New Compounds: Peptide Derivatives of the Antitumor Agent *N*-Phosphonoacetyl-L-aspartic Acid

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Abstract □ Two peptide forms of the antitumor transition state analogue *N*-phosphonoacetyl-L-aspartic acid (N^2-phosphonoacetyl-N^4-glycylglycinamidoethyl-L-asparagine and N^1-glycylglycinamidoethyl-N^2-phosphonoacetyl-L-isoasparagine) have been synthesized to obtain potential medicinal agents useful as prodrugs or in a lysosomotropic carrier approach. The bridging unit, ethylenediamine, used for synthetic purposes might be of general interest.

Keyphrases □ *N*-Phosphonoacetyl-L-aspartic acid—peptide derivatives, synthesis, ethylenediamine bridging unit, potential prodrugs and lysosomal carriers □ Peptide derivatives—*N*-phosphonoacetyl-L-aspartic acid, synthesis, ethylenediamine bridging unit, potential prodrugs and lysosomal carriers □ Ethylenediamine—use as a bridging unit in syntheses of peptide derivatives, *N*-phosphonoacetyl-L-aspartic acid

Previous work (1, 2) showed that peptide derivatives of artificial inhibitors are useful alternatives for inhibiting the growth of cells having membrane systems unable to transport the free forms of these molecules. This paper describes the synthesis of two peptide derivatives of *N*-phosphonoacetyl-L-aspartic acid (I), a transition state inhibitor with antitumor activity (3, 4). The new compounds synthesized, N^2-phosphonoacetyl-N^4-glycylglycinamidoethyl-L-asparagine (VIII)[1] and N^1-glycylglycinamidoethyl-N^2-phosphonoacetyl-L-isoasparagine (XIII) are possible medical agents that could be used as prodrugs or in a lysosomotropic carrier approach (5). Two intermediates synthesized during this work, 1-*N*-(*tert*-butoxycarbonyl)ethylenediamine (II) and 1-*N*-(benzyloxycarbonylglycylglycyl)ethylenediamine (IX), might have numerous practical applications when bonding a drug to a peptide backbone is necessary.

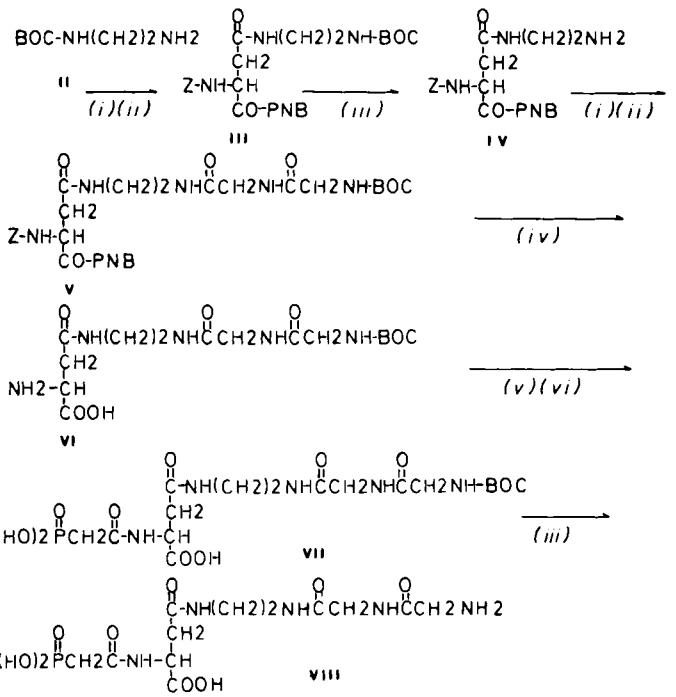
DISCUSSION

Investigations in a model system (1) showed that peptide derivatives having a free *N*-terminal α-amino group were preferentially taken up by cells. The bonding of *N*-phosphonoacetyl-L-aspartic acid (I) to the C-terminal group of a peptide unit must, therefore, be carried out with the help of a bridge. Ethylenediamine was chosen as the bridging unit because the molecule bears two reactive amino groups and, due to its small size, was not expected to create major steric interferences in the intracellular transport of the bonded forms of I.

In the particular case of I, the two carboxyl functions appeared to be suitable attachment points. Because of this, isomeric forms of linked I were considered, since the ultimate biological response depends on the structure. In the synthesis of N^2-phosphonoacetyl-N^4-glycylglycinamidoethyl-L-asparagine (VII), the β-linked form of I (Scheme I), the bridging molecule was first connected to a precursor of the inhibitor. We synthesized 1-*N*-*tert*-butoxycarbonylethylenediamine (II) which was joined to *N*-benzyloxycarbonyl-L-aspartic acid α-*p*-nitrobenzyl ester to give N^2-benzyloxycarbonyl-N^4-(2-*tert*-butoxycarbonylaminoethyl)-L-asparagine *p*-nitrobenzyl ester (III). Compound III was partially unblocked with trifluoroacetic acid to unmask the amino group of the bridge. The resulting compound, N^2-benzyloxycarbonyl-N^4-(2-aminoethyl)-L-asparagine *p*-nitrobenzyl ester (IV), coupled with *N*-*tert*-butoxycarbonylglycylglycine (V) was then treated with hydrogen in the presence of Pd-C to give N^4-(*tert*-butoxycarbonylglycylglycinamidoethyl)-L-asparagine (VI). The final product (VIII) (26% yield) was obtained by phosphonoacetylation of VI, treatment with trifluoroacetic acid, and preparative TLC.

In the synthesis of N^1-(glycylglycinamidoethyl)-N^2-phosphonoacetyl-L-isoasparagine (XIII), the α-linked isomer of I (Scheme II), ethylenediamine was connected to *N*-benzyloxycarbonylglycylglycine to give 1-*N*-(benzyloxycarbonylglycylglycyl)ethylenediamine (IX). Compound IX coupled with *N*-*tert*-butoxycarbonyl-O^4-benzylhydrogen-L-aspartic acid gave N^1-benzyloxycarbonylglycylglycinamidoethyl-N^2-*tert*-butoxycarbonyl-O^4-benzylhydrogen-L-isoasparagine (X), which was then partially unblocked with trifluoroacetic acid. Compound XI, obtained in the previous step, was phosphonoacetylated and totally unblocked with hydrogen bromide in acetic acid (45%). A final preparative TLC gave XIII (32% yield).

It was found, using a paper disk assay (1, 2), that VIII and XIII did not inhibit the growth of *Escherichia coli*. Further investigations showed that the derivatives are actively transported into the microbial cells, but not cleaved[2].



Scheme I

[1] Recommendations of the IUPA-IUB Commission of Biochemical Nomenclature [*J. Biol. Chem.*: **247**, 977 (1972); *Eur. J. Biochem.* **53**, 1 (1975)] have been followed for the biochemical names of the derivatives described in the text.

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

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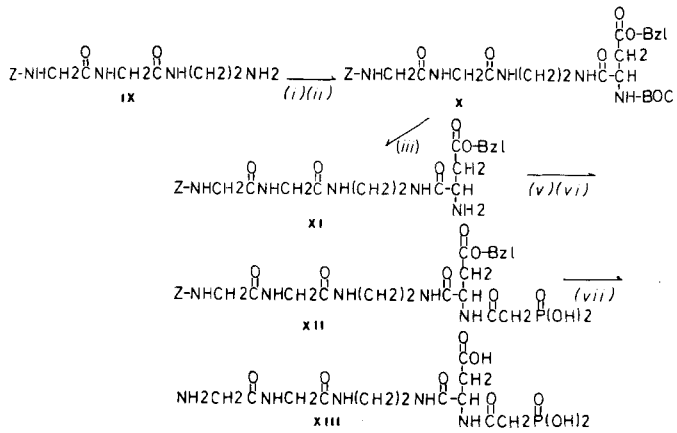

Scheme II

EXPERIMENTAL

For analytical purposes, ~50 μg of the products was spotted on silica gel plates[3]. Three elution systems, ethanol–25% aqueous ammonia (77:23) (system A), chloroform–methanol (2:1) (system B), and butanol–water (85:15) (system C), were used in this work. Spots were detected by ninhydrin and by chlorine and starch–iodine when applicable. Phosphorus-containing spots were detected by the Bandurski and Axelrod procedure (6). Free amino group analyses were made by the dansylation procedure (7). 1-*N*-dansyl-ethylenediamine was obtained by the labeling of *N*-(*tert*-butoxycarbonyl)ethylenediamine with dansyl chloride followed by acid hydrolysis (8). Resolution of the dansylation products was performed by TLC on polyamide sheets (9). High-voltage analytical paper electrophoresis (30 V/cm)[4] was carried out at pH 3.55 (pyridine–acetic acid–water, 1:10:289). Melting points are uncorrected.

1-*N*-(*tert*-Butoxycarbonyl)ethylenediamine (II)—Ethylenediamine (1.8 g, 0.03 mol) and 2-*tert*-butoxycarbonyloxyimino-2-phenylacetonitrile (10.4 g, 0.03 mol) were mixed in 50 mL of dry dioxane and cooled. The mixture was allowed to stand 2 h at room temperature, and the solvent was evaporated under reduced pressure; the residue was dissolved in 50 mL of ethyl acetate. The organic phase was extracted with 100 mL of hot water (50°C), and the aqueous layer was left overnight at 4°C. The resulting precipitate was removed by filtration and recrystallized from water to give II as white needles (0.6 g: 12%), mp 102–103°C; R_f: 0.87 (A), 0.15 (B), 0.05 (C); IR(KBr): 3350 (NH_2), 2940 (—CH_2—), 1690 [C=O (amide I)], 1620 (NH_2) 1535 [amide II (NH)], 1475 (—CH_2—), 1405–1395 (doublet), 1370 and 1255 [$C(CH_3)_3$], 1180 (C—O), 1030 (C—N), and 490 and 480 [$C(CH_3)$] cm^{-1}.

Anal.—Calc. for $C_7H_{16}N_2O_2$: C, 52.50; H, 10.00; N, 17.50; O, 20.00. Found: C, 52.32; H, 10.16; N 17.46.

N^2-Benzyloxycarbonyl-N^4-(2-*tert*-butoxycarbonylaminoethyl)-L-asparagine *p*-Nitrobenzyl Ester (III)—*N*,*N*′-dicyclohexylcarbodiimide (4.12 g, 0.02 mol) was added to a solution of *N*-hydroxysuccinimide (2.28 g, 0.02 mol) and 8.04 g (0.01 mol) of *N*-benzyloxycarbonyl-L-aspartic acid α-*p*-nitrobenzyl ester [obtained by the procedure of Schröder and Klieger (10)] in 40 mL of *N*,*N*-dimethylformamide and cooled. The mixture was allowed to stand at 4°C overnight. The dicyclohexylurea was removed by filtration and washed with 10 mL of *N*,*N*-dimethylformamide. To the filtrate, at room temperature, was added 1-*N*-*tert*-butoxycarbonylethylenediamine (3.2 g, 0.02 mol) in 30 mL of water containing sodium bicarbonate (3.36 g, 0.04 mol). After 12 h, the mixture was acidified to pH 5.0 at 4°C with citric acid and extracted twice with 25 mL of cold (5°C) ethyl acetate. The resulting organic phase was washed twice with 50 mL of saturated sodium chloride; a further concentration *in vacuo* gave a yellow clear oil which was triturated with ether. Recrystallization from ether–light petroleum (1:1) gave a white solid (8.06 g, 74%), mp 90–92°C; R_f: 0.94 (A), 0.69 (B), 0.48 (C); quantitative amino acid analysis of the hydrolyzed product (24 h in 5.7 M HCl at 105°C) gave 1.12 mol of aspartic acid/mol of the product.

Anal.—Calc. for $C_{26}H_{33}N_4O_9$: C, 57.25; H, 6.06; N, 10.28; O, 26.42. Found: C, 57.12; H, 6.12; N, 10.26.

N^2-Benzyloxycarbonyl-N^4-(2-aminoethyl)-L-asparagine *p*-Nitrobenzyl Ester (IV)—Compound III (5.45 g, 0.01 mol) was allowed to stand with 30 mL of anhydrous trifluoroacetic acid for 30 min at room temperature. The excess acid was removed under reduced pressure, and the oily residue was partitioned between 50 mL of cold (4°C) ethyl acetate and 50 mL of 1 *M* sodium carbonate. The organic layer was washed quickly with cold water until neutral and concentrated to dryness (35°C) to give a white solid (3.64 g; 82%), mp 98–100°C; R_f: 0.83 (A), 0.21 (B), 0.11 (C); quantitative amino acid analysis of the hydrolyzed peptide gave 0.97 mol of aspartic acid/mol of the product; amino group analysis by dansylation revealed a single fluorescent spot migrating to the same position as a sample of 1-*N*-dansyl-ethylenediamine.

Anal.—Calc. for $C_{21}H_{25}N_4O_7$: C, 56.63; H, 5.62; N, 12.58; O, 25.17. Found: C, 56.67; H, 5.65; N, 12.49.

N^2 - Benzyloxycarbonyl -N^4- (*tert*-butoxycarbonylglycylglycinamidoethyl)-L-asparagine *p*-Nitrobenzyl Ester (V)—*N*,*N*′-dicyclohexylcarbodiimide (2.06 g, 0.01 mol) was added to a solution of *tert*-butoxycarbonylglycylglycine (2.32 g, 0.01 mol) and 1.04 g (0.01 mol) of *N*-hydroxysuccinimide in 20 mL of *N*,*N*-dimethylformamide and cooled. The mixture was allowed to stand at 4°C overnight. The dicyclohexylurea was removed by filtration and washed with 5 mL of *N*,*N*-dimethylformamide. To the filtrate, at room temperature, was added 4.45 g (0.01 mol) of IV and 2.02 g (0.02 mol) of triethylamine. After standing for 24 h, 50 mL of water was added to the mixture, and the pH was adjusted to 5.0 with citric acid. The precipitated gum was extracted three times with 25 mL of cold (5°C) ethyl acetate. The organic layer was washed twice with 50 mL of saturated sodium chloride, and the solvent was evaporated under reduced pressure. The residue was crystallized from ether–light petroleum (1:1) to give a white solid (3.5 g, 53%), mp 137–139°C; R_f: 0.64 (A), 0.37 (B), 0.17 (C); quantitative amino acid analysis of the hydrolyzed peptide gave 1.04 mol of aspartic acid and 1.98 mol of glycine/mol of the product.

Anal.—Calc. for $C_{30}H_{38}N_6O_{11}$: C, 54.71; H, 5.77; N, 12.76; O, 26.74. Found: C, 54.71; H, 5.69; N, 12.74.

N^4-(*tert*-butoxycarbonylglycylglycinamidoethyl)-L-asparagine (VI)—Compound V (6.58 g, 0.01 mol) was dissolved in a 25-mL mixture of *N*,*N*-dimethylformamide–methanol (1:2) in the presence of 0.5 g of 10% Pd-C. The mixture was hydrogenated for 6 h at room temperature and atmospheric pressure. The catalyst was removed by filtration, and the solution concentrated to dryness under reduced pressure. The residue was dissolved in 20 mL of methanol, and the product precipitated by the addition of 100 mL of ether. Recrystallization from methanol–ether (1:1) gave a white solid (3.47 g; 89%), mp 192–194°C; R_f: 0.41 (A), 0.17 (B), 0.06 (C); amino acid analysis gave 0.95 mol of aspartic acid and 2.1 mol of glycine/mol of the product; amino group analysis by dansylation revealed only one spot migrating to the same position as *N*-dansyl-aspartic acid.

Anal.—Calc. for $C_{15}H_{27}N_5O_7$: C, 46.27; H, 6.94; N, 17.99; O, 28.79. Found: C, 46.17; H, 6.91; N, 18.04.

N^2-Phosphonoacetyl-N^4-glycylglycinamidoethyl-L-asparagine (VIII)—Triethylamine (1.2 g, 0.02 mol) was added to a solution of VI (3.89 g, 0.01 mol) in 20 mL of cold (4°C) dioxane–*N*,*N*-dimethylformamide (4:1). Phosphonoacetylchloride (1.51 g, 0.01 mol) in 15 mL of dioxane obtained by the method of Balsiger (11), was added with stirring to the aforementioned solution in a dropwise manner. After the addition was complete, the mixture was filtered. The precipitate was washed with 5 mL of dioxane–*N*,*N*-dimethylformamide (4:1). The filtrate was made alkaline with ammonia and extracted three times with 20 mL portions of ether. The aqueous layer was lyophilized, and the residue was treated during 1 h with 25 mL of anhydrous trifluoroacetic acid. The crude peptide salt was precipitated by the addition of 150 mL of anhydrous ether. The precipitate was decanted, transferred to a centrifuge tube, and repeatedly washed with ether, centrifuging each time.

Ten-milligram scale preparative fractionation of the material was performed by preparative TLC. The crude product contained in 100 μL of water was applied in a horizontal band near the base of the plate. Elution was performed using solvent system A. Under those conditions, the expected phosphonoacetylated product 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 (1.22 g, 26.5% yield extrapolated); R_f: 0.06 (A).

The material migrated also as a single ninhydrin- and phosphorus-positive spot towards the anode when subjected to paper electrophoresis (0.3 cm for 2 h). Amino acid analysis gave 1.16 mol of aspartic acid and 2.07 mol of glycine/mol of the product. Amino group analysis revealed only one spot migrating to the same position as *N*-dansyl-glycine. The extent of racemization of the product was estimated by the enzymatic quantitative determination of L-aspartic acid released during the acid hydrolysis of the peptide. One mole of the product gave 0.95 mol of L-

[3] Merck Kieselgel 60-5721.
[4] Whatman 3-mm paper and Camag 67701 apparatus.

aspartic acid, as determined by conversion to ureidosuccinic acid with L-aspartate carbamoyltransferase (12).

Anal.—Calc. for $C_{12}H_{22}N_5O_9P \cdot 3NH_3$ (the triammonium salt of the peptide): C, 31.17; H, 6.71; N, 24.24; O, 31.17; P, 6.71. Found: C, 31.20; H, 6.76; N, 24.09; P, 6.83.

1-*N*-(Benzyloxycarbonylglycylglycyl)ethylenediamine (IX)—*N,N'*-dicyclohexylcarbodiimide (2.06 g, 0.01 mol) was added to a solution of *N*-benzyloxycarbonylglycylglycine (2.66 g, 0.01 mol) and 1.04 g (0.01 mol) of *N*-hydroxysuccinimide in 20 mL of *N,N*-dimethylformamide and cooled. After standing overnight at 4°C the mixture was filtered and the precipitate washed with 10 mL of *N,N*-dimethylformamide. To the filtrate was added a solution of 0.6 g (0.01 mol) of ethylenediamine in 10 mL of water adjusted to pH 8.5. The reaction mixture was allowed to stand overnight at room temperature and then was filtered. The filtrate was adjusted to pH 9.5 with 2 M NaOH and extracted three times with 25 mL of ethyl acetate. The organic layer was washed twice with 20 mL of saturated sodium chloride and concentrated *in vacuo*. The oily residue was mixed with 5 mL of absolute ethanol and three drops of 12 M HCl and allowed to crystallize in a desiccator under reduced pressure (1.24 g, 36%), mp 176–178°C; R_f: 0.61 (A), 0.11 (B), 0.06 (C); amino acid analysis gave 2.16 mol of glycine/mol of the product; amino group analysis gave one fluorescent spot running at the same position as 1-*N*-dansyl-ethylenediamine.

Anal.—Calc. for $C_{14}H_{20}N_4O_4 \cdot HCl$: C, 48.77; H, 6.10; N, 16.26; O, 18.58; Cl, 10.30. Found: C, 48.59; H, 6.04; N, 16.40.

N^1 - Benzyloxycarbonylglycylglycinamidoethyl - N^2 - *tert*-butoxycarbonyl-O^4-benzylhydrogen-L-isoasparagine (X)—*N,N'*-dicyclohexylcarbodiimide (2.06 g, 0.01 mol) was added to a solution of *N*-hydroxysuccinimide (1.04 g, 0.01 mol) and 3.27 g (0.01 mol) of *N*-*tert*-butoxycarbonyl-β-benzyl-L-aspartic acid [obtained by the procedure of Laufer and Blout (13)] in 20 mL of *N,N*-dimethylformamide and cooled, the mixture was allowed to stand at 4°C overnight and then was filtered. Compound IX (3.44 g, 0.01 mol) in 10 mL of water containing 2.02 g (0.02 mol) of triethylamine was added to the aforementioned mixture. After 12 h of reaction, at room temperature, the solution was acidified to pH 5.0 with citric acid, and the precipitate was extracted twice with 30 mL of cold (4°C) ethyl acetate; the organic layer was washed twice with 50 mL of saturated sodium chloride and concentrated *in vacuo*. Recrystallization of the residue from ether–light petroleum gave the product as a white powder (2.15 g; 62), mp 127–129°C; R_f: 0.83 (A), 0.71 (B), 0.54 (C); amino acid analysis gave 1.91 mol of glycine and 1.03 mol aspartic acid/mol of the product.

Anal.—Calc. for $C_{30}H_{39}N_5O_9$: C, 58.73; H, 6.36; N, 11.42; O, 23.49. Found: C, 58.62; H, 6.31; N, 11.44.

N^1 - Benzyloxycarbonylglycylglycinamidoethyl - O^4 - benzylhydrogen-L-isoasparagine (XI)—Compound X (5.9 g, 0.01 mol) was treated for 30 min at room temperature with 35 mL of anhydrous trifluoroacetic acid. The excess acid was removed under reduced pressure and the free base of the desired product was obtained by the same procedure as described for IV (3.4 g, 67%), mp 159–161°C; R_f: 0.44 (A), 0.36 (B), 0.25 (C); amino acid analysis gave 1.1 mol of aspartic acid and 1.98 mol of glycine/mol of the product; amino group analysis gave one single spot migrating at the same position as *N*-dansyl-aspartic acid.

Anal.—Calc. for $C_{25}H_{31}N_5O_7$: C, 58.48; H, 6.04; N, 13.65; O, 21.23. Found: C, 58.39; N, 5.93; N, 13.61.

N^1 - Glycylglycinamidoethyl - N^2 - phosphonoacetyl - L - isoasparagine (XIII)—Phosphonoacetylation of XI (2.56 g, 0.05 mol) was carried out by the same procedure as described for VI in the preparation of VII. The lyophilized intermediate (XII) was then treated with 15 mL of 35% hydrogen bromide in glacial acetic acid for 12 h at room temperature. The crude peptide salt was then precipitated by the addition of 100 mL of cold (4°C) anhydrous ether and further treated as in the preparation of VIII. After the final preparative TLC, XIII was obtained as a white hygroscopic powder (0.87 g, 38%); R_f: 0.10 (A). The material migrated also as a single ninhydrin- and phosphorus-positive spot toward the anode when subjected to paper electrophoresis (0.4 cm for 2 h). Amino acid analysis gave 0.98 mol of aspartic acid and 2.04 mol of glycine/mol of the product. Amino group analysis gave one single fluorescent spot migrating to the same position as *N*-dansyl-glycine. Extent of racemization of the product was determined using the procedure described for VIII; the aspartic acid residue of the product appears to be 97% under the L-form.

Anal.—Calc. for $C_{12}H_{31}N_8O_9P \cdot 3NH_3$, (the triammonium salt of the peptide): C, 31.17; H, 6.71; N, 24.24; O, 31.17; P, 6.71. Found: C, 31.24; H, 6.64; N, 24.32; P, 6.59.

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