Synthesis and Inhibitory Activity of Polyaspartic Acid Derivatives[1]

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Polyaspartic acids were used as carriers for biologically active decarboxylated amino acid residues. Among the numerous polydecarboxypeptides the α,β-poly-DL-aspartic acid 3-aminopropylamide (IXa), which was prepared easily and inexpensively from anhydropoly-DL-aspartic acid (VII) and 1,3-diaminopropane, showed fairly selective antibacterial and antifungal activity as well as activity against Sarcoma 180 in mice. Work to relate structure to this activity included the synthesis of four structural and optical isomers of IXa; neither α-polyaspartic acid 3-aminopropylamides (XI) nor β-polyaspartic acid 3-aminopropylamides (XIII), L and D isomers, were as effective antimicrobial agents as IXa. The four decarboxydipeptides IV and VI, L and D isomers, were completely inactive. Polysuccinimide VII was cytotoxic against cell line KB with an ED_{50} value of 19 μg/ml.

Polypeptides were used recently as "carriers" for biologically active compounds, such as enzymes[3] and nitrogen mustards.[4] A few α-polyglutamic acid derivatives are also reported as having antibacterial properties.[5] In this paper the synthesis and inhibitory activity of decarboxypeptides, derived from aspartic acid and decarboxyamino acids, as well as their polymers, referred to as polydecarboxypeptides, are reported.[6]

The best inhibitory activities were exhibited by polymers of decarboxypeptides of aspartic acid with decarboxy-α,γ-diaminobutyric acid (1,3-diaminopropane) and these compounds will be used to demonstrate synthetic procedures.

The decarboxypeptides in both the L and D series were obtained through aminolysis of carbobenzoxyaspartic acid esters with a large excess of 1,3-diaminopropane followed by removal of the carbobenzoxy group. For example, β-benzyl carbobenzoxyaspartate[7] (I) and 1,3-diaminopropane gave N-(3-aminopropyl)-N^2-carbobenzoxy-L- and -D-asparagines (V) (Chart I). Catalytic hydrogenation of V afforded N-(3-aminopropyl)-L- and -D-asparagines (VI), which were isolated as dihydrochlorides.

The preparation of carbobenzoxy-3-aminopropylamine (II) allowed the synthesis of decarboxypeptides without danger of side reactions. Monocarbobenzoxylation of 1,3-diaminopropane with carbobenzoxy chloride, even in the presence of a large excess of the diamine, and low temperature presented great difficulty; the main product was always N,N′-dicarbobenzoxy-1,3-diaminopropane. When dibenzyl carbonate[8] was used for acylation of the diamine, II was obtained in good yield as the hydrochloride salt. At the melting point temperature it was converted to 2-oxohexahydropyrimidine and was identical with a previously reported preparation.[9] When carbobenzoxy-3-aminopropylamine was liberated from its hydrochloride with sodium carbonate, the free base reacted with CO_2 and the carbobenzoxy-3-aminopropylcarbamic acid salt of carbobenzoxy-3-aminopropylamine separated out of the solution.

N-α-Aspartyl-3-aminopropylamines (IV) in the L and D forms were prepared by coupling of β-benzyl N-carbobenzoxyaspartate (I) to II using the mixed anhydride procedure; the resulting decarboxypeptide derivatives III gave, after catalytic hydrogenation, IV which were isolated as dihydrochlorides.

A series of decarboxypeptides of type IX were prepared easily and inexpensively from anhydropoly-DL-aspartic acid[10] (VII) by opening of the succinimide ring with amine derivatives. The aminolysis was carried out in dimethylformamide solution, in which the anhydropoly-DL-aspartic acid is soluble, with a large excess of the amine derivatives. This procedure was necessary to avoid extensive cross-linking when diamines were used. The opening of the succinimide ring was expected to proceed similarly to the ring opening effected with ammonia,[10] which gave a polypeptide containing approximately 43% asparaginyl and 57% isoasparaginyl residues. The diamines used were 1,3-diaminopropane, 1,2-diaminopropane, 1,4-diaminobutane, and 1,6-diaminohexane; the polydecarboxypeptides obtained were IXa–d, respectively. In addition aminoethanol, hydrazine and N,N-dimethylhydrazine were also used for aminolysis of VII, yielding polydecarboxypeptide IXe, α,β-poly-DL-aspartic acid hydrazide (IXf), and dimethylhydrazide (IXg), respectively. All of these polyaspartic acid derivatives were purified by dialysis and then lyophilized.

Both L- and D-polydecarboxypeptides XI were prepared from the corresponding poly-α-aspartic acid benzyl esters[11] by aminolysis with 1,3-diaminopropane. The resulting water-soluble poly-N-(3-aminopropyl)-asparagines were also purified by dialysis.

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(2) To whom inquiries should be addressed.

(3) I. H. Silman and E. Katchalski, *Ann. Rev. Biochem.*, **35**, 633 (1966).

(4) C. W. Mosher, R. H. Iwamoto, E. M. Acton, and L. Goodman, *J. Med. Chem.*, **7**, 650 (1964); M. Szekerke, R. Wade, and F. Bergel, *J. Chem. Soc.*, 1907 (1965).

(5) K. Kovacs and A. Kotai, *Acta Chim. Acad. Sci. Hung.*, **21**, 453 (1959); K. Kovacs, A. Kotai, and I. Szabo, *Nature*, **185**, 266 (1960); K. Kovacs, A. Kotai, I. Szabo, and R. Mecseki, *ibid.*, **192**, 190 (1961). In addition, A. Kotai, K. Kovacs, and J. Csaszar [*Acta Chim. Acad. Sci. Hung.*, **21**, 461 (1959)] reported the preparation of an α,β-poly-DL-aspartic acid derivative (IX, $n = 2$, X = NH_2) without its biological activity.

(6) Several decarboxylated amino acids and derivatives of decarboxylated amino acids, *e.g.*, histamine, putrescine [R. G. Ham, *Biochem. Biophys. Res. Commun.*, **14**, 34 (1964)], and spermine [H. Tabor, C. W. Tabor, and S. M. Rosenthal, *Ann. Rev. Biochem.*, **30**, 579 (1961)], are bioactive compounds and some of these exhibit antibacterial activity; polyelectrolytes such as polyacrylic acid [W. Regelson, S. Kuhar, M. Tunis, J. Fields, J. Johnson, and E. Gluesenkamp, *Nature*, **186**, 778 (1960)] and polylysine [T. Richardson, J. Hodgett, A. Lindner, and M. A. Stahmann, *Federation Proc.*, **18**, 309 (1959); M. Sela and E. Katchalski, *Advan. Protein Chem.*, **14**, 391 (1959)] show antitumor activity. Combination of the two types of compounds led to the synthesis of polydecarboxypeptides.

(7) A. Berger and E. Katchalski, *J. Am. Chem. Soc.*, **73**, 4084 (1951).

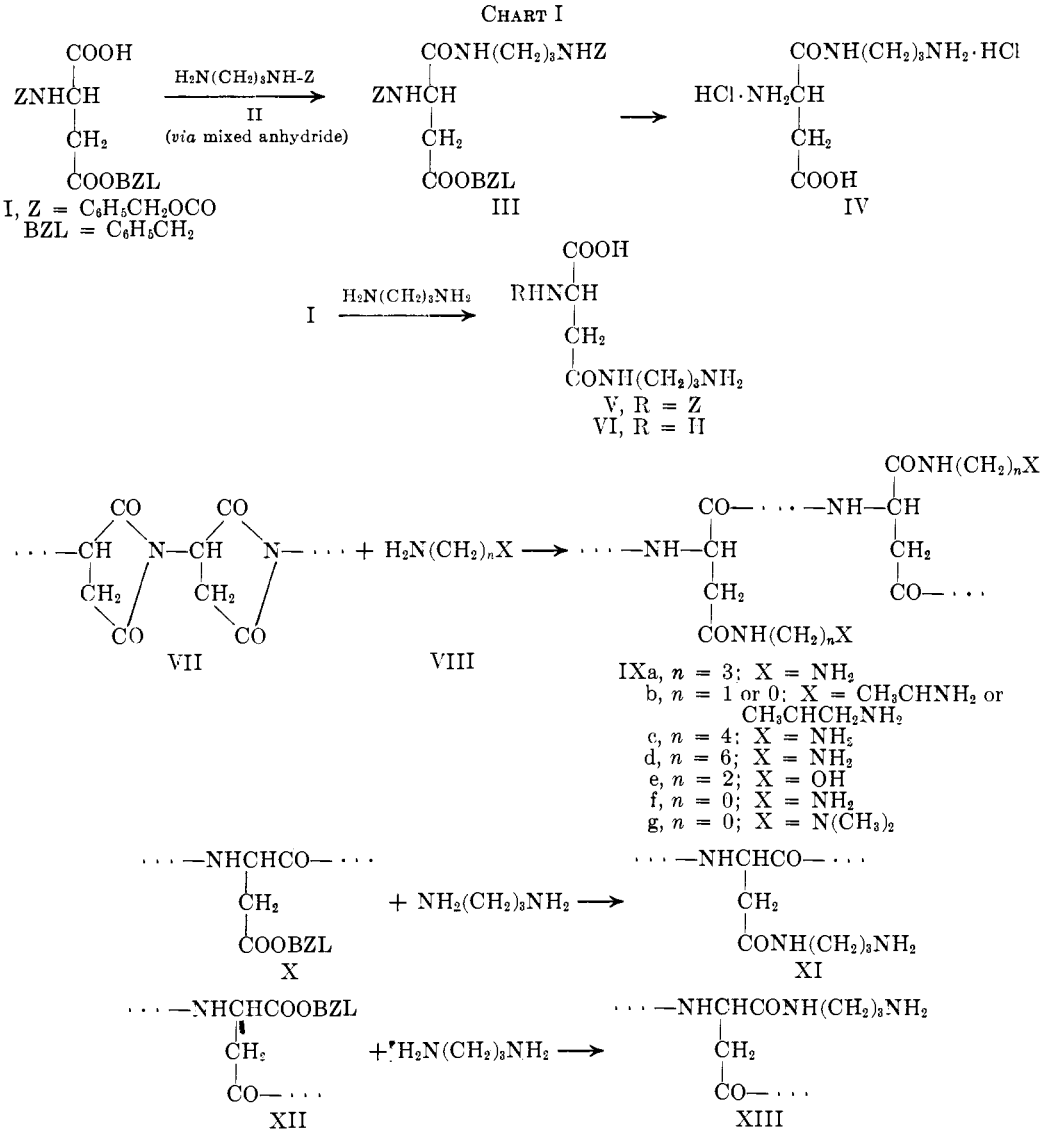
(8) C. A. Bischoff, *Ber.*, **36**, 159 (1903).

(9) E. Fischer, *Ann.*, **232**, 224 (1886).

(10) J. Kovacs, I. Konyves, and A. Pusztai, *Experientia*, **9**, 459 (1953); J. Kovacs and I. Konyves, *Naturwissenschaften*, **41**, 333 (1954); J. Kovacs, H. N. Kovacs, I. Konyves, J. Csaszar, T. Vajda, and H. Mix, *J. Org. Chem.*, **26**, 1084 (1961).

(11) R. Karlson, K. Norland, G. Fasman, and E. Blout, *J. Am. Chem. Soc.*, **82**, 2268 (1960).

CHART I



L- and D-poly-β-aspartic acid benzyl esters[12] were similarly converted with 1,3-diaminopropane to polydecarboxypeptides XIII. These decarboxypeptides and polydecarboxypeptides were investigated for antimicrobial activity;[13] several of them were also tested for tumor inhibitory properties.

The polydecarboxypeptides IXa–g were the simplest to synthesize and these preparations were available initially. Only IXa exhibited significant antimicrobial activity as demonstrated by the selective inhibition of several gram-positive bacteria and fungi in serial broth dilution assays. Minimal inhibitory concentrations (MIC) for the fungal pathogens *Cryptococcus neoformans* and *Histoplasma capsulatum* were 10 and 50 μg/ml, respectively. For *Staphylococcus aureus* the MIC was 50 μg/ml, whereas for *Mycobacterium smegmatis* 100 μg/ml was required for inhibition.

The presence in IXa of D- and L- as well as α- and β-aspartyl residue suggested an investigation of the structural features responsible for the antimicrobial activity. The L and D isomers of the poly-α-aspartyl derivative XI possessed less antibacterial activity than IXa. D-XI, however, paralleled IXa in the inhibition of *C. neoformans* but the L isomer was only one-tenth as effective as IXa. The L isomer of the poly-β-aspartic acid derivative XIII was similar to IXa in the inhibition of *S. aureus*, but less effective against *M. smegmatis* and *C. neoformans*. D-XIII displayed comparatively minimal activity against the test microorganisms. The four monomers, the L and D isomers of IV and VI, were completely inactive. From the results obtained, it was concluded that the superior activity of IXa was probably attributable to the manner of association of the α and β residues in the same macromolecule.

Experimental work recently completed, and to be published elsewhere,[14] disclosed that IXa retains its antimicrobial activity in the presence of human serum, and that it can act synergistically with several antibiotics, *i.e.*, chloramphenicol, benzylpenicillin, oxytetracycline, and tetracycline. Mice infected with

(12) J. Kovacs, R. Ballina, and R. Rodin, *Chem. Ind.* (London), 1955 (1963).

(13) Tests for antibacterial activity were carried out using Antibiotic Medium 3 (Difco), and Sabouraud Maltose Broth (Difco) was employed for the determination of antifungal activity. Concentrations of the compound under test ranging from 1 to 1000 μg/ml were incorporated into the appropriate medium. The inoculated tubes were incubated at 37° and inhibition end points were recorded after 48 hr.

(14) M. A. Pisano, B. A. Shidlovsky, H. N. Kovacs, and J. Kovacs, "Antimicrobial Agents and Chemotherapy 1966," American Society for Microbiology, Ann Arbor, Mich., in press.

S. aureus were protected with a single dose (intraperitoneal) of IXa in levels of 5–10 mg/kg.

Cytotoxic effects of the different preparations were determined against cell line KB at the Cancer Chemotherapy National Service Center. The ED_{50} value for IXf was 25 μg/ml, while for the 1,2-diaminoethane derivative of α,β-poly-DL-aspartic acid (IX, n = 2; X = NH_2) it was 35 μg/ml. The best activity was displayed by anhydropoly-DL-aspartic acid (VII) with an ED_{50} value of 19 μg/ml. *In vivo* tests with Sarcoma 180 bearing mice revealed that IXa was active at a level of 25 mg/kg ip with a C/T of 3.09.[15]

Experimental Section

Melting points are uncorrected. The microanalyses were carried out by Drs. G. Weiler and F. B. Strauss, Oxford, England. Optical rotations were measured with a Rudolph polarimeter, Model 200S-340-80Q3. Viscosity measurements were made with a Cannon-Ubbelohde viscometer.

Carbobenzoxy-3-aminopropylamine Hydrochloride (II). Method A.—Dibenzyl carbonate (5 g, 0.0206 mole) was added dropwise with stirring to 50 ml of 3-aminopropylamine. The reaction mixture was stirred at room temperature for 4 days, then concentrated *in vacuo* to a small volume. The residue was triturated with ether, and the insoluble solid material (1 g) was filtered and redissolved in ethanolic HCl. On addition of ether, 700 mg of carbobenzoxy-3-aminopropylamine hydrochloride, mp 180–182°, was obtained. To the ether solution petroleum ether (bp 30–60°) was added until an oily material separated; this was triturated several times with hexane. The residue was dissolved in ethanol and excess ethanolic HCl was added to the solution when some 3-aminopropylamine hydrochloride precipitated. After filtration, ether was added to the solution and 2.62 g of carbobenzoxy-3-aminopropylamine hydrochloride crystallized out; mp 180–182°. From the mother liquor an additional 400 mg of material was isolated; total yield 3.7 g (74%). Recrystallization raised the melting point to 184–186°; infrared (KBr), 2.93 (s), 3.3 (s, broad), 5.91 (s), 6.2 (w), 6.5 (s) μ.

Anal. Calcd for $C_{11}H_{17}ClN_2O_2$: C, 53.98; H, 7.00; N, 11.45. Found: C, 53.77; H, 7.20; N, 11.72.

Method B.—Carbobenzoxy-3-aminopropylamine hydrochloride was also obtained by acylation of 1,3-diaminopropane with carbobenzoxy chloride (1) at pH 5, or (2) by using a large excess of diamine at −60°.

(1) To a solution of 8.3 ml (0.1 mole) of 1,3-diaminopropane in 100 ml of 2 *N* HCl 15.5 ml (0.1 mole) of carbobenzoxy chloride in 200 ml of hexane was added dropwise under ice cooling. The pH of the solution was kept at about 5 by concurrent addition of a solution of 27.2 g (0.2 mole) of $NaOAc \cdot 3H_2O$ in 70 ml of water. This procedure took 17 hr after which time the reaction mixture was stirred for an additional 6 hr. During this time N,N′-dicarbobenzoxy-1,3-diaminopropane separated from the reaction mixture. The aqueous solution was separated from the hexane phase, filtered, made alkaline with about 200 ml of 5 *N* NaOH, and then extracted five times with ether. The ether extract was saturated with CO_2 and the white crystalline precipitate was filtered, washed with ether, and dissolved in alcoholic HCl. The insoluble unreacted 3-aminopropylamine hydrochloride was filtered and from the alcohol solution carbobenzoxy-3-aminopropylamine hydrochloride was precipitated by addition of ether; yield 3.4 g (14%), mp 184–185°, no depression on admixture with a sample prepared under A.

(2) A solution of 100 ml of 1,3-diaminopropane in 100 ml of water was cooled to −60°. A hexane solution (280 ml) containing 20 ml (22 g, 0.129 mole) of carbobenzoxy chloride was added dropwise to the stirred reaction mixture, and the temperature then was allowed to rise to 0°. After 2 hr the stirring was continued at room temperature for 1 additional hour. Crystalline N,N′-dicarbobenzoxy-1,3-diaminopropane was filtered (19 g), the aqueous phase was made alkaline with 4 *N* NaOH and then extracted ($CHCl_3$). The $CHCl_3$ solution was dried and evaporated and the residue was dissolved in alcohol. On addition of alcoholic HCl to the solution some 1,3-diaminopropane hydrochloride separated, which was quickly filtered. From the filtrate 3.6 g of monocarbobenzoxy derivative separated, which after recrystallization from alcohol–acetone melted at 184–186° and was identical with the preparation described under A. The N,N′-dicarbobenzoxy-1,3-diaminopropane by-product, (19 g, 85%), mp 98–100°, was recrystallized from $CHCl_3$–hexane, which raised the melting point to 111–113°.

Anal. Calcd for $C_{19}H_{22}N_2O_4$: C, 66.65; H, 6.47; N, 8.11. Found: C, 66.75; H, 6.53; N, 8.01.

The **picrate** was prepared from 150 mg of carbobenzoxy-3-aminopropylamine hydrochloride by mixing with 20 ml of saturated picric acid solution. The heavy yellow precipitate was filtered (400 mg) and recrystallized from aqueous alcohol; mp 78–80°.

Anal. Calcd for $C_{17}H_{19}N_5O_9 \cdot H_2O$: C, 44.80; H, 4.65; N, 15.30. Found: C, 44.65; H, 4.84; N, 15.05.

Carbobenzoxy-3-aminopropylcarbamic Acid Salt of Carbobenzoxy-3-aminopropylamine.—A solution of 250 mg of carbobenzoxy-3-aminopropylamine hydrochloride in 1.5 ml of water was mixed with 2 ml of saturated aqueous Na_2CO_3. After a few minutes crystalline material separated out, which was filtered and washed with cold water, acetone, and ether; yield 100 mg; mp 122–125°; infrared (KBr), 2.9 (s), 3.2 (sh), 3.3 (w), 5.9 (s), 6.05 (w), 6.35 (sh), 6.5 (s) μ.

Anal. Calcd for $C_{28}H_{32}N_4O_8$: C, 60.00; H, 7.00; N, 12.16. Found: C, 60.02; H, 7.00; N, 11.82.

Carbobenzoxy-3-aminopropylamine can be liberated from its hydrochloride with strong NaOH; the oily free amine is water and ether soluble and absorbs CO_2 rapidly from the air to give the crystalline carbamic acid salt described above.

Carbobenzoxy-3-aminopropylamine hydrochloride gave 2-oxohexahydropyrimidine on heating at 160°, which after recrystallization from alcohol melted at 260°, and was identical with a preparation reported in the literature.[9]

N-Carbobenzoxy-N′-α-(β-benzyl-N-carbobenzoxy-D-asparty l)-1,3-diaminopropane (III).—To 301 ml of dry toluene, 4.296 g (0.012 mole) of β-benzyl-N-carbobenzoxy-D-aspartate and 1.68 ml (0.012 mole) of dry triethylamine were added and cooled to −10°. To this mixture 1.56 ml (0.012 ml) of isobutyl chloroformate was added with stirring, and the temperature was maintained at −10 to −5° for 0.5 hr. Then 2.9 g (0.012 mole) of carbobenzoxy-3-aminopropylamine hydrochloride in 6 ml of water and 12 ml of 1 *N* NaOH were added. The temperature was allowed to rise to 0°; the mixture was then diluted with 30 ml of ethyl acetate and stirred in an ice bath for 1.5 hr and at room temperature for 2.5 hr. The ethyl acetate solution was washed with water, 0.1 *N* NaOH, water, 0.1 *N* HCl, and water, dried ($MgSO_4$), and concentrated to a small volume. The crystalline product was filtered and recrystallized from ethyl acetate–ether; yield 3.65 g (54%), mp 145–146°, $[\alpha]^{24}D$ −10.54° (*c* 2.58, $CHCl_3$).

Anal. Calcd for $C_{36}H_{38}N_4O_7$: C, 65.81; H, 6.08; N, 7.67. Found: C, 65.97; H, 6.12; N, 7.56.

L **Isomer (III)** was prepared similarly in 68% yield, mp 128–130°, which after recrystallization from ethyl acetate melted at 146–147°, $[\alpha]^{24}D$ 10.67° (*c* 2.58, $CHCl_3$).

Anal. Found: C, 65.85; H, 6.13; N, 7.92.

N-α-D-Aspartyl-3-aminopropylamine Dihydrochloride (IV).—N-Carbobenzoxy-N′-α-(β-benzyl-N-carbobenzoxy-D-asparty l)-1,3-diaminopropane (III) (0.583 g, 0.00106 mole) was hydrogenated in 130 ml of methanol–water (1:1) containing 0.25 ml of concentrated HCl. When the hydrogen uptake stopped (50 ml), the 10% Pd–C catalyst was filtered and washed with methanol and water, and the combined solution was evaporated *in vacuo*. The residue was dissolved in a small amount of methanol and, on addition of 2-propanol and ether, a fluffy hygroscopic material precipitated; yield 247 mg (88%); after drying *in vacuo* at 56°, $[\alpha]^{23}D$ −8.40° (*c* 2.665, water).

Anal. Calcd for $C_7H_{17}Cl_2N_3O_3$: Cl, 27.03. Found: Cl, 26.86.

L **Isomer (IV).**—Hydrogenation of the L isomer of III (2.12 g, 0.00399 mole) yielded 65% dihydrochloride, $[\alpha]^{23}D$ 9.4° (*c* 2.219, water).

Anal. Found: Cl, 26.87, 27.26.

When the hydrogenation was performed in a methanol-water (1:1) suspension (until complete solution was obtained, 2 days), the free base was obtained after evaporation of the filtered solution. Paper chromatography of the solid residue (100 μg) in 1-butanol–acetic acid–water (4:1:1) for 16 hr gave, after development with ninhydrin, a strong violet color with an R_f value

(15) *In vivo* tests with Sarcoma 180 in mice were conducted by Dr. E. Grunberg, Director of the Department of Chemotherapy of Hoffmann-La Roche, Inc.

TABLE I
PREPARATION OF POLYDECARBOXYPEPTIDES

Polydecarboxypeptide	Starting material	Amine component	Reaction conditions	Yield, %
IXb[a]	VII	1,2-Diaminopropane	5 g of VII was added to 50 ml of 1,2-diaminopropane	34
IXc[a]	VII	Putrescine	Same as for IXa (Experimental Section)	46
IXd[b]	VII	1,6-Diaminohexane	Same as for IXa (Experimental Section)	53
IXe	VII	2-Aminoethanol	5 g of VII in 50 ml of DMF was added to 50 ml of 2-aminoethanol	68
IXf[c]	VII	Hydrazine	Same as for IXa (Experimental Section); anhydrous hydrazine was used	32
IXg[d]	VII	N,N-Dimethylhydrazine	2 g of VII was refluxed in 13 g of N,N-dimethylhydrazine for 45 min, then evaporated	48
L-XI[e]	L-X[f]	1,3-Diaminopropane	1 g of X in 20 ml of DMF was added to 20 ml of 1,3-diaminopropane	35
D-XI	D-X	1,3-Diaminopropane	Same as for L-XI	
D-XIII[g]	D-XII[h]	1,3-Diaminopropane	Same as for XI	72
L-XIII	L-XII	1,3-Diaminopropane	Same as for XI	53

[a] The analytical values indicated the presence of about 50% free aspartyl residues with 1 mole of water. [b] The analytical values indicated the presence of about 30% free aspartyl residues with 1 mole of water. [c] The analytical values indicated the presence of 0.5 H_2O/aspartyl residues. [d] About 30% free aspartyl residues are present. [e] Estimated molecular weight was about 60,000 [$\eta_{sp/c}$ (*c* 1, dichloroacetic acid) was 0.44]. [f] Estimated molecular weight was about 35,000 [$\eta_{sp/c}$ (*c* 1, dichloroacetic acid) was 0.27]. [g] Estimated molecular weight was about 20,000 [$\eta_{sp/c}$ (*c* 1, dichloroacetic acid) was 0.18]. [h] Estimated molecular weight was about 10,000 [$\eta_{sp/c}$ (*c* 1, dichloroacetic acid) was 0.09].

of 0.16 for both L and D isomers. This result is in agreement with the observation that α-aspartyl peptides gave violet colors with ninhydrin, while β peptides gave brown colors.

N-3-Aminopropyl-N²-carbobenzoxy-D-asparagine (V).—A solution of 1 g (0.00285 mole) of N-carbobenzoxy-D-aspartic acid β-benzyl ester, mp 107–108°, $[\alpha]^{24}D$ −11.97° (*c* 10, acetic acid), in 10 ml of dry methanol was added dropwise to 10 ml of 1,3-diaminopropane with vigorous stirring. After standing overnight 50 ml of dry ether was added to the reaction mixture when 800 mg (84%) of solid material precipitated. It was filtered and washed with hexane; mp 204–205°. Recrystallization from water–alcohol (1:10) raised the melting point to 208–210°, $[\alpha]^{30}D$ −10.99° (*c* 4.53, acetic acid).

Anal. Calcd for $C_{15}H_{21}N_3O_5 \cdot H_2O$: C, 52.75; H, 6.79; N, 12.31. Found: C, 52.62; H, 6.71; N, 12.08.

L Isomer (V) was prepared similarly. One gram of the L-benzyl ester (mp 106–108°, $[\alpha]^{24}D$ 10.9° (*c* 10, acetic acid)) yielded 905 mg (94%) of crude V, mp 205–206°, which after two recrystallizations from hot water melted at 211–212°, $[\alpha]^{24}D$ 10.8° (*c* 5.02, acetic acid).

Anal. Found: C, 52.59; H, 6.81; N, 12.68.

The **hydrochloride** of N-3-aminopropyl-N²-carbobenzoxy-L-asparagine was prepared by dissolving 500 mg of V in excess methanolic HCl; 450 mg of crude hydrochloride was precipitated with dry ether. After recrystallization from dry ethanol–ether it melted at 178–180°.

Anal. Calcd for $C_{15}H_{22}ClN_3O_5$: C, 50.07; H, 6.16; N, 11.67. Found: C, 49.71; H, 6.31; N, 11.31.

N-3-Aminopropyl-D-asparagine (VI).—N-3-Aminopropyl-N²-carbobenzoxy-D-asparagine (1.8 g) was hydrogenated with prehydrogenated 10% Pd–C catalyst in water–ethanol suspension. The hydrogenation was continued until a clear solution was obtained; this was filtered and evaporated *in vacuo* at room temperature. The residue was dissolved in ethanol and evaporated *in vacuo*. This was repeated three times, and 975 mg of a hygroscopic white solid was obtained which melted around 146–148° (unsharp). A paper chromatogram of 100 μg of this free base in 1-butanol–acetic acid–water (4:1:1) gave a brown spot with an R_f value of 0.13 when developed with ninhydrin.

The free base (242 mg) was dissolved in methanolic HCl and the solution evaporated *in vacuo* at 20°. The residue was triturated with ether and acetone yielding 335 mg of hygroscopic dihydrochloride which was dissolved in methanol and reprecipitated with 2-propanol and ether, filtered, and dried *in vacuo* (P_2O_5); $[\alpha]^{24}D$ −24.2° (*c* 1.425, 1 *N* HCl).

Anal. Calcd for $C_7H_{17}Cl_2N_3O_3$: Cl, 27.05. Found: Cl, 27.25.

The **L isomer (VI)** was similarly obtained in 97% yield; infrared (KBr), 2.95 (s), 3.15 (s), 3.38 (s), 6.05 (s), 6.42 (s) μ. The free base was converted into the dihydrochloride; infrared (KBr), 2.85 (s), 3.0 (s), 5.7 (s), 6.0 (s), 6.35 (s), 6.65 (s) μ; $[\alpha]^{24}D$ 25.8° (*c* 2.53, 1 *N* HCl).

Anal. Found: Cl, 26.88.

Polydecarboxypeptide IXa.—A general procedure for the aminolysis of anhydropoly-DL-aspartic acid (VII) is outlined below, using 3-aminopropylamine (VIII, *n* = 3; X = NH_2). Anhydropoly-DL-aspartic acid (35 g) was dissolved in 250 ml of dimethylformamide and was added dropwise to 350 ml of 3-aminopropylamine with constant stirring. The stirring was continued for 7 hr at room temperature; to the solution 600 ml of ether and 600 ml of petroleum ether were added gradually. A gummy material precipitated, which was washed with dry ether several times and was allowed to stand under ether overnight. The ether was discarded, the residue was dissolved in a small amount of methanol and reprecipitated with ether. This procedure was repeated three times. The solubility of the polymer in methanol decreased during this procedure. After trituration with 500 ml of ethanol, a light-colored powdery material was obtained. Finally it was washed with ether and petroleum ether and dried *in vacuo;* yield 41 g (79.5%). This polymer was completely soluble in water, giving an alkaline solution; infrared (KBr), broad peaks at 2.9 (s), 3.2 (m), 3.33 (m), 6.05 (s), 6.5 (s) μ. Reduced specific viscosity $\eta_{sp/c}$ (*c* 1, Cl_2CHCO_2H) was 0.34.[16]

A portion of this compound (10 g) was dissolved in a small amount of water and dialyzed against a constant flow of distilled water (in a 2000-ml measuring cylinder, 100 ml of water flow/20 sec) for 2 hr, then lyophilized; yield 7.1 g (71%); $\eta_{sp/c}$ (*c* 1, Cl_2CHCO_2H) was 0.32.[16]

Anal. Calcd for $C_{18}H_{33}N_7O_8$: C, 45.5; H, 7.0; N, 20.6. Found: C, 46.6; H, 7.8; N, 19.7.

The analytical values were calculated for a polymer in which every third unit is a free aspartyl residue with 1 mole of water. The fairly large deviation of the analytical values for the polypeptide and for those described below can be attributed to the ability of such polymers to bind water, other solvents, and salts to a varying degree; it is sometimes impossible to remove these impurities without causing some degradation of the polymer itself.

Hydrochloride of polydecarboxypeptide IXa was prepared from 10 g of the undialyzed material by dissolving it in a small amount of water and neutralizing with 4 *N* HCl to pH 2.5. The hydrochloride was dialyzed as described above for 2 hr; yield 8.4 g, after lyophilization; $\eta_{sp/c}$ (*c* 1, Cl_2CHCO_2H) was 0.31.[16]

Anal. Calcd for $C_{18}H_{33}N_7O_8 \cdot 2HCl$: C, 39.4; H, 6.2; N, 17.9; Cl, 12.9. Found: C, 40.0; H, 7.0; N, 17.4; Cl, 12.0.

(16) These viscosity values would indicate approximate molecular weights of 35,000–50,000 assuming Doty's curve for poly-γ-benzyl glutamate [P. Doty, J. H. Bradbury, and A. M. Holtzer, *J. Am. Chem. Soc.*, **78**, 947 (1956)] is applicable to this type of polymer. The starting material VII has an estimated molecular weight of 16,000–18,000 by the same method.

The preparation of polydecarboxypeptides IXb–g, XI, and XIII is described in Table I.

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The Role of the 5′-Hydroxyl Group of Adenosine in Determining Substrate Specificity for Adenosine Deaminase

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The relationship between structural alterations in the carbohydrate moiety of adenosine and the resulting changes in substrate activity was examined with adenosine deaminase. Of the 43 analogs studied, 16 were deaminated, all of them at slower rates than the natural substrate. With the exception of adenosine 2′- or 3′-monophosphate, modifications at the 2′ or 3′ positions, including the simultaneous removal of the 2′- and 3′-hydroxyl groups or changes in their steric configuration, did not abolish substrate activity. Replacement of the bridge oxygen with a sulfur atom allowed deamination, but modifications at the 1′ position prevented it. Replacement or substitution of the 5′-hydroxyl group with a variety of other groups, or removal of the 4′-hydroxymethyl group, invariably led to loss of substrate activity. Very low activity was retained when an amino group replaced the 5′-hydroxyl group, or when, in the absence of the 5′-hydroxyl, a hydroxyl group was present at carbon 3′ in a configuration *cis* to the base moiety. These data show that the 2′- or 3′-hydroxyl groups of adenosine are not required for substrate activity, but that the 5′-hydroxyl group is essential for binding to the enzyme unless its function can be assumed to a very limited extent by an amino or possibly other hydrogen bonding groups, or by a hydroxyl group at the 3′ position *cis* to the base. The implication of these observations for the design of adenosine analogs of interest in chemotherapy is discussed.

Nucleoside analogs, particularly of the adenosine variety, have long been of interest in chemotherapy. One of the factors which severely limits the usefulness of many of these analogs is their ready degradation by adenosine deaminase. It is of interest, therefore, to determine which modifications in the nucleoside molecule decrease or abolish the susceptibility of an analog to deamination. Numerous investigations of this nature have been carried out in recent years, utilizing structural analogs of adenosine modified in the base or in the sugar moiety or in both.[1] In particular, the contribution to binding made by the 2′- and 3′-hydroxyl groups of the carbohydrate moiety has received close attention, and the conclusions reached have varied, depending upon the types of analogs available for testing.[2]

The availability of new adenosine analogs, all modified in the sugar moiety and particularly in the 5′ position, prompted us to examine further the relationship between structural alterations in the carbohydrate portion of adenosine and the ensuing changes in susceptibility to deamination.

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Materials and Methods

The deamination of adenosine, 2′-deoxyadenosine, and of their structural analogs was followed spectrophotometrically at 265 mμ.[3] The measurements were carried out at 25° with a Gilford absorbance recorder (Model 2000) and a Beckman monochromator.

Adenosine deaminase, type I, from intestinal mucosa (Lot 95B-9022) was purchased from Sigma Chemical Co. As specified by the supplier, this preparation deaminates adenosine 5′-monophosphate (5′-AMP) at less than 0.01% the rate at which adenosine is deaminated. This activity is held to be due to the presence of phosphatase. Nucleoside phosphorylase, myokinase, xanthine oxidase, and guanase activity is also below 0.01% of the activity found in the crude extract. The enzyme was diluted in 0.05 *M* phosphate buffer, pH 7.5, to a concentration of 0.280 unit/ml (1 unit being defined as that amount of enzyme which causes the deamination of 1 μmole of adenosine to inosine/min at pH 7.5 and 25°). The assays were carried out by adding 0.1 ml of this solution to 2.9 ml each of 5×10^{-5} *M*, 1×10^{-4} *M*, and 2×10^{-4} *M* substrate in 0.05 *M* phosphate buffer, pH 7.5. Since the rates of deamination at the two highest substrate concentrations differed little, the enzyme appears to be essentially saturated at these levels. Progress of the deamination was recorded until the reaction was virtually completed. The rates of deamination of those analogs which were deaminated very slowly were determined by increasing the amount of enzyme from 5 to 100 times (see Table I). All of the compounds which were apparently not deaminated by the initial low enzyme concentrations were reexamined in the presence of 30 times the amount of enzyme. Since the activity of the enzyme in the buffer solution changes markedly upon standing (in the cold), a new solution was prepared after every 4 hr, and the rate of deamination of adenosine was determined alongside each analog determination.

The buffer-insoluble compound X was first dissolved in 0.02 ml of dimethyl sulfoxide (DMSO),[4] and its rate of deamination was compared to the rate of deamination of adenosine in a mixture containing an equal amount of DMSO.

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