**Communications to the Editor**

## NITROPEPTIN, A NEW DIPEPTIDE ANTIBIOTIC POSSESSING A NITRO GROUP

Sir:

During the course of our screening program for new antibiotics effective against blast of rice, we have recently isolated nitropeptin (**1**), a dipeptide antibiotic possessing a nitro group, from the culture broth of *Streptomyces xanthochromogenus* 6257-$MC_1$. The producing strain was isolated from a soil sample collected at Matsushima-cho, Miyagi prefecture, Japan, and was classified as *S. xanthochromogenus* 6257-$MC_1$ by taxonomic studies and direct comparison with a type strain. This communication describes the isolation, structural elucidation and biological properties of **1**.

Fermentation was carried out at 28°C for 72 hours in a 50-liter jar fermentor containing 30 liters of a medium consisting of glycerol 2.5%, peptone 2.0% and $CaCO_3$ 0.4% (pH 7.2). The maximum titer, 15 $\mu$g/ml, was estimated by the paper disc method using *Pyricularia oryzae* as a test organism. The fermentation broth was filtered with a filter aid and the antibiotic in the filtrate was adsorbed on a column of Amberlite IRA-401 ($OH^-$, 2 liters) and eluted with 0.4 N HCl. The active eluate was neutralized and passed through a column of activated charcoal (300 ml). The effluent was adjusted to pH 2.0, adsorbed on a column of activated charcoal (700 ml) and eluted with 50% aq acetone. The eluate was concentrated, and then applied to a column of DEAE-Sephadex A-25 ($Cl^-$, 600 ml). After washing with water, the antibiotic was eluted with 0.05 M NaCl. The active eluate was desalted by activated charcoal in the same manner as above, and concentrated to dryness. Further purifications were carried out by Diaion CHP-20 and Sephadex G-10 column chromatographies. Lyophilization of the active fraction yielded the sodium salt of **1** (120 mg).

The physico-chemical properties of **1** are summarized in Table 1. Nitropeptin is a water-soluble, acidic compound, and shows on characteristic UV absorption in neutral and acidic solutions. However an absorption peak appears at 242 nm ($\varepsilon$ 9,800) in alkaline solution. The molecular formula of **1** was determined to be $C_{11}H_{18}N_3O_7Na$ on the basis of elemental analysis, fast atom bombardment mass spectrum (FAB-MS) and $^{13}C$ NMR spectral data.

Acid hydrolysis (6 N HCl, 110°C, 17 hours) of

Table 1. Physico-chemical properties of nitropeptin sodium salt.

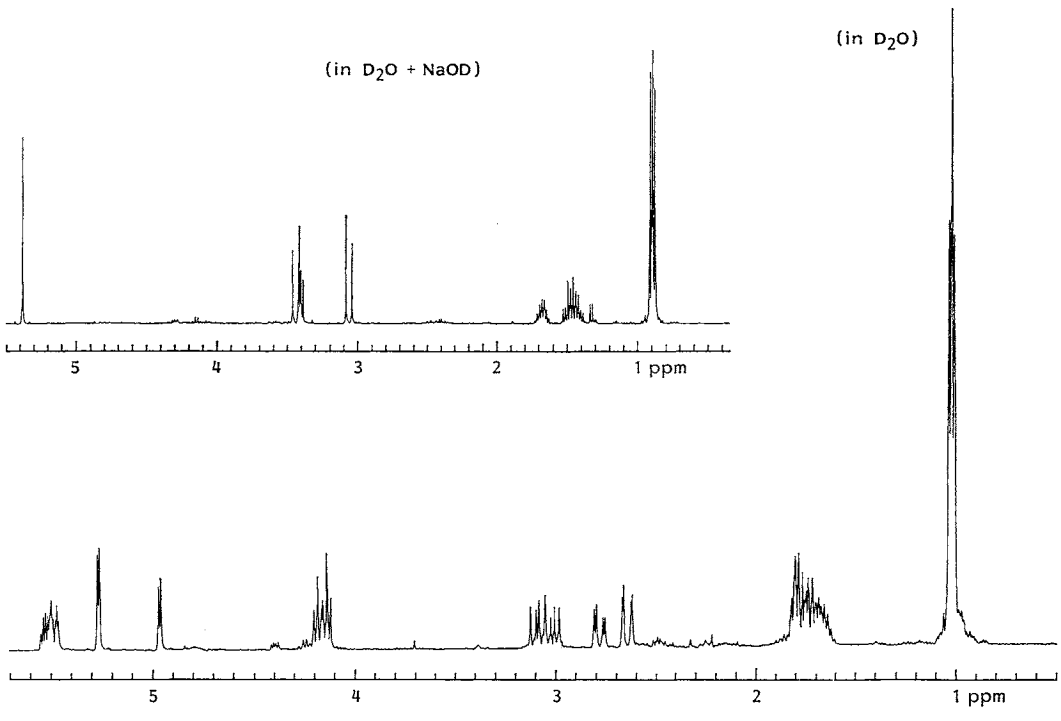
| | |
|---|---|
| Appearance | White amorphous powder |
| MP | 166~168°C (dec) |
| $[\alpha]_D^{22}$ | $-27.2°$ ($c$ 1.0, $H_2O$) |
| *Anal* | Found: C 39.02, H 5.93, N 12.08, O 35.02, Na 6.53. |
| | Calcd for $C_{11}H_{18}N_3O_7Na \cdot \frac{1}{2}H_2O$: |
| | C 39.29, H 5.65, N 12.50, O 35.71, Na 6.85. |
| FAB-MS ($m/z$) | Positive: 328 $(M+1)^+$, 350 $(M+Na)^+$ |
| | Negative: 326 $(M-1)^-$ |
| Solubility | Soluble: Water |
| | Slightly soluble: MeOH, acetone |
| | Insoluble: Benzene, $CHCl_3$ |
| High-voltage paper electrophoresis | Rm (glu) 0.67 (pH 6.4, 3,500 V, 15 minutes) |
| UV | End absorption in $H_2O$ and 0.1 N HCl, 242 nm ($\varepsilon$ 9,800) in 0.1 N NaOH |
| IR (KBr) $cm^{-1}$ | 3400~3200, 2950, 1660, 1600, 1540, 1390, 1290 and 1140 |
| Color reaction | +: Ninhydrin, $H_2SO_4$ |
| | −: Sakaguchi |
| Silica gel TLC (Rf) | Butanol - Acetic acid - $H_2O$ (2:1:1): 0.68 |
| | Butanol - MeOH - $H_2O$ (4:1:2): 0.32 |

**1** gave L-leucine which was identified by NMR, MS and amino acid analysis. Its absolute configuration was established to be L by the optical rotation value, $[\alpha]_D^{22}$ $-8.0°$ ($c$ 0.3, $H_2O$). Treatment of **1** with 2,4-dinitrofluorobenzene gave a 2,4-dinitrophenyl (DNP) derivative, and then it was hydrolyzed with 6 N HCl to give DNP-leucine which was identified by silica gel TLC in comparison with an authentic sample.

The $^1H$ signals in Fig. 1 and the $^{13}C$ resonances of **1** are listed in Tables 2 and 3, respectively. The $^1H$ and $^{13}C$ NMR spectra were analyzed mainly through 2D COSY, $^{13}C$-$^1H$ shift correlation and $^1H$ spin decoupling experiments. The $^1H$ and $^{13}C$ NMR spectra of **1** revealed two sets of signals suggesting the presence of two diastereoisomers in $D_2O$ solution. These spectral data revealed two fragments corresponding to leucine [$\delta_C$ 53.3 (53.3)†, 41.0 (41.0), 25.1 (25.2), 22.4 (22.4), 22.7 (22.7)] and the unknown amino acid [$\delta_C$ 57.4 (56.9), 85.9 (86.3), 36.2 (38.9)]. The assignments of carbonyl carbon signals at $\delta_C$ 171.2 (171.2), 172.3 (173.4) and 176.9 (176.5) and the relationship between the $\alpha'$-proton and the carbonyl carbon of the leucine moiety were established by $^{13}C$-$^1H$ long range selective proton decoupling experiments as shown in Fig. 2.

In order to prove the functional groups represented by X, Y and Z in Fig. 2, the following chemical reactions were carried out. Reduction of **1** with platinum dioxide in aqueous acetic acid gave **2** (Scheme 1) as a mixture of diastereoisomers, secondary ion mass spectrum (SI-MS) $m/z$ 276 $(M+1)^+$, *Anal* Calcd for $C_{11}H_{21}N_3O_5 \cdot 1\frac{1}{2}H_2O$: C 43.70, H 7.95, N 13.91; Found: C 43.48, H 7.70, N 13.66. The structure of **2** was determined to be *N*-$\alpha$-leucyl-$\beta$-aminoglutamic acid from its $^{13}C$ NMR spectral data [$D_2O$, $\delta_C$: $CH_3$, 22.9 (22.8), $CH_3$, 21.8 (22.0); $\gamma$-CH, 25.0 (25.4); $\beta$-$CH_2$, 40.7 (40.7); $\alpha$-CH, 53.2 (53.1); $\alpha'$-CH, 53.6 (55.1); $\beta'$-CH, 50.2 (49.8); $\gamma'$-$CH_2$, 34.5 (35.2); C=O, 168.4 (168.3), 172.1 (171.8), 175.6 (175.9)]. Thus, the signal at $\delta_C$ 85.9 (86.3) in **1** was replaced by a resonance at $\delta_C$ 50.2 (49.8) ascribed to an amino methine in **2**. Therefore, it was deduced that nitropeptin had a nitro group at the $\beta'$-position in the unknown amino acid residue. The $^{13}C$ chemical shift of the $\beta'$-methine carbon in **1** is in agreement with that of the nitromethine group in 2-

Fig. 1. 400 MHz $^1H$ NMR spectrum of nitropeptin.




† Values in parenthesis denote $^{13}C$ chemical shifts due to the minor isomer.

Table 2. $^1$H NMR chemical shifts of nitropeptin (ppm, $J$; Hz).

| Proton | In $D_2O$ | | In $D_2O$+NaOD |
|---|---|---|---|
| | Major isomer | Minor isomer | |
| $CH_3$ | 0.99 (3H, d, $J$=6.0) | 0.99 (3H, d, $J$=6.0) | 0.88 (3H, d, $J$=6.2) |
| $CH_3$ | 1.00 (3H, d, $J$=6.0) | 1.00 (3H, d, $J$=6.0) | 0.92 (3H, d, $J$=6.2) |
| $\gamma$-CH | 1.66 (1H, m) | 1.66 (1H, m) | 1.67 (1H, m) |
| $\beta$-$CH_2$ | 1.76 (2H, m) | 1.76 (2H, m) | 1.46 (2H, m) |
| $\alpha$-CH | 4.10 (1H, t, $J$=7.6) | 4.15 (1H, t, $J$=7.4) | 3.42 (1H, t, $J$=7.2) |
| $\gamma'$-$CH_2$ | 2.57 (1H, dd, $J$=17.0, 3.0), | 2.71 (1H, dd, $J$=16.4, 5.0), | 3.06 (1H, d, $J$=16.2), |
| | 3.02 (1H, dd, $J$=17.0, 12.2) | 2.94 (1H, dd, $J$=16.4, 10.2) | 3.44 (1H, d, $J$=16.2) |
| $\beta'$-CH | 5.44 (1H, m) | 5.47 (1H, m) | |
| $\alpha'$-CH | 5.23 (1H, d, $J$=3.2) | 4.92 (1H, d, $J$=4.8) | 5.38 (1H, s) |

Observed at 400 MHz.

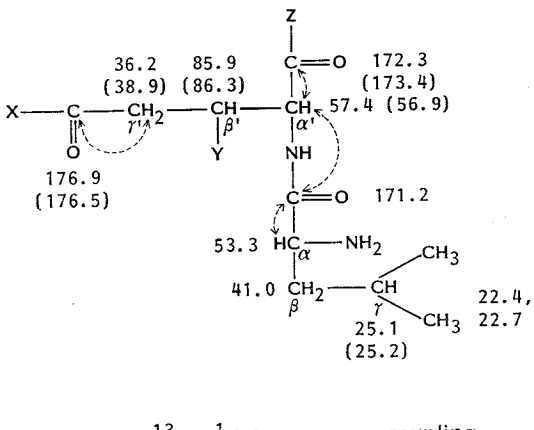
Table 3. $^{13}$C NMR chemical shifts of nitropeptin (ppm).

| Carbon | In $D_2O$ | | In $D_2O$+NaOD |
|---|---|---|---|
| | Major isomer | Minor isomer | |
| $\alpha$-CH | 53.3 | 53.3 | 54.4 |
| $\beta$-$CH_2$ | 41.0 | 41.0 | 44.3 |
| $\gamma$-CH | 25.1 | 25.2 | 25.3 |
| $CH_3$ | 22.4, | 22.4, | 22.3, |
| | 22.7 | 22.7 | 23.5 |
| $\alpha'$-CH | 57.4 | 56.9 | 56.8 |
| $\beta'$-CH | 85.9 | 86.3 | 120.1 |
| $\gamma'$-$CH_2$ | 36.2 | 38.9 | 39.4 |
| -NH*C*O- | 171.2 | 171.2 | 177.9 |
| >C*H*C*O*- | 172.3 | 173.4 | 175.6 |
| -$CH_2$*C*O- | 176.9 | 176.5 | 177.9 |

Observed at 100 MHz.

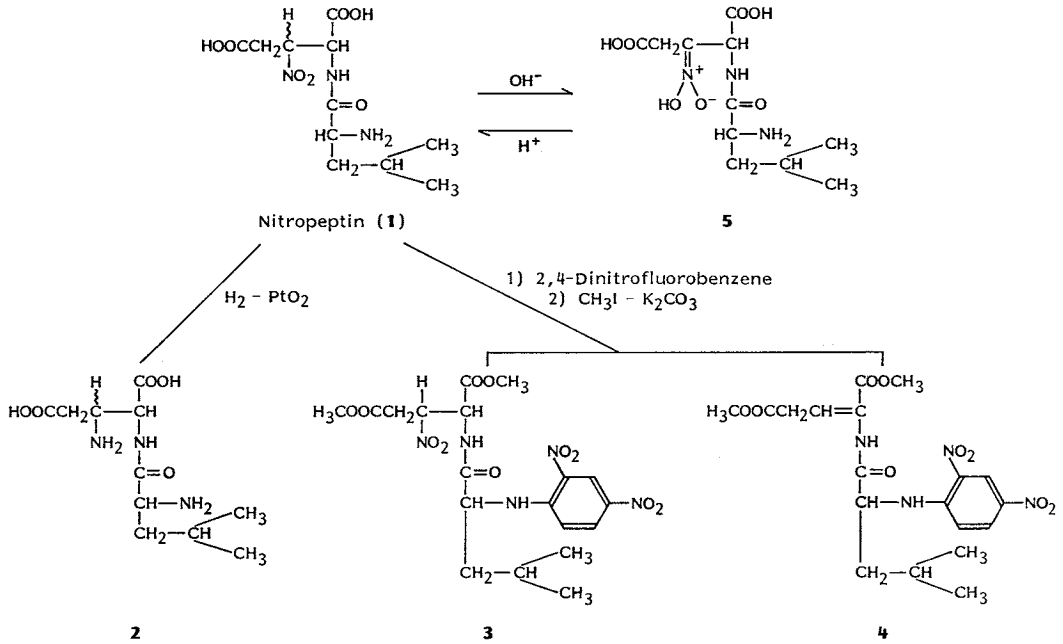
Fig. 2.

$^{13}$C NMR chemical shifts (ppm). ( ): Minor isomer.




nitro-2-methylpropane[1] ($\delta_c$ 85.2). Treatment of **1** with 2,4-dinitrofluorobenzene and subsequent methylation with methyl iodide - potassium carbonate in dimethylformamide solution gave a DNP-derivative dimethyl ester (**3**), fast atom bombardment-high resolution mass spectra (FAB-HRMS) $m/z$ obscured: 500.1622 $(M+1)^+$, Calcd for $C_{19}H_{26}N_5O_{11}$: 500.1621, and a dehydro DNP-derivative dimethyl ester (**4**), FAB-MS $m/z$ 453 $(M+1)^+$; mp 159°C; $[\alpha]_D^{20}$ +96.0 ($c$ 0.5, MeOH); *Anal* Calcd for $C_{19}H_{24}N_4O_9$: C 50.44, H 5.31, N 12.39; Found: C 50.60, H 5.20, N 12.52. The structure of **4** was determined to be *N*-DNP-leucyl-2-amino-2-pentenedioic acid methyl ester from its $^{13}$C NMR spectrum data [$CDCl_3$, $\delta_c$: $CH_3$, 21.6, 22.9; $\gamma$-CH, 25.2; $\beta$-$CH_2$, 33.9; $\gamma'$-$CH_2$, 42.0; $OCH_3$, 52.4, 52.8; $\alpha$-CH, 57.7; $\alpha'$>C=, 126.9; $\beta'$-CH=, 128.4; C=O, 164.0, 169.8, 170.5; DNP, 147.2 (C-1), 131.7 (C-2), 124.0 (C-3), 137.6 (C-4), 130.7 (C-5), 114.7 (C-6)]. From these results, the X, Y and Z functions of **1** were deduced to be a hydroxy, a nitro and a hydroxy group, respectively.

Scheme 1.




The $^{1}H$ and $^{13}C$ NMR spectra measured in $D_2O$ solution added with NaOD supported the presence of the nitro group in **1**. As listed in Tables 2 and 3, the $\beta'$-methine protons corresponding to the signals at $\delta_H$ 5.44 (5.47) disappeared, and the chemical shift of the $\beta'$-methine carbon changed from $\delta_C$ 85.9 (86.3) to $\delta_C$ 120.1. In addition, all signals were simplified in $^{1}H$ and $^{13}C$ NMR spectra to be ascribed to the aci-nitro form (**5**) of **1** in Scheme 1. In agreement with this structure, **1** showed a UV absorption at 242 nm ($\varepsilon$ 9,800) in 0.1 N NaOH solution, whereas no characteristic UV absorption was observed in neutral and acidic solution. These phenomena of NMR and UV spectra observed in alkaline solution were reversed by neutralization. The signal at $\delta_C$ 120.1 in **5** was assigned to the unsaturated carbon of an aci-nitro group, and UV absorption at 242 nm is derived by the unsaturated bond of the aci-nitro group. The slow exchange of the $\beta'$-proton of **1** in $D_2O$ solution (50% exchange in *ca.* 5 hours) and the presence of **1** as a diastereomeric mixture (*vide supra*) are explained in terms of this nitro-aci-nitro tautomerism.

From the results described above, the structure of nitropeptin was determined to be *N*-L-leucyl-$\beta$-nitroglutamic acid as depicted in Scheme 1.

Among known antibiotics, bovinocidin[2], 1-amino-2-nitrocyclopentanecarboxylic acid[3,4] and antibiotics[5~9] containing nitro sugar were reported as antibiotics possessing an aliphatic nitro group. Therefore, nitropeptin is a very unique compound among the natural products.

Nitropeptin showed little antibacterial activity on nutrient media. It exhibited activity against *Escherichia coli* K-12 on a synthetic medium, but the activity was decreased by supplementation of L-glutamine. From these biological and structural properties, the compound may be regarded as an antimetabolite of glutamine. The protective effect against the rice plant desease caused by *Pyricularia oryzae* was observed in a green house test (94% at 200 ppm). The $LD_{50}$ for nitropeptin in mice was 50~100 mg/kg (iv).

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