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Synthesis and Antibacterial Activities of Theanine-containing Oligopeptides

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Theanine, an antimetabolite of L-glutamic acid, is a weak antibacterial agent. In the hope of obtaining theanine derivatives which have more potent biological activities, a series of theanyl-L-alanine oligopeptides, H–Tea-L-Ala$_n$–OH ($n=1,2,3$), and several derivatives of these peptides with blocking groups at the amono-and/or carboxyl-termini were prepared, and their antibacterial and anti-glutamic decarboxylase activities were examined. Theanyl-L-alanine and theanyl-L-alanyl-L-alanyl-L-alanine inhibited the growth of *Staphylococcus aureus* and *Escherichia coli* most strongly, the percent inhibitions being 2 to 23 times higher against *S. aureus* or 3 to 4 times higher against *E. coli* than those of theanine. Structural modifications at the end groups of the peptides, especially N-acylation, caused marked reductions in the antibacterial activities. None of the theanine and theanyl-L-alanine oligopeptides possessed any significant ability to inhibit bacterial glutamic decarboxylase even at a concentration of 50 μmol/ml.

Keywords——theanine; oligopeptides; transport system; antibacterial activity; glutamic decarboxylase

Many results obtained by various methods on amino acid and peptide utilization by microorganisms have indicated that amino acid and peptide uptakes occur by different processes. For example, Leach and Snell showed that peptide transport of *Lactobacillus casei* was not inhibited by free amino acid.[2] Mutants of several bacterial species have also been isolated that fail to transport a free amino acid but readily take up the amino acid when in peptide form.[3]

On the basis of these nutritional and biochemical findings, Gilvarg *et al.* proposed that peptides are taken up *via* peptide transport systems and hydrolyzed by intracellular peptidases to give amino acids.[4] The feasibility of using this transport system to pass impermeant substances into the bacterial cells by connecting them to simple and small peptides has been confirmed with several amino acid metabolites or antimetabolites.[5] These results on the membrane transport of metabolites or antimetabolites could be important in the design of novel antibacterial agents.

Lichtenstein and Grossowicz showed that DL-theanine [DL-(γ-glutamyl)-ethylamine] acts as an antagonist of L-glutamic acid, inhibiting the growth of *Staphylococcus aureus*.[6] However, its activity is not high, probably due to its poor ability to enter the cells.

In the hope of converting this antimetabolite into more effective derivatives by utilizing the peptide transport system, we investigated a series of oligopeptides in which theanine is

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2) F.R. Leach and E.E. Snell, *Biochem. Biophys. Acta*, **34**, 292 (1959); *idem*, *J. Biol. Chem.*, **235**, 3523 (1960).
3) J. Guardiola and M. Iaccarino, *J. Bacteriol.*, **103**, 1034 (1971).
4) J.W. Payne and Ch. Gilvarg, *Advan. Enzymol.*, **35**, 187 (1971); Z. Barak and Ch. Gilvarg, *J. Biol. Chem.*, **249**, 143 (1974).
5) E. Bayer, K.H. Gugel, K. Hagel, H. Hagenmaier, S. Jessipow, W.A. Konig, and H. Zahner, *Helv. Chim. Acta*, **55**, 224 (1972); D.L. Pruess, J.P. Scanell, H.A. Ax, M. Kellet, F. Weiss, C. Demny, and A. Stempel, *J. Antibiot., Ser., A*, **26**, 261 (1973); T.E. Fickel, and Ch. Gilvarg, *Nature, New Biol.* (London), **241**, 161 (1973); B.N. Ames, G.F. Ames, L.D. Young, D. Isuchiya, and J. Lecocq, *Proc. Natl. Acad. Sci. U.S.*, **70**, 456 (1973).
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linked to alanine or alanine peptides at the N-terminus. Several derivatives of these peptides with blocking groups at the amino- and/or carboxyl-termini were also investigated in order to clarify the structural requirements for the transport system.[4,7]

The peptides studied here are compounds **1a—d**, **2a**, **b**, **d**, and **3a—d**, which have the general formula R_1–Tea–L-Ala_n–OR_2 (n=1,2,3) (Table I).

The protected theanylalanine peptides, **1a**, **2a**, and **3a**, were synthesized, starting from L-alanine benzyl ester, by a combination of the active ester method and the mixed anhydride method. As an example, the synthetic scheme for **3d** is presented in Fig. 1.

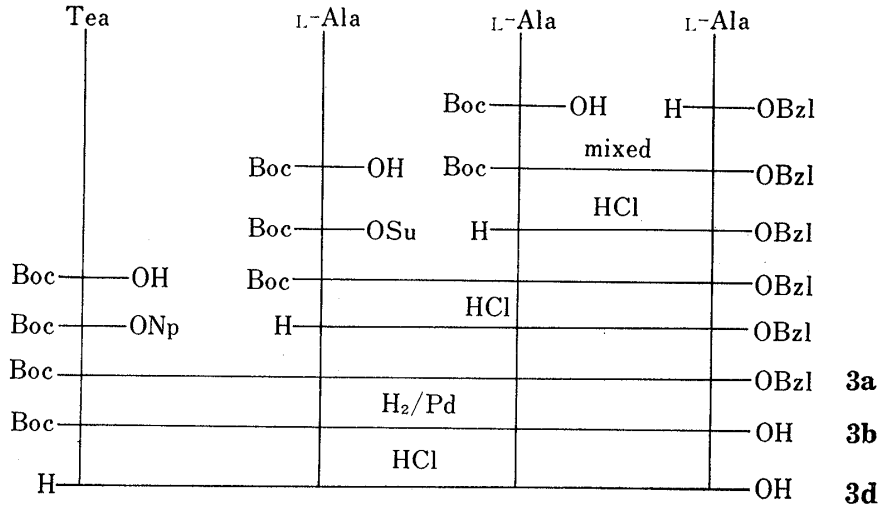

Fig. 1. Synthetic Scheme for **3d**

H–L-Ala–L-Ala–OBzl, obtaining by removal of the Boc group of Boc–L-Ala–L-Ala–OBzl with HCl-dioxane, was condensed with Boc–L-Ala–OSu by the active ester method. The resulting Boc–L-Ala–L-Ala–L-Ala–OBzl was deprotected with HCl-dioxane and coupled with Boc–Tea–ONp to afford Boc–Tea–L-Ala–L-Ala–OBzl (**3a**). Similarly, H–L-Ala–OBzl and H–L-Ala–L-Ala–OBzl were coupled with Boc–Tea–ONp to give Boc–Tea–L-Ala–OBzl (**1a**) and Boc–Tea–L-Ala–L-Ala–OBzl (**2a**), respectively.

Deblocking of these Boc-peptide esters was carried out as follows. The Boc group of **1a** and **3a** was acidolytically removed to afford **1c** and **3c**, respectively. The benzyl group was removed by catalytic hydrogenation over 5% Pd on charcoal to yield **1b—3b**, which were deprotected with HCl-dioxane to afford **1d—3d**. Purification of the products by ion exchange chromatography on Dowex 50W×2 provided each of the free di- to tetra-peptides (**1d—3d**) as a mixture of diastereomers. The two isomers of each compound were indistinguishable, giving a single spot on silica gel plates with several solvent systems. The compounds were tested for biological activities without resolution, since it has been shown that there is no difference in biological response (growth inhibition against bacteria and anticonvulsant effects in mice) between DL-and L-theanine.[8]

The synthetic peptides were tested for their inhibitory activities on the growth of *Staphylococcus aureus* and *Escherichia coli*, and the inhibitory activities were compared with those of L-alanine, theanine, and L-alanine+theanine. These data are summarized in Table I.

The di- and tetra-peptides, **1d** and **3d**, inhibited the growth of both bacteria most strongly; the 50% inhibition concentrations against *S. aureus* and *E. coli* were 6.8 and 18.6 μmol/ml for **1d** and 18.2 and 20.6 μmol/ml for **3d**, respectively. Unexpectedly, the tripeptide **2d** showed slightly higher inhibitory activity than theanine.

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Table I. Inhibitory Effects of Theanine-containing Peptides on Bacterial Growth

Compound	Concn. μmol/ml	Growth inhibition (%)[a]	
		S. aureus[b]	*F. coli*[c]
L-Alanine	10	0	0
	20	0	1.9
	30	0	10.0
Theanine	10	1.8	7.8
	20	21.9	16.4
	30	39.0	31.7
L-Alanine + theanine[d]	10	10.6	0
	20	10.4	1.4
	30	41.4	29.0
Boc–theanine	10	0	0
	20	0	0
	30	20.6	13.5
1b	10	0	0
	20	0	0
	30	15.5	13.5
1c	10	0	0
	20	15.8	13.5
	30	57.5	50.0
1d	10	55.7	27.9
	20	64.9	51.0
	30	83.1	97.6
2b	10	0	0
	20	0	0
	30	0	15.0
2d	10	4.8	12.0
	20	18.9	20.0
	30	34.9	58.5
3b	10	0	0
	20	0	0
	30	0	24.1
3c	10	0	0
	20	25.8	8.7
	30	47.4	30.8
3d	10	38.4	19.6
	20	50.2	48.4
	30	78.0	97.4

a) The growth inhibition (%) was determined as described in "Experimental."
b) *Staphylococcus aureus* 209 P.
c) *Escherichia coli* B RIMD 0509003.
d) An equimolar mixture of each component.

It is interesting that blocking of either the amino or the carboxyl terminal of the peptides decreased the antibacterial activity. Blocking of the amino group caused a remarkable inactivation. These results are compatible with an earlier observation that blocking of the α-amino group of peptides abolished their nutritional activities for microorganisms.[9]

The peptides **1d** and **3d** exhibited obviously greater antibacterial activities than the equimolar mixture of theanine and L-alanine. This result suggests that the uptake of theanine

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into the cell is favored by the peptide formation; presumably the peptides are hydrolyzed to free theanine and alanine inside the cell by a peptidase.

Unfortunately, theanine, **1d** and **3d** all failed to show inhibitory activity against glutamic decarboxylase of *E. coli* even at a concentration of 50 μmol/ml.

However, our present investigation shows that the attachment of an antimetabolite to a carrier peptide may be a useful method for obtaining potent antibacterial agents.

Table II. Analytical Data for the Products

R_1–Tea–L-Ala–OR_2 (**1**) R_1–Tea–L–Ala–L-Ala–OR_2 (**2**)

R_1–Tea–L-Ala–L-Ala–L-Ala–OR_2 (**3**)

a, R_1=Boc, R_2=Bzl; **b**, R_1=Boc, R_2=H;
c, R_1=H, R_2=Bzl; **d**, R_1=R_2=H

Compound	Formula	Analysis (%) Calcd C	H	N	Found C	H	N
Boc–Tea–OH	$C_{12}H_{22}N_2O_5 \cdot 1/2H_2O$	50.87	8.18	9.87	51.27	7.80	9.87
Boc–Tea–ONp	$C_{18}H_{25}N_3O_7$	54.68	6.37	10.63	54.32	6.12	10.63
1a	$C_{22}H_{33}N_3O_6$	60.67	7.64	9.65	60.33	7.52	9.88
2a	$C_{25}H_{38}N_4O_7 \cdot 1/2H_2O$	58.24	7.62	10.87	58.58	7.38	10.87
3a	$C_{28}H_{43}N_5O_8$	58.22	7.50	12.12	57.93	7.68	11.91
H–L-Ala–L-Ala–OBzl·HCl	$C_{13}H_{18}N_2O_3 \cdot HCl$	54.45	6.68	9.77	54.57	6.83	9.50
Boc–L-Ala–L-Ala–L-Ala–OBzl	$C_{21}H_{31}N_3O_6$	59.84	7.41	9.97	59.89	7.47	9.88
H–L-Ala–L-Ala–L-Ala–OBzl·HCl	$C_{16}H_{23}N_3O_4 \cdot HCl \cdot 1/2H_2O$	52.37	6.87	11.46	52.39	6.70	11.43
2b	$C_{18}H_{32}N_4O_7 \cdot H_2O$	49.75	7.89	12.89	50.15	7.52	12.68
3b	$C_{21}H_{37}N_5O_8 \cdot H_2O$	49.89	7.77	13.85	49.49	7.48	13.52
1c·HCl	$C_{17}H_{25}N_3O_4 \cdot HCl$	54.91	7.05	11.30	55.38	7.35	11.44
3c·HCl	$C_{23}H_{35}N_6O_5 \cdot HCl \cdot 2H_2O$	50.22	7.33	12.73	50.55	7.08	13.13

Experimental

Melting points are uncorrected. Optical rotations were determined on a Union PM-101 polarimeter. Thin-layer chromatography was performed on silica gel (Kiesel gel 60, Merck) with the following solvent systems: Rf_1, $CHCl_3$–MeOH–AcOH (95:5:3), by volume); Rf_2, $CHCl_3$–MeOH–pyridine (95:5:3); Rf_3, *n*-BuOH–AcOH–H_2O (3:1:1). Amino acid analysis was performed with a Hitachi 034 liquid chromatograph. The analytical data for the products are listed in Table II. Abbreviations used are those recommended in *J. Biol. Chem.*, **247**, 977 (1972). Other abbreviations used are: ONp=*p*-nitrophenyl ester; OSu=N-hydroxysuccinimide ester; DCC=N,N-dicyclohexylcarbodiimide; Tea=DL-theanyl.

Boc-Tea-OH——This compound was prepared by the reaction of theanine[8,10] (8.7 g, 0.05 mol) and di-*tert*-butyldicarbonate (Fluka) (12 g, 0.05 mol) in *tert*-BuOH (10 ml) according to the Moroder *et al.*[11] The product was recrystallized from AcOEt; yield 11.0 g (81%), mp 97—98.5°, Rf_1 0.23, Rf_2 0.16.

Boc-Tea-ONp——DCC (18.0 g, 0.086 mol) was added to a stirred solution of Boc–Tea–OH (22.0 g, 0.08 mol) and *p*-nitrophenol (13.4 g, 0.096 mol) in DMF (180 ml) at −10° and the mixture was stirred for 30 min and then at room temperature for 4 hr. After standing overnight in a refrigerator, the precipitated dicyclohexylurea was removed by filtration and the filtrate was evaporated to dryness *in vacuo*. The residue was mixed with H_2O (1.5 l) and the solution was left overnight in a refrigerator to form a gelatinous solid, which was collected by filtration and recrystallized from EtOH; yield 24.9 g (79%), mp 116—117.5°, Rf_1 0.55, Rf_2 0.55, Rf_3 0.70.

Boc-Tea-L-Ala-OBzl (**1a**)——Boc–Tea–ONp (9.1 g, 0.023 mol) was added to a solution of Et_3N (3.1 ml,

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11) L. Moroder, A. Hallet, E. Wünsch, O. Keller, and G. Wersin, *Z. Physiol. Chem.*, **357**, 1651 (1976).

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0.023 mol) and H-L-Ala–OBzl·TosOH[12] (8.1 g, 0.023 mol) in dioxane (100 ml). The solution was stirred at room temperature for 2 days and then evaporated to dryness. The residue was dissolved in AcOEt (100 ml) and the solution was washed with 4% $NaHCO_3$, 10% citric acid, and H_2O, then dried over Na_2SO_4. The residue was recrystallized from AcOEt–ether–light petroleum; yield 7.5 g (75%), mp 104—106°, Rf_1 0.49, Rf_2 0.59, Rf_3 0.89.

Boc-Tea-L-Ala-L-Ala-OBzl (**2a**)——a) H-L-Ala-L-Ala–OBzl·HCl: Boc-L-Ala-L-Ala–OBzl[13] (3.5 g, 0.01 mol) in 2 N HCl-dioxane (50 ml) was left to stand at room temperature for 5 hr. The reaction mixture was evaporated to dryness *in vacuo* with repeated addition of MeOH to remove excess HCl, and the residue was recrystallized from MeOH; yield 2.1 g (73%), mp 118—120°, Rf_1 0.07, Rf_2 0.12, Rf_3 0.58.

b) Boc–Tea-L-Ala-L-Ala–OBzl (**2a**): Boc–Tea–ONp (18.2 g, 0.04 mol) was added to a solution of Et_3N (7 ml, 0.05 mol) and H-L-Ala-L-Ala–OBzl·HCl (11.5 g, 0.046 mol) in a mixture of dioxane (200 ml) and H_2O (80 ml). The reaction was carried out as described for the preparation of **1a** to give **2a**, which was recrystallized from MeOH–light petroleum; yield 14.0 g (69%), mp 168—170°, Rf_1 0.69, Rf_2 0.63, Rf_3 0.84.

Boc-Tea-L-Ala-L-Ala-L-Ala-OBzl·HCl (**3a**)——a) Boc-L-Ala-L-Ala-L-Ala–OBzl: Boc-L-Ala–OSu[14] (7.7 g, 0.027 mol) was added to a solution of H-L-Ala-L-Ala–OBzl·HCl (6.0 g, 0.025 mol) and Et_3N (3.6 ml, 0.025 mol) in $CHCl_3$ (20 ml). The mixture was stirred at room temperature for 3 days and evaporated to dryness *in vacuo*. The residue was dissolved in AcOEt (70 ml) and the solution was washed with 4% $NaHCO_3$, 10% citric acid and H_2O, then dried over Na_2SO_4, and evaporated to dryness. Ether–light petroleum was added to the residue to afford a white crystalline material, which was collected by filtration and recrystallized from AcOEt–ether-light petroleum; yield 7.2 g (71%), Rf_1 0.56, Rf_2 0.38, $[\alpha]_D^{22}$ −90° (c=0.5, MeOH).

b) H-L-Ala-L-Ala-L-Ala–OBzl·HCl: Boc-L-Ala-L-Ala-L-Ala·OBzl (19.5 g, 0.04 mol) was treated with 2 N HCl-dioxane (150 ml) as described for the preparation of H-L-Ala-L-Ala–OBzl·HCl to give H-L-Ala-L-Ala-L-Ala–OBzl·HCl, which was recrystallized from AcOEt–ether–light petroleum; yield 13.6 g (77%), mp 151—153°, Rf_3 0.63, $[\alpha]_D^{22}$ −100° (c=0.5, MeOH).

c) Boc–Tea-L-Ala-L-Ala-L-Ala–OBzl (**3a**): Boc-Tea-ONp (6.8 g, 0.017 mol) was condensed with H-L-Ala-L-Ala-L-Ala–OBzl·HCl (5.5 g, 0.017 mol) in the manner described for the preparation of **2a** to give **3a**, which was recrystallized from AcOEt–EtOH–light petroleum; yield 7.2 g (73%), mp 210—212°, Rf_1 0.04, Rf_3 0.84.

Boc-Tea-L-Ala-OH (**1b**)——A solution of **1a** (1.3 g, 0.003 mol) in a mixture of MeOH (15 ml) and H_2O (3 ml) was hydrogenated over a 5% Pd-C catalyst (0.2 g) for 3 hr. After removal of the catalyst, the filtrate was concentrated *in vacuo* to give **1b** as an oil; yield, quantitative; Rf_1 0.04, Rf_3 0.78.

Boc-Tea-L-Ala-L-Ala-OH (**2b**)——**2a** was hydrogenolyzed in the manner described above to give **2b**, which was recrystallized from AcOEt–ether–light petroleum; yield 70%, mp 149—150.5°, Rf_1 0.08, Rf_3 0.56.

Boc-Tea-L-Ala-L-Ala-L-Ala-OH (**3b**)——**3a** was hydrogenolyzed in the manner described above to give **3b**, which was recrystallized from EtOH, yield 57%, mp 169—172°, Rf_3 0.36.

H-Tea-L-Ala-OBzl·HCl (**1c·HCl**)——**1a** (1.0 g, 0.003 mol) was treated with 2 N HCl-dioxane (30 ml) as described for the preparation of H-L-Ala-L-Ala–OBzl·HCl to give **1c**·HCl, which was recrystallized from EtOAc–ether; yield 0.92 g (83%), mp 172—174°, Rf_2 0.28, Rf_3 0.78.

H-Tea-L-Ala-L-Ala-L-Ala-OBzl·HCl (**3c·HCl**)——This compound was prepared and crystallized in the same manner as **1c**·HCl; yield 85%, mp 192—194°, Rf_1 0.05, Rf_3 0.57.

H-Tea-L-Ala-OH (**1d**)——A solution of **1b** (1.0 g, 0.003 mol) in 2 N HCl-dioxane (15 ml) was left to stand at room temperature for 3 hr and then repeatedly evaporated to dryness *in vacuo* with the addition of MeOH to remove excess HCl. The residue was dissolved in 0.2 M pyridine-formate buffer, pH 3.1, and applied to a Dowex 50W×2 column (pyridine form, 2.0×140 cm). The column was eluted with the same solvent. The fractions containing the compound of Rf_3 0.93 were combined and lyophilized to furnish pure **1a** as a white powder; yield 0.4 g (54%), Rf_3 0.93. Amino acid ratios in an acid hydrolysate (6 N HCl, 16 hr at 105°): glutamic acid 1.00, alanine 0.95.

H-Tea-L-Ala-L-Ala-OH (**2d**)——The deprotection of **2b** and the subsequent purification were carried out in the manner described for the preparation of **1d**. The pure **2d** was obtained as a hygroscopic powder; yield 55%, Rf_1 0.05, Rf_3 0.53. Amino acid ratios in an acid hydrolysate: glutamic acid 1.00, alanine 2.08.

H-Tea-L-Ala-L-Ala-L-Ala-OH (**3d**)——This compound was prepared in 74% yield from **3b** in the same manner as **1d**. The pure **3d** was obtained as a white powder; Rf_3 0.36. Amino acid ratios in an acid hydrolysate: glutamic acid 1.00, alanine 2.88.

Assay for the Antibacterial Activity——Five milliliters of the basal medium[6] with the test sample was inoculated with 0.1 ml of 10^{-3} dilution of 18 hr broth cultures. Growth was measured by estimating the

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turbidity after incubation at 37° for 48 hr. The per cent inhibition was calculated as followed: % inhibition = $100 \times (A-B)/A$; where A is the turbidity in the absence of test sample and B is that with the test sample.

Inhibition Test for Glutamic Acid Decarboxylase——Glutamic decarboxylase (250 μg) of *E. coli* (Kyowa Hakko Co.) was incubated with a mixture of 50 mM DL-[1-^{14}C]glutamate (0.05 Ci/mol, New England Nuclear Co.), 5 mM glutathione, 0.05 mM pyridoxal phosphate and test sample in 50 mM phosphate buffer, pH 6.0 (total volume 0.5 ml), at 37° for 1 hr, and the radioactivity of $^{14}CO_2$ produced was measured according to the method of Endo *et al.* with slight modifications.[15]

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