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Synthesis of a Peptide Form of *N*-δ-(Phosphonoacetyl)-L-ornithine

ITS ANTIBACTERIAL EFFECT THROUGH THE SPECIFIC INHIBITION OF *ESCHERICHIA COLI* L-ORNITHINE CARBAMOYLTRANSFERASE*

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_N_-δ-(Phosphonoacetyl)-L-ornithine is a potent inhibitor of the *Escherichia coli* L-ornithine carbamoyltransferase (K_i = 0.77 μM, pH 8.0, 37°C). Nevertheless, the analog does not cross the bacterial membrane. Therefore we have designed a tripeptide, glycylglycyl-_N_-δ-(phosphonoacetyl)-L-ornithine, to take advantage of the broad specificity of the oligopeptide permease system of the bacterium. A lag effect, related to the tripeptide concentration, was observed in the growth of the wild type P4X strain. At high concentration (≥ 0.75 mM) the peptide appears to be bacteriostatic and the cells which escape this action were characterized genetically as mutants devoid of the oligopeptide transport system. It was shown that the *in vivo* cellular target of the toxic tripeptide is solely restricted to L-ornithine carbamoyltransferase and that the tripeptide is probably split in the cell to permit an effective inhibition by _N_-δ-(phosphonoacetyl)-L-ornithine. Resistance of the wild type cells to moderate levels (< 0.75 mM) of the phosphonic analog is accompanied by a derepression of the L-ornithine carbamoyltransferase activity. The P4XB2 strain, which is an *arg* R regulatory mutant, has a reduced lag effect in the presence of the tripeptide and appears to react to the intoxication by a further adjustment of the L-ornithine carbamoyltransferase cellular level.

The synthesis of potential stable phosphonic analogs of the transition state in the reaction catalyzed by L-ornithine carbamoyltransferase (EC 2.1.3.3.) were recently reported (1-3). Although combining most of the structural features of the two substrates or the two products of the reaction into a single molecule (Fig. 1), *N*-δ-(phosphonoacetyl)-L-ornithine (PALO[1]) is not precisely a true transition state analog (1, 4). Nevertheless, the molecule is a powerful inhibitor of L-ornithine carbamoyltransferase. The inhibition constant for PALO with the *Escherichia coli* enzyme is 0.77 μM at pH 8.0 and 37°C (1). Hoogenraad (2) reported a K_i of 0.24 μM for the rat liver enzyme at pH 7.4 and 30°C. The potency of the inhibitor has prompted us to use it as a highly specific tool for metabolic studies in bacteria. In similar studies, *N*-(phosphonoacetyl)-L-aspartate, an inhibitor of L-aspartate carbamoyltransferase, was found to effectively block *de novo* pyrimidine nucleotide biosynthesis and cellular proliferation of simian virus 40-transformed hamster cells (5).

Unfortunately, PALO failed to inhibit the growth of the microorganisms that we have tested. This failure was shown to be due to a permeability barrier imposed by the bacterial membrane.[2]

We have solved the problem, at least for *E. coli*, by integrating PALO into a tripeptide, glycylglycyl-*N*-δ-(phosphonoacetyl)-L-ornithine. It was expected that the broad specificity of the oligopeptide permease system of *E. coli* (6-8) would enable this compound to enter the cell. This paper is concerned mainly with the synthesis of the above-mentioned tripeptide and its interaction with *E. coli* strains.

EXPERIMENTAL PROCEDURES

The "Experimental Procedures," including the GlyGlyPALO synthesis, are presented in the miniprint supplement following this paper.[3]

RESULTS AND DISCUSSION

Growth Inhibition of E. coli—Table I shows that PALO is a growth inhibitor when present as component of the tripeptide GlyGlyPALO. A reduced sensitivity to the toxic peptide was observed for the *arg* R strain. On the other hand, the wild type strain and the regulatory mutant were equally sensitive to triornithine, a toxic tripeptide which specifically blocks protein biosynthesis (29). A growth inhibition of the wild type strain in liquid minimal medium and in the presence of GlyGlyPALO was observed as a lag of growth dependent on the concentration of the toxic tripeptide. The growth data were represented on semilogarithmic coordinates (Fig. 3). It can be seen that once appreciable growth is initiated, it occurs with the same generation time as in the uninhibited culture. Moreover, the lag lengthens with increasing concentrations of GlyGlyPALO until a limiting value of about 7 h is reached (Fig. 4). Under those conditions, only a reduced lag effect was

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[1] The abbreviations used are: PALO, *N*-δ-(phosphonoacetyl)-L-ornithine; GlyGlyPALO, glycylglycyl-*N*-δ-(phosphonoacetyl)-L-ornithine; NHOsu, *N*-hydroxysuccinimide; DCCI, dicyclohexylcarbodiimide; DMF, *N*,*N*-dimethylformamide; Z, benzyloxycarbonyl; tBoc, *t*-butyloxycarbonyl; F_3Ac, anhydrous trifluoroacetic acid; dns, dansyl; TOR and GGP, triornithine- and GlyGlyPALO-resistant mutants.

[2] D. Gigot and M. Penninckx, unpublished data.

[3] Portions of this paper (including "Experimental Procedures" and Fig. 2) are presented in a miniprint at the end of this article. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 78-2230, cite author(s), and include a check or money order for $1.00 per set of photocopies.

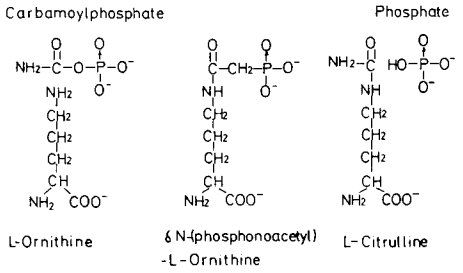

FIG. 1. The structure of PALO and the substrates and the products of the reaction catalyzed by L-ornithine carbamoyltransferase.

TABLE I

Growth inhibition by PALO, free and in peptide form

About 5×10^7 bacteria in 0.1 ml of a minimal medium culture were plated on a minimal medium Petri dish. Filter paper discs (6-mm diameter) containing the indicated amount of test substance were placed in the center. Incubation was at 37°C for 24 h. Mutations TOR and GGP were isolated, respectively, by resistance to triornithine and GlyGlyPALO (see "Experimental Procedures" and "Results").

Compound		Strain (diameter of the inhibition zone)							
		P4X	P4XB2	TOR-2	TOR-3	TOR7	GGP1	GGP5	GGP8
	μmol	*mm*							
PALO	2	0	0	0	0	0	0	0	0
PALO + glycine	2	0	0	0	0	0	0	0	0
	4								
GlyGlyPALO	0.8	36	10	0	0	0	0	0	0
Orn Orn Orn	2	27	25	0	0	0	0	0	0

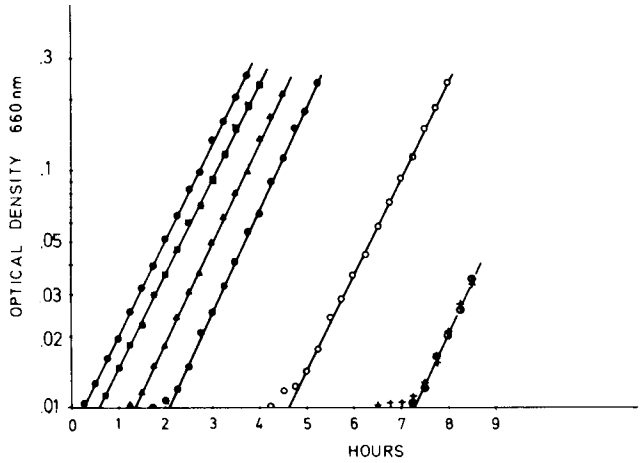

FIG. 3. Semilogarithmic plot of the growth curves of P4X on minimal medium supplemented with glucose and methionine in the presence of GlyGlyPALO. The initial innoculum amounted for about 5×10^6 cells/ml. The tripeptide concentrations are indicated in micromoles per ml. (●) 0; (■) 0.03; (▲) 0.075; (⊕) 0.15; (○) 0.30; (+) 0.75; (◑) 1.5.

observed for the *arg* R strain at high concentration of the toxic tripeptide.

Mode of Action on the P4X Strain—The lag effect produced by high concentrations of GlyGlyPALO could be due to a bacteriostatic or bactericidal action of the peptide. These possibilities were tested by a determination of the viable count during exposure of the cells to the toxic peptide (Fig. 5). The viable count remained nearly constant in the 5 h after the addition of 1.5 μmol/ml of the peptide. The reference situation, without exposure to the toxic peptide, showed that the cell population doubled every 50 min. GlyGlyPALO appears thus to exert a bacteriostatic effect on *E. coli*. Nevertheless, the check of the viable counts after 10 h incubation revealed a significant increase of the cell population. This effect was shown to be related to the spontaneous presence in the initial cell population of a significant number of mutants devoid of the oligopeptide permease system (*opp*$^-$). Viable counts made in the presence of 100 μg/ml of triornithine (29) revealed that the titer of the *opp*$^-$ cells amounted to 2.5×10^4 per 1.5×10^7 of the original inoculum.

Moreover, during the exposure to GlyGlyPALO, the titer of the *opp*$^-$ cells rose continually and could actually account for the observed resumption of growth. Spontaneous high titers of *opp*$^-$ cells in *E. coli* strains were previously observed (29, 30). As also noted for the triornithine toxicity (29), both the dependence of the lag on GlyGlyPALO concentration and the maximum lag are functions of the size and previous history of the inoculum. As a general rule, the observed P4X cells

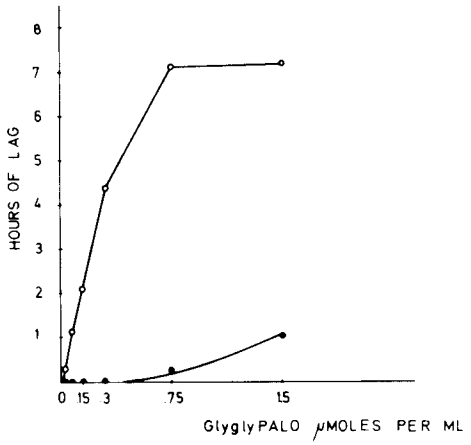

FIG. 4. Inhibition of the growth of *E. coli* strains by GlyGlyPALO. Inhibition is expressed as the number of hours before exponential growth is resumed relative to a control without the analog. (○) wild type P4X; (●) *arg* R mutant P4XB2.

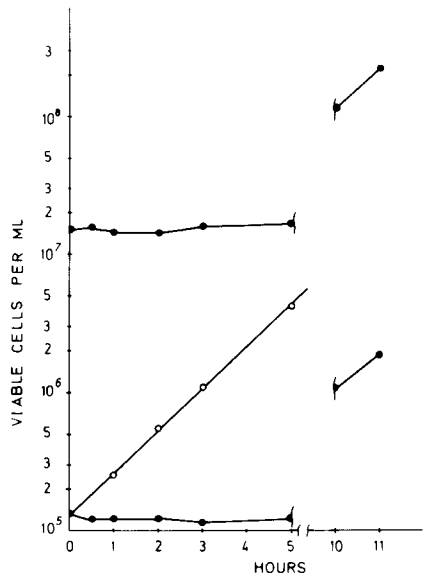

FIG. 5. Bacteriostatic action of high GlyGlyPALO concentration on the P4X strain. The bacterium at the indicated initial cell densities was incubated at 37°C on minimal medium supplemented with glucose and methionine in the presence of 1.5 μmol/ml of GlyGlyPALO: (○) count without addition of the toxic peptide; (●) count with addition of the toxic peptide. The innoculum was taken from an exponential phase culture. At the indicated times, aliquots were suitably diluted and plated on the counting medium. Colonies were scored as described under "Experimental Procedures," in the miniprint supplement.

resistant to the toxic effect of 1.5 μmol/ml of GlyGlyP ALO all appeared to be the *opp*$^-$ type. Plate count experiments made on those cells in the absence, or in the presence of triornithine in the counting medium, gave the same number of bacteria irrespective of the exposure to the toxic tripeptide in the bacterial culture. At the highest GlyGlyPALO concentration used in the experiment depicted in Fig. 3, a 7-h lag period was found to take place before resumption of growth. This is exactly what can be calculated taking into account the proportion of *opp*$^-$ mutants in the inoculum and knowing that *opp*$^-$ mutants exhibit the same growth rate as the wild type strain. The mechanisms of the resistance of P4X to lower GlyGlyPALO concentrations and of P4XB2 strain is of completely different nature and are discussed below.

In other experiments, spontaneous triornithine-resistant mutants were selected by virtue of their insensitivity to 100 μg/ml of triornithine. These mutants were shown also to be insensitive to GlyGlyPALO (Table I). Isolated colonies, insensitive to GlyGlyPALO, appeared spontaneously in the inhibition zone of the paper disc assay for the P4X strain. Some colonies (GGP) were purified and shown to be insensitive to triornithine (Table I). This peptide is only translocated by way of the oligopeptide permease system (31). Therefore, the cross-resistance of our mutant strains to triornithine and to the phosphonic tripeptide suggested a common mechanism of the entry for the two inhibitors. This was confirmed by a transduction-mediated mapping experiment. The closest marker to the *trp* operon is the *opp* gene (31); the determination of the linkage of TOR and GGP to the *trp* marker was thus chosen as a test of identity for the two characters. Table II shows in all the analyzed cases a close linkage (98% and more) of GGP and TOR to *trp*.

Mechanism of Action—Fig. 6 shows the selectivity of the inhibitory effect of GlyGlyPALO. The inhibition on minimal solid medium was completely antagonized in the presence of 50 μg/ml of L-arginine. The presence of L-ornithine had no effect on the inhibition while L-citrulline had the same effect as L-arginine. In the presence of 100 μg/ml of L-citrulline and 1.5 μmol/ml of GlyGlyPALO, the lag for P4X was nearly absent (15 min) as compared with the 7 h value without the amino acid addition (not shown). The *E. coli* strain JEF8C23 is only able to obtain carbamoylphosphate for pyrimidine biosynthesis by the L-ornithine carbamoyltransferase-catalyzed phosphorolysis of L-citrulline (13). A paper disc agar diffusion assay (4 μmol of the toxic tripeptide) with 5×10^7 cells of the strain on a minimal medium supplemented with

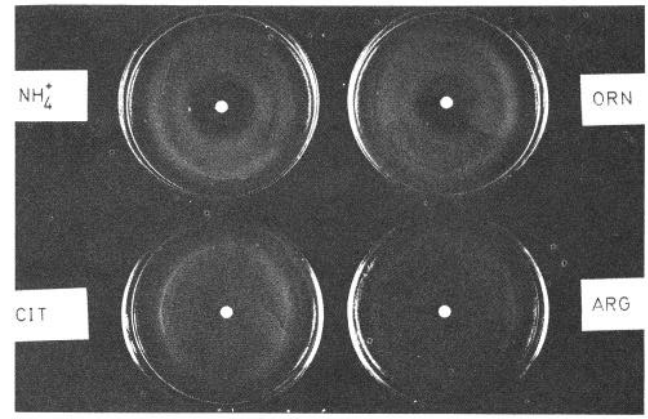

FIG. 6. Specificity of the action of GlyGlyPALO on *E. coli*. 4 to 5 $\times 10^7$ P4X cells were plated on each Petri dish. One micromole of GlyGlyPALO (in 10 μl) was placed on a filter paper disc (6-mm diameter) in the center of each plate. The plates were incubated for 24 h at 37°C. NH_4^+ is the symbol for the minimal medium supplemented with glucose and methionine. This medium contains ammonium as sole nitrogen source. *ORN*, *CIT*, and *ARG* pictures the same medium with L-ornithine, L-citrulline, and L-arginine as respective nitrogen sources.

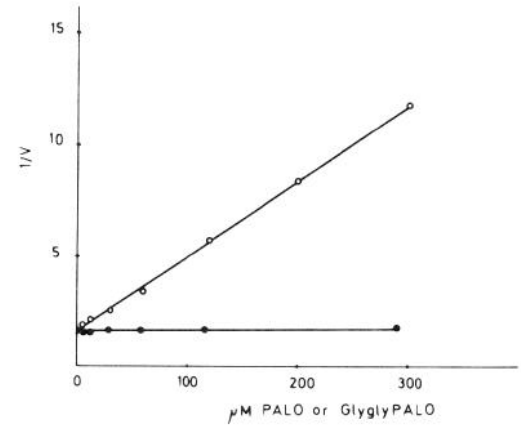

FIG. 7. *In vitro* inhibition of L-ornithine carbamoyltransferase by PALO in the free and peptide form. Reciprocal velocity with respect to the inhibitor concentration: (○) PALO; (●) GlyGlyPALO. The respective concentration of L-ornithine and carbamoylphosphate were taken equal to 10 and 1.5 mM. *V* is micromoles of citrulline per 10-min incubation.

TABLE II

Linkage of TOR and GGP to Trp determined by phage 363-mediated co-transduction

Trp$^+$ transductants were selected on minimal medium supplemented with 0.5% glucose and the required amino acids for the phage receptor 58-161 strain, except tryptophan. After 44 h of growth at 37°C, colonies were picked at random and plated on master plates containing the same medium. Replica plating was carried out with sterile velvet pads after allowing the colonies to grow for 24 h at 37°C. Growth of the TOR and GGP transductants was determined after a similar period of incubation. The above-mentioned medium, containing 100 μg of triornithine or 1.5 μmol of GlyGlyPALO/ml was used for the respective selection of TOR and GGP transductants.

Origin of particle	No. of Trp$^+$ scored	TOR co-transductants		GGP co-transductants	
		No.	Percentage	No.	Percentage
TOR1	118	117	99	116	98
TOR5	120	118	98	120	100
GGP2	115	114	99	114	99
GGP6	117	117	100	115	98

L-citrulline showed an inhibition zone of 27-mm diameter. The inhibition was completely alleviated by the presence of uracil. This shows that the "healing" effect of citrulline and arginine in the presence of the inhibitor is due to their suppressing the need for an active ornithine carbamoyltransferase. The cellular target of the inhibitor is thus solely restricted to this enzyme. In a similar study, the toxicity of *N*-(phosphonoacetyl)-L-aspartate, an inhibitor for L-aspartate carbamoyltransferase, was found to be prevented by uridine for mammalian cell lines showing thus a specific action on the uridylic acid biosynthesis (5).

The Dixon plot presented in Fig. 7 shows that 300 μM GlyGlyPALO does not exert any detectable inhibitory effect on *E. coli* L-ornithine carbamoyltransferase at saturating concentrations of the substrates (10). Under those conditions, the apparent K_i for PALO is 47 μM. This observation suggests that PALO must be freed from the glycylglycyl carrier by the intracellular array of peptidases (8) before it exerts an inhibitory effect.

No significant degradation of the toxic peptide took place

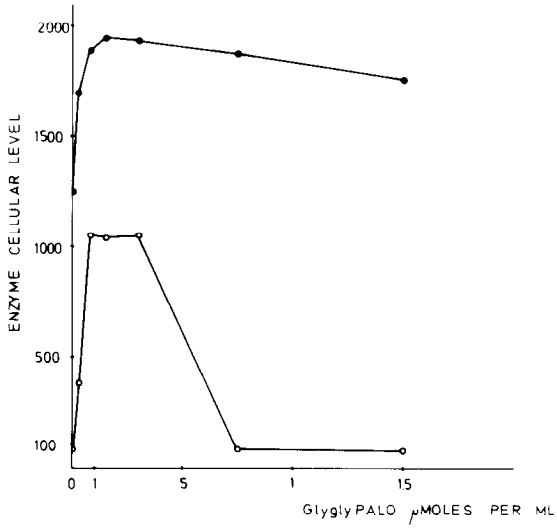

FIG. 8. Response of the *E. coli* L-ornithine carbamoyltransferase cellular level to GlyGlyPALO. About 5×10^6 cells/ml were inoculated as in Fig. 3 and allowed to grow at different GlyGlyPALO concentrations until a cell population of 3.5 to 4.5×10^8 individuals/ml was attained. Crude extracts were prepared by sonication of the respectively collected cells and the enzyme specific activity (enzyme cellular level) was estimated as described under "Experimental Procedures," in the miniprint. (○) P4X; (●) P4XB2. The enzyme specific activity is expressed in micromoles of citrulline $\times$ h^{-1} $\times$ mg $protein^{-1}$.

in the growth medium during the 7 h lag period (Fig. 3). Indeed, using amino acid analysis (see "Experimental Procedures"), we have found no evidence for the presence of GlyGlyPALO amino acids constituents in the supernatant fluid of the culture at the end of this period. This observation is consistent with the previous reports pointing to the absence of peptidases secretion by *E. coli* (8).

With the aim of understanding the mechanism of resistance to low concentrations of GlyGlyPALO found in the wild type at the end of the lag periods and in the *arg* R mutant at nearly all times (see Fig. 4), we studied the variation of the cellular L-ornithine carbamoyltransferase level in response to increasing amounts of the toxic peptide. Fig. 8 shows that P4X cells which have recovered from the toxic effect of low concentrations of the inhibitor have higher levels of the enzyme than cells grown in the absence of the inhibitor. The maximum level reached is about 80% of that obtained in the *arg* R strain on minimal medium. This value is attained at an inhibitor concentration of 0.15 μmol/ml in the growth medium. Those recovering P4X cells are not regulatory mutants because their inoculation in subcultures without GlyGlyPALO gives rise to cell populations with wild type enzyme levels. The wild type derived cells which recovered from high GlyGlyPALO concentrations have their normal enzyme level and are in fact, as seen above, of the opp^- type. The *arg* R strain reacts to the GlyGlyPALO addition by a further rise of the L-ornithine carbamoyltransferase cellular level to a maximum 2-fold value relative to the basal level of the mutant.

The actual mechanism of this unexpected reaction exhibited by the *arg* R strain still remains unexplained and is under present investigation. In the case of *N*-(phosphonoacetyl)-L-aspartate action on mammalian cell lines, the only reported mechanism of resistance seems to be related to the appearance of stable cell lines with a large increase in L-aspartate carbamoyltransferase activity (5). Regulatory mutants derived from *E. coli* wild type cells were not observed in our current experiments with GlyGlyPALO because of their probable masking by permeaseless mutants. Finally, GlyGlyPALO could also be a tool for selecting mutants of the intracellular enzymatic system of oligopeptide degradation (8, 31).

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Supplementary Material

to

SYNTHESIS OF A PEPTIDE FORM OF N-δ-(PHOSPHONOACETYL)-L-ORNITHINE

ITS ANTIBACTERIAL EFFECT THROUGH THE SPECIFIC INHIBITION OF

ESCHERICHIA COLI L-ORNITHINE CARBAMOYLTRANSFERASE.

M. Penninckx and D. Gigot

Experimental Procedures.

Chemicals. Purchased from the following sources were:
Z-glycylglycine, N-hydroxysuccinimide, N-δ-tBoc-L-ornithine, L-ornithine, L-citrulline, L-arginine, carbamoylphosphate, Tris-HCl, Dansyl chloride: all from Sigma.
Anhydrous trifluoroacetic acid, Thionyl chloride, HBr 40% in glacial acetic acid, triethylamine, ninhydrin, methylene chloride, petroleum ether, diethyl ether, ethyl acetate, acetone, N,N-dimethylformamide: all from Merck.
Triornithine: Miles-Yeda. N-α-Z-L-ornithine: ICN.
Phosphonoacetic acid was synthesized by acid hydrolysis of triethylphosphonoacetate (Eastman-Kodak). The product was recrystallised in glacial acetic acid (9). Escherichia coli L-ornithine carbamoyltransferase was purified as described (10).

Strains. All the strains were derived from Escherichia coli K12. Strain F4X met B (11) was used as the reference strain in this work.
All the triornithine-resistant mutants (TOR) are spontaneous mutants of Escherichia coli which were obtained by virtue of their insensitivity to 100μg/ml of triornithine. As described in the results, glyglyPALO-resistant strains (GGP) were obtained as isolated colonies in the inhibition zone around a paper disc impregnated with the toxic phosphonic derivative. The individual isolates are referred to by numbers after the TOR and GGP designates.
The strain F4XB2 met B arg R is a regulatory mutant for the L-arginine biosynthesis (12). F4X met B thr car B8 (JEF8023) is a strain devoid of carbamoylphosphate synthetase activity and which is able to synthesize carbamoylphosphate using the L-ornithine carbamoyltransferase-catalyzed phosphorolytic cleavage of L-citrulline (13).
The strain 58-161 met B thr leu trp his (14) was used in the transduction experiments.

Growth experiments. The minimal 132 medium has been described (15). When used in a solid form, the medium was supplemented with 1.5% final agar. The final concentration of glucose in the minimal medium was 0.5%. Metabolites were supplied at the following concentrations:
L-ornithine, L-citrulline, L-arginine, L-tryptophan: 100μg/ml.
L-methionine, DL-histidine: 50 g/ml. DL-threonine, DL-leucine: 200μg/ml.
Uracil: 100μg/ml. For the growth experiments with the strain JEF8023, L-citrulline was added at 1mg/ml final concentration.
The growth of the cells in liquid medium was followed at 660nm on a Beckman B spectrophotometer. In general, the innoculum was taken from an exponential phase culture of the bacteria growing in the minimal medium supplemented with the required metabolites. In the plate count experiments for viable count, the innocula were taken from cultures in the exponential phase of growth. After suitable dilution, 0.1ml aliquots were plated on the solid minimal medium supplemented with glucose, L-methionine and eventually triornithine (see results) at the above-mentioned concentration. Colonies were scored after 24 hours incubation at 37°C.

Phage transduction. Lysates of 363 were prepared according to the method of Glansdorff (16). Transduction experiments were carried out by infecting 1.5ml of exponentially growing cells (4 to 5.10^8/ml) in nutrient broth containing $CaCl_2$, 2mM, with an equal number of phage 363 particles. Adsorption of phages on the recipient strain 58-161 was performed by incubation at 37°C for 20 minutes.

Enzyme activity determination. Initial velocity determination of the forward L-ornithine carbamoyltransferase-catalysed reaction were made at pH 8.0 in 150mM Tris.HCl buffer at 37°C as described (10).
The citrulline produced was measured according to the colorimetric procedure of Archibald (17). The enzyme specific activity was determined in the presence of 10mM L-ornithine and 1.5mM carbamoylphosphate.
Protein assays were performed by the Lowry method (18) with bovine serum albumin as standard. Ornithine carbamoyltransferase specific activity is expressed as μmol citrulline produced x mg $protein^{-1}$ x h^{-1}.

Thin layer silica gel chromatography. For the analytical purposes, about 50μg of the products to be tested were spotted on the plates (Merck Kieselgel 60-5721). Two elution systems, ethanol-aqueous ammonia (25%) (77:23) (system A) and chloroform-methanol (2:1) (system B) were used throughout this work.

High voltage paper electrophoresis of the free peptide. The procedure was carried out on Whatman n°3 MM paper. The electrophoresis was run at 400 Volts (30 Volts/cm) and pH 3.55 in pyridine-acetic acid-water (1:10:289) in a Camag 67701 apparatus. Phosphorus-containing material was detected by the Bandurski and Axelrod method (19).

Amino group analysis by the dansylation procedure. The general method of Gray (20) for the labeling of peptides was followed. However, the final tripeptide in our synthesis was obtained as an ammonium salt. Therefore, a supplementary drying step in the presence of sodium bicarbonate was necessary to ensure the removal of the last traces of ammonia before labeling with dansyl chloride. Samples of dns glycine and of N-δ-dns ornithine were obtained by the respective labelling of glycine and of N-α-C-L-ornithine with dansyl chloride (21) followed by acid hydrolysis (12 hours, 6N HCl at 105°C) for the ornithine derivative. Resolution of the dansylation products was performed by two dimensional thin layer chromatography on polyamide sheets (Cheng-Chin Trading Co. Ltd.).
The triple development procedure was used for the resolution of dns glycine (22). A fourth solvent (0.07M Na_2PO_4 in 25% aqueous ethanol) was used for the resolution of δ-dns ornithine. Under these conditions, the ornithine derivative was found to run at approximatively the same position as ε-dns lysine.

Synthesis of glyglyPALO. The condensation of an activated ester derivative of Z-glycylglycine with N-δ-(phosphonoacetyl)-L-ornithine could have been a direct way for the synthesis of the desired peptide. However, in our hands, this method failed to give appreciable yields. Therefore we chose the alternative way depicted in Fig.2. The general recommandations of Anderson et al. (23) for the N-hydroxysuccinimide active ester procedure were followed.

1. N-hydroxysuccinimidyl ester of Z-glycylglycine.
N-N dicyclohexylcarbodiimide (1.03g, 5mmol) was added to a solution of Z-glycylglycine (1.33g, 5mmol) and 0.57g (5mmol) of N-hydroxysuccinimide in 10ml of N-N dimethylformamide with cooling. The reaction mixture was allowed to stand at 4°C overnight. The formed dicyclohexylurea was filtered and washed with N,N-dimethylformamide. The filtrate was concentrated in vacuo to yield a pale yellow oil which soon crystallized after trituration with methylene chloride-petroleum ether (1:1). Recrystallization from the same solvent system yielded 1.67g (92%), mp 143-144°C (uncorrected); thin layer chromatography Rf=0.96 (system B).

2. Z-glycylglycyl-(N-δ-tBoc)-L-ornithine.
N-δ-tBoc-L-ornithine (1.16g, 5mmol), 0.84g (10mmol) of sodium bicarbonate and 10ml of water were mixed. Then, a solution of 1.81g (5mmol) of the N-hydroxysuccinimidyl ester of Z-glycylglycine in 15ml N,N-dimethylformamide was added at room temperature. After 12 hours, the reaction mixture was filtered and acidified to pH 2.7 at 4°C with diluted hydrochloric acid. The precipitated gum was extracted three times with 25ml of cooled (4°C) ethyl acetate. The resulting organic phase was washed twice with 50ml of NaCl-saturated water and dried briefly over sodium sulfate.
A further concetration in vacuo gave an oil which was triturated with ether. Recrystallization from ether-petroleum ether (1:1) yielded 1.73g (72%) mp 123-124°C (uncorrected) α_D^{25} -3,1° ± 0.8 (c=3.0, dioxane).

Anal: Calcd. for $C_{22}H_{32}N_4O_8$: C, 55; H, 6.66; N, 11,66; O, 26.66

Found: C, 54.85; H, 6.70; N, 11.62

Thin layer chromatography: Rf=0.90 (system A); Rf=0.22 (system B).
The single spots were ninhydrin negative and revealed by the sulfochromic spray for urethane N-protected compounds (24).
Quantitative amino acid analysis of the hydrolyzed peptide (24 hours in 6N HCl at 105°C) gave a gly/orn ratio of 1.97.

3. Z-glycylglycyl-L-ornithine.
Z-glycylglycyl-(N-δ-tBoc)-L-ornithine (2.40g, 5mmol) was mixed with 15ml anhydrous trifluoroacetic acid (25) in a round-bottom flask fitted with a calcium chloride drying tube. After standing for 60 minutes at room temperature, the solution was poured into cold anhydrous ether (250ml) and the mixture was stored in the cold for 1 hour. The precipitated trifluoroacetic acid salt of the tripeptide with the tertbutyloxycarbonyl (tBoc) group removed was collected by filtration, washed with ether and dried under reduced pressure over sodium hydroxide; yield 2.21g (92%) α_D^{25} -6,9° ± 1,7° (c=1.0 H_2O). Thin layer chromatography: Rf=0.40 (system A) Rf=0.08 (system B). The single spot was ninhydrin and Z positive. Amino-acid analysis gave a gly/orn ratio of 2.17. A satisfactory elementary analysis was carried out using the free base of the peptide which was obtained by dipolar precipitation (26).

Anal: Calcd. for $C_{17}H_{24}N_4O_6$: C, 53.68; H, 6.31; N, 14.74; O, 25.26

Found: C, 53.48; H, 6.18; N, 14.68

Amino group analysis by dansylation revealed a fluorescent spot migrating to the same position as a sample of N-δ-dns ornithine.
No material was observed at the place of dns glycine.

4. Glycylglycyl-N-δ-(phosphonoacetyl)-L-ornithine.
Triethylamine (1.22g, 12mmol) was added to a solution of the trifluoroacetate of Z-glycylglycyl-L-ornithine (2.20g, 4mmol) in 7ml of cooled (4°C) dioxane-N,N-dimethylformamide (4:1). Phosphonoacetyl chloride (0.63g, 4mmol) in 5ml dioxane was prepared by the method of Balsiger et al. (9).
The acyl chloride was then added under stirring to the above-mentioned solution with the aid of a dropping funnel. After completion of the reaction, the medium was filtered. The precipitate on the filter was washed with 5ml of dioxane-N,N-dimethylformamide (4:1). The filtrate was made alcaline with ammonia (2.5% final) and extracted three times with 20ml portions of ether to remove the solvents. The aqueous layer was lyophilized and the residue was decarbobenzoxylated in a stoppered flask by the addition of 10ml of 35% HBr in glacial acetic acid. The mixture was gently swirled for two hours at room temperature, a procedure that effected complete dissolution. The crude peptide hydrobromide was precipitated by the addition of 100ml cooled anhydrous ether. The precipitate was decanted, trasferred to a centrifuge tube and repeatedly washed with ether, centrifuging each time. The obtained product was impure as judged by thin layer chromatography and high voltage electrophoresis. A ten milligram scale preparative fractionation of the material was performed by silica gel plate chromatography. The crude product contained in 100μl of water was applied in a horizontal band near the base of the plate. Elution was performed using the solvent system A. Under those conditions, the phosphonoacetylated tripeptide migrated slowly. The material was recovered by extraction of the desired gel portion with a solution of 5% ammonia.
The aqueous phase was further lyophilized and the final product was obtained as a white hygroscopic powder: yield 37%.
Thin layer chromatography: Rf=0.06 (system A). Single ninhydrin and phosphorus positive spot. The material migrated also as a single ninhydrin and phosphorus positive spot towards the anode when subjected to paper electrophoresis (0.4cm for 2 hours). Amino-acid analysis indicated a gly/orn ratio of 1.96. Nitrogen and phosphorus elementary analysis were in accordance with a triammonic salt of the peptide.

Anal: Calcd. for $C_{11}H_{30}N_5O_8P$: N, 23.39; P, 7.40

Found: N, 23.07; P, 7.47

Amino end group analysis of the peptide by the dansylation method revealed the presence of glycine only. The extent of racemization of the final product was estimated by the enzymatic quantitative determination of L-ornithine liberated by acid hydrolysis of the peptide.
22.40μmol of the product gave 21.96μmol L-ornithine, as determined by conversion to L-citrulline with L-ornithine carbamoyltransferase.
At least 98% of the ornithine residue thus appeared to be the L form (27). To have obtained such a large proportion of L-ornithine by this method eliminates doubts which might have arisen as to whether racemization occurs during peptide hydrolysis (28).

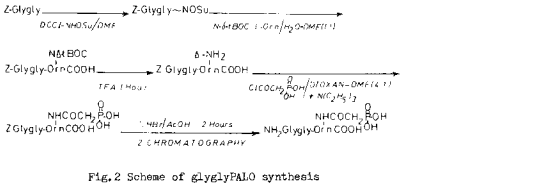

Fig.2 Scheme of glyglyPALO synthesis

Synthesis of a peptide form of N-delta-(phosphonoacetyl)-L-ornithine. Its antibacterial effect through the specific inhibition of Escherichia coli L-ornithine carbamoyltransferase.

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