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Amino Acids and Peptides. II.[1,2] Synthesis of Stereoisomeric Alanine containing Peptide Derivatives and Their Effects on Germination of *Bacillus thiaminolyticus* Spores

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Stereoisomeric alanine containing peptides were synthesized. L-Ala-L-Ala and L-Ala-Gly induced germination of *Bacillus thiaminolyticus* spores and L-Ala-D-Ala and Gly-D-Ala inhibited L-alanine or L-Ala-L-Ala induced germination. The relationship between the structure of alanylpeptides and the effects on germination were also studied. Antibacterial activity of synthetic alanylpeptides against gram positive and gram negative organisms was also studied.

The synthesis of stereoisomeric alanine containing peptide derivatives is under way in our laboratory to study their microbiological activities. The germination of *Bacillus* and *Clostridium* species is induced by various substances, in which L-alanine is the most effective for germination.[4] Kawasaki, *et al.*[5] reported that free amino and carboxyl groups and the α hydrogen atom in L-alanine were important for induction of germination. It is also interesting that L-alanine induced germination is quantitatively inhibited by D-alanine.[6] This investigation deals with the synthesis of alanine containing peptide derivatives and their effects on germination of *Bacillus thiaminolyticus* spores. Furthermore, antibacterial activity of synthetic alanylpeptides is also described in this report continued from previous paper.[1]

Materials and Methods

Ac-L-Ala and Ac-D-Ala[7] were synthesized from L or D-alanine and acetic anhydride in cold $NaHCO_3$ solution. Stereoisomeric alanylalanine, L-Ala-L-Ala, L-Ala-D-Ala, D-Ala-L-Ala, and D-Ala-D-Ala and L-Ala-$NHCH_3$·HCl were synthesized as described in previous report.[1] L-Ala-Gly, D-Ala-Gly,[8] Gly-L-Ala, and Gly-D-Ala[9] were prepared as follows, N^{α}-benzyloxycarbonyl amino acid and amino acid methyl ester were coupled with dicyclohexylcarbodiimide (DCC) to form N-protected dipeptide methyl ester, which was saponified with 1 N NaOH and hydrogenated over palladium catalyst to give the desired dipeptide. They were purified by recrystallization from H_2O and EtOH. Their melting point (Melting Point Apparatus, Model MP-21, Yamato) and optical rotation (automatic polarimeter, Model DIP-180, Japan Spectroscopic Co. Ltd.) were identical with those in literatures. Stereoisomeric alanine tripeptides, L-Ala-L-Ala-L-Ala, D-Ala-D-Ala-D-Ala, L-Ala-L-Ala-D-Ala, D-Ala-D-Ala-L-Ala, L-Ala-D-Ala-D-Ala, D-Ala-L-Ala-L-Ala, L-Ala-D-Ala-L-Ala, and D-Ala-L-Ala-D-Ala were synthesized by saponification with 1 N NaOH and hydrogenation over palladium catalyst of the corresponding N^{α}-benzyloxycarbonyl tripeptide methyl ester[10] which was constructed by DCC coupling of the C-terminal dipeptide methyl ester with N-protected L or D-alanine. Purification of tripeptides

1) Part I: Y. Okada, S. Tani, Y. Yawatari, and M. Yagyu, *Chem. Pharm. Bull.* (Tokyo), **24**, 1925 (1976).
2) Abbreviations used are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 3485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972).
3) Location: *Ikawadani-machi, Tarumi-ku, Kobe, 673, Japan.*
4) G.W. Gould, "Bacterial Spore," ed. by G.W. Gould and A. Hurst, Academic Press, Inc., London, 1969, pp. 398—444.
5) C. Kawasaki, M. Kondo, and K. Teshima, *J. Food Hyg. Soc. Japan*, **8**, 207 (1967).
6) C. Kawasaki, M. Kondo, and K. Teshima, *J. Food Hyg. Soc. Japan*, **9**, 201 (1968).
7) S.M. Birnbaum, L. Levintow, R.B. Kingsley, and J.P. Greenstein, *J. Biol. Chem.*, **194**, 455 (1952).
8) D. Felicetti and H. Hanson, *Z. Physiol. Chem.*, **351**, 1260 (1970).
9) G. Losse and H. Schmidt, *Chem. Ber.*, **91**, 1068 (1958).
10) S. Visser, J. Roeloffs, K.E.T. Kerling, and E. Havinga, *Recl. Trav. Chim. Pays-Bas*, **87**, 559 (1968).

Table I. Physical Constant and Elemental Analysis of Synthetic Peptides

Compound	mp (°C)	$[\alpha]_D^{25}$	Analysis (%) Calcd. C	H	N	Found. C	H	N
		(c=1, MeOH)						
Z-Ala-Ala-Ala-OMe	166—167	−66.0	57.0	6.64	11.1	57.3	6.64	11.1
-D- -D- -D-	166—167	+71.7				57.2	6.72	11.2
-L- -L- -D-	163—164	−23.1				57.1	6.89	11.0
-D- -D- -L-	162—164	+21.8				57.2	6.91	11.2
-L- -D- -D-	149—152	+34.0				57.1	6.71	11.1
-D- -L- -L-	152—153	−36.1				57.2	6.81	11.2
-L- -D- -L-	139—140	−3.8				57.1	6.75	11.2
-D- -L- -D-	136—138	+2.2				57.3	6.69	11.0
		(c=1, MeOH)						
Z-Ala-Ala-Ala-OH	221—223	−59.1	55.9	6.35	11.5	56.0	6.50	11.6
-D- -D- -D-	224—225	+58.7				55.5	6.25	11.3
-L- -L- -D-	190—191	−38.8				55.9	6.43	11.6
-D- -D- -L-	191—193	+36.8				55.8	6.43	11.4
-L- -D- -D-	191—192	+23.2				55.8	6.28	11.4
-D- -L- -L-	190—192	−24.5				56.0	6.61	11.6
-L- -D- -L-	oil							
-D- -L- -D-	oil							
		(c=1, H_2O)						
H-Ala-Ala-Ala-OH·1/2H_2O	240—241 (decomp.)	−70.0	45.0	7.54	17.5	45.1	7.64	17.2
-D- -D- -D- 1/2H_2O	240—241 (decomp.)	+67.0				44.9	7.51	17.1
-L- -L- -D- 1/2H_2O	242—243 (decomp.)	−17.9				45.3	7.30	17.5
-D- -D- -L- 1/2H_2O	242—243 (decomp.)	+17.8				45.2	7.41	17.3
-L- -D- -D-	224—225	+97.3	46.7	7.41	18.2	46.5	7.39	18.0
-D- -L- -L-	224—225	−97.3				46.7	7.59	18.1
-L- -D- -L- 2/3H_2O	216, 250 (decomp.)	+50.9	44.5	7.57	17.3	44.5	7.76	17.1
-D- -L- -D- 2/3H_2O	216, 250 (decomp.)	−51.0				44.3	7.51	17.1

was achieved by recrystallization from H_2O and EtOH. Physical constants and analytical data on the purified tripeptides and their intermediates are presented in Table I. All peptides were homogeneous and free from alanine as indicated by thin-layer chromatography on silica gel G (Merck), (*n*-BuOH/AcOH/H_2O, 4: 1: 5; upper phase and *n*-BuOH/pyridine/AcOH/H_2O, 4: 1: 1: 2) and by amino acid analysis (Model JLC-6AH, JEOL Co., Ltd.). *Bacillus thiaminolyticus* Matsukawa et Misawa was gift of Professor M. Kondo, Faculty of Pharmaceutical Sciences of Osaka University.

Germination was estimated either by measuring changes in absorbance of the bacterial suspension containing 10 mM germinant at 610 nm or by determining changes in heat resistance.[11] Occurrence of free alanine or glycine from peptides during germination reaction (pH 7.8; 37°; 60 min) was not observed on an amino acid analyzer.

Results and Discussion

Combination of L-alanine which is germinative and D-alanine which has an inhibitory effect on L-alanine induced germination gives four kinds of stereoisomeric alanylalanine derivatives, L-Ala-L-Ala, L-Ala-D-Ala, D-Ala-L-Ala and D-Ala-D-Ala. Their effects on germination were estimated and these data are summarized in Table II.

11) K. Watabe, T. Ichikawa, and M. Kondo, *Japan. J. Microbiol.*, **18**, 173 (1974).

Table II. Effects of Ala–Ala on Germination of *Bacillus thiaminolyticus* Spores

Germinant	Germinability[a]	Inhibition of[b] germination
L-Ala	100	0
L-Ala-L-Ala	>90	0
L-Ala-D-Ala	0	>90
D-Ala-L-Ala	0	5—10
D-Ala-D-Ala	0	20—25
D-Ala	0	100

a) relative germinability (L-Ala=100%)
b) relative potency of inhibitory effect (D-Ala=100%)

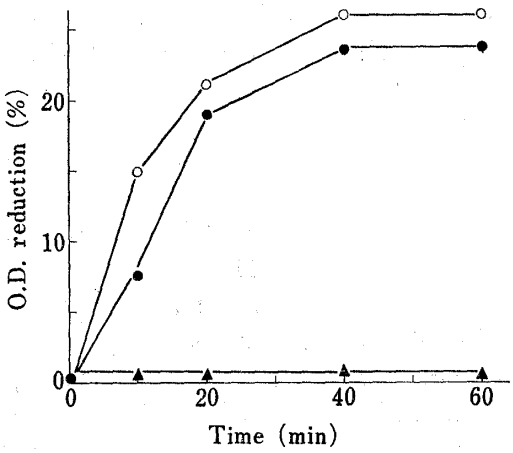

Fig. 1. Germination of Spores induced by L-Ala-L-Ala

—○—: L-Ala
—●—: L-Ala-L-Ala
—▲—: D-Ala+L-Ala

L-Ala-L-Ala out of four stereoisomeric alanylalanine derivatives did induce germination as well as L-alanine so far as reduction of optical density, loss of heat resistant cells and release of dipicolinic acid were concerned. Fig. 1 shows germination pattern of L-Ala-L-Ala and L-alanine.

There has been the hypothesis regarding germination mechanism that a free carboxyl group, a free amino group and α hydrogen atom of L-alanine are required for its germinability and those three points contribute to binding with receptor site of some enzyme which has been proposed to effect germination.[12,13] But the fact that dipeptide, L-Ala-L-Ala, still has germinability and it might still be able to bind with receptor site of enzyme to induce germination is conflicting with the hypothesis described above.

On the contrary, D-alanine containing alanylalanine (L-D, D-L, D-D) did not exhibit any germinability but showed inhibitory effects on L-alanine or L-Ala-L-Ala induced germination. It was also hypothesized with regard to inhibitory mechanism that in the presence of L-alanine, the affinity of D-alanine to the receptor is so much stronger than that of L-alanine that D-

Table III. Effects of Alanine Derivatives on Germination of *Bacillus thiaminolyticus* Spores

Germinant	Germinability[a]	Inhibition of[b] Germination
L-Ala-L-Ala	>90	0
L-Ala-Gly	85—90	0
Gly-L-Ala	<5	0
Ac-L-Ala	<5	0
L-Ala–$NHCH_3 \cdot HCl$	<5	0
Ac-D-Ala	0	<5
D-Ala-Gly	0	<5
Gly-D-Ala	0	80
L-Ala-D-Ala	0	>90

a) relative germinability (L-Ala=100%)
b) relative potency of inhibitory effect (D-Ala=100%)

12) E. Freese and M. Cashal, "Spores," Vol. 3, ed. by L.L. Campbell and H.O. Halvorson, Ann Arbor, Michigan, 1965, pp. 144—151.
13) R.J. O'Connor and H.O. Halvorson, *Biochim. Biophys. Acta*, **48**, 47 (1961).

alanine prevents the approach of L-alanine to the receptor.[14] Out of three D-alanine containing alanylalanine, L-Ala-D-Ala had the strongest inhibitory effect, whereas D-Ala-L-Ala had a slight inhibitory effect. The reason why L-Ala-D-Ala shows the strongest effect is not known yet.

In order to study the relationship between the structure of the alanylpeptides and the effect on germination, the activity of Ac-L-Ala, Ac-D-Ala, L-Ala–Gly, D-Ala–Gly, Gly-L-Ala, Gly-D-Ala and L-Ala–$NHCH_3$·HCl on germination was estimated and the results are summarized in Table III.

L-Ala–Gly stimulated germination, whereas Ac-L-Ala, Gly-L-Ala and L-Ala-$NHCH_3$·HCl did not show any remarkable germinability. The activity of L-Ala–OMe·HCl was also estimated but it was revealed that about 5% of methyl ester was saponified nonenzymatically during germination reaction to liberate free alanine to induce germination. From these results, it will be deduced that a free amino group in L-alanine is very important for the induction of germination because substitution of hydrogen atom on amino group of L-alanine, Ac-L-Ala and Gly-L-Ala, almost diminishes the germinability, although L-Ala–Gly retains the ability to induce germination. The fact that L-Ala-$NHCH_3$·HCl in which carboxyl group in L-alanine is protected as methylamide does not exhibit any remarkable germinability suggests that a free carboxyl group is required in germinant for its effectiveness.

With regard to D-alanine containing peptide derivatives, they did not exhibit any germinability as well as D-alanine containing alanylalanine. Out of them, Gly-D-Ala inhibited L-Ala or L-Ala-L-Ala induced germination, on the contrary, D-Ala–Gly and Ac-D-Ala did not show any remarkable inhibitory effects on L-alanine or L-Ala-L-Ala induced germination. The phenomenon that Gly-D-Ala inhibits germination and D-Ala–Gly does not show any remarkable inhibitory effect seems to be compatible with the fact that L-Ala-D-Ala is the strongest inhibitor for germination out of three D-alanine containing stereoisomeric alanylalanine and D-Ala-L-Ala shows only a little inhibitory effect on germination. Regarding inhibition of germination, a free carboxyl group of D-alanine is important for inhibitory effect on germination and the presence of a free amino group in the molecule is also required for its inhibitory effect. It was also deduced that L-Ala-D-Ala and Gly-D-Ala might still be able to bind with receptor of some enzyme to prevent the approach of L-alanine or L-Ala-L-Ala to receptor.

Elongation of stereoisomeric alanylalanine with L-alanine or D-alanine forms eight kinds of stereoisomeric trialanylpeptides. They were synthesized and the activity of them on germination was estimated. All trialanylpeptides did not show any detectable germinability or inhibitory effects on L-Ala-L-Ala induced germination of *Bacillus thiaminolyticus* spores. This result suggests that the tripeptides may be prevented from arrival at the enzyme site or binding with the receptor site of the enzyme is prevented because of steric hindrance.

Although all compounds obtained above did not show any antibacterial activity against gram positive organisms, *Staphylococcus aureus* and *Sarcina lutea*, and gram negative organisms, *Escherichia coli* and *Pseudomonas aeruginosa*, in the concentration of 100 μg/ml, it was revealed that some alanine peptides effected on germination of *Bacillus thiaminolyticus* spores.

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14) K. Watabe, T. Nishihara, and M. Kondo, *Japan. J. Microbiol.*, **18**, 181 (1974).