Chemical Synthesis and Characterization of *n*-Fattyacyl Mono-Aminoacyl Derivatives of Colistin Nonapeptide[†]

Shiro CHIHARA, Akira ITO, Masahiro YAHATA,
Takashi TOBITA and Yasuo KOYAMA

Kayaku Antibiotics Research Laboratory, Funato, Itabashi, Tokyo 174

Received January 25, 1974

In order to examine the antimicrobial activity of *n*-fattyacyl mono-aminoacyl derivatives of colistin nonapeptide (CNP), twenty-one derivatives were synthesized and characterized on their chemical, physicochemical and antimicrobial properties. Five α -amino acids, glycine, L-alanine, DL-aminobutyric acid, DL-norvaline and DL-norleucine, and two ω -amino acids, β -alanine and γ -aminobutyric acid, were first acylated with each of three kinds of acid chloride, *n*-octanoyl, *n*-decanoyl and *n*-dodecanoyl chloride, and then esterified with *p*-nitrophenol prior to the coupling of those *n*-fattyacyl amino acids to the terminal threonine of CNP. The coupling reaction was carried out in an aqueous solvent buffered at pH 5.0 without any protection of the γ -amino groups of CNP, as reported previously.

All of the derivatives obtained in hydrochloride salts were hygroscopic white amorphous powder showing decomposing points at 180~230°C. Each purified preparation exhibited a ninhydrin positive spot on a paper chromatogram, and each spot coincided with respective inhibitory zone at *R_f* 0.54~0.72 on a bioautogram obtained with *Escherichia coli* NIHJ or *Bacillus subtilis*. The antimicrobial spectra of the fifteen *n*-fattyacyl α -aminoacyl CNPs against gram-negative bacteria were narrower and the activities are less than those of the corresponding *n*-fattyacyl derivatives of CNP or colistin. Of *n*-fattyacyl ω -aminoacyl derivatives, *n*-dodecanoyl β -alanyl-CNP showed unique antimicrobial spectrum against gram-negative bacteria, in which colistin insensitive *Serratia marcescens* and *Proteus morganii* were inhibited at the concentrations of 25 and 50 mcg/ml, respectively. In conclusion, the elongation of the peptide chain in the CNP molecule by an mono-amino acid acylated with *n*-fatty acid resulted in the reduction of the antimicrobial activity except for a case of β -alanine; the activity seems to depend on the kind of amino acid introduced.

In a previous paper,¹⁾ it was reported that the antimicrobial spectra of α -N-fattyacyl colistin nonapeptide (CNP) derivatives were affected by either the carbon number of the fattyacyl moiety introduced or such a slight diversity in the chemical structure of the fattyacyl moiety as normal and iso type. These fattyacylated derivatives of CNP were considerably inferior to colistin in the antimicrobial activity against gram-negative bacteria such as *Escherichia coli*. This could be due to the nature of CNP itself which is the product of the elimination of the terminal amino acid, α,γ -diaminobutyric acid (Dab), of colistin. Therefore, a series of the *n*-fattyacyl mono-

aminoacyl derivatives of CNP introduced with an amino acid of five α - and two ω -amino acids was synthesized to determine the antimicrobial character.

The synthesis was carried out at first by the acylation of seven amino acids with each of three normal fatty acids such as *n*-octanoic, *n*-decanoic and *n*-dodecanoic acid, then followed by the coupling of these fattyacylated α - or ω -amino acids to the α -amino group of terminal threonine in CNP. The synthesized twenty-one preparations of *n*-fattyacyl mono-aminoacyl-CNP exhibited characteristic antimicrobial spectra. As a whole, these derivatives introduced with a mono-amino acid to the N-terminal threonine of CNP molecule were weakened in their antimicrobial activity

[†] Studies on Colistin Nonapeptide Derivatives Part III.

FIG. 1. Synthetic Reaction of *n*-Fattyacyl Mono-Aminoacyl Colistin Nonapeptide Derivatives.

* *n*-Fattyacyl mono-amino acid *p*-nitrophenyl ester.

and narrowed in the extent of the antimicrobial spectrum. However, β -alanine derivative, *n*-dodecanoyl β -alanine-CNP exhibited a very interesting antimicrobial spectrum, unlike other CNP derivatives synthesized hitherto, in which *Serratia marcescens* and *Proteus morganii* belonging to the colistin insensitive gram-negative bacteria¹⁾ were inhibited at the concentrations of 25 and 50 mcg/ml.

This paper is concerned with the synthesis of a series of *n*-fattyacyl mono-aminoacyl-CNPs and the characterization of their chemical, physicochemical properties and biological activities.

MATERIALS AND METHODS

Colistin nonapeptide (CNP). CNP hydrochloride was prepared by the method previously described.²⁾

Reagents. Glycine was purchased from Junsei Pure Chemicals Co. Ltd., and L-alanine, DL- α -aminobutyric acid, γ -aminobutyric acid, DL-norvaline and DL-norleucine were obtained from Tokyo Kasei Kogyo Co. Ltd. Thionylchloride, *n*-octanoic acid, *n*-decanoic acid, *n*-dodecanoic acid, *p*-nitrophenol and dicyclohexylcarbodiimide (DCC) were obtained from Tokyo Kasei Kogyo Co. Ltd., while dimethylformamide (DMF), ethylacetate and other organic solvents from Junsei Pure Chemical Co. Ltd.

***n*-Fattyacid chlorides and *n*-fattyacyl amino acids.** *n*-Octanoyl, *n*-decanoyl and *n*-dodecanoyl chloride were synthesized^{1,5)} by the chlorination with thionylchloride of corresponding fatty acids. Twenty-one kinds of *n*-fattyacylated amino acids were synthesized by a usual manner³⁾ with equimolar amounts of amino acid and acid chloride at alkaline pH.

Synthesis of *n*-fattyacyl mono-aminoacyl *p*-nitrophenyl

esters. The twenty-one *n*-fattyacyl mono-aminoacyl *p*-nitrophenyl esters listed in Table I were prepared⁴⁾ with each *n*-fattyacyl amino acid and *p*-nitrophenol by dehydrative esterification with DCC. The reaction conditions were as follows. *n*-Fattyacyl mono-amino acid and *p*-nitrophenol in the amount of the molar ratio of 1.0: 1.2 were dissolved in a small volume of DMF, and then the esterification was initiated by the addition of 1.2 moles of DCC with stirring under cooling in an ice bath. After 2~4 hr, formed DC-urea was removed by filtration. The filtrate was evaporated *in vacuo* to remove DMF, and a crude preparation of the *p*-nitrophenyl ester of *n*-fattyacyl mono-amino acid was obtained. Recrystallization with a small volume of a suitable solvent resulted in pale yellow crystals. The results of the elemental analysis and the melting points of all preparations synthesized are given in Table I.

Reaction conditions for the synthesis of *n*-fattyacyl mono-aminoacyl-CNPs. One mm (1.1 g) of CNP hydrochloride was dissolved in 1000 ml of an aqueous solvent containing 300~500 ml of 1/5 M acetate buffer, pH 5.0, and 500~700 ml of acetone or dioxane. Then 2~5 mm of *n*-fattyacyl amino acid *p*-nitrophenyl ester dissolved in a small volume of acetone was gradually added to the solution and allowed to react under vigorous stirring for 24 hr at a fixed temperature.

Purification of synthesized *n*-fattyacyl mono-aminoacyl-CNPs. After the reacted mixture was adjusted to pH 1.0, *p*-nitrophenol and acyl amino acid liberated were extracted with ethylacetate. The residual acidic aqueous layer was adjusted to pH 8.0, repeatedly extracted with *n*-butanol and then the *n*-butanol layer was reextracted with a small volume of dil. HCl. The acidic aqueous solution of the reaction products was adjusted to pH 5.0 and lyophilized. A crude preparation of *n*-fattyacyl mono-aminoacyl-CNP was obtained as white powder. This crude preparation was further purified by counter current distribution,²⁾ with the use of the following solvent system; *n*-butanol, *sec*-butanol, 0.1 N HCl, 6: 30: 40. The distribution pattern of *n*-fattyacyl mono-aminoacyl-CNP was checked with ninhydrin reagent and peak fractions corresponding to the derivative were collected. The acidic aqueous layer obtained was adjusted to pH 5.0 by the addition of a small volume of IRA 410 (OH type) and lyophilized. Thus, *n*-fattyacyl mono-aminoacyl-CNP hydrochloride was obtained as white amorphous powder.

Quantitative estimation of amino acid and fatty acid constituting *n*-fattyacyl mono-aminoacyl-CNPs. Each preparation of *n*-fattyacyl mono-aminoacyl-CNPs was hydrolyzed with 6 N HCl at 110°C for 24 hr under a flow of nitrogen gas and the acid hydrolyzate was separated with ether extraction into two fractions, amino acid and fatty acid. Fatty acid was esterified with diazomethane

TABLE I. *n*-FATTYACYL AMINO ACID *p*-NITROPHENYL ESTERS

<i>p</i> -Nitrophenyl <i>n</i> -octanoyl glycinate.	mp 104~106°C.
Found: C. 59.99, H. 7.07, N. 8.76,	Calcd: C. 59.61, H. 6.88, N. 8.63.
<i>p</i> -Nitrophenyl <i>n</i> -decanoyl glycinate.	mp 94~96°C.
Found: C. 61.02, H. 8.12, N. 7.55,	Calcd: C. 61.7, H. 7.48, N. 8.00.
<i>p</i> -Nitrophenyl <i>n</i> -dodecanoyl glycinate.	mp 92~94°C.
Found: C. 63.10, H. 8.12, N. 7.57,	Calcd: C. 65.00, H. 8.43, N. 6.89.
<i>p</i> -Nitrophenyl <i>n</i> -octanoyl L-alaninate.	mp 79~81°C.
Found: C. 61.02, H. 7.47, N. 7.80,	Calcd: C. 60.70, H. 7.19, N. 8.33.
<i>p</i> -Nitrophenyl <i>n</i> -decanoyl L-alaninate.	mp 84~86°C.
Found: C. 63.13, H. 7.75, N. 7.75,	Calcd: C. 62.62, H. 7.74, N. 7.69.
<i>p</i> -Nitrophenyl <i>n</i> -dodecanoyl L-alaninate.	mp 80~82°C.
Found: C. 64.05, H. 8.26, N. 7.17,	Calcd: C. 64.26, H. 8.22, N. 7.14.
<i>p</i> -Nitrophenyl <i>n</i> -octanoyl DL- α -aminobutyrate.	mp 86~87°C.
Found: C. 61.57, H. 7.53, N. 8.53,	Calcd: C. 61.70, H. 7.48, N. 8.00.
<i>p</i> -Nitrophenyl <i>n</i> -decanoyl DL- α -aminobutyrate.	mp 92~93°C.
Found: C. 64.19, H. 8.18, N. 7.40,	Calcd: C. 63.47, H. 7.99, N. 7.40.
<i>p</i> -Nitrophenyl <i>n</i> -dodecanoyl DL- α -aminobutyrate.	mp 88~89°C.
Found: C. 64.82, H. 8.68, N. 7.10,	Calcd: C. 65.00, H. 8.43, N. 6.89.
<i>p</i> -Nitrophenyl <i>n</i> -octanoyl DL-norvalinate.	mp 76~78°C.
Found: C. 62.82, H. 7.69, N. 7.36,	Calcd: C. 62.62, H. 7.74, N. 7.69.
<i>p</i> -Nitrophenyl <i>n</i> -decanoyl DL-norvalinate.	mp 78~79°C.
Found: C. 64.81, H. 8.22, N. 7.15,	Calcd: C. 64.26, H. 8.22, N. 7.14.
<i>p</i> -Nitrophenyl <i>n</i> -dodecanoyl DL-norvalinate.	mp 75~77°C.
Found: C. 66.75, H. 8.54, N. 6.68,	Calcd: C. 65.69, H. 8.63, N. 6.66.
<i>p</i> -Nitrophenyl <i>n</i> -octanoyl DL-norleucinate.	mp 82~83°C.
Found: C. 63.83, H. 8.14, N. 7.16,	Calcd: C. 63.64, H. 7.74, N. 7.42.
<i>p</i> -Nitrophenyl <i>n</i> -decanoyl DL-norleucinate.	mp 76~77°C.
Found: C. 64.63, H. 8.27, N. 6.88,	Calcd: C. 65.00, H. 8.43, N. 6.89.
<i>p</i> -Nitrophenyl <i>n</i> -dodecanoyl DL-norleucinate.	mp 68~69°C.
Found: C. 66.27, H. 8.87, N. 6.58,	Calcd: C. 66.33, H. 8.81, N. 6.45.
<i>p</i> -Nitrophenyl <i>n</i> -octanoyl β -alaninate.	mp 79~81°C.
Found: C. 61.02, H. 7.47, N. 7.70,	Calcd: C. 60.70, H. 7.19, N. 8.83.
<i>p</i> -Nitrophenyl <i>n</i> -decanoyl β -alaninate.	mp 86~87°C.
Found: C. 63.13, H. 7.75, N. 7.75,	Calcd: C. 62.62, H. 7.74, N. 7.69.
<i>p</i> -Nitrophenyl <i>n</i> -dodecanoyl β -alaninate.	mp 83~85°C.
Found: C. 64.05, H. 8.02, N. 7.17,	Calcd: C. 64.62, H. 8.22, N. 7.14.
<i>p</i> -Nitrophenyl <i>n</i> -octanoyl γ -aminobutyrate.	mp 63~65°C.
Found: C. 61.92, H. 7.73, N. 8.26,	Calcd: C. 61.70, H. 7.48, N. 8.00.
<i>p</i> -Nitrophenyl <i>n</i> -decanoyl γ -aminobutyrate.	mp 73~74°C.
Found: C. 63.84, H. 8.19, N. 7.64,	Calcd: C. 63.47, H. 7.99, N. 7.40.
<i>p</i> -Nitrophenyl <i>n</i> -dodecanoyl γ -aminobutyrate.	mp 75~77°C.
Found: C. 65.38, H. 8.65, N. 7.37,	Calcd: C. 65.00, H. 8.43, N. 6.89.

and analyzed with a Hitachi gas liquid chromatograph type K53 under the same experimental conditions as reported in the previous paper;²⁾ amino acids were analyzed with an amino acid analyzer, Japan Electron Optics Laboratory JLC-6AS.

Quantitative estimation of N-terminal amino acid of synthesized n-fattyacyl mono-aminoacyl-CNPs. Preparation of dinitrophenylated (DNP) *n*-fattyacyl mono-aminoacyl derivatives of CNP and the quantitative estimation of DNP-amino acids contained in the DNP-

n-fattyacyl mono-amino acyl-CNP were carried out by the same methods as described previously.⁵⁾

Paper chromatography and bioautography of synthesized derivatives. Paper chromatography was carried out on a paper sheet, Toyo Roshi No. 51 paper with a solvent system of *n*-butanol, pyridine, acetic acid and water, 15: 10: 3: 12, while bioautography was performed on a nutrient agar plate sowing *E. coli* NIHJ or *Bacillus subtilis* PCI 219 as a test organism.

RESULTS

Chemical synthesis and purification of n -fattyacyl mono-aminoacyl-CNPs

For the synthesis of a series of the n -fattyacyl mono-aminoacyl derivatives of CNP, five α -amino acids, glycine, L-alanine, DL- α -aminobutyric acid, DL-norvaline and DL-norleucine, and two ω -amino acids, β -alanine and γ -aminobutyric acid were employed. All of them were first acylated with each of n -octanoyl, n -decanoyl and n -dodecanoyl chloride and then coupled to the α -amino group of the N-terminal threonine of CNP molecule without any protection for the four γ -amino groups residing in CNP. When the n -fattyacyl amino acid was directly coupled to CNP with DCC reagent,⁴⁾ the products were so complicated that no clear antimicrobial activity against *E. coli* and *B. subtilis* was recognized. Therefore, the coupling reaction was performed with the active p -nitrophenyl ester of n -fattyacyl amino acid as listed in Table I.

As to the synthetic and purification procedures, a case of n -octanoyl glycyl-CNP is presented as follows.

One mm (1.1 g) of CNP hydrochloride was dissolved in a mixture of 500 ml of 1/5 M acetate buffer, pH 5.0, and 500 ml of acetone, and stirred at 25°C. To the solution 5 mm of n -octanoyl glycine p -nitrophenyl ester dissolved in 50 ml of acetone was gradually added and allowed to react for 24 hr. The reaction was terminated by acidifying the mixture with 1N HCl to pH 1.0 and acetone removed *in vacuo* after that liberated p -nitrophenol and n -octanoyl glycine were extracted five times with a half volume of ethylacetate. Then the residual acidic aqueous layer containing reaction products and a considerable amount of CNP was adjusted to pH 8.0 with 1N NaOH, and repeatedly extracted with a half volume of n -butanol. Synthesized n -octanoyl glycyl-CNP was preferentially transferred into the n -butanol layer. The n -butanol layers were collected, then the reaction products were re-extracted with 50 ml of dil. HCl. The acidic

aqueous layer obtained was adjusted to pH 5.0 by the addition of a small volume of Amberlite IRA 410 (OH type), followed by lyophilization, and then a crude preparation was obtained. Further purification of this preparation was performed by a counter current distribution apparatus with a solvent system of n -butanol, *sec*-butanol, 0.1 N HCl, 6: 30: 40, where 1.3 g of the crude preparation was charged to 5 tubes from fifth to ninth and continuously extracted for 200 times. The distribution pattern of the target compound was searched by ninhydrin reaction for an aliquot of the underlayer in each extraction tube and two ninhydrin-positive peaks appeared. The minor peak corresponded to CNP of K_d 0.02,²⁾ while the major one was of K_d 0.25 to the target compound. The acidic aqueous layer in extraction tubes corresponding to the latter peak was collected and lyophilized at pH 5.0 adjusted by the addition of a small volume of Amberlite IRA 410 (OH type). The purified preparation of n -octanoyl glycyl-CNP hydrochloride was obtained in hygroscopic white amorphous powder.

Other twenty n -fattyacyl mono-aminoacyl-CNP hydrochlorides were synthesized under the conditions described in Table II and purified by the same manner as in the above example.

Physicochemical and chemical properties of synthesized n -fattyacyl mono-aminoacyl-CNP derivatives

As shown in Table III, all of n -fattyacyl mono-aminoacyl-CNP hydrochlorides so far synthesized were obtained in hygroscopic white amorphous powder decomposed between 180 ~ 230°C and optically levorotatory. On an ascending paper chromatogram, each preparation showed a ninhydrin positive spot in the range of R_f 0.54 ~ 0.72, which agreed with the inhibitory zone on a bioautogram using *E. coli* or *B. subtilis*.

The amino acid and the fatty acid component of all purified preparations were listed in Table IV, in which three amino acids, Leu, Thr and Dab were brought from the CNP

TABLE II. REACTION CONDITIONS FOR THE SYNTHESIS OF *n*-FATTYACYL MONO-AMINOACYL-CNP DERIVATIVES

	CNP	Acylamino acid <i>p</i> -NPE ^{a)}	Solvent	Reaction temp.	Yield
<i>n</i> -Octanoyl glycyl-CNP	1 mm	5 mm	50% A ^{b)}	25°C	10.6%
<i>n</i> -Decanoyl glycyl-CNP	1	4	70 A	25	3.4
<i>n</i> -Dodecanoyl glycyl-CNP	1	2	70 A	25	7.0
<i>n</i> -Octanoyl L-alanyl-CNP	1	4	60 A	5	9.0
<i>n</i> -Decanoyl L-alanyl-CNP	1	2	70 A	20	8.4
<i>n</i> -Dodecanoyl L-alanyl-CNP	1	2.5	60 A	20	7.6
<i>n</i> -Octanoyl DL- α -aminobutyryl-CNP	1	4	60 A	5	15.2
<i>n</i> -Decanoyl DL- α -aminobutyryl-CNP	1	2.5	70 A	5	10.2
<i>n</i> -Dodecanoyl DL- α -aminobutyryl-CNP	1	2	60 A	5	11.1
<i>n</i> -Octanoyl DL-norvalyl-CNP	1	3	70 A	5	25.3
<i>n</i> -Decanoyl DL-norvalyl-CNP	1	2	60 A	25	8.7
<i>n</i> -Dodecanoyl DL-norvalyl-CNP	1	4	50 A	20	10.8
<i>n</i> -Octanoyl DL-norleucyl-CNP	1	2	60 A	20	13.7
<i>n</i> -Decanoyl DL-norleucyl-CNP	1	2	60 A	25	14.1
<i>n</i> -Dodecanoyl DL-norleucyl-CNP	1	2.5	60 A	5	6.5
<i>n</i> -Octanoyl β -alanyl-CNP	1	4	70 A	25	5.3
<i>n</i> -Decanoyl β -alanyl-CNP	1	5	60 A	25	7.7
<i>n</i> -Dodecanoyl β -alanyl-CNP	1	5	60 A	15	14.0
<i>n</i> -Octanoyl γ -aminobutyryl-CNP	1	3	70 D ^{b)}	25	14.8
<i>n</i> -Decanoyl γ -aminobutyryl-CNP	1	2.5	70 A	5	16.7
<i>n</i> -Dodecanoyl γ -aminobutyryl-CNP	1	2.5	70 D	25	14.6

^{a)} *p*-NPE: *p*-Nitrophenyl ester.^{b)} A: Acetone, D: Dioxane.TABLE III. PHYSICOCHEMICAL PROPERTIES OF *n*-FATTYACYL MONO-AMINOACYL-CNP DERIVATIVES

	mp (Decomp.)	$[\alpha]_D^{25}$ (In water)	<i>R</i> _f ^{a)}
<i>n</i> -Octanoyl glycyl-CNP	207 ~ 210°C	-29.6°	0.54
<i>n</i> -Decanoyl glycyl-CNP	220 ~ 225	-27.5	0.57
<i>n</i> -Dodecanoyl glycyl-CNP	204	-26.7	0.63
<i>n</i> -Octanoyl L-alanyl-CNP	195 ~ 204	-27.0	0.56
<i>n</i> -Decanoyl L-alanyl-CNP	185 ~ 197	-29.0	0.57
<i>n</i> -Dodecanoyl L-alanyl-CNP	190 ~ 201	-25.8	0.56
<i>n</i> -Octanoyl DL- α -aminobutyryl-CNP	210 ~ 221	-47.4	0.62
<i>n</i> -Decanoyl DL- α -aminobutyryl-CNP	200 ~ 211	-33.3	0.61
<i>n</i> -Dodecanoyl DL- α -aminobutyryl-CNP	216 ~ 221	-42.4	0.65
<i>n</i> -Octanoyl DL-norvalyl-CNP	218 ~ 222	-29.7	0.63
<i>n</i> -Decanoyl DL-norvalyl-CNP	195 ~ 203	-35.1	0.66
<i>n</i> -Dodecanoyl DL-norvalyl-CNP	218 ~ 226	-42.2	0.66
<i>n</i> -Octanoyl DL-norleucyl-CNP	180 ~ 187	-31.8	0.66
<i>n</i> -Decanoyl DL-norleucyl-CNP	200 ~ 209	-36.1	0.69
<i>n</i> -Dodecanoyl DL-norleucyl-CNP	210 ~ 220	-29.3	0.64
<i>n</i> -Octanoyl β -alanyl-CNP	215 ~ 218	-24.8	0.65
<i>n</i> -Decanoyl β -alanyl-CNP	198 ~ 205	-35.3	0.72
<i>n</i> -Dodecanoyl β -alanyl-CNP	205 ~ 209	-18.0	0.70
<i>n</i> -Octanoyl γ -aminobutyryl-CNP	182 ~ 186	-25.8	0.68
<i>n</i> -Decanoyl γ -aminobutyryl-CNP	186 ~ 191	-17.0	0.60
<i>n</i> -Dodecanoyl γ -aminobutyryl-CNP	182 ~ 186	-27.2	0.61

^{a)} Solvent system: *n*-Butanol: Pyridine: Acetic acid: Water, 15: 10: 3: 12.

TABLE IV. AMINO ACIDS AND FATTY ACID COMPOSITION OF *n*-FATTYACYL MONO-AMINOACYL-CNP DERIVATIVES

	Leu	Thr	Dab	Gly	Fatty acid
<i>n</i> -Octanoyl glycyl-CNP	2.00	1.63	4.88	0.88	1.16
<i>n</i> -Decanoyl glycyl-CNP	2.00	1.64	5.12	0.96	0.86
<i>n</i> -Dodecanoyl glycyl-CNP	2.00	1.64	5.04	0.88	0.60
				<u>L-Ala</u>	
<i>n</i> -Octanoyl L-alanyl-CNP	2.00	1.87	5.23	1.10	1.07
<i>n</i> -Decanoyl L-alanyl-CNP	2.00	1.85	5.42	1.08	1.11
<i>n</i> -Dodecanoyl L-alanyl-CNP	2.00	1.95	5.23	1.10	1.29
				<u>DL-α-Ab^{a)}</u>	
<i>n</i> -Octanoyl DL- α -aminobutyryl-CNP	2.00	1.76	5.08	1.08	0.96
<i>n</i> -Decanoyl DL- α -aminobutyryl-CNP	2.00	1.73	5.13	1.06	1.02
<i>n</i> -Dodecanoyl DL- α -aminobutyryl-CNP	2.00	1.67	4.73	1.09	0.64
				<u>DL-Norval^{a)}</u>	
<i>n</i> -Octanoyl DL-norvalyl-CNP	2.00	1.81	5.10	1.04	1.14
<i>n</i> -Decanoyl DL-norvalyl-CNP	2.00	2.17	4.58	1.33	0.68
<i>n</i> -Dodecanoyl DL-norvalyl-CNP	2.00	1.86	5.14	0.98	1.05
				<u>DL-Norleu^{a)}</u>	
<i>n</i> -Octanoyl DL-norleucyl-CNP	2.00	1.83	4.92	1.10	1.14
<i>n</i> -Decanoyl DL-norleucyl-CNP	2.00	1.72	5.06	1.00	1.02
<i>n</i> -Dodecanoyl DL-norleucyl-CNP	2.00	1.66	5.06	0.95	1.02
				<u>β-Ala</u>	
<i>n</i> -Octanoyl β -alanyl-CNP	2.00	1.97	5.17	1.32	0.88
<i>n</i> -Decanoyl β -alanyl-CNP	2.00	1.74	4.64	0.95	1.05
<i>n</i> -Dodecanoyl β -alanyl-CNP	2.00	1.94	4.68	1.08	0.74
				<u>γ-Ab^{a)}</u>	
<i>n</i> -Octanoyl γ -aminobutyryl-CNP	2.00	1.90	5.44	1.02	0.68
<i>n</i> -Decanoyl γ -aminobutyryl-CNP	2.00	1.93	5.38	1.10	0.83
<i>n</i> -Dodecanoyl γ -aminobutyryl-CNP	2.00	1.64	5.00	0.70	0.64

^{a)} Ab: Aminobutyric acid, Norval: Norvaline, Norleu: Norleucine.

moiety, and *n*-fatty acid and one amino acid other than the above three from the *n*-fattyacyl aminoacyl moiety newly introduced. The molar ratio of 4 kinds of amino acid and a fatty acid contained in each purified preparation agreed with the theoretical value (Leu: Thr: Dab: introduced amino acid: *n*-fattyacid, 2: 2: 5: 1: 1). Thus, it is obvious that the synthesized derivatives were combined in an equimolar amount of CNP and *n*-fattyacyl aminoacyl moiety within an experimental error. In order to estimate the purity of the synthesized derivatives on the basis of the molar ratio of *n*-fattyacyl aminoacyl moiety introduced to both the α - and γ -amino groups of a CNP molecule, the purified preparations were dinitrophenylated with 2,4-dinitrofluorobenzene, hydrolyzed with 6 N HCl for 18 hr at 110°C and

the content of DNP-Thr and γ -DNP-Dab were quantitatively analyzed by the same method as previously reported.²⁾ As shown in Table V,

TABLE V. MOLAR RATIO OF *N*-TERMINAL AMINO ACIDS OF *n*-FATTYACYL MONO-AMINOACYL-CNP DERIVATIVES

	γ -DNP-Dab	DNP-Thr
<i>n</i> -Octanoyl glycyl-CNP	4.00	0.49
<i>n</i> -Dodecanoyl L-alanyl-CNP	4.00	0.68
<i>n</i> -Dodecanoyl		
DL- α -aminobutyryl-CNP	4.00	0.48
<i>n</i> -Dodecanoyl DL-norvalyl-CNP	4.00	0.41
<i>n</i> -Dodecanoyl DL-norleucyl-CNP	4.00	0.33
<i>n</i> -Dodecanoyl β -alanyl-CNP	4.00	0.41
<i>n</i> -Dodecanoyl		
γ -aminobutyryl-CNP	4.00	0.38
Theoretical value	4.00	0.00

TABLE VI. ANTIMICROBIAL SPECTRA OF *n*-FATTYACYL α -AMINOACYL-CNP DERIVATIVES

	Glycyl-CNP			L-Alanyl-CNP			DL- α -Aminobutyryl-CNP			DL-Norvalyl-CNP			DL-Norleucyl-CNP		
	C-8	C-10	C-12	C-8	C-10	C-12	C-8	C-10	C-12	C-8	C-10	C-12	C-8	C-10	C-12
<i>M. lysodeikticus</i>	>100	50	100	>100	>100	>100	—	—	—	>100	>100	>100	>100	25	50
<i>St. aureus</i> FDA 209P	>100	>100	100	>100	>100	>100	>100	50	100	50	50	50	>100	>100	100
<i>S. lutea</i> PCI 1001	—	50	100	>100	>100	>100	100	>100	>100	25	100	100	50	12	12
<i>B. subtilis</i> PCI 219	50	25	25	25	25	12	25	12	25	25	50	12	50	12	12
<i>B. cereus</i> IFO 3001	>100	>100	50	>100	>100	100	100	>100	100	50	50	25	>100	50	>100
<i>E. coli</i> NIHJ	100	50	50	50	>100	25	50	100	50	25	100	50	100	100	25
<i>E. coli</i> K12	>100	100	50	50	>100	25	100	50	100	50	100	100	50	25	50
<i>A. aerogenes</i> IAM 1063	50	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<i>Ser. marcescens</i> IAM 1022	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<i>Pr. vulgaris</i> IFO 3045	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<i>Pr. mirabilis</i> IFO 3849	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<i>Pr. morgani</i> IFO 3848	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<i>Pr. rettgeri</i>	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
<i>Sal. enteritidis</i>	100	>100	>100	25	>100	25	100	>100	>100	>100	>100	>100	25	12	25
<i>Sal. infantis</i>	>100	>100	>100	>100	50	25	>100	100	>100	100	>100	100	>100	50	50
<i>Sh. flexneri</i>	>100	>100	100	>100	>100	25	50	>100	>100	100	>100	50	50	25	25
<i>Ps. aeruginosa</i> IFO 3910	>100	100	100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	100	>100
<i>Ps. desmolytica</i>	—	—	—	>100	25	25	>100	>100	>100	>100	>100	50	50	12	50
<i>X. oryzae</i>	—	—	—	—	—	—	50	50	>100	50	25	25	100	50	100

the amount of DNP-Thr contained in seven preparations ranged, except for 0.68 mole in *n*-dodecanoyl L-alanyl-CNP, in 0.33~0.49 mole equivalent to 4 mole of γ -DNP-Dab. Therefore, the efficiency of the coupling toward the α -amino group of the N-terminal Thr of CNP is regarded as about 50~60% in each synthesis. The remaining part contained in the preparation as a contaminant seemed to be occupied by the *n*-fattyacyl mono-aminoacylated derivative at one γ -amino group of CNP molecule in view of the *Kd* value of the peak of the counter current distribution pattern and the *Rf* value on paper chromatogram.

Although further consideration is needed for the purity of these synthesized derivatives, they were subjected to the observations for antimicrobial profile and toxicity.

Antimicrobial spectra of n-fattyacyl mono-aminoacyl-CNP derivatives

Of the preparations of *n*-fattyacyl mono-aminoacyl-CNP synthesized, 15 derivatives introduced with any one of five kinds of α -amino acid, the carbon number of which ranged from 2 (glycine) to 6 (norleucine) and fatty-

acylated with *n*-octanoic, *n*-decanoic or *n*-dodecanoic acid, were first examined for their antimicrobial spectra. In regard to the three kind of glyceryl derivatives, their antimicrobial activity varied depending on the kind of the fattyacyl moiety, although they were all effective against *B. subtilis* at concentrations of 25~50 mcg/ml. The antimicrobial activity of these derivatives against gram-negative bacteria was restricted to *E. coli* NIHJ, *E. coli* K12 and *Aerobacter aerogenes*, while other gram-negative bacteria such as *Salmonella enteritidis*, *Salmonella infantis* and *Shigella flexneri* which were inhibited by *n*-decanoyl and *n*-dodecanoyl-CNP at concentrations of 6~25 mcg/ml,¹⁾ were susceptible to these three *n*-fattyacyl glyceryl derivatives at 100 mcg/ml or more. *n*-Fattyacyl L-alanyl- and *n*-fattyacyl DL- α -aminobutyryl-CNPs, though *n*-dodecanoyl L-alanyl-CNP was the lowest in purity, showed essentially the same antimicrobial profiles as did *n*-fattyacyl glyceryl-CNP derivatives, showing the antimicrobial activity at concentrations of 12~100 mcg/ml against *B. subtilis*, *E. coli* NIHJ, *E. coli* K12, *Sal. enteritidis*, *Sal. infantis*, *Sh. flexneri* and *Pseudomonas desmolytica*.

TABLE VII. ANTIMICROBIAL SPECTRA OF *n*-FATTYACYL ω -AMINOACYL-CNP DERIVATIVES

	β -Alanyl-CNP			γ -Aminobutyryl-CNP		
	C-8	C-10	C-12	C-8	C-10	C-12
<i>M. lysodeikticus</i>	>100	12	>100	50	>100	>100
<i>St. aureus</i> FDA 209P	>100	>100	100	>100	>100	100
<i>S. lutea</i> PCI 1001	—	50	—	>100	—	—
<i>B. subtilis</i> PCI 219	>100	6	12	50	50	12
<i>B. cereus</i> IFO 3001	100	>100	50	>100	>100	50
<i>E. coli</i> NIHJ	50	100	25	100	50	12
<i>E. coli</i> K 12	>100	50	50	25	>100	50
<i>A. aerogenes</i> IAM 1063	50	>100	25	>100	50	12
<i>Ser. marcescens</i> IAM 1022	100	>100	25	>100	>100	>100
<i>Pr. vulgaris</i> IFO 3045	100	>100	100	>100	>100	>100
<i>Pr. mtlabilis</i> IFO 3849	>100	>100	>100	>100	>100	>100
<i>Pr. morganii</i> IFO 3848	>100	>100	50	>100	>100	>100
<i>Pr. rettgeri</i>	>100	>100	>100	>100	>100	>100
<i>Sal. enteritidis</i>	>100	12	25	100	>100	100
<i>Sal. infantis</i>	50	25	50	50	100	50
<i>Sh. flexneri</i>	50	25	25	100	50	25
<i>Ps. aeruginosa</i> IFO 3901	100	50	50	100	100	100
<i>Ps. desmolytica</i>	—	—	—	—	—	—
<i>X. oryzae</i>	—	—	—	—	—	—

n-Fattyacyl DL-norleucyl derivatives, in comparison with *n*-fattyacyl DL-norvalyl-CNP derivatives, possessed broader antimicrobial spectra and more effective antimicrobial activity against gram-positive and negative bacteria such as *Micrococcus lysodeikticus*, *Sarcina lutea*, *B. subtilis*, *E. coli* K12, *Sal. enteritidis*, *Sal. infantis*, *Sh. flexneri* and *Ps. desmolytica*. This seemed to reflect the enhancement of the antimicrobial activity with the increase in the carbon number of amino acid introduced to CNP molecule. However, the antimicrobial activity of all *n*-fattyacyl α -aminoacyl-CNP derivatives were fairly lowered than those of corresponding *n*-fattyacyl-CNP derivatives.

Of ω -aminoacyl series, *n*-fattyacyl β -alanyl-CNP derivatives were noticeable one and their antimicrobial spectra extended with the increase in the carbon number of the acyl moiety. *n*-Octanoyl β -alanyl-CNP exhibited a minimum inhibitory concentration value of 50 mcg/ml against *E. coli* NIHJ, *Aerobacter aerogenes*, *Sal. infantis* and *Sh. flexneri*, while *n*-decanoyl β -alanyl derivative inhibited *M. lysodeikticus*, *S. lutea*, *B. subtilis*, *E. coli* K12, *Sal. enteritidis*, *Sal. infantis*, *Sh. flexneri* and *Pseudomonas aeruginosa* at concentrations of 6~50 mcg/ml. In contrast with them, *n*-dodecanoyl β -alanyl derivative showed the broadest antimicrobial spectrum towarded gram-negative bacteria and two bacteria, *Ser. marcescens* and *Pr. morganii*, both of which were insusceptible to colistin even at a concentration of 100 mcg/ml, were inhibited by this derivative at concentrations of 25 and 50 mcg/ml, respectively.

On the contrary, three *n*-fattyacyl γ -aminobutyryl-CNPs did not inhibit the above two bacteria at a concentration of 100 mcg/ml, rather they were similar to *n*-fattyacyl α -aminoacyl derivatives in the antimicrobial profile. These results suggest that the extension of antimicrobial spectrum against colistin insensitive gramnegative bacteria such as *Serratia* and *Proteus* is not a common character to any ω -amino acid introduced but limited in the case of β -alanine.

Acute toxicity of *n*-fattyacyl mono-aminoacyl-CNP derivatives

The acute toxicity of a series of *n*-fattyacyl mono-aminoacyl-CNP derivatives was examined by intravenous dose against male mice. As shown in Table VIII, the acute toxicity of

TABLE VIII. ACUTE TOXICITY OF *n*-FATTYACYL MONO-AMINOACYL-CNP DERIVATIVES

	LD ₅₀ (mg/kg)
<i>n</i> -Octanoyl glyceryl-CNP	70
<i>n</i> -Decanoyl glyceryl-CNP	66
<i>n</i> -Dodecanoyl glyceryl-CNP	58
<i>n</i> -Octanoyl L-alanyl-CNP	78
<i>n</i> -Decanoyl L-alanyl-CNP	94
<i>n</i> -Dodecanoyl L-alanyl-CNP	53
<i>n</i> -Octanoyl DL- α -aminobutyryl-CNP	37
<i>n</i> -Decanoyl DL- α -aminobutyryl-CNP	25
<i>n</i> -Dodecanoyl DL- α -aminobutyryl-CNP	54
<i>n</i> -Octanoyl DL-norvalyl-CNP	65
<i>n</i> -Decanoyl DL-norvalyl-CNP	20
<i>n</i> -Dodecanoyl DL-norvalyl-CNP	17
<i>n</i> -Octanoyl DL-norleucyl-CNP	50
<i>n</i> -Decanoyl DL-norleucyl-CNP	37
<i>n</i> -Dodecanoyl DL-norleucyl-CNP	53
<i>n</i> -Octanoyl β -alanyl-CNP	35
<i>n</i> -Decanoyl β -alanyl-CNP	25
<i>n</i> -Dodecanoyl β -alanyl-CNP	74
<i>n</i> -Octanoyl γ -aminobutyryl-CNP	31
<i>n</i> -Decanoyl γ -aminobutyryl-CNP	112
<i>n</i> -Dodecanoyl γ -aminobutyryl-CNP	66

these derivatives ranged in LD₅₀ from 17 mg/kg of *n*-dodecanoyl norvalyl-CNP to 112 mg/kg of *n*-decanoyl γ -aminobutyryl-CNP, showing higher toxicity than CNP itself,²⁾ but lower one than colistin. Of all derivatives synthesized, *n*-dodecanoyl and *n*-decanoyl norvalyl-CNP, *n*-decanoyl α -amino-butyryl-CNP and *n*-decanoyl β -alanyl-CNP indicated fairly high acute toxicity as the LD₅₀ values of 17, 20, 25 and 25 mg/kg, respectively.

DISCUSSION

CNP²⁾ is a large peptide fragment and obtained from colistin by enzymatic cleavage. Although it has almost no antimicrobial activity, the introduction of a suitable acyl group such as one of normal fatty acids of carbon

number 6 to 14 to the terminal threonine of CNP results in the regain of the antimicrobial activity.^{1,5)} Increase in the carbon number of the *n*-fatty acid moiety gave rise to an extension of the antimicrobial spectrum toward gram-positive bacteria, whereas the antimicrobial activity against gram-negative bacteria is generally weaker than that of colistin.¹⁾ As mentioned in this report, introduction of a mono-amino acid, instead of a Dab in colistin molecule, to the N-terminal position of CNP in a form of *n*-fattyacyl amino acid gave a rather narrowed antimicrobial spectrum against gram-negative bacteria as compared with the corresponding *n*-fattyacyl-CNP. However, *n*-dodecanoyl β -alanyl-CNP could inhibit *Ser. marcescens* and *Pr.morganii*, both belonging to the colistin-insensitive gram-negative bacteria.¹⁾ This fact suggests that the kind of introduced amino acid is responsible for the antimicrobial activity, whereas the length of the peptide chain is not responsible. Therefore, the N-terminal amino acid introduced to CNP or possibly N-terminal Dab in colistin molecule in addition to an acyl moiety may have a certain role in the appearance of the characteristic antimicrobial activity, though in more restricted nature than that of the acyl moiety as shown in a series of fattyacylated derivatives of CNP.

As to the mechanism of bacterial susceptibility to antibiotics of the polymyxin group, the involvement of cell permeability⁶⁾ has been presumed. However, it is known that even the cell permeability of such gram-negative bacteria as *Pr. milabilis* which are insensitive to polymyxin could be affected by polymyxin B,^{7,8)} and moreover that the spheroplasts of this bacterium are readily bursted by polymyxin B.⁸⁾ Nakajima⁹⁾ revealed that colistin decapeptide, the deacylated product of colistin, retained about 1/20 of the antimicrobial activity compared with that of colistin. Contrary to colistin, this decapeptide was incapable of liberating 260 nm absorption materials from cells of *E. coli*.¹⁰⁾ On the other hand, this decapeptide was not different from colistin in the ability of the formation of an insoluble

complex with cell ribosomes¹¹⁾ and DNA¹²⁾ obtained from the bacterial cells of the above strain. The diversity in the mode of action between colistin and colistin decapeptide seemed to be dependent on the difference in the chemical structure relevant to the fattyacyl moiety in both molecules. However, in view of the results obtained by the present authors, it is inferred that the terminal amino acid introduced into the molecule of CNP, as in the case of colistin decapeptide, functions as an acyl moiety so as to select susceptible bacterial cells in penetrating into the inner membrane of the cell envelope.

Polymyxin antibiotics, though a few component amino acids of their peptide moiety are different from each other, seem to differ scarcely in their antimicrobial activity against *E. coli*.¹³⁾ Vogler *et al.*¹⁴⁾ synthesized two types of polymyxin analogs, one of which has a ring peptide moiety composed of eight amino acids, and the other corresponding to ordinary polymyxins has a ring peptide moiety composed of seven amino acids. Comparison of both analogs shows that the antimicrobial activity against some gram-negative bacteria of the former is 1/6~1/30 lower than that of the latter. In each of the two types of the analogs, two series of compounds differentiated in the linkage between the ring and the straight chain part of peptide were synthesized; one was mediated through the α -amino group of a Dab in the ring peptide, while the other was mediated through its γ -amino group. These twin derivatives varied only at the point of the linkage corresponding to each of two polymyxin analogs which are scarcely differed in their antimicrobial activity. Furthermore, Kurihara *et al.*¹⁵⁾ have reported that two colistin analogs, in which one was replaced by lysine instead of all six Dabs residing in the colistin decapeptide moiety, while the other, an unclosed derivative of the above analog, had the chain form in the peptide moiety, were as well active as colistin against twelve gram-negative bacterial strains. Recently, Chapman and Golden¹⁶⁾ have presented a tentative conformation for polymyxin B₁, in which it

holds the antiparallel pleated sheet structure of the skeletal peptide chain supported by five hydrogen bonds. Further conformational evidence of colistin or various CNP derivatives will clarify more the relationship between chemical structure and antimicrobial activity.

Acknowledgement. The authors wish to express their thanks to Teijiro Uemura, the president of Iwate University, for his valuable suggestions and Miss Isoko Haneda for her assistance in the microbiological analysis of antibiotics.

REFERENCES

- 1) S. Chihara, A. Ito, M. Yahata, T. Tobita and Y. Koyama, *Agr. Biol. Chem.*, **38**, 521 (1974).
 - 2) S. Chihara, T. Tobita, M. Yahata, A. Ito and Y. Koyama, *ibid.*, **37**, 2455 (1973).
 - 3) "Methods in Enzymology," Vol III, ed. by S.P. Colowick and N.O. Kaplan, Academic Press Inc., New York, 1957, p. 555.
 - 4) N.F. Alberton, "Organic Reactions," Vol. XII, ed. by A.C. Cope, John Wiley & Sons, Inc., New York, 1962, p. 162~355.
 - 5) S. Chihara, A. Ito, M. Yahata, T. Tobita and Y. Koyama, *Agr. Biol. Chem.*, **37**, 2709 (1973).
 - 6) B.A. Newton, *Bacteriol. Rev.*, **20**, 14 (1956).
 - 7) M. Teuber, *J. Bacteriol.*, **98**, 347 (1969).
 - 8) I. J. Sud and D. S. Feingold, *Antimicro. Agents & Chemoth.*, **5**, 417 (1972).
 - 9) K. Nakajima, *Chem. Pharm. Bull.*, **15**, 229 (1967).
 - 10) K. Nakajima and J. Kawamata, *Biken J.*, **8**, 233 (1965).
 - 11) K. Nakajima and J. Kawamata, *ibid.*, **9**, 45(1966).
 - 12) K. Nakajima and J. Kawamata, *ibid.*, **8**, 225(1965).
 - 13) K. Hayashi and T. Suzuki, *Bull. Inst. Chem. Res., Kyoto Univ.*, **43**, 259 (1965).
 - 14) K. Vogler, R.O. Studer, P. Lanz, W. Lergier and E. Böhni, *Experientia.*, **17**, 223 (1961).
 - 15) T. Kurihara, H. Takeda and H. Ito, *YAKUGAKU ZASSHI*, **92**, 129 (1972).
 - 16) T.M. Chapman and M.R. Golden, *Biochem. Biophys. Res. Commun.*, **46**, 2040 (1972).
-