ISOLATION OF A58365A AND A58365B, ANGIOTENSIN CONVERTING ENZYME INHIBITORS PRODUCED BY *STREPTOMYCES CHROMOFUSCUS*†

JON S. MYNDERSE*, SUSAN K. SAMLASKA††, DAVID S. FUKUDA, RENE H. DU BUS and PATRICK J. BAKER

The Lilly Research Laboratories, Eli Lilly & Company, Lilly Corporate Center, Indianapolis, Indiana 46285, U.S.A.

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A58365A and A58365B, angiotensin converting enzyme inhibitors, were isolated from the culture filtrate of *Streptomyces chromofuscus* NRRL 15098. A58365A and A58365B are homologous nitrogen-containing bicyclic structures of molecular formulae $C_{12}H_{13}NO_6$ and $C_{13}H_{15}NO_6$.

Recently, several inhibitors of angiotensin converting enzyme (ACE) have been discovered from fermentation sources[1~11]. A previous paper in the present series described the development of a high volume, agar-based screen for the detection of ACE inhibitors produced by fermentations and the discovery of ACE inhibitory activity in the culture broth of *Streptomyces chromofuscus* NRRL 15098[12] (culture A58365.1). The conditions for the biosynthesis of the ACE inhibitors A58365A and A58365B produced by this microorganism have also been described[13]. This paper describes the isolation and characterization of the two ACE inhibitors.

Materials and Methods

Assay of Angiotensin Converting Enzyme Inhibitory Activity

The qualitative agar-plate method of O'CONNOR and SOMERS[12] was employed for monitoring purification of ACE inhibitors. Dried preparations were assayed in the quantitative spectrophotometric assay[12] to determine inhibitor potency.

HPLC Assay of A58365A and A58365B

HPLC determinations of A58365A and A58365B utilized a 4 mm×30 cm Waters Associates μBondapak C_{18} reversed phase column with a mobile phase consisting of CH_3CN - HCOOH - H_2O (6.0: 0.3: 93.7). Broths were adjusted to pH 2.0 and filtered (0.45 μm) prior to injection. Partially purified materials required no sample preparation. Detection was by either UV absorption at 325 nm or by fluorescence measurement. For accurate quantitation a Schoeffel model FS970 spectrofluorometer with excitation at 327 nm and a 370 nm emission cutoff filter coupled to a Hewlett-Packard 3390A integrator was used. With a mobile phase flow rate of 2.5 ml/minute, A58365A had a retention time of 4.88 minutes; A58365B, 12.47 minutes.

General Methods

UV spectra were run on a Cary model 118 spectrophotometer. Fluorescence spectra were obtained with an Amino-Boman Spectrophotofluorometer. IR spectra were recorded on a Nicolet MX-1 FT-IR spectrometer. ^1H NMR spectra were obtained with a Bruker model WH-360 NMR

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†† Present address: Ortho Pharmaceutical Corp., Raritan, New Jersey 08869, U.S.A.

spectrometer. Optical rotations were determined on a Perkin-Elmer model 241 polarimeter. EI, FD and FAB mass spectra were run on a Varian-MAT 731 spectrometer.

Isolation and Purification of A58365A and A58365B

Whole broth (305 liters) was acidified to pH 2.0 with concentrated hydrochloric acid and then filtered with 2% Hyflo filter aid. The filtered broth (285 liters; 15.8 μg/ml A58365A, 0.38 μg/ml A58365B) was adjusted to pH 7.0 with 5 N NaOH. Diaion HP-20 macroreticular resin (30 liters) was added to the neutral broth, stirred for two hours, and then filtered off to remove large quantities of neutral components introduced by use of OM peptone (Amber Laboratories) in the fermentation medium[13]. The effluent was reacidified to pH 2.0 with 5 N HCl, chilled, then filtered to remove inactive precipitates. The acidified broth was applied to a 10 cm×6.1 m column containing 20 liters of HP-20 resin and the effluent was discarded. The column was washed with 60 liters of 0.3% aqueous formic acid and the effluent was discarded. The ACE inhibitors were then eluted with a 100-liter gradient from H_2O - HCOOH (99.7: 0.3) to CH_3CN - H_2O - HCOOH (20: 79.7: 0.3) and 2-liter fractions were collected. Fractions 27~48, containing factor A, were pooled and concentrated under reduced pressure to a volume of 750 ml. Elution was continued with 20 liters of CH_3CN - H_2O - HCOOH (20: 79.7: 0.3). Fractions 56~63, containing factor B, were pooled and concentrated to a volume of 350 ml.

The factor A-containing concentrate was applied to a 9.3 cm×80 cm (5 liters) column of Dowex 50W-X2 (H^+) resin and eluted with 17 liters of deionized water; 2-liter fractions were collected. Fractions 9~15 were collected, pooled and concentrated to about 200 ml. The factor A concentrate (pH 2~3) was filtered and the filtrate chromatographed by reversed phase high performance liquid chromatography (RPHPLC) on a 8 cm×1 m column (Jobin Yvon Chromatospac Prep 100) containing 2.5 kg (approx 4.2 liters) of octadecylsilanized Whatman LP-1 silica gel. The column was developed with HCOOH - H_2O (2 liters, 0.3: 99.7), CH_3CN - HCOOH - H_2O (5 liters, 1.0: 0.3: 98.7), and finally CH_3CN - HCOOH - H_2O (20 liters, 2.5: 0.3: 97.2), collecting 500-ml fractions. Fractions 32~44, containing factor A, were pooled and concentrated to 200 ml under reduced pressure.

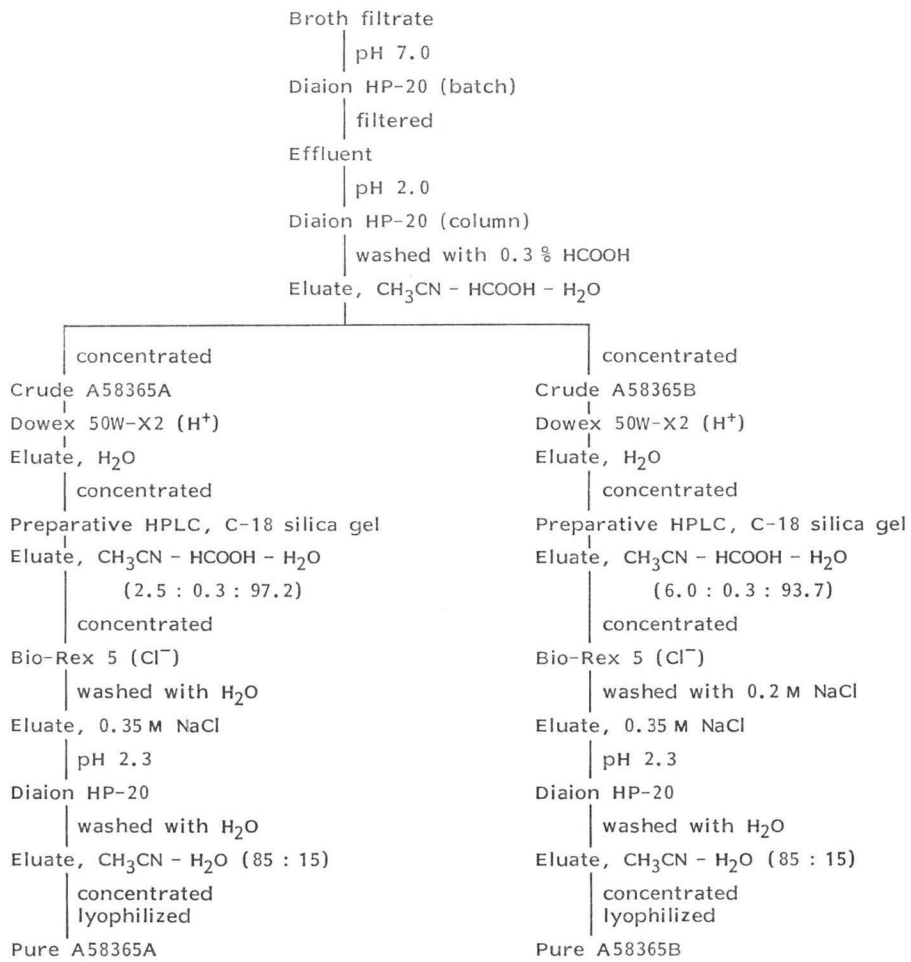
The factor A concentrate from HPLC was applied to a 2.5 cm×30 cm (180 ml) column of Bio-Rex 5 (Cl^-) anion exchange resin (100~200 mesh). The resin was washed with deionized water, then the inhibitor was eluted with 2.2 liters of 0.35 M NaCl, collecting 20-ml fractions. Fractions 106~140, containing pure factor A, were pooled. The pool was adjusted to pH 2.3 with 1 N hydrochloric acid and applied to a 2.8 cm×19 cm (120 ml) column of Diaion HP-20 resin equilibrated in 0.01 N hydrochloric acid. The column was washed with 100 ml of dil aqueous HCl (pH 2.3), followed by 200 ml of deionized water (pH 5.9). Factor A was finally eluted with 340 ml of CH_3CN - H_2O (15: 85). One hundred eighty ml of eluate was collected, concentrated under reduced pressure, and lyophilized to give 1.31 g of pure A58365A.

The factor B-containing concentrate from the HP-20 resin column was applied to a 9.3 cm×80 cm (5 liters) column of Dowex 50W-X2 (H^+) cation exchange resin. The column was eluted with 32 liters of deionized water, collecting 1-liter fractions. Fractions 10~14 were combined and concentrated to 250 ml under reduced pressure. The factor B concentrate was subjected to RPHPLC on the same column as factor A. The column was eluted with HCOOH - H_2O (0.3: 99.7), CH_3CN - HCOOH - H_2O (6.0: 0.3: 93.7), and then CH_3CN - HCOOH - H_2O (15.0: 0.3: 84.7). A single 500-ml fraction containing A58365B was collected and concentrated under reduced pressure to 100 ml. The concentrate was applied to a 2.0 cm×25 cm column of Bio-Rex 5 (Cl^-) anion exchange resin (100~200 mesh). The column was eluted with 200 ml of 0.2 M NaCl followed by 1,600 ml of 0.35 M NaCl, collecting 10-ml fractions. Fractions 193~210, containing factor B, were pooled, acidified (to pH 2.3 with 1 N HCl), and applied to a column of HP-20 resin (8 mm×20 cm, 10 ml) equilibrated in 0.01 N HCl. The column was washed with 300 ml of dil aqueous HCl (pH 2.3), washed with 14 ml of deionized water, and then eluted with 44 ml of CH_3CN - H_2O (15: 85). Fractions containing pure, desalted factor B were pooled (8 ml), concentrated under reduced pressure, and lyophilized to give 2.9 mg of pure A58365B.

Table 1. Spectral properties of A58365A.

UV λ_{max} nm (ε)	Neutral, acid	232 (6,000), 325 (7,600)
	Base	243 (7,200), 353 (7,400)
Fluorescence	Neutral, acid	Excitation maximum 325 nm Emission maximum 397 nm
	Base	Excitation maximum 350 nm Emission maximum 424 nm

Fig. 1. Isolation of A58365A and A58365B.



Results and Discussion

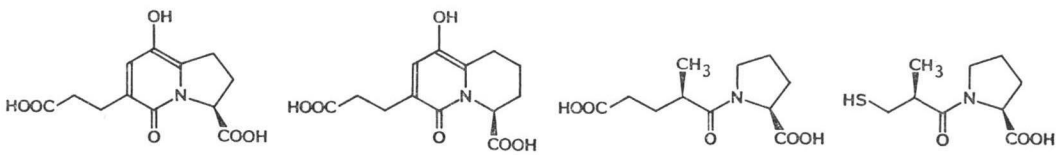
A58365A was first isolated (1 mg) from 100 liters of broth containing approximately 1 μg/ml of the ACE inhibitor by means of ion suppression chromatography on Diaion HP-20 nonionic macroreticular resin and repeated RPHPLC on C-18 silica resin. Rapid ACE-inhibition assay by the agar-plate method of O'CONNOR and SOMERS[12] facilitated monitoring of the purification of the enzyme inhibitor.

The UV spectrum of A58365A (Table 1) exhibited a bathochromic shift in base, consistent with our observation that acidic conditions suppress ionization of the compound and thus permit retention

Table 2. Physico-chemical properties of A58365A and A58365B.

	A58365A	A58365B
Appearance	White amorphous powder	White amorphous powder
$[\alpha]_D^{25}$	−199.5° (*c* 1.0, H_2O)	−141.2° (*c* 0.16, H_2O)
Electrometric titration *pK*	5.7, 7.5, 12.3	6.2, 7.7, 13.0
Molecular formula	$C_{12}H_{13}NO_6$ (MW 267)	$C_{13}H_{15}NO_6$ (MW 281)
Anal Calcd	C 53.93, H 4.90, N 5.24	
Found	C 53.72, H 5.10, N 5.16	
Mass spectrometry	FD-MS *m/z* 267 (M), 223 (M−CO_2)	FD-MS *m/z* 281 (M)
	FAB-MS *m/z* 268 (M+H)	EI-MS *m/z* 281 (M)
HRMS Calcd	268.08211 (M+H)	281.08994 (M)
Found	268.08189 (FAB-MS)	281.09035 (EI-MS)
UV λ_{max}^{MeOH} nm (ε)	232 (6,000), 325 (7,600)	233 (3,800), 332 (4,500)
IR (KBr) cm^{-1}	3500~2700, 1719, 1660, 1526, 1410, 1285, 1210	3700~2700, 1715, 1657, 1525, 1414, 1281, 1223
^1H NMR (360 MHz, D_2O) δ	2.30 (1H, m), 2.57 (1H, m), 2.64 (2H, t), 2.74 (1H, dt), 2.81 (1H, dt), 3.05 (1H, dd), 3.15 (1H, ddd), 5.01 (1H, dd), 7.33 (1H, s)	1.67 (1H, m), 1.86 (1H, m), 2.08 (1H, m), 2.35 (1H, m), 2.66 (2H, t), 2.76 (1H, m), 2.78 (2H, m), 2.93 (1H, m), 5.10 (1H, dd), 7.35 (1H, s)

Fig. 2. Structures of angiotensin converting enzyme inhibitors A58365A (**1**), A58365B (**2**), 2-methylglutaryl-L-proline (**3**) and captopril (**4**).



1 **2** **3** **4**

on reversed phase HPLC supports. In the course of the isolation, intense blue fluorescence was observed upon exposure of A58365A to long wavelength UV light (see Table 1).

Sensitive, specific HPLC assays were developed for the ACE inhibitors, based upon the observed UV absorption and fluorescence characteristics. Using the HPLC assays, an improved isolation scheme (Fig. 1) was developed.

The filtered broth (285 liters) contained 15.8 μg/ml of the A factor and 0.38 μg/ml of the B factor. The overall recovery was 29% for A58365A and 2.7% for A58365B. Factors A and B were obtained as white lyophilized powders which were freely soluble in water, dimethyl sulfoxide, and methanol; sparingly soluble in acetone; and insoluble in solvents such as chloroform and hexane. The two factors did not exhibit any antimicrobial activity. Solutions of factor B, like factor A, exhibited intense blue fluorescence under ultraviolet light. The physico-chemical properties of A58365A and A58365B are shown in Table 2.

The structure elucidation of the two new angiotensin converting enzyme inhibitors is described elsewhere[14]. A58365A was determined to be 3-carboxy-1,2,3,5-tetrahydro-8-hydroxy-5-oxo-6-indolizinepropanoic acid (**1**); A58365B was the homologous 4-carboxy-1,3,4,6-tetrahydro-9-hydroxy-6-oxo-2*H*-quinolizine-7-propanoic acid (**2**) (see Fig. 2). A58365A is, in fact, a naturally occurring conformationally restricted analog of 2-methylglutaryl-L-proline (**3**), which was a part of the structure-

activity relationship studies leading to captopril (**4**)[15]. The *in vitro* and *in vivo* activities of the A58365 ACE inhibitors and modification products will be reported in a future publication.

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