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On the Transport of Tripeptide Antibiotics in Bacteria

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The two tripeptide antibiotics L-2-amino-4-methylphosphinobutyryl-alanyl-alanine (L-phosphinothricyl-alanyl-alanine) and L-(N^5-phosphono)methionine-*S*-sulfoximinyl-alanyl-alanine, both inhibitors of the glutamine synthetase, are transported into the cell of *Escherichia coli* K 12 *via* the oligopeptide transport system. The uptake by this system is proved first of all by cross-resistance with tri-L-ornithine using oligopeptide-transport-deficient mutants, and secondly by antagonism tests demonstrating competitive reversal of the action of the antibiotic by several peptides which have been shown to be transported *via* the oligopeptide transport system, *e.g.* tri-L-alanine, tetra-L-alanine, tri-L-lysine, tri-L-serine, tri-glycine, glycyl-glycyl-L-alanine and the synthetic tripeptide L-azaadenylaminohexanoyl-alanyl-alanine. On the other hand, there is no effect on the action of the antibiotic in antagonism tests with compounds which use different transport systems, such as L-alanyl-alanine, L-lysyl-lysine, glutathione and the synthetic amino acid azaadenylaminohexanoic acid, *i.e.* 2-amino-6-(7-amino-3*H*-*v*-triazolo-[4,5-*d*]-pyrimidin-3-yl)hexanoic acid.

Another inhibitor of the glutamine synthetase, L-methionine-*S*-dioxide (methioninesulfone) could be converted into a tripeptide form by linkage to L-alanyl-alanine analogously to the tripeptide antibiotics described above. Whereas the free L-methionine-*S*-dioxide seems to be transported *via* the methionine transport system, the tripeptide form is transported *via* the oligopeptide transport system. Thus, this glutamine synthetase inhibitor can be taken up by the cell *via* two different transport mechanisms. Our results indicate that this could provide a synergistic effect.

The syntheses of the new tripeptides L-azaadenylaminohexanoyl-alanyl-alanine and L-methionine-*S*-dioxidyl-alanyl-alanine were performed by dicyclohexylcarbodiimide couplings of the unusual *N* -protected L-α-amino acids azaadenylaminohexanoic acid and L-methionine-*S*-dioxide to L-alanyl-alanine-*tert*-butyl ester followed by common deprotection steps. Tri-L-ornithine was synthesized without carboxyl protection *via* two successive couplings of hydroxybenzotriazol esters of N^{α}-butoxycarbonyl-N^{δ}-benzyloxycarbonyl-L-ornithine to N^{δ}-benzyloxycarbonyl-L-ornithine.

Due to special transport systems bacteria are capable of taking up nutrients even from very dilute solutions, resulting in an accumulation of solutes inside the cell. Likewise, the bactericidal effect of various antibiotics can be obtained at extremely low concentrations of the drug. In some cases the minimal inhibitory concentration is found to be lower *in vivo* than *in vitro* [2]. Therefore, it seems reasonable to investigate the possibility of an active accumulation of antibiotics. One cannot expect that bacteria maintain transport systems for antibiotics; however, it is quite possible that certain antibiotics are able to 'misuse' existing transport systems the bacterium needs to take up various nutrients. This raises the question of which transport system can be used by which antibiotic.

As yet only a few experimental results concerning the transport of antibiotics are available [2,3]. For

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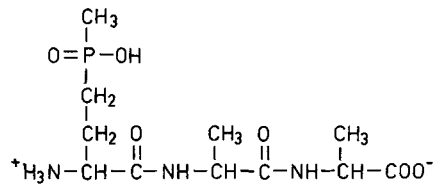
Abbreviations. NMR, nuclear magnetic resonance; Met(O), L-methionine-*S*-oxide (methioninesulfoxide); $Met(O_2)$, L-methionine-*S*-dioxide (methioninesulfone); azaadenylaminohexanoic acid or Aza, 2-amino-6-(7-amino-3 *H*-*v*-triazolo[4,5-*d*]-pyrimidin-3-yl)-hexanoic acid (this compound was called AZA in a previous communication [24]); Tü, strain collection, Institut für Mikrobiologie I, Tübingen. Abbreviations for amino acid derivatives and peptides follow the IUPAC-IUB Commission of Biochemical Nomenclature symbols, see *Eur. J. Biochem. 27*, 201–207 (1972).



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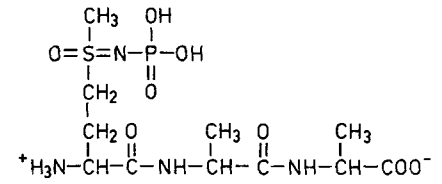

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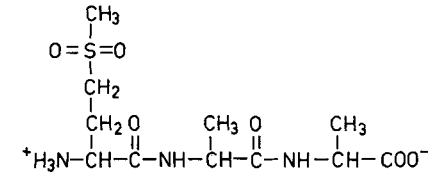

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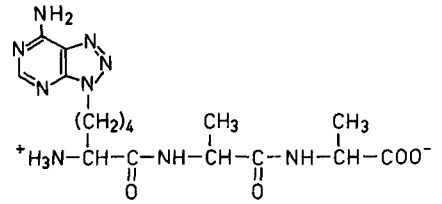

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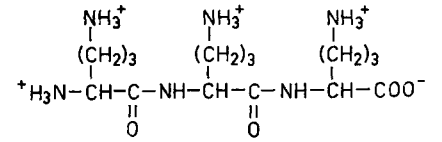

(V)

Fig. 1. *Structures of the tested tripeptides.* $^{+}H_2$-L-Phosphinothricyl-alanyl-alanine-O^{-} (I), $^{+}H_2$-L-(N^5-phosphono)-methionine-*S*-sulfoximinyl-alanyl-alanine-O^{-} (II), $^{-}H_2$-L-methionine-*S*-dioxidyl-alanyl-alanine-O^{-} (III), $^{+}H_2$-L-Aza-Ala-Ala-O^{-} (IV) and $^{+}H_2$-L-Orn-Orn-Orn-O^{-} (V)

some amino acid antimetabolites, the uptake *via* the transport systems of amino acids has been proved. Recent results by Hantke and Braun [4] show that the long-known antagonism between sideromycins and sideramines [5–7] can be explained by a competition for the same membrane binding site, which is part of a specific transport system. An energy dependence has been shown for the accumulation of tetracycline and chloramphenicol [2]. However, nothing is known about the transport systems which are involved in these cases.

Bayer *et al.* [8] have pointed out that the tripeptide antibiotic L-phosphinothricyl-alanyl-alanine [8–12] (I, Fig. 1) exhibits much greater antibacterial activity against intact cells than does the constituent amino acid, phosphinothricin. In contrast, in the cell-free system only the amino acid shows effective inhibition of the *Escherichia coli* glutamine synthetase. It has been suggested that the uptake of the antibiotic into the cell is strongly favored by the tripeptide form, which is then hydrolyzed to free phosphinothricin and alanine. This leads to the assumption that the tripeptide form, but not the free inhibitor, is capable of invading the cell *via* a transport system, most likely *via* the oligopeptide system [13, 21].

A similar transport effect could explain the greater antibacterial activity of the tripeptide antibiotic L-(N^5-phosphono)methionine-*S*-sulfoximinyl-alanyl-alanine (II, Fig. 1) [22], compared to the amino acid L-(N^5-phosphono)methionine-*S*-sulfoximine, which also inhibits the glutamine synthetase of *E. coli*. The structure of the tripeptide is closely related to L-phosphinothricyl-alanyl-alanine.

The possibility that this transport system could be used to bring normally impermeant substances into the bacterial cells after linkage of the compound to a peptide has been demonstrated already. Fickel and Gilvarg [17] investigated the uptake of the impermeant threonine precursor, homoserine phosphate, into *E. coli* W TL 3 in a peptide form as lysyl-lysyl-homoserine phosphate. Ames *et al.* [20] explored the transport of histidinol phosphate ester, a histidine biosynthetic intermediate, into *Salmonella typhimurium* cells as glycyl-glycyl-histidinol phosphate. These results on the transport of cell metabolites could be of great importance for the problem of the uptake of antibiotics. The attachment of inhibitors to a carrier may be very useful for several reasons.

a) Drugs showing a very poor uptake in the free form could be actively transported in the carrier-linked form.

b) Intrinsic resistance due to permeability changes as a result of plasmid-mediated drug resistance could be overcome.

c) The action spectrum of an inhibitor possessing initially only a small potential could be broadened.

d) The simultaneous application of an inhibitor and its derivative entering the cell on different pathways but affecting the same target could provide synergistic effects.

In this communication we demonstrate that the antibiotics L-phosphinothricyl-alanyl-alanine and L-(N^5-phosphono)methionine-*S*-sulfoximinyl-alanyl-alanine can enter the bacterial cell *via* the transport system for oligopeptide nutrients. Furthermore we describe experiments aiming at the idea of enabling antibiotics to invade the bacterium *via* more than one transport system. As a first model we investigated the inhibitor of glutamine synthetase, L-methionine-*S*-dioxide and the synthetic tripeptide L-methionine-*S*-dioxidyl-alanyl-alanine (III, Fig. 1).

MATERIALS AND METHODS

Strain and Tests

E. coli K 12 served as the test organism. Agar plate diffusion tests and antagonism tests were performed on minimal medium according to Davis and Mingioli [23] but with the addition of 15 g/l agar. The plates were seeded with 1.3×10^7 cells/ml. The evaluation of the agar plate diffusion tests and antagonism tests followed the method of Zähner *et al.* [5]. The L-phosphinothricyl-alanyl-alanine-resistant mutants were isolated from agar plates containing about 0.2 µg L-phosphinothricyl-alanyl-alanine/ml agar. They were characterized according to their sensitivity against L-phosphinothricyl-alanyl-alanine and tri-L-ornithine.

Substances

L-(N^5-Phosphono)methionine-*S*-sulfoximinyl-alanyl-alanine (II, Fig. 1) was a gift of Dr A. Stempel (Hoffmann-La Roche Inc., Nutley, U.S.A.). L-Phosphinothricyl-alanyl-alanine (I, Fig. 1) was tested in a solution containing about 1 mg/ml tripeptide originating from a fermentation with *Streptomyces viridochromogenes* Tü 494. The chemical synthesis of the new tripeptides Aza-Ala-Ala (IV, Fig. 1) and L-methionine-*S*-dioxidyl-alanyl-alanine (III, Fig. 1) and a new synthesis of tri-L-ornithine (V, Fig. 1) are described below in a separate section. Glutathione was purchased from Boehringer (Mannheim). Glycine, L-alanine, and L-methionine were products of Merck (Darmstadt) and L-lysyl-lysine and tri-L-lysine were from Miles (Kankakee, U.S.A.). Tri-L-alanine, tetra-L-alanine, tri-L-serine, triglycine, glycyl-glycyl-L-alanine and glycyl-L-phenylalanyl-phenylalanine were purchased from Sigma (St Louis, U.S.A.). L-Alanyl-alanine was obtained from Schuchard (München). The peptides have been checked by thin-layer chromatography on silica gel plates developed in the solvent system 1-butanol/acetic acid/water (3:1:1, v/v/v). All peptides revealed single ninhydrin-positive spots. Bacto-Agar was from Difco Lab. (Detroit, U.S.A.) and D-glucose from Deutsche Maizena Werke (Hamburg).

All solvents used for peptide synthesis and chromatography were dried, fractionated, and stored over molecular sieves of 30 and 40 nm (Merck, Darmstadt). For recording spectra 'Uvasol' solvents were used. Catalytic hydrogenations were performed on Pd catalysts (10% Pd on charcoal) at 760 Torr (101 kPa) H_2 pressure and 25 °C. All reagents used are puriss. grade. L-Methionine-*S*-dioxide puriss. was purchased from Fluka (Buchs, Schweiz) and contained as an impurity methionine-*S*-oxide. L-Ornithine was a product of Merck (Darmstadt). Anion-exchanger Dowex 1×2 (Nr. 41011) was from Serva (Heidelberg).

Chromatography and Analyses

All substances and intermediates were tested for purity by thin-layer chromatography using plates Kieselgel 60 F_{254} no. 5714 of Merck (Darmstadt). The following solvent systems were used: system A = 1-butanol/acetic acid/water (3:1:1, v/v/v); B = 1-butanol/acetic acid/water (4:1:1, v/v/v); C = chloroform/methanol/acetic acid/water (60:45:6:4, v/v/v). Amino acid analyses were made from samples hydrolyzed at 110 °C for 22 h in 6 N HCl in sealed evacuated ampoules. The elution times of the unusual amino acids were determined by a Beckman-Multichrom automatic amino acid analyzer using standard conditions and refering to elution diagrams of standard mixtures. Elemental analyses were made in the microanalytical laboratory of the Chemisches Institut. Melting points were determined in capillaries according to Tottoli and are not corrected.

Spectra

Pulse Fourier transform nuclear magnetic resonance spectra with proton decoupling were recorded on a Bruker HFX-90 multinuclear NMR spectrometer (22.624 MHz for ^{13}C) by accumulation (Fabritek 1074 computer) of ^{13}C pulse interferograms (pulse width 5 µs; 0.4 s/scan) followed by Fourier transformation of the accumulated interferograms (Digital PDP-8-I calculator). The chemical shift values, δ (ppm), refer to tetramethylsilane with $\delta = 0$ ppm and have an error of $\pm$ 0.1 ppm. Some ^{13}C NMR spectra were taken also on a WP-60 NMR spectrometer of Bruker-Physik (Karlsruhe). Because of the low solubility of some intermediates the accumulation times for 'off-resonance' spectra were > 12 h.

Ultraviolet spectra were recorded from 1 mM solutions at room temperature on a Cary 15 (Varian). The optical rotation was measured on a Zeiss OLD-5 polarimeter at 365, 405, 436, 546 and 578 nm and $[\alpha]_D$ was determined by extrapolation. Infrared spectra in KBr (concentration 1–2 mg/200 mg KBr) were recorded on Perkin/Elmer 021 and 221 spectrometers.

SYNTHESES OF TRIPEPTIDES

Synthesis of L-Aza-Ala-Ala

The azapurinyl-substituted L-α-amino acid azaadenylaminohexanoic acid (Aza) [24,25] has been synthesized in a series of pyrimidinyl and purinyl-substituted amino acids [26,27]. Amino acids with pyrimidine and purine ring systems in the side chain are of some interest because of their potential antibacterial and cytostatic activity. The lability of the heterocycle in strongly acidic media had to be taken

into consideration for the synthesis of peptides containing the aza-purinyl-substituted amino acid.

Secondly the N^{α}-benzyloxycarbonyl derivatives from the multi-step synthesis of the α-amino acid azaadenylaminohexanoic acid as coupling component were directly available [26,27]. The removal of the benzyloxycarbonyl group with HBr in acetic acid results in destruction of the heterocycle; therefore the deprotection by catalytic hydrogenation was chosen. Fortunately, the 8-azaadenine system of azaadenylaminohexanoic was found to be stable towards the acidolytic reagents trifluoroacetic acid or trifluoroacetic acid/dichloromethane (1 : 1, v/v) used for the removal of the *tert*-butyl and butyloxycarbonyl groups. Furthermore it was found that the amino function of the heterocycle gives no side reactions during peptide coupling due to its low reactivity; therefore this group needs no particular protection. The reaction scheme shown in Fig. 2 summarizes the synthesis of the tripeptide L-Aza-Ala-Ala.

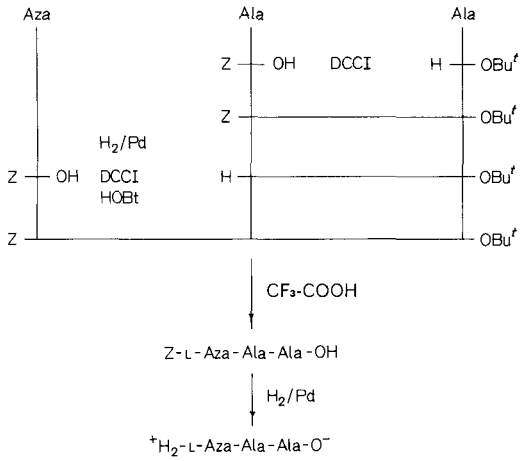

Fig. 2. *Reaction scheme of the synthesis of the tripeptide* L-*Aza-Ala-Ala.* DCCI = dicyclohexylcarbodiimide, HOBt = *N*-hydroxybenzotriazol

The coupling of N^{α}-benzyloxycarbonyl-L-alanine [28] with L-alanine *tert*-butyl ester [29] followed by hydrogenation of the resulting protected dipeptide Z-L-Ala-Ala-OBu^t [30] yielded L-Ala-Ala-OBu^t. Azaadenylaminohexanoic acid [24,25] coupled to the dipeptide ester using dicyclohexylcarbodiimide gave the protected tripeptide Z-L-Aza-Ala-Ala-OBu^t in good yield. The coupling reactions had to be performed in dimethylformamide because of the low solubility of Z-L-Aza-OH in less polar solvents. Using dicyclohexylcarbodiimide as coupling agent in very polar solvents *O*-acyl shifts and racemization are often observed; however, these side reactions can be totally suppressed by addition of *N*-hydroxybenzotriazol according to König and Geiger [31]. The removal of the *tert*-butyl protecting group by trifluoroacetic acid followed by catalytic hydrogenation yielded the free tripeptide L-Aza-Ala-Ala (IV, Fig. 1), which was purified by gel chromatography on Sephadex G-25. All peptide derivatives have been fully characterized by several independent chromatographic and spectroscopic methods. Fig. 3 shows the assignement of the ^{13}C NMR resonances of the final product L-Aza-Ala-Ala.

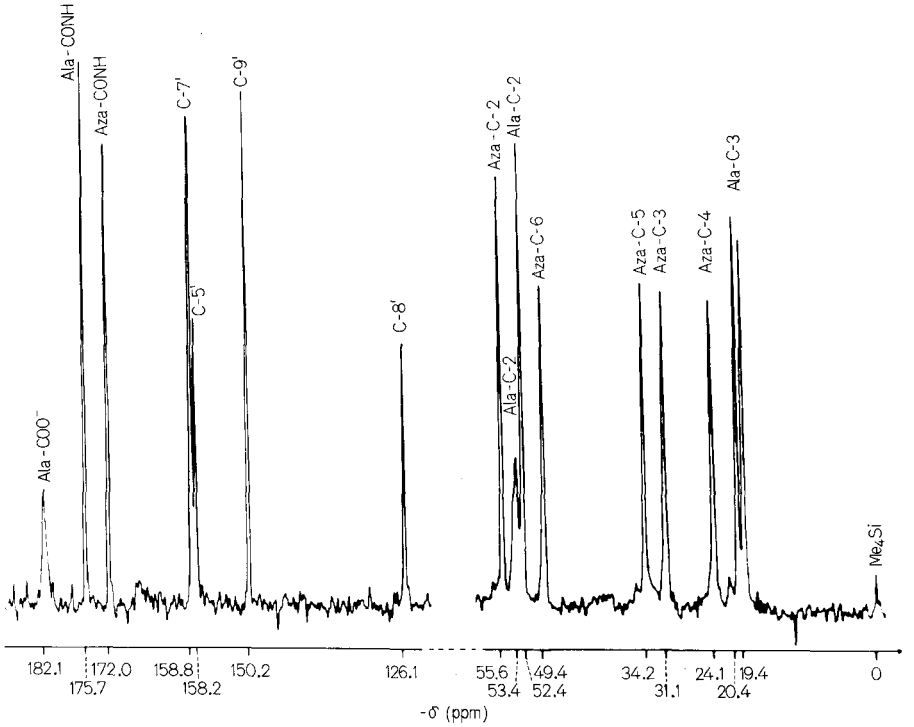

Fig. 3. *Assignment of the 90-MHz pulse Fourier transform nuclear magnetic resonance spectra with proton decoupling of the tripeptide* L-*Aza-Ala-Ala in* ^2H_2O

Synthesis of L-Methionine-S-dioxidyl-alanyl-alanine

The tripeptide L-Met(O_2)-Ala-Ala (III, Fig. 1) with the N-terminal amino acid L-methionine-*S*-dioxide [Met(O_2) or L-methionine sulfone] was synthesized according to the reactions summarized in Fig. 4. The synthesis of the *N-tert*-butyloxycarbonyl derivative

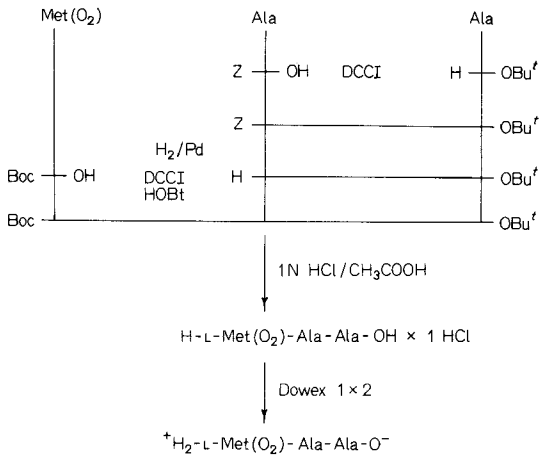

Fig. 4. *Reaction scheme of the synthesis of the tripeptide* L-*methionine-S-dioxidyl-alanyl-alanine.* DCCI = dicyclohexylcarbodiimide, HOBt = *N*-hydroxybenzotriazol

of L-methionine-*S*-dioxide required the relatively long reaction time of 30 h at pH 10 according to the pH-stat method of Schnabel [32]. The polar reaction product could not be dissolved in common solvents like ethyl acetate or dichloromethane but was successfully worked up in a mixture of 1-butanol/ethyl acetate (1:1, v/v).

The coupling of *N-tert*-butyloxycarbonyl-L-methionine-*S*-dioxide with L-alanyl-alanine *tert*-butyl ester yielded the protected tripeptide N^{α}-*tert*-butyloxycarbonyl-L-methionyl(*S*-dioxide)-alanyl-alanine *tert*-butyl ester. The N and C-terminal protecting groups were removed simultaneously by treatment with 1.2 N hydrogen chloride in acetic acid for 2 h. The free tripeptide $^{+}H_2$-L-Met(O_2)-Ala-Ala-O^{-} is very soluble in water and was obtained by neutralisation of its hydrochloride using an anion-exchange resin and characterized by its ^{13}C NMR spectrum (Fig. 5).

Synthesis of Tri-L-ornithine

A new synthesis of the antibiotically active tripeptide L-Orn-Orn-Orn was performed without any carboxyl protection. As shown in the reaction scheme (Fig. 6) this approach requires only seven reaction steps starting with ornithine.

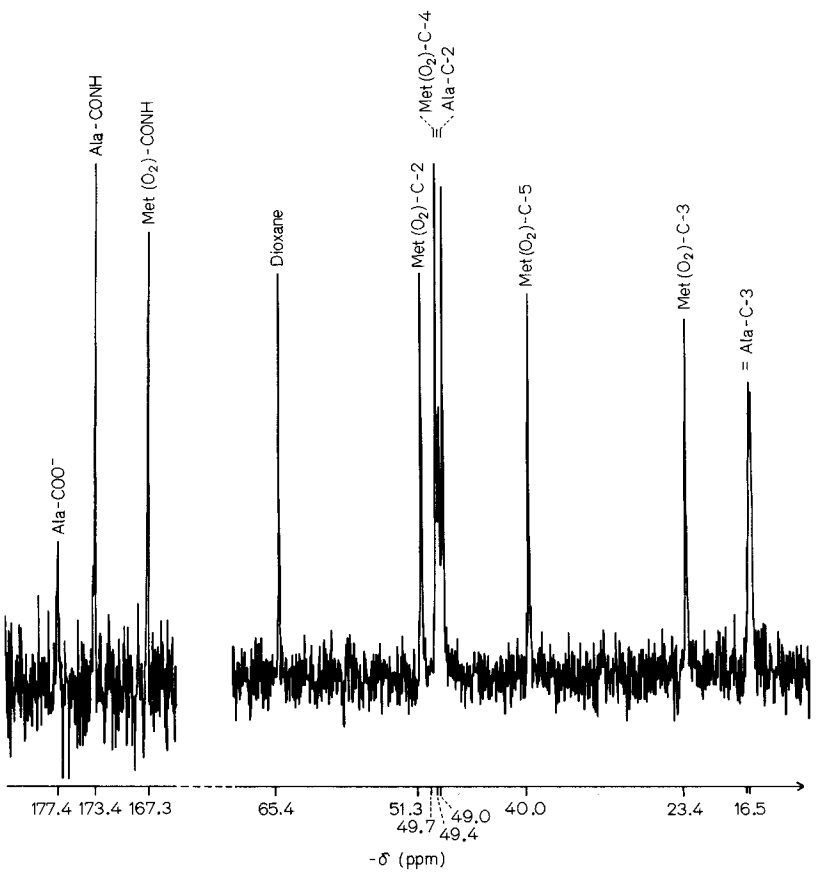

Fig. 5. ^{13}C *NMR spectrum of* L-*methionine-S-dioxidyl-alanyl-alanine in* $^{2}H_2O/^{2}HCl$ *at* $p^{2}H$ *1.0 (internal standard dioxane)*

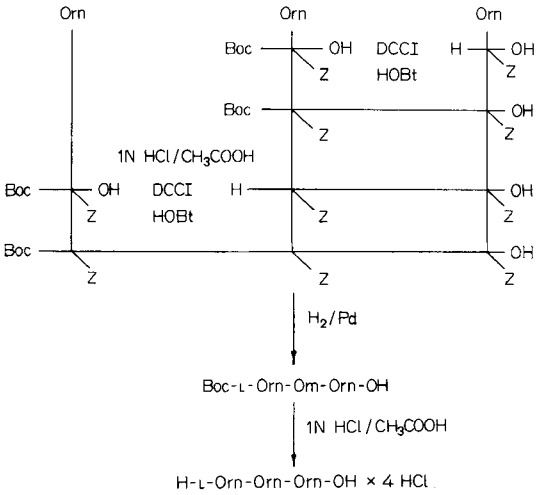

Fig. 6. *Reaction scheme of the synthesis of tri-L-ornithine.* DCCI = dicyclohexylcarbodiimide, HOBt = *N*-hydroxybenzotriazol

By preactivation [33] the *N*-hydroxybenzotriazol ester of N^{α}-*tert*-butyloxycarbonyl-N^{δ}-benzyloxycarbonyl-L-ornithine [34, 35] was obtained and coupled in heterogeneous reaction to N^{δ}-benzyloxycarbonyl-L-ornithine [34] yielding the N-protected dipeptide. The selective removal of the N^{α}-*tert*-butyloxycarbonyl groups followed by an analogous coupling reaction yielded N^{α},N^{δ}-protected tri-ornithine from the N^{δ}-diprotected di-ornithine. Deprotection by hydrogenation and acid hydrolysis yielded the hygroscopic hydrochloride of tri-L-ornithine, which was characterized by its ^{13}C NMR spectrum (Fig. 7). Tri-L-ornithine and various other ornithine-containing tripeptides have also been synthesized using a recently developed repetitive technique which combines the use of soluble supports and solid polymer reagents for peptide synthesis [36].

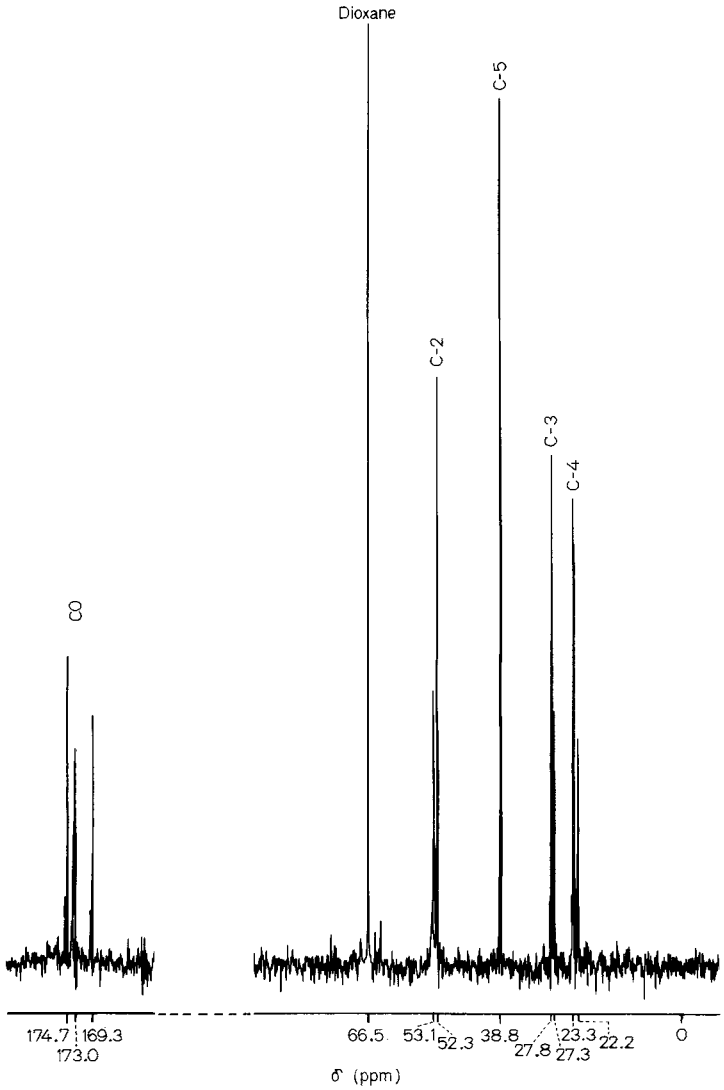

Fig. 7. ^{13}C *NMR spectrum of tri-L-ornithine in* $^{2}H_2O/^{2}HCl$ *at* $p^{2}H$ *0.5 (internal standard dioxane)*

EXPERIMENTAL PROCEDURES

N^{α}-*Benzyloxycarbonyl-L-alanyl-alanine* tert-*butyl Ester*

0.036 mol (8.05 g) N^{α}-Z-L-Ala-OH [28] and 0.036 mol (6.55 g) L-Ala-OBu^{t}HCl [29] were dissolved in 90 ml absolute dichloromethane. After cooling to −5 °C, 0.036 mol (4.17 g) *N*-ethylmorpholine and 0.04 mol (8.2 g) *N,N'*-dicyclohexylcarbodiimide were added and the mixture stirred for 2 h at −5 °C and 12 h at 25 °C. Four drops of acetic acid were added and the reaction mixture filtered after 30 min. The solution was extracted with 4 × 30 ml of 10% aqueous solution of citric acid, of 1 M $NaHCO_3$ and of water. The organic layer was dried over Na_2SO_4 and after removal of dichloromethane the colourless oily residue was dissolved in dry ethyl ether. After 24 h in the cold small amounts of additionally precipitated dicyclohexylurea were filtered off and hexane added to the filtrate. After standing at −30 °C for several days the crystalline product was filtered off and washed with hexane. The crystals of the dipeptide become oily in the air; however, *in vacuo* over P_2O_5 they solidified again. Yield 12 g (95%); m.p. 69–71 °C (lit. 70–71 °C [30]); R_F (A) 0.82; $[\alpha]_{578}^{25} = -47.0$ ($c = 1.275$, ethanol). Calcd for $C_{18}H_{26}N_2O_5$ (350.42): C, 61.70; H, 7.48; N, 7.99%. Found: C, 61.48; H, 7.45; N, 7.72%.

L-*Alanyl-alanine* tert-*butyl Ester*

0.017 mol (6 g) N^{α}-Z-L-Ala-Ala-OBu^t dissolved in 100 ml ethanol were hydrogenated after addition of 200 mg Pd catalyst and two drops of acetic acid. A stream of hydrogen was introduced for 12 h at 760 Torr (101 kPa) and the ethanol evaporated after filtering off the catalyst. The etheral solution of the residual colourless oil was cooled to 0 °C and a dry etheral solution of HCl carefully added until no precipitation occurred after further addition of HCl and a slight acidic reaction (pH 2–3) was indicated on pH paper. After 12 h at −30 °C the colourless crystals of the dipeptide ester hydrochloride were filtered off and stored *in vacuo* over P_2O_5. Yield 3.8 g (87.4%), m.p. 195 °C (dec.); R_F (A) 0.40.

L-*2-Benzyloxycarbonyl-azaadenylaminohexanoyl-L-alanyl-L-alanine* tert-*butyl Ester*

A solution of 0.01 mol (2.6 g) L-Ala-Ala-OBu^t · HCl and 0.0103 mol (4.1 g) Z-L-Aza-OH [24,25] in 40 ml dry dimethylformamide was cooled to −10 °C. After addition of 0.01 mol (1.19 g) *N*-ethylmorpholine, 0.015 mol (2.03 g) *N*-hydroxybenzotriazole [31] and 0.011 mol (2.35 g) *N,N'*-dicyclohexylcarbodiimide the reaction mixture was stirred for 2 h at −5 °C and 12 h at 25 °C.

Acetic acid (1 ml) was added, the solution filtered after 30 min and the filtrate evaporated to dryness. The oily residue was dissolved in 300 ml ethyl acetate/1-butanol (1:1, v/v) and the organic layer washed with the following NaCl-saturated solutions: 10% citric acid 4 × 25 ml, 2 × 25 ml water, 5 × 25 ml 1 M $NaHCO_3$, 3 × 25 ml 1 M Na_2CO_3 and 4 × 25 ml water. The organic layer was dried over Na_2SO_4 and concentrated by evaporation *in vacuo*. Upon addition of ether Z-L-Aza-Ala-Ala-OBu^t precipitated after 24 h at −30 °C and was recrystallized from hot ethyl acetate/ethanol. Yield 4.45 g (72%); m.p. 167 °C; R_F (A) 0.68; $[\alpha]_{578}^{25} = -24.95$ ($c = 0.465$, ethanol/$CHCl_3$ 1:3, v/v); ultraviolet absorbance (in methanol) $\lambda_{max} = 278$ nm ($\varepsilon = 12100\ M^{-1}\ cm^{-1}$); infrared spectrum (KBr): 3320 (NH); 3070; 2950; 1725 (ester-CO); 1700 (urethane-CO); 1660–1640 (amide-I); 1605 (azapurine and phenyl); 1585; 1575–1510 (amide-II); 1455; 1395 and 1370 (*tert*-butyl); 1325; 1260; 1155; 1055; 955; 875; 850 and 700 cm^{-1}.

Amino acid analysis yielded five ninhydrin-positive peaks after hydrolysis in 6 N HCl (110 °C, 22 h): alanine (80.9 min; found 2.03 mol; theoretical 2.00 mol); lysine (157.1 min; found 0.158 mol; theoretical 0 mol), two unknown peaks at 163.6 and 190.4 min and NH_3 at 167.5 min; at the elution time of L-azaadenylaminohexanoic acid (≈ 135 min) no peak.

Calcd for $C_{28}H_{39}N_9O_6$ (597.66): C, 56.27; H, 6.58; N, 21.09%. Found: C, 56.28; H, 6.50; N, 20.89%.

L-*2-Benzyloxycarbonyl-azaadenylaminohexanoyl-L-alanyl-L-alanine*

3.85 mmol (2.3 g) Z-L-Aza-Ala-Ala-OBu^t were dissolved in 10 ml dry trifluoroacetic acid and stirred in a dry atmosphere for 60 min at 25 °C.

After evaporation of trifluoroacetic acid the residue was repeatedly dissolved in acetone and evaporated. Upon addition of ethyl acetate the oily colourless residue crystallized spontaneously. After 12 h at 0 °C Z-L-Aza-Ala-Ala-OH was filtered off, washed with hexane and recrystallized from hot ethyl acetate/ethanol. Yield 1.9 g (91%); m.p. 243 °C; R_F (A) 0.54; infrared (KBr): 3500–2500 (OH, NH_2H); 3280 (NH) 3030; 1705 (CO_2H); 1680 (urethane-CO); 1670–1640 (amide-I); 1580; 1560–1520 (amide-II); 1455; 1400; 1330; 1235; 1055 and 800 cm^{-1}. Calcd for $C_{24}H_{31}N_9O_6$ (541.56): C, 53.22; H, 5.77; N, 23.28%. Found: C, 53.48; H, 5.78; N, 23.51%.

L-*Azaadenylaminohexanoyl-L-alanyl-L-alanine*

1.85 mmol (1.0 g) Z-L-Aza-Ala-Ala-OH were dissolved in 60 ml dimethylformamide and 15 ml water. Upon addition of 200 mg Pd catalyst (10% Pd on charcoal) and 1 ml acetic acid the compound was hydrogenated at 25 °C and 760 Torr (101 kPa) for

12 h. The mixture was filtered and the solvent removed *in vacuo*. The colourless glass-like residue was dissolved in a small amount of water and chromatographed on a Sephadex G-25 column (62 × 2.7 cm). The free tripeptide $^{+}H_2$-L-Aza-Ala-Ala-O^{-} was eluted as a symmetrically shaped peak and lyophilized. Yield 0.62 g (82%); m.p. 216 °C; R_F (C) 0.64.

On amino acid analysis; the unhydrolysed peptide showed a single symmetrical and ninhydrin-positive peak in the amino acid analyzer. Under standard conditions this peak was eluted at the elution time of phenylalanine (142 min). After total hydrolysis under the usual conditions five ninhydrin-positive peaks were found in the elution diagram: alanine (82.5 min; calcd 2.0 mol, found 2.1 mol), lysine (158.9 min; calcd 0 mol, found 0.09 mol; two unknown substances at 166.7 and 193.4 min and NH_3 at 170.3 min, at the elution time of azaadenylaminohexanoic acid (135 min) no peak was found. $[\alpha]_{578}^{25} = 8.7$ ($c = 0.3$, water); ultraviolet spectrum: (in 0.1 N HCl) $\lambda_{max} = 264$ nm ($\varepsilon = 12800\ M^{-1}\ cm^{-1}$) (phosphate buffer pH 12) $\lambda_{max} = 277$ nm ($\varepsilon = 12200\ M^{-1}\ cm^{-1}$); infrared spectrum (KBr): 3600–2700 (broad); 3350 (NH); 2920; 2100 (NH_3); 1680–1640 (amide-I region); 1600 (heteroaromate); 1580 (CO_2^-); 1455; 1400; 1325; 1270; 1160; 1040; 920; 855; 800; and 720 cm^{-1}.

^{13}C-NMR (in ^2H_2O): $-\delta =$ 182.1, 175.7, 172.0 (CO); 158.8 (C-7′); 158.2 (C-5′); 150.2 (C-9′); 126.1 (C-8′); 55.6 (Aza-C-2); 53.4 (Ala-C-2); 52.4 (Ala-C-2); 49.4 (Aza-C-6); 34.2 (Aza-C-5); 31.1 (Aza-C-3); 24.1 (Aza-C-4); 20.4, 19.4 ppm (Ala-C-3).

Calcd for $C_{16}H_{25}N_9O_4$ (407.43): C, 47.16; H, 6.19; N, 30.94%. Found: C, 47.46; H, 6.34; N, 30.89%. Prior to the elemental analysis the substance was dried for 12 h over P_2O_5 at 0.1 Torr (13 Pa) and 95 °C.

N$^{\alpha}$-tert-*Butyloxycarbonyl-L-methionine*-S-*dioxide*

0.036 mol (6.5 g) L-Met(O_2) were suspended in 20 ml water and 10 ml dioxane. After addition of 0.045 mol (5.8 g) *tert*-butyloxycarbonyl-azide the slowly reacting mixture was kept at pH 10.25 with a pH-stat titrator (4 N NaOH supply). After 30 h the solution was extracted with 3 × 50 ml ether and acidified with 2 N HCl to pH 2.2 after cooling to 0 °C. The aqueous solution was saturated with NaCl and extracted with 3 × 50 ml 1-butanol and 3 × 50 ml 1-butanol/ethylacetate (1:1, v/v).

The combined organic extracts were washed with 5 × 25 ml 2% acetic acid saturated with NaCl. The solution was dried over Na_2SO_4 and evaporated to dryness. The colourless residue was dissolved in hot ethylacetate with addition of 1-butanol. After cooling the addition of ether yielded a voluminous precipitate which was removed by filtration. In this precipitate some Met(O_2) and Boc-Met(O)-OH originating from an impurity in the commercial starting material were found in thin-layer chromatography besides the desired Boc-L-Met(O_2)-OH.

The filtrate was evaporated *in vacuo* and the residue crystallized spontaneously after dissolving in dichloromethane. Boc-L-Met(O_2)-OH was filtered off after 12 h at 0 °C and washed with small amounts of ether. Yield 5 g (57%); m.p. 113–114 °C; R_F (A) 0.47; $[\alpha]_{578}^{25} = +3.3$ ($c = 0.6$, ethanol); infrared spectrum (KBr): 3335 (NH); 3500–2800 (OH); 1740 (free CO_2H); 1690 (amide-I, urethane); 1530 (amide-II); 1328 and 1125 (SO_2); 1398 and 1370 (*tert*-butyl); 1060; 970; 890; 850; 805; 780 and 740 cm^{-1}; ^{13}C-NMR: (in $C_2^2H_6SO$ $-\delta =$ 173.2 (CO_2H); 155.5 (Boc-CO); 78.4 (Boc-C^t); 52.1 (C-2); 50.8 (C-4); 40.2 (C-5); 28.1 (Boc-CH_3); 24.0 ppm (C-3).

Calcd for $C_{10}H_{19}NO_6S$ (281.32): C, 42.69; H, 6.81; N, 4.98; S, 11.40%. Found: C, 42.48; H, 6.70; N, 5.14; S, 11.17%.

N$^{\alpha}$-tert-*Butyloxycarbonyl-L-methioninesulfonyl-L-alanyl-L-alanine* tert-*butyl Ester*

To a solution of 7.79 mmol (82.19 g) Boc-L-Met(O_2)-OH and 7.79 mmol (1.9 g) L-Ala-Ala-OBu^t · HCl in 20 ml dimethylformamide, 11.7 mmol (1.58 g) dry *N*-hydroxybenzotriazol, 7.79 mmol (0.89 g) *N*-ethylmorpholine and 8.56 mmol (1.92 g) *N*,*N*′-dicyclohexylcarbodiimide were added successively at −5 °C. The reaction mixture was stirred 3 h at −5 °C and 12 h at 25 °C. Acetic acid (1 ml) was added and the mixture filtered after 30 min. The slightly yellow filtrate was evaporated *in vacuo* and the oily residue was dissolved in 100 ml ethyl acetate and 25 ml 1-butanol. This solution was washed with the following NaCl-saturated solutions: 5 × 20 ml 10% citric acid, 1 × 20 ml water, 3 × 20 ml 1 M $NaHCO_3$, 2 × 20 ml 1 M Na_2CO_3, 3 × 20 ml 1 M $NaHCO_3$ and 3 × 20 ml water. The organic phase was dried over Na_2SO_4, filtered and evaporated *in vacuo*. The solid residue was dissolved in warm ethyl acetate. After 30 h at −30 °C the precipitated white crystals were filtered off. Additional Boc-L-Met(O_2)-Ala-Ala-OBu^t was obtained from the mother liquor by precipitation with hexane. Yield 3.1 g (83%); m.p. 161–162 °C; R_F (A) 0.67; Amino acid analysis: original Met(O_2) was used to determine the ninhydrin colour yield; a mixture of Met(O_2) with other amino acids showed the following elution times (min): aspartic acid (41.5); Met(O_2) (42.6); threonine (49.5); serine (51.6); methionine (122.4); NH_3 (171.1). Directly after this standard elution diagram the tripeptide hydrolysate was chromatographed and only the two expected peaks were obtained: Met(O_2) (42.8): found 0.98 mol; calcd 1.00 mol; alanine (82.4): found 2.07 mol; calcd 2.00 mol. $[\alpha]_{578}^{25} = -44.35$ ($c = 0.685$, ethanol); infrared spectrum (KBr): 3350 (NH); 2980; 2940; 1725 (ester-CO); 1695 (urethane-CO); 1670–1645 (amide-I re-

gion); 1550–1515 (amide-II region); 1455; 1395; 1370 (*tert*-butyl); 1305; 1125 (SO_2); 1250; 1160; 1050; 975; 875; 845; 755 cm^{-1}. ^{13}C-NMR (in C^2HCl_3, $-\delta$ = 172.1, 171.8, 170.9, (ester and peptide CO); 155.7 (urethane-CO); 82.0 (butyl-C^t); 80.4 (Boc-C^t); 52.6 [Met(O_2)-C-21]; 50.9 [Met(O_2)-C-4]; 49.2 (Ala-C-2); 48.8 (Ala-C-2); 40.7 [Met(O_2)-C-5]; 28.4 (*tert*-butyl-CH_3); 28.0 (Boc-CH_3); 25.0 [Met(O_2)-C-3]; 18.6 and 18.3 ppm (Ala-C-3).

Calcd for $C_{20}H_{37}N_3O_8S$ (479.59): C, 50.08; H, 7.78; N, 8.76; S, 6.69%. Found: C, 49.82; H, 7.78; N, 8.64; S, 6.71%.

L-Methionine-S-dioxidyl-L-alanyl-L-alanine

2.7 mmol (1.3 g) Boc-L-Met(O_2)-Ala-Ala-OBu^t were dissolved at 25 °C in 20 ml 1.2 N HCl in acetic acid. After 130 min the clear solution was evaporated with exclusion of moisture. The residue was taken up twice in acetone and evaporated again. The colourless hygroscopic tripeptide hydrochloride was transformed to the neutral free $^+H_2$-L-Met(O_2)-Ala-Ala-O^- with the aid of an anion-exchange resin (Dowex 1 × 2, OH^- form) in a batch procedure. Yield 0.75 g (86%); m.p. 203–205 °C (dec.); R_F (B) 0.53. Amino acid analysis: Met(O_2) found 1.04 mol, calcd 1.00 mol; alanine found 2.10 mol, calcd 2.00 mol. $[\alpha]_{578}^{25} = -14.05$ (c = 0.79, water); infrared spectrum (KBr): 3500–2700 (broad); 3350 (NH); 2950; 2550; 2020 (NH_3^+); 1640 (amide-I); 1595 (CO_2^-); 1510 (amide-II); 1450; 1410; 1355; 1330; 1135 (SO_2); 1290; 1055; 980; 955 and 860 cm^{-1}. ^{13}C-NMR (in $^2H_2O/^2HCl$, $-\delta$ = 177.4, 173.4, 167.3 (CO); 51.3 [Met(O_2)-C-2]; 49.7 [Met(O_2)-C-4]; 49.4 (Ala-C-2); 40.0 [Met(O_2)-C-5]; 23.4 [Met(O_2)-C-3]; 16.5 ppm (2 Ala-C-3).

Calcd for $C_{11}H_{21}N_3O_6S$ (323.37): C, 40.85; H, 6.55; N, 12.99; S, 9.92%. Found: C, 40.17; H, 6.27; N, 12.06; S, 9.97%.

Prior to the elemental analysis the substance was dried for 12 h *in vacuo* over P_2O_5 at 0.1 Torr (13 Pa) and 95 °C.

N$^\alpha$-tert-*Butyloxycarbonyl*-N$^\delta$-*benzyloxycarbonyl-di-L-ornithine*

18.8 mmol (6.87 g) crystalline Boc-Orn(Z)-OH [34, 35] were dissolved in 20 ml dichloromethane. At −20 °C 18.8 mmol (2.87 g) *N*-hydroxybenzotriazol in 40 ml tetrahydrofuran and 18.8 mol (3.71 g) *N,N'*-dicyclohexylcarbodiimide in 20 ml dichloromethane were added for preactivation [33]. The precipitated dicyclohexyl urea was filtered off after 20 min and the filtrate was added to a suspension of 18.8 mmol (5 g) $^+H_2$-Orn(Z)-O^- [34] in 60 ml dimethylformamide and stirred. The pH was kept at 7–8 for 4.5 h by addition of *N*-methylmorpholine. The unreacted Orn(Z) was filtered off and the solution acidified with diluted HCl to pH 2. The oily phase was separated and the aqueous phase extracted twice with dichloromethane. After drying over Na_2SO_4 the combined organic phases were evaporated to dryness. The residual oil was dissolved in ethyl acetate and the solution shaken with water; *N*-hydroxybenzotriazol precipitated and was filtered off. The organic phase was dried over Na_2SO_4 and evaporated. The N$^\alpha$-butyloxycarbonyl group was removed by treatment with 1.2 N HCl in acetic acid. After 25 min the acetic acid was evaporated *in vacuo* and the residue dissolved in water and extracted three times with ethyl acetate. Residual Orn(Z) could be collected from the aqueous solution after some hours. The ethyl acetate extract was dried over Na_2SO_4 and evaporated to yield $^+H_2$-L-Orn(Z)-Orn(Z)-O^- as a colourless oil, which showed a single spot on thin-layer chromatography. Yield 5.6 g (60%).

N$^\alpha$-tert-*Butyloxycarbonyl*-N$^\delta$-*benzyloxycarbonyl-L-triornithine*

10.9 mmol (5.6 g) $^+H_2$-L-Orn(Z)-Orn(Z)-O^- were dissolved in 100 ml dichloromethane and reacted with preactivated Boc-Orn(Z)-OH from 11.4 mmol (4.17 g) Boc-L-Orn(Z)-OH, 11.4 mmol (1.74 g) *N*-hydroxybenzotriazol and 10.9 mmol (2.5 g) *N,N'*-dicyclohexylcarbodiimide at pH 8 for 2 h under stirring. After removal of the solvent the residue was dissolved in ethyl acetate and the precipitated *N*-hydroxybenzotriazol filtered off. Boc-L-Orn(Z)-Orn(Z)-Orn(Z)-OH was precipitated and recrystallized from ethyl acetate solution by addition of light petroleum. Yield 3.9 g (43%); R_F (B) 0.83; m.p. 69–71 °C.

Tri-L-ornithine × 4 HCl

The protecting benzyloxycarbonyl groups were removed by hydrogenation of 460 µmol (400 mg) Boc-L-Orn(Z)-Orn(Z)-Orn(Z)-OH in 30 ml methanol; 60 mg Pd catalyst (10% Pd on charcoal) and some drops of acetic acid were added. After 12 h 31 ml H_2 had been consumed at 760 Torr (101 kPa) corresponding to the theoretically required amount. The catalyst was filtered off and the solvent removed *in vacuo*. The residue was dissolved in 20 ml 1.2 N HCl in acetic acid for removal of the N$^\alpha$-butyloxycarbonyl group. The resulting tripeptide hydrochloride precipitated and was collected by centrifugation, washed with ether, taken up in water and lyophilised yielding a colourless hygroscopic residue. Yield 210 mg (90.5%); m.p. 134–136 °C. Amino acid analysis: ornithine found 3.05 mol, calcd 3.00 mol; infrared spectrum (KBr): 3350 (NH); 2860; 2400; 2170 (NH_2^+); 1680 (amide-I region); 1620 (CO); 1520; 1430; 1220; 1140; 1090; 1040; 970; 940; 860; 740 cm^{-1}; ^{13}C-NMR (in ^2H_2O) – δ = 174.7, 173.0, 169.3 (CO); 53.1, 52.3 (C-2); 38.8 (3 C-5); 27.8, 27.4 (C-3); 23.3, 22.2 ppm (C-4).

Table 1. *Antagonism tests with inhibitors and various peptides*
Test strain: *E. coli* K 12; medium: minimal medium according to Davis and Mingioli [23] with the addition of 15 g/l agar; concentration of peptides: 5 mM; (comp.) competitive inhibition; (++) noncompetitive inhibition; (−) no inhibition.
$Met(O_2)$ = L-methionine-*S*-dioxide; $Met(O_2)$-$(Ala)_2$ = L-methionine-*S*-dioxidyl-alanyl-alanine; II = L-(N^5-phosphono)methionine-*S*-sulfoximinyl-alanyl-alanine; I = L-phosphinothricyl-alanyl-alanine (see Fig. 1)

Peptide	Inhibition by				
	I (1.2 µM)	II (0.5 mM)	$(Orn)_3$ (20 mM)	$Met(O_2)$-$(Ala)_2$ (50 mM)	$Met(O_2)$ (50 mM)
$(Ala)_2$	−	−	−	−	−
$(Ala)_3$	comp.	comp.	comp.	comp.	−
$(Ala)_4$	comp.	comp.	comp.	comp.	−
$(Lys)_2$	−	−	−	−	−
$(Lys)_3$	comp.	comp.	comp.	comp.	−
$(Ser)_3$	comp.	comp.	comp.	comp.	−
$(Gly)_3$	comp.	comp.	comp.	comp.	−
$(Gly)_2$-Ala	comp.	comp.	comp.	comp.	−
Gly-$(Phe)_2$	++	++	++	++	−
Glutathione	−	−	−	−	−
Aza	−	−	−	−	−
Aza-$(Ala)_2$	comp.	comp.	++	comp.	−

Calcd for $C_{15}H_{36}N_6O_4Cl_4$ (505.33): C, 35.58; H, 7.17; N, 16.60%. Found: C, 35.49; H, 7.14; N, 16.50%.

RESULTS

The uptake of the two tripeptide antibiotics L-2-amino-4-methylphosphinobutyryl-alanyl-alanine and L-(N^5-phosphono)methionine-*S*-sulfoximinyl-alanyl-alanine into cells of *E. coli* K 12 *via* the oligopeptide transport system was investigated by antagonism tests with various peptides. The results of the antagonism tests are summarized in Table 1. The characteristic pattern of inhibition of the two tripeptide antibiotics coincides exactly with that of tri-L-ornithine. All tripeptides and tetrapeptides which are accepted by the oligopeptide transport system abolish the inhibitory effects of L-phosphinothricyl-alanyl-alanine and L-(N^5-phosphono)methionine-*S*-sulfoximinyl-alanyl-alanine. The pattern of the zones of inhibition corresponds to that of competitive antagonism in all cases tested with the exception of Gly-Phe-Phe. However, there is no effect on the antibiotic action in antagonism tests with peptides which use different transport systems. As an example Fig. 8 illustrates the result of an antagonism test showing the competitive antagonism between L-phosphinothricyl-alanyl-alanine and tri-L-alanine.

The spontaneous L-phosphinothricyl-alanyl-alanine-resistant mutants can be divided into two groups according to their sensitivity to tri-L-ornithine. Group (a) exhibits full cross-resistance to tri-L-ornithine, L-(N^5-phosphono)methionine-*S*-sulfoximinyl-alanyl alanine and L-methionine-*S*-dioxidyl-alanyl-alanine.

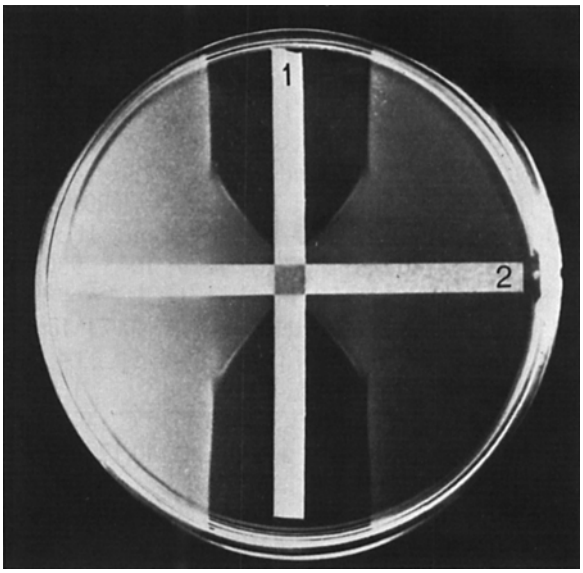

Fig. 8. *Agar-diffusion test, according to Zähner* et al. *[5] with* E. coli *K12 as test organism on minimal medium agar plates, showing the competitive antagonism between L-phosphinothricyl-alanyl-alanine and tri-L-alanine.* Incubation for 18 h at 37 °C. Filter strip 1 was impregnated with 1.2 µM L-phosphinothricyl-alanyl-alanine, filter strip 2 with 5 mM tri-L-alanine

This group should be identical with the oligopeptide transport-deficient (*Opp*$^-$) mutants of Barak and Gilvarg [21]. Group (b) is still sensitive to tri-L-ornithine, but shows cross-resistance to L-(N^5-phosphono)methionine-*S*-sulfoximinyl-alanyl-alanine and a very low sensitivity to L-methionine-*S*-dioxidyl-alanyl-alanine. This group (b) is probably a peptidase-deficient mutant. The results of the agar diffusion tests on plates seeded with the wild-type

Table 2. *Sensitivity of* E. coli *K12 and* L*-phosphinothricyl-alanyl-alanine-resistant mutants to inhibitors*
Agar diffusion test; diameter of the filter disks: 6 mm; medium: minimal medium according to Davies and Mingioli [23] with the addition of 15 g/l agar; ( ) incomplete inhibition zone; (/) no inhibition zone.
$Met(O_2)$ = L-methionine-*S*-dioxide; $Met(O_2)$-$(Ala)_2$ = L-methionine-*S*-dioxidyl-alanyl-alanine; II = L-(N^5-phosphono)methionine-*S*-sulfoximinyl-alanyl-alanine; I = L-phosphinothricyl-alanyl-alanine (see Fig. 1); mutant Ia = L-phosphinothricyl-alanyl-alanine-resistant mutant of *E. coli* K 12, oligopeptide-transport-deficient; mutant Ib = L-phosphinothricyl-alanyl-alanine-resistant mutant of *E. coli* K 12 with defective endopeptidase

Inhibitor	Concn	Diameter of inhibition zone with		
		E. coli K 12	mutant Ia	mutant Ib
	mM	mm		
I	4	43	24	30
	0.4	35	/	/
	0.13	26	/	/
II	0.5	29	/	/
$Met(O_2)$	10	(12)	(12)	(12)
	25	(13)	(13)	(14)
	50	(17)	(17)	(20)
$Met(O_2)$-$(Ala)_2$	10	20	/	(9)
	25	22	/	12
	50	25	/	13
$(Orn)_3$	20	22 (29)	/	18 (25)

strain and with a representative of each of the resistant mutants are listed in Table 2.

The unusual heterocyclic amino acid azaadenylaminohexanoic acid was examined with respect to its possible antibacterial activity; however, there was no activity found against intact cells of *E. coli* K 12. To exclude permeation difficulties as a reason for the ineffectiveness, an Ala-Ala residue was linked to the azaadenylaminohexanoic acid molecule to enable an active transport into the cell *via* the oligopeptide transport system. Nevertheless, the tripeptide Aza-Ala-Ala (IV, Fig. 1) was also inactive against *E. coli* K 12, though it antagonizes the antibiotic activities of all inhibitors listed in Table 1, indicating at least an antagonism at the binding site of the oligopeptide transport permease.

We also investigated the transport of the glutamine synthetase inhibitor L-methionine-*S*-dioxide [37] and the tripeptide L-methionine-*S*-dioxidyl-alanyl-alanine into cells of *E. coli* K 12. The determinations on the uptake of these two substances into the cells were done as described for the tripeptide antibiotics L-phosphinothricyl-alanyl-alanine and L-(N^5-phosphono)-methionine-*S*-sulfoximinyl-alanyl-alanine. The results obtained with L-methionine-*S*-dioxide and L-methionine-*S*-dioxidyl-alanyl-alanine (Tables 1 and 2) show

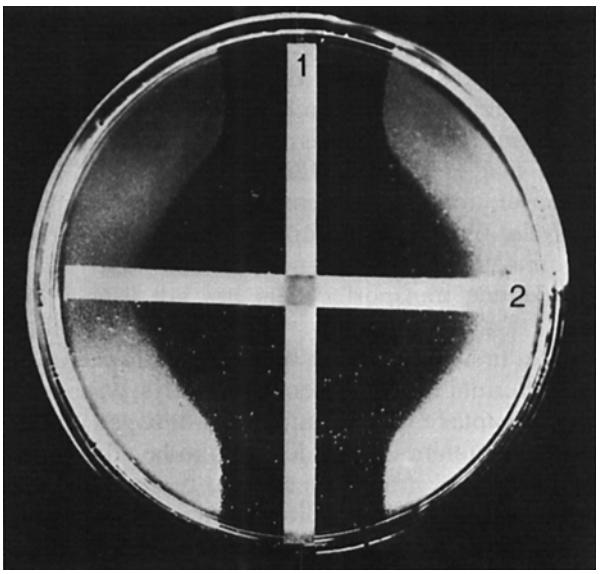

Fig. 9. *Agar-diffusion test, as described in Fig. 8, showing synergistic effect filter strip 1 was impregnated with 50 mM* L*-methionine-S-dioxidyl-alanyl-alanine, filter strip 2 with 50 mM* L*-methionine-S-dioxide*

that L-methionine-*S*-dioxide enters the cell differently as an amino acid and as a tripeptide. For L-methionine-*S*-dioxidyl-alanyl-alanine it is proved that the tripeptide enters the cell *via* the oligopeptide transport system. For L-methionine-*S*-dioxide the results indicate an uptake *via* the methionine transport system. After addition of methionine to the minimal medium L-methionine-*S*-dioxide no longer shows inhibitory effects, whereas the inhibition due to L-methionine-*S*-dioxidyl-alanyl-alanine is not affected by the addition of methionine. The inhibitory effect of L-methionine-*S*-dioxide on *E. coli* K 12 cells is also relieved by methionine, using the test arrangements of an antagonism test. A synergistic effect is found when the two inhibitors are given simultaneously, using the antagonism test arrangements, where one filter paper strip is impregnated with L-methionine-*S*-dioxide and the other with L-methionine-*S*-dioxidyl-alanyl-alanine (Fig. 9).

DISCUSSION

The results of the antagonism tests and the investigations of cross-resistance clearly show that the two tripeptide antibiotics L-phosphinothricyl-alanyl-alanine and L-(N^5-phosphono)methionine-*S*-sulfoximinyl-alanyl-alanine are transported into cells of *E. coli* K 12 *via* the oligopeptide transport system and hydrolyzed inside the cell by a peptidase. The molecular structures of the two tripeptides fulfill the specific requirements of the oligopeptide transport

system [14,15]. Further studies will show whether among the large number of antibiotics there are more which are taken up by the oligopeptide transport system. Hantke and Braun [4] have described a special membrane protein which is essential for the adsorption of the phages T5, T1, $\Phi 80$ and the colicin M and, moreover, for the transport of ferrichrome and albomycin. Whether this utilization of a binding protein by quite different substances is also valid for the oligopeptide transport system has not been proved yet.

As a first model for an inhibitor which can invade the bacterium *via* two different pathways, we investigated the uptake of L-methionine-*S*-dioxide. Whereas free L-methionine-*S*-dioxide seems to be taken up *via* the methionine transport system, the carrier-linked form, L-methionine-*S*-dioxidyl-alanyl-alanine, is shown to be taken up *via* the oligopeptide transport system. Inside the cell both substances inhibit specifically the glutamine synthetase, as has been proved by antagonism tests with glutamine. From a pharmacological point of view it is very important to find out whether the simultaneous application of L-methionine-*S*-dioxide and L-methionine-*S*-dioxidyl-alanyl-alanine results in a synergistic effect (Fig. 9). It should be noted, however, that high doses of alanine inhibit glutamine synthetase [38,39]. Since the uptake of L-methionine-*S*-dioxidyl-alanyl-alanine results in a concomitant transport of alanine which is liberated by a peptidase, we can not decide unequivocally whether the observed synergism is based on a synergism originating from an additional transport possibility for the inhibitor L-methionine-*S*-dioxide or from an effect caused by the increase in alanine. Further examinations of this problem are in progress.

In a detailed study, Lacey [40] has discussed the basis of synergism, but for the particular case of a combination of inhibitors which have the same target site while being transported by different uptake systems he considers as an additive effect. A discrepancy between our results and the conclusion of Lacey [40] exists. The experimental indication of a synergism between L-methionine-*S*-dioxide and L-methionine-*S*-dioxidyl-alanyl-alanine should be followed by studies on a larger number of inhibitors which, due to modification, can enter the bacterium *via* two different transport system.

The oligopeptide transport system provides a basic and, within certain limits, widely applicable carrier system. It is possible that exploitation of the oligopeptide transport system could form the basis for the design of novel, therapeutically useful, antibacterial agents.

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