## Communications to the Editor

# ISOLATION OF CHITINOVORIN D

Sir:

The isolation of chitinovorins A, B and C from the culture broth of *Flavobacterium chitinovorum* sp. nov. PB-5016 has been reported previously[1]. After that, seven additional chitinovorins-producing strains which all belong to the genus *Flavobacterium* were isolated in our searching studies for new $\beta$-lactam antibiotics from bacteria. One of them, *Flavobacterium* sp. PB-5246, was found to produce a more strongly basic antibiotic named chitinovorin D together with chitinovorins A, B and C. In this paper, the isolation and characterization of chitinovorin D are presented (hereafter, chitinovorin is abbreviated as CTV).

The strain PB-5246 was cultivated using a medium consisting of yeast extract 0.5% and glucose 1.0%, pH 6.5, by jar fermentation in the same manner as described in the preceding paper[1]. The antibiotics in the culture filtrate (150 liters) were adsorbed on an activated carbon (Wako Chemicals) at pH 7.0 and eluted with 60% acetone at pH 3.5. The active eluate on an assay plate using *Escherichia coli* LS-1 (a super sensitive mutant to $\beta$-lactam antibiotics) was concentrated and freeze-dried to give a crude powder. It was dissolved in water and applied to a column of SP-Sephadex C-25 (Pharmacia Fine Chemicals) at pH 2.0. The column was eluted with water and then a gradient of $0\sim1.0$ M NaCl in 50 mM phosphate buffer, pH 7.0. The fractions containing CTV C, CTV A and B, and CTV D were separately eluted in that order. The crude powder (480 mg) of CTV D was obtained from the last fraction by adsorption on an activated carbon, elution with 60% acetone, pH 3.5, and freeze-dry. The crude powder was then chromatographed on a column of MCI gel CHP-20P (Mitsubishi Kasei Kogyo) with 20 mM phosphate buffer, pH 4.0. The resulted active eluate was desalted using an activated carbon and subjected to preparative HPLC on a Nucleosil 10 $C_{18}$ column (Macherey-Nagel) with 20 mM phosphate buffer, pH 5.0. The fraction of CTV D was desalted using an activated carbon and then a Sephadex G-10 column. Concentration and freeze-dry gave a pure preparation (25 mg) of CTV D hydrochloric acid salt.

CTV D is more strongly basic than CTV A and B. When they were compared by paper electrophoresis using DFF buffer solution, pH 4.0[2], at 10 v/cm for 2 hours, CTV D migrated 28 mm to the cathod, whereas CTV A and B 17 mm. Comparison by HPLC on Nucleosil 10 $C_{18}$ column ($4\times250$ mm) with a mobile phase of methanol - 20 mM phosphate buffer solution, pH 6.0 (4:96) showed the following retention volumes: CTV C (3.0 ml), CTV A (7.2 ml), CTV B (7.8 ml) and CTV D (13.2 ml).

CTV D hydrochloric acid salt is obtained as a hygroscopic colorless powder. It is readily soluble in water and substantially insoluble in ethanol, acetone and ethyl acetate. It shows positive reactions with ninhydrin and Sakaguchi reagents. In the UV spectrum, a maximum at 262 nm ($\varepsilon$ 6,200) is shown. In the IR spectrum, absorptions at 1772 ($\beta$-lactam carbonyl), 1725 (hydrogen-bonded ester carbonyl), 1655 and 1540 $cm^{-1}$ (amide I and II) are noticed. In the CD spectrum, two Cotton effects positive at 258 nm and negative at 229 nm are observed. The above described properties of CTV D are quite similar to those of CTV A and B. Secondary ion mass spectrometry on CTV D hydrochloric acid salt showed a peak, $m/z$ 959 (M+1), from which a molecular formula $C_{37}H_{62}N_{14}O_{14}S$ was suggested.

CTV D was hydrolyzed with 6 N HCl at 110°C for 20 hours and the hydrolysate was analyzed by an automatic amino acid analyzer, giving the following results. Found (in ratio): $\alpha$-Aminoadipic acid (Aad) (1.00), Gly (0.45), Ala (1.65) and compound **5** (2.32) (The name compound **5** has been used for (4*S*,3*R*)-7-guanidino-4-amino-3-hydroxyheptanoic acid in the preceding paper[1]). This means that Gly is produced from a cephem nucleus[3], and the CTV D molecule contains one mol of Aad and two mol each of Ala and **5** residues. To clarify the chiralities of these constituent amino acids, the acid hydrolysate was L-leucylated and analyzed by HPLC[4]. The peaks of L-Leu-L-Ala, L-Leu-D-Aad and L-Leu-**5** were confirmed by comparison with the respective authentic specimens.

CTV D was 2,4-dinitrophenylated and then

Table 1. $^1$H NMR data of chitinovorin D ($D_2O$, ref: external TMS).

| $\delta$ | Multiplicity, $J$, intensity | Assignment |
|---|---|---|
| 0.71 | d, 7.0, 3H | 3″-$CH_3$ |
| 0.87 | d, 7.0, 3H | 3⁗-$CH_3$ |
| 0.7~1.4 | m, 12H | 16-$CH_2$, 17-$CH_2$, 5′-$CH_2$, 6′-$CH_2$, 5‴-$CH_2$, 6‴-$CH_2$ |
| 1.78 | t-like, 2H | 15-$CH_2$ |
| ~1.8 | —[a], 1H | 2′-$CH_2$ |
| 2.01 | dd, 15.5, 3, 1H | 2′-$CH_2$ |
| 1.6~1.9 | —[a], 2H | 2‴-$CH_2$ |
| 2.51 | t-like, 2H | 7′-$CH_2$ |
| 2.53 | t-like, 2H | 7‴-$CH_2$ |
| 2.64 | d, 17.7, 1H | 2-$CH_2$ |
| 2.98 | d, 17.7, 1H | 2-$CH_2$ |
| 3.07 | t-like, 1H | 18-CH |
| ~3.10 | m, 1H | 4′-CH |
| 3.19 | m, 1H | 4‴-CH |
| ~3.3 | m, 2H | 3′-CH, 3‴-CH |
| 3.44 | q, 7.0, 1H | 2⁗-CH |
| 3.56 | q, 7.0, 1H | 2″-CH |
| 4.04 | d, 12.8, 1H | 10-$CH_2$ |
| 4.22 | d, 12.8, 1H | 10-$CH_2$ |
| 4.66 | s, 1H | 6-CH |
| 7.48 | s, 1H | 12-CH |

[a] Distinct observation was impossible because of overraping of signals.

Table 2. $^{13}$C NMR data of chitinovorin D ($D_2O$, ref: internal acetonitrile $\delta$=1.7).

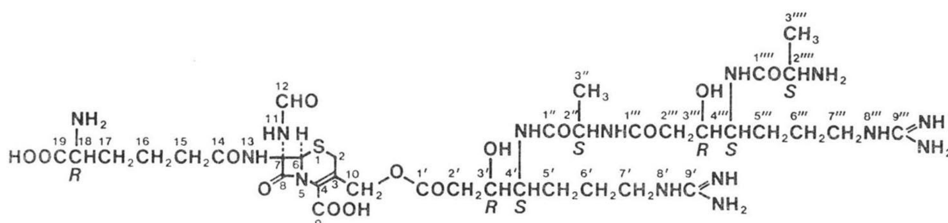
| $\delta$ (multiplicity) | Assign-ment | $\delta$ (multiplicity) | Assign-ment |
|---|---|---|---|
| 17.5 (q) | 3″ | 63.9 (d) | 6 |
| 17.6 (q) | 3⁗ | 65.0 (t) | 10 |
| 21.4 (t) | 16 | 70.8 (d) | 3′ |
| 25.2 (t) | 6‴[a] | 71.3 (d) | 3‴ |
| 25.4 (t) | 6′[a] | 77.5 (s) | 7 |
| 26.4 (t) | 2 | 115.5 (s) | 3 |
| 27.0 (t) | 5‴[b] | 132.8 (s) | 4 |
| 27.4 (t) | 5′[b] | 157.5 (s) | 9′ |
| 30.6 (t) | 17 | 157.5 (s) | 9‴ |
| 35.2 (t) | 15 | 160.0 (s) | 8 |
| 39.4 (t) | 2′ | 164.2 (d) | 12 |
| 40.0 (t) | 2‴ | 168.7 (s) | 9 |
| 41.5 (t) | 7′ | 171.6 (s) | 1⁗ |
| 41.5 (t) | 7‴ | 174.2 (s) | 1′ |
| 49.9 (d) | 2⁗ | 174.3 (s) | 1″[d] |
| 51.3 (d) | 2″ | 175.1 (s) | 19 |
| 53.8 (d) | 4′[c] | 176.2 (s) | 1‴[d] |
| 54.5 (d) | 4‴[c] | 177.6 (s) | 14 |
| 55.2 (d) | 18 | | |

[a,b,c,d] May be interchanged.

hydrolyzed with 6 N HCl at 110°C for 20 hours. DNP-Aad and DNP-Ala were detected by TLC from the ethyl acetate extract of the hydrolysate. Further, CTV D was processed by one step of Edman degradation, and the residual peptide was 2,4-dinitrophenylated and then hydrolyzed. DNP-**5** was detected in the hydrolysate. These results indicated that Aad and Ala → **5** (7-guanidino-4-alanylamino-3-hydroxyheptanoic acid) are present at *N*-terminuses.

The $^1$H and $^{13}$C NMR data of CTV D in $D_2O$ at 24°C recorded with a Varian XL-200 spectrometer are listed in Tables 1 and 2. The assignments were made by proton decoupling experiments and comparison with the $^1$H and $^{13}$C spectra of CTV A[1]. From the comparison it is evident that CTV D is a 7$\alpha$-formylaminocephem having the same 7$\beta$-side chain as that of CTV A, since all of the corresponding signals are observed. Besides the signals corresponding to the constituents of CTV A, CTV D exhibits the $^1$H and $^{13}$C signals ascribable to additional two constituents, Ala and **5**. Compared with the $^1$H signals of Ala residue of CTV A, the methin and methyl signals of one of the Ala residues of CTV D exhibit significant down-field shift (*ca.* +0.2 ppm) and up-field shift (*ca.* −0.1 ppm), respectively. While, the shifts found for those of the other Ala residue are very slight. Taking into account the result of degradative experiments, the above facts imply the structure in which the Ala → **5** moiety is linked to the amino group of the Ala residue of a

Scheme 1. Structure of chitinovorin D.



CTV A moiety by an amide linkage. The proposed structure for CTV D shown in Scheme 1 is supported by the result of full signal assignment described in the Tables 1 and 2.

The antimicrobial activity of CTV D is about a half of the activity of CTV A, so far as compared by pulp disk agar diffusion method using several strains of *E. coli*.

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