# ISOLATION OF DEALANYLALAHOPCIN, A NEW AMINO ACID, AND ITS BIOLOGICAL ACTIVITY

EIJI HIGASHIDE, TSUNEO KANAMARU, HIROSHI FUKASE and SATOSHI HORII

Central Research Division, Takeda Chem. Ind., Ltd.
Yodogawa-ku, Osaka 532, Japan

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A new amino acid, dealanylalahopcin, was isolated from a culture filtrate of *Streptomyces albulus* subsp. *ochragerus*; it was also formed by the enzymatic hydrolysis of alahopcin using microbial $\alpha$-amino acid ester hydrolase. The amino acid was obtained as colorless needles and its molecular formula is $C_6H_{10}N_2O_5$. It showed very weak antibacterial activity against Gram-positive and Gram-negative bacteria, and weak inhibitory activity against the collagen prolylhydroxylase.

In a previous paper[1], we reported that alahopcin, a new dipeptide antibiotic was isolated from a culture filtrate of *Streptomyces albulus* subsp. *ochragerus*. The antibiotic has unique biological properties in its antibacterial activity, inhibitory activity against collagen prolylhydroxylase and stimulatory effect on the production of humoral immune response to bacterial $\alpha$-amylase in mice.

While investigating a fermentation broth of the alahopcin producer, we found an amino acid, tentatively called B-52653C, which inhibited collagen prolylhydroxylase and had very weak antibacterial activity. The amino acid was identified as dealanylalahopcin produced by an enzymatic hydrolysis of alahopcin.

In this report, the isolation, enzymatic formation, and chemical and biological properties of dealanylalahopcin are described.

## Materials and Methods

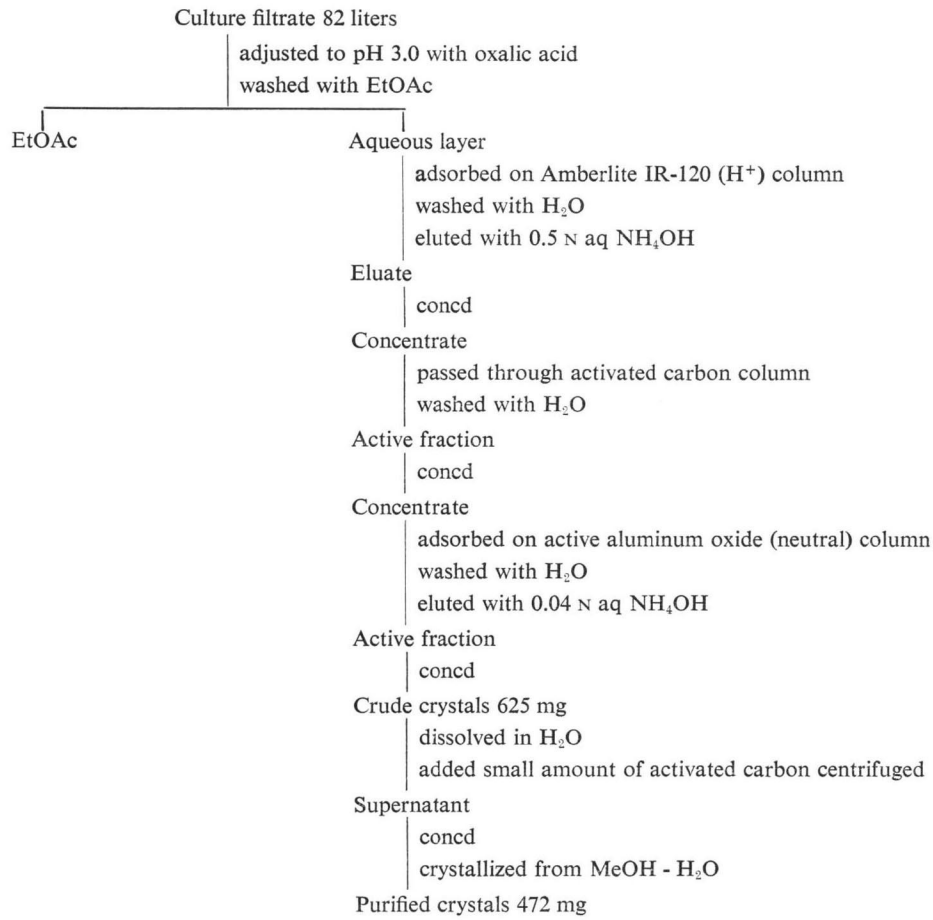
### Fermentation

The fermentation procedure of *Streptomyces albulus* subsp. *ochragerus* and the method for determining antibacterial activity were as described in the previous report[1].

### Preparations of Lyophilized Microbial Cells

One loopful of bacterial cells grown on slant cultures was inoculated into 300 ml of culture medium I in one liter flasks, containing Bacto-tryptone (Difco) 0.5%, Bacto-yeast extract (Difco) 0.3%, Casamino Acids (Difco) 0.3%, glucose 0.5% and alahopcin 0.004%, pH 7.0; the flasks were incubated at 30°C for 2 days on a rotary shaker. Cells were harvested by centrifugation under refrigeration at 10,000 rpm for 20 minutes and washed twice with 1/15 M phosphate buffer, pH 6.5, to lyophilize them. Suspensions of spores and hyphae of actinomycetes were inoculated into 30 ml of culture medium I in 200 ml flasks, which were then incubated at 28°C for 2 days on a rotary shaker. 30 ml of cultivated media were transferred to 300 ml of culture medium II in one liter flasks, containing Bacto-tryptone 0.5%, Bacto - yeast extract 0.3%, Casamino Acids (Difco) 0.3%, glucose 0.1%, soluble starch 1.4%, and alahopcin 0.004%, pH 7.0; the flasks were incubated at 28°C for 4 days on a rotary shaker. Cells were harvested by centrifugation under refrigeration at 8,000 rpm for 20 minutes and washed twice with 1/15 M phosphate buffer, pH 6.0, to lyophilize them.

Fig. 1. Procedure for isolating dealanylalahopcin.




Preparation of Immobilized $\alpha$-Amino Acid Ester Hydrolase

Preparation of crude extracts of $\alpha$-amino acid ester hydrolase produced by *Acetobacter turbidans* ATCC 9325 and immobilization of the enzyme to Sepharose-4B were carried out by the method of TAKAHASHI *et al.*[2].

Determination

B-52653C: B-52653C and alahopcin were determined by high-performance liquid chromatography (HPLC) with a Waters Associates Instrument equipped with a model 6000A solvent-delivery system, model U6K injector, Model 440 detector (at 254 nm), and a column (4×300 mm) of Unicil QC18 (Gasukuro Kogyo Co.) at a flow rate of 1.0 ml/minute and using 0.01 M $(NH_4)_2HPO_4$ as a mobile phase.

Collagen Prolylhydroxylase: Collagen prolylhydroxylase was partially purified from the extracts of 14-day-old chick embryos by a modified method of KIVIRIKKO *et al.*[3] and HALME *et al.*[4]. The enzyme activity was determined by the $^{14}CO_2$ release assay of RHOADS *et al.*[5] using $(Pro\text{-}Pro\text{-}Gly)_5$ as a substrate.

## Results and Discussion

### Production and Isolation of B-52653C

Two liters of the seed culture were transferred to 100 liters of the fermentation medium in a 200-liter

Table 1. Rf value of dealanylalahopcin on thin-layer chromatogram.

| Solvent | Rf | TLC plate* |
|---|---|---|
| 1-BuOH - MeOH - 10% citric acid (4: 2: 2) | 0.21 | a |
| 1-BuOH - AcOH - $H_2O$ (3: 1: 1) | 0.13 | a |
| 1-PrOH - pyridine - AcOH - $H_2O$ (15: 10: 3: 12) | 0.61 | a |
| 1-BuOH - AcOH - $H_2O$ (4: 1: 2) | 0.10 | b |

*a: Cellulose F (Art 5718, Merck)
b: Silica gel (60F-254, Merck)

silica gel plates with the solvents shown in Table 1.

fermentor. The fermentation was carried out at 27°C for 90 hours with aeration of 100 liters per minute and agitation of 200 rpm.

The fermentation broth was filtered with a filter aid and 82 liters of the filtrate were obtained. From the culture filtrate B-52653C was isolated by the procedure shown in Fig. 1. 472 mg of B-52653C were obtained by a combination of purification procedures: ion-exchange, activated carbon and active aluminum oxide chromatography, and crystallization. The purity was checked by thin-layer chromatography (TLC) on

## Physico-chemical Properties

B-52653C was obtained as colorless needles and melted with decomposition at 160~170°C, $[\alpha]_D^{25}$ +50.8° (*c* 0.5, $H_2O$), +55.6° (*c* 1.0, 0.1 N HCl), −4.0° (*c* 1.0, 0.1 N NaOH). The molecular formula was established to be $C_6H_{10}N_2O_5$ from elemental analysis [Calcd for $C_6H_{10}N_2O_5$: C 37.90, H 5.30, N 14.73, MW 190.15; Found: C 37.64, H 5.67, N 14.74; MW (neutral equiv) 192] and mass spectrum (SIMS) [*m/z* 191 $(M+H)^+$]. It is soluble in water, dimethyl sulfoxide, and acetic acid, and hardly soluble or insoluble in methanol, ethanol, ethyl acetate, and chloroform. It is positive to ninhydrin and shows a characteristic color; dark yellowish green on a cellulose thin-layer plate and reddish brown on a silica gel thin-layer plate. Rfs of the chromatograms are presented in Table 1. The UV absorption spectrum in water shows end absorption at 210~360 nm; the IR absorption and the NMR spectrum at 90 MHz are shown in Figs. 2 and 3, respectively.

## Hydrolysis of Alahopcin

Some microorganisms hydrolyzed alahopcin to give alanine and an amino acid (**1**). Lyophilized

Fig. 2. IR spectrum of dealanylalahopcin (KBr).

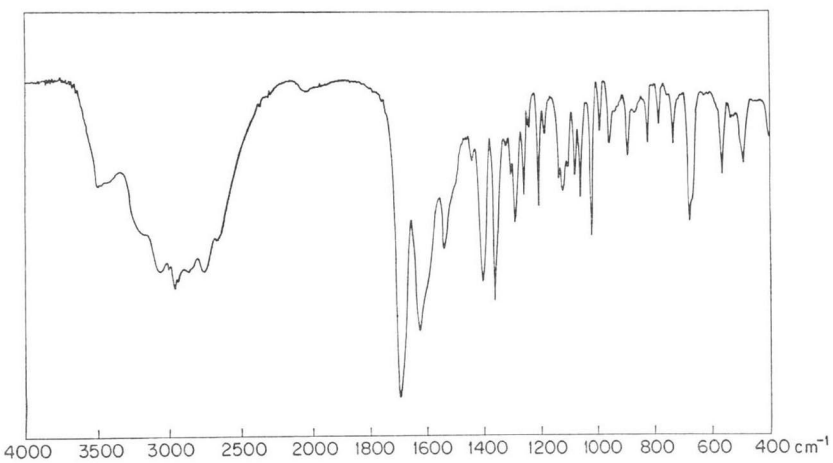


Fig. 3. $^1$H NMR spectrum of dealanylalahopcin (90 MHz, $D_2O$+DCl).

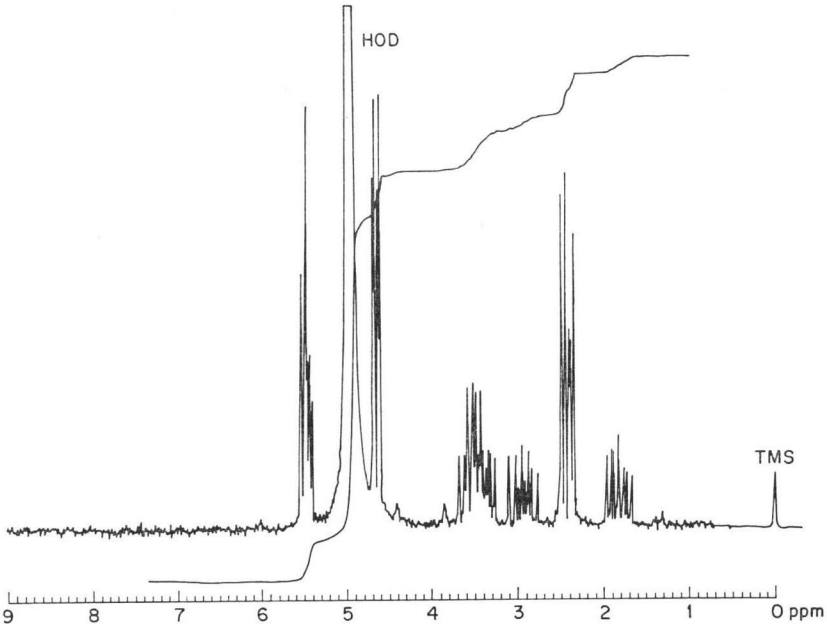



Table 2. Hydrolysis of alahopcin by microbial cells.

| Microorganism | Ratio of hydrolysis (%) |
|---|---|
| (A) *Proteus rettgeri* IFO 13501 | 88.0 |
| (A) *Pseudomonas maltophilia* IFO 12020 | 28.4 |
| (A) *Mycoplasma dimorpha* IFO 13213 | 57.7 |
| (A) *Escherichia coli* IFO 3542 | 79.2 |
| (A) *Aeromonas hydrophila* IFO 3820 | 82.8 |
| (A) *Xanthomonas citri* IFO 3835 | 84.1 |
| (B) *Streptomyces hygroscopicus* subsp. *limoneus* KCC S-0911 | 37.1 |
| (B) *Streptoverticillium cinnamoneum* IFO 13713 | 30.0 |
| (B) *Micromonospora chalcea* subsp. *izumensis* IFO 12988 | 17.7 |
| (B) *Nocardia mediterranei* ATCC 31064 | 21.4 |
| (B) *Actinosynnema mirum* KCC A-0225 | 37.4 |

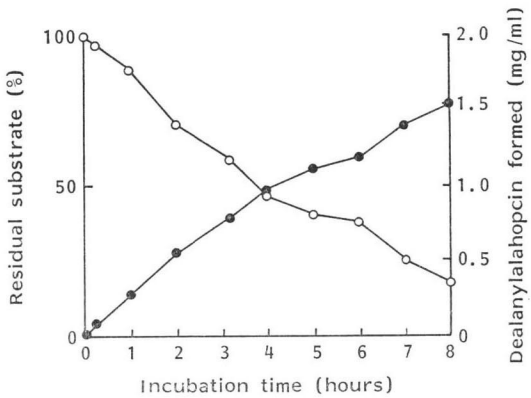
Reaction mixture of hydrolysis: lyophilized cells 10 mg (A) or 20 mg (B), 1/15 M phosphate buffer (pH 6.5) 10 ml, substrate (alahopcin) 10 mg.

Reaction was carried out at 35°C for 4 hours under shaking and supernatants were obtained by centrifugation. Alahopcin was determined by HPLC and the ratio of the hydrolysis was calculated on the basis of weight percent.

Fig. 4. Hydrolysis of alahopcin and formation of dealanylalahopcin by immobilized enzyme.

Reaction: 3 g of alahopcin were reacted with 10 ml of the immobilized $\alpha$-amino acid ester hydrolase from *A. turbidans* (specific activity: 20 units/ml gel) in 1.2 liters of distilled water (adjusted to pH 6.0) at 5°C for 8 hours.

○ Alahopcin, ● dealanylalahopcin.




cells of 6 strains of bacteria and 5 strains of actinomycetes were incubated with alahopcin in 1/15 M phosphate buffer, pH 6.5, at 37°C for 4 hours. The formation of alanine and **1** were detected by TLC and the hydrolysis ratio of alahopcin was determined by HPLC. As shown in Table 2 the hydrolysis activities of the bacteria (average

Table 3. Antibacterial spectrum of dealanylalahopcin.

| Test organisms | MIC ($\mu$g/ml) |
|---|---|
| *Escherichia coli* NIHJ JC-2 | 31.25 |
| *Proteus vulgaris* IFO 3988 | 1,000 |
| *Proteus mirabilis* IFO 3848 | 125 |
| *Proteus morganii* IFO 3168 | 500 |
| *Klebsiella pneumoniae* IFO 3317 | 62.5 |
| *Citrobacter freundii* IFO 12681 | 62.5 |
| *Salmonella typhimurium* IFO 12529 | 15.6 |
| *Serratia marcescens* IFO 12648 | 125 |
| *Enterobacter cloacae* IFO 12937 | 62.5 |
| *Bacillus subtilis* PCI 219 | 1,000 |
| *Bacillus cereus* FDA 5 | 62.5 |

Method: serial agar dilution method.
Medium: synthetic agar[1].

70%) were stronger than those of the actinomycetes (average 28.7%). Acylase I from porcine kidney (Sigma Chem. Co.) also had the same activity as the enzyme from *Xanthomonas citri* IFO 3835, but acylase I from *Aspergillus* sp. (Sigma Chem. Co.) had no activity.

Immobilized $\alpha$-amino acid ester hydrolase also hydrolyzed alahopcin. As shown in Fig. 4, 83% of alahopcin was hydrolyzed and from 3 g of the substrate, 1.52 g of **1** was formed in a yield of 84.2% (w/w). After the reaction, the immobilized enzyme was removed by filtration. The filtrate was adsorbed on Amberite IR-68 ($OH^-$) column (300 ml) and eluted with 0.3 N acetic acid. The eluate was concentrated and chromatographed on an activated carbon column (350 ml) with water. The fractions containing **1** were combined, concentrated under reduced pressure, and methanol was added to the concentrate to give 1.2 g of colorless needles. The physico-chemical properties and IR absorption spectrum of these crystals **1** were identical with those of B-52653C.

The structure of **1** (B-52653C) was determined to be (2*S*,3*R*)-2-amino-4-formyl-4-(hydroxyaminocarbonyl)butyric acid (dealanylalahopcin) by chemical and spectrometric studies[6].

## Biological Activity

The antibacterial spectrum of dealanylalahopcin is shown in Table 3. It showed very weak antibacterial activity against Gram-positive and Gram-negative bacteria, and had about one hundredth the activity of alahopcin.

On the other hand, the amino acid showed a weak inhibitory activity ($ID_{50}$ 2.2 mM) against the collagen prolylhydroxylase; the activity was about 60% that of alahopcin. In addition, the adjuvant of dealanylalahopcin has stimulatory effect on the production of humoral immune response to bacterial $\alpha$-amylase in mice (unpublished data). Compared with alahopcin, its antibacterial activity was quite low, and its enzyme inhibitory and adjuvant activity were much less so. These biological activity suggest that the latter activities of alahopcin may depend on dealanylalahopcin.

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