

DISCOVERY, PURIFICATION AND CHARACTERIZATION  
OF THE ANGIOTENSIN CONVERTING ENZYME INHIBITOR,  
L-681,176, PRODUCED BY *STREPTOMYCES* SP. MA 5143a

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L-681,176, an inhibitor of angiotensin converting enzyme was found in the culture filtrate of *Streptomyces* sp. MA 5143. The  $I_{50}$  of the crystalline inhibitor is about 1.3  $\mu\text{g/ml}$  and the inhibition is reversed by zinc sulfate. In rats, L-681,176 exhibits a dose-related inhibition of the pressor response to angiotensin I with an  $ID_{50}$  of 142 mg/kg when administered intravenously. The structure of L-681,176 is similar to that of marasminine but lacking one carboxyl group. The maximum yield of L-681,176 occurs after three to four days growth at 28°C.

Angiotensin converting enzyme (peptidyl dipeptide hydrolase EC 3.4.15.1) generates angiotensin II from angiotensin I<sup>1,2)</sup>. Angiotensin II has been implicated as a true vasoconstrictor while angiotensin I is not<sup>3)</sup>. The discovery and elucidation of pharmacologic action of the nonapeptide inhibitor SQ 20881 which was obtained originally from *Bothrops jararaca* venom have underscored the importance of converting enzyme as a target for therapeutic treatment of essential hypertension<sup>4-7)</sup>. Since then many potent and orally active inhibitors such as SQ 14225<sup>8)</sup> and MK-421<sup>9)</sup> have been synthesized. In the process of searching for new types of biologically active metabolites from microorganisms, we have discovered an inhibitor of angiotensin converting enzyme. This inhibitor is designated as L-681,176.

### Materials and Methods

#### Culture and Medium Conditions

The microorganism used in this study, *Streptomyces* sp. MA 5143a, was obtained from a soil isolation program at the MSDRL, CIBE Laboratories, Madrid, Spain. The seed medium contained 0.1% dextrose, 1% soluble starch, 0.3% beef extract, 0.5% yeast autolysate, 0.5% N-Z-amine, 0.005%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.018%  $\text{KH}_2\text{PO}_4$ , 0.019%  $\text{Na}_2\text{HPO}_4$  and 0.05%  $\text{CaCO}_3$ , pH 7.0 to 7.2. The production medium contained 1.5% corn steep liquor, 0.4%  $(\text{NH}_4)_2\text{SO}_4$ , 0.6%  $\text{CaCO}_3$ , 2% soluble starch, 0.1% corn meal, 0.5% 4S-soy bean meal, 0.5% glucose, 0.03%  $\text{KH}_2\text{PO}_4$  and 0.25% lard oil. The pH of the medium is about 6.7. A lyophilized culture was inoculated into a 250-ml flask containing 55 ml of seed medium. Two ml of this two-day old seed culture was then inoculated into a 250-ml production flask containing 50 ml of production medium. The flask was incubated for 4 days at 28°C on a rotary shaker.

#### Assay of Angiotensin Converting Enzyme Inhibitory Activity

The assay method of HAYAKARI *et al.*<sup>10)</sup> was used. Hippuryl-L-histidyl-L-leucine (HHL, Sigma Co.) was used as a substrate and rat lung homogenate was used as an enzyme source. In the routine assay, 0.2 ml of reaction mixture containing 1 mM of HHL, 0.5 M NaCl and 0.06 M potassium phosphate buffer, pH 8.3 was mixed with 10  $\mu\text{l}$  of 50% aqueous methanol or a 50% methanolic extract of fermentation broth and 25  $\mu\text{l}$  of diluted enzyme suspension. The mixture was incubated at 37°C for 30 minutes and the enzyme reaction was stopped by placing the tube in a boiling water bath for 10 minutes. One ml of 0.2 M potassium phosphate buffer pH 8.3 was added, followed by 0.5 ml of 3% 2,4,6-trichloro-*s*-

triazine in dioxane after which the tube was vortexed immediately. The yellow color was measured at 382 nm.

#### The Pressor Response to Angiotensin-I

This assay was run in two rats each at various dosage levels. The detailed protocols were as described by GROSS *et al.*<sup>11)</sup>. The inhibition of a pressor response elicited by 100 ng/kg iv of angiotensin I was assessed. SQ 14225 or captopril were used as reference standards. L-681,176 was injected intravenously at a dose range of 1~30 mg/kg.

### Results and Discussion

#### Production of L-681,176 by *Streptomyces* sp. MA 5143a

The growth of *Streptomyces* sp. MA 5143a was monitored by both cell volume and the utilization of reducing sugar. The production of L-681,176 was monitored by the inhibition of angiotensin converting enzyme, and purified L-681,176 was used as a standard. The activity was also confirmed by thin-layer chromatography and the reversal of inhibition by zinc sulfate. The production pattern of L-681,176 is shown in Fig. 1. The production of L-681,176 was initiated when the majority of reducing sugar was utilized and was greatly accelerated after cell growth had ceased. The concentration of L-681,176 reached its maximum at about 78 hours and began to decrease after 100 hours.

#### Isolation and Characterization of L-681,176

The procedure for the isolation is summarized in Fig. 2. The culture broth (1 liter) was filtered through Celite. The filtered broth was then passed through a Dowex-50 (NH<sub>4</sub><sup>+</sup>) column at pH 3.5 and the column was eluted with 2% pyridine after washing the column with water. The activity in the pyridine eluate was adsorbed to Dowex-50X4 (200~400 mesh) equilibrated in pyridinium acetate buffer (pH 3.0~3.5) and eluted stepwise with pyridinium acetate buffers of increasing pH. The activity was eluted at pH 4.0 to 4.5. The enriched fraction crystallized spontaneously from water. The yield of crystalline material was about 126 mg. It was homogeneous as determined by thin-layer chromatography on silica gel using butanol - methanol - water (2: 1: 1) as a solvent system. Its purity was confirmed by

Fig. 1. The production of L-681,176 versus the incubation time of *Streptomyces* sp. MA 5143a at 28°C.

The production of L-681,176 is indicated by solid circles (●). The level of reducing sugar is indicated by solid squares (■). Cell volume was indicated by solid triangles (▲).

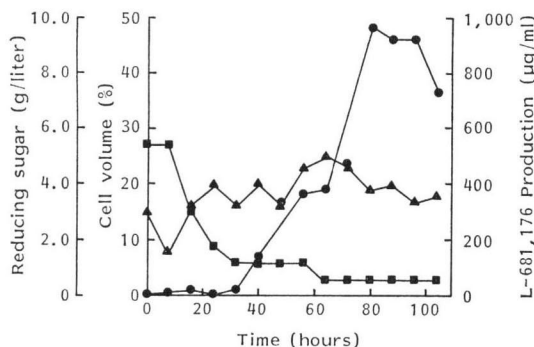


Fig. 2. Purification steps.

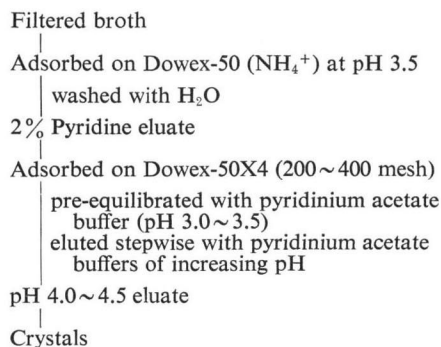
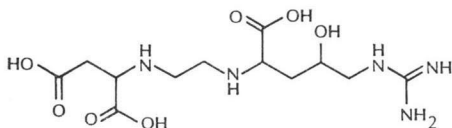


Fig. 3. Proposed structure of L-681,176.

Table 1. Reversal of enzyme inhibition by Zn<sup>++</sup>.

Sample	Concentration	ZnSO <sub>4</sub>	Inhibition (%)
L-681,176	1.3 μg/ml or 21 μl of broth	—	51
L-681,176	"	5 × 10 <sup>-5</sup> M	0
L-681,176	"	5 × 10 <sup>-6</sup> M	7
SQ 14225	0.2 μg/ml	—	96

NMR and mass spectrum. The molecular weight of this crystalline material is 349 with a molecular formula of C<sub>12</sub>H<sub>23</sub>N<sub>5</sub>O<sub>7</sub>. It shows only end absorption in ultraviolet region and it gives a positive SAKAGUCHI test which detects a guanidine derivative. This compound is very polar and is water soluble. The IR spectrum indicates that it has a broad carboxylate absorption at 1570 cm<sup>-1</sup> and two other absorption bands at 1675 cm<sup>-1</sup> and 1635 cm<sup>-1</sup>, which are probably due to the monoalkyl guanidine group. The determination of the structure of L-681,176 is the subject of a separate paper<sup>12)</sup>. L-681,176 is similar to marasmin<sup>12)</sup> but lacks one carboxyl group as shown in Fig. 3. This inhibitor appears to be a novel compound which may serve as a lead for a new type of angiotensin converting enzyme inhibitor.

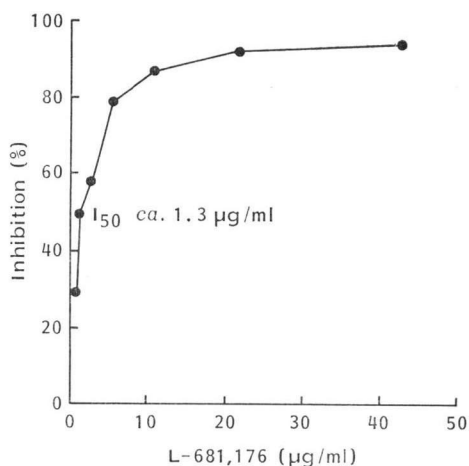
#### *In Vitro* Activity Against Angiotensin Converting Enzyme

L-681,176 was found to possess a dose-related inhibition of converting enzyme as shown in Fig. 4. The I<sub>50</sub> was estimated to be about 1.3 μg/ml (3.7 × 10<sup>-6</sup> M). The inhibition can be reversed completely by zinc sulfate (5 × 10<sup>-5</sup> M) as shown in Table 1. In this particular experiment, the inhibitor was preincubated with zinc sulfate for 5 minutes at 37°C before the enzyme reaction was initiated. SQ 14225 was used as a positive control. It gave 96% inhibition at 0.2 μg/ml and its I<sub>50</sub> is estimated to be about 0.01 μg/ml (2.1 × 10<sup>-8</sup> M). It appears that L-681,176 inhibits angiotensin converting enzyme by chelating mechanism.

#### *In Vivo* Activity Against the Pressor Response to Angiotensin I

L-681,176 was tested intravenously at a dose range of 1~30 mg/kg and found to possess a dose-related inhibition in rats of the pressor response to angiotensin I with an ID<sub>50</sub> of 142 mg/kg. SQ 14225 was used as a positive control and was active with an ID<sub>50</sub> of 60.5 μg/kg.

Fig. 4. Inhibition of angiotensin converting enzyme versus the concentration of L-681,176.



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