L of trace metal solution (1.0 mg/mL $FeSO_4$·7 H_2O, 1.0 mg/mL $MnCl_2$·4 H_2O, 1.0 mg/mL $ZnSO_4$·7 H_2O, 1.0 mg/mL $CuSO_4$·5 H_2O, 1.0 mg/mL $CoCl_2$·6 H_2O]. The fermentation was run at 30 °C and 400 rpm for 5 days prior to harvesting.

Isolation of 8(*S*)-Amino-2(*R*)-methyl-7-oxononanoic Acid (1). Isolation of **1** from two 10-L fermentations was accomplished by bioassay-guided fractionation. The centrifuged and 0.2-μm filtered fermentations were chromatographed over 500 g of Amberlite 200 strong cation exchange resin (Rohm and Hass). After loading, 2-L fractions of deionized H_2O, 0.25 M, and 0.5 M NH_4-OH were eluted. Activity was quantitatively recovered by combining the two NH_4OH fractions, which yielded 3.5 g dry wt. The sample was redissolved in 50 mL of H_2O for flash chromatography on 50 g of Bakerbond octadecyl 40-μm resin. Stepwise 50-mL elutions of 10, 20, and 50% aqueous MeOH yielded 480 mg (40% activity recovered) by combining the last two fractions. Preparative HPLC (21.4 × 250 mm, 8 μm, Rainin Dynamax C-18 column, 10 mL/min using a gradient of 0−40% CH_3CN in H_2O with 0.05% TFA) yielded 39 mg active material (ca. 80% pure by proton NMR). Final purification by semipreparative HPLC (10 × 250 mm, 5 μm, Rainin Microsorb C-18 column, 4 mL/min using a gradient of 0−25% CH_3CN in H_2O with 0.05% TFA) yielded 16 mg (ca. 95% pure by proton NMR) of **1** as the TFA salt for NMR and biological analysis. Overall recovered activity yield was 18%.

8(*S*)-Amino-2(*R*)-methyl-7-oxononanoic acid (1): colorless oil; HRFABMS m/z $[MH]^+$ 202.1467 ($C_{10}H_{19}NO_3$ + H req. 202.1443, Δ +2.4 mmu); $[\alpha]^{24}_D$ −13 (c 0.003, H_2O). ^{1}H, ^{13}C, PCOSYX, HMQC, and HMBC NMR, see Table 1.

Bioassays. The following assays were carried out in a 96-well microtiter plate format using 10 mM aqueous solution of **1** as the test solution.

Recombinant Human LTA$_4$ Hydrolase Assay (rhLTA$_4$). The rhLTA$_4$ assay was prepared as previously described[11] and stored at −20 °C as a 3.5 mg/mL stock solution in 50 mM TRIS, 150 mM NaCl, 2.5 mM β-mercaptoethanol, 50% v/v glycerol buffer, pH 8.0. LTA$_4$ substrate was prepared from the methyl ester in THF (Biomol) by the addition of 30 molar equivalents of LiOH at room temperature for 18 h and stored at −80 °C until used. Enzyme was diluted to 4.7 μg/mL in assay buffer [100 mM potassium phosphate pH 7.4 with 5 mg/mL fatty acid free bovine serum albumin (BSA) and 10% DMSO]. An 85-μL (400 ng) aliquot of the diluted enzyme was pre-incubated with 15 μL of test sample in assay buffer for 30 min at room temperature. The LTA$_4$ substrate was diluted immediately before use in assay buffer without DMSO to a concentration of 5 ng/mL, and 50 μL (250 pg) was added to the assay. The assay solution was mixed, and, after a 15-min incubation at room temperature, the reaction was stopped by the addition of 10 μL of 1 μM (*R,S*)-kelatorphan. The LTB$_4$ was measured in a solid-phase ELISA assay using a commercially available LTB$_4$ acetylcholinesterase conjugate (LTB$_4$-Tracer, Cayman Chemical Co.) and a rabbit anti-LTB$_4$ polyclonal antibody generated at Searle. Corning ELISA plates were precoated with mouse anti-rabbit IgG and blocked with a 1 mg/mL solution of BSA; 20 μL of the stopped enzyme reaction or standards were added to 50 μL of diluted LTB$_4$-Tracer and 80 μL of diluted rabbit anti-LTB$_4$ in the precoated ELISA plate, and the plate was incubated at 4 °C overnight. The plates were washed and 100 μL of combined

Ellman's reagent (0.5 mM 5,5′-dithiobis(2-nitrobenzoic acid) and substrate (1.0 mM acetylthiocholine in 10 mM of potassium phosphate buffer, pH 7.4) was added, and the plates were read at 405 nm after a 2-h incubation at room temperature. LTB_4 was quantified from a LTB_4 standard curve generated with each experiment.

Peptidase Assay. The aminopeptidase activity of $rhLTA_4$ was measured using a modification of the procedure published by Orning et al.[12] Aminopeptidase activity was determined spectrophotometrically by measuring the production of *p*-nitroanaline from the substrate L-leucine-*p*-nitroanilide (Leu-*p*NA). Test compounds were pre-incubated with 1 μg of $rhLTA_4$ in 100 mM MOPS, 0.1% BSA, pH 7.0 buffer for 60 min in a volume of 150 μL; 50 μL of a 120-μg/mL stock solution of $rhLTA_4$ (1 μg) was added and mixed and the absorbance measured at 405 nM every 15 min for 1 h at room temperature. The rates were determined from the slope of the absorbance vs time.

Human Whole Blood Assay. Human blood collected in heparin was diluted 1:4 with RPMI-1640 media, and 200 μL was added in 96-well mictrotiter plates. Serially diluted test solution was added, and the plate was incubated for 15 min at 37 °C in a humidified incubator. Calcium ionophore A23187 (20 μg/mL final conc) was added, and the incubation was continued for 10 min. The assay was terminated by centrifugation (833 g, 10 min, 4 °C). Supernatants were analyzed for LTB_4 and TxB_2 by ELISA using commercially available reagents.

ACE Assay. In polystyrene 96-well microtiter plates were mixed 10 μL of DMSO or test compound in 10 μL of DMSO, 100 μL of buffer (0.05 M HEPES, pH 7.5, containing 0.3 M NaCl), 50 μL of diluted enzyme [ACE from rabbit lung (Sigma A-6778), dissolved in 2 units in 1.0 mL buffer and stored at −20 °C, diluted 1/20 in buffer before assay]. Then the mixture was preincubated for 10 min at 37 °C. Next was added 40 μL substrate [*N*-(3-(2-furyl)acryloyl)-Phe-Gly-Gly (Sigma F-7131), 2.5 mM solution in buffer], which was then mixed and incubated at 37 °C for 10 min. Decrease in absorbance at 340 nm was read. Captopril was included as a standard inhibitor on each plate.

Acknowledgments We thank Mr. William C. Hutton, Dr. John J. Kotyk, and Ms. Cinda S. Page of Monsanto's NMR facility for development of the PCOSYX, HMQC, and HMBC experiments and for their assistance. We thank Dr. Kevin L. Duffin, Mr. James P. Doom, and Mr. Ralph Scheibel of Monsanto's mass spectrometry facility for providing LRMS and HRMS data. We thank Ms. Vicki L. Chlanda for FTIR analysis. For secondary assay data, we thank Ms. Kathryn A. Houseman and Ms. Elizabeth I. Harding. We are also grateful to Ms. Margaret Miller-Wideman, Mr. Joseph K. McLaughlin, Dr. Ping T. Wang, and Mr. Michael J. Prinsen for fermentation and microbiology support.

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NP9603986