Polyglutamyl and Polylysyl Derivatives of the Lysine Analogues of Folic Acid and Homofolic Acid[1,2]

Laurence T. Plante,* Elizabeth J. Crawford, and Morris Friedkin

Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111.
Received March 26, 1976

A series of N^{ϵ}-poly-α-glutamyl and N^{ϵ}-polylysyl derivatives of N^{α}-pteroyllysine and N^{α}-homopteroyllysine, analogues of the naturally occurring γ-polyglutamyl forms of folate, was prepared and tested as substrates for dihydrofolate reductase and as substrates and inhibitors of thymidylate synthetase. N^{α}-Dihydropteroyl-N^{ϵ}-(tri-α-glutamyl)lysine was 1.8 times as active as N^{α}-dihydropteroyl glutamate (dihydrofolate) as a substrate for L1210 murine leukemia dihydrofolate reductase. N^{α}-Dihydropteroyl-N^{ϵ}-(di-α-lysyl)lysine was 1.2 times as active as dihydrofolate in spite of its strong positive charge. The most active compound tested, N^{ϵ}-(*tert*-butyloxycarbonyl)lysine, was 3.5 times as active as dihydrofolate. None of the enzymatically prepared N^{α}-tetrahydropteroyllysine derivatives tested was as active as N^{α}-tetrahydropteroyl glutamate (tetrahydrofolate) as a substrate for *E. coli* thymidylate synthetase. However, there was a progressive increase in activity with the addition of each α-glutamyl residue, the N^{ϵ}-(penta-α-glutamyl)lysine being 88% as active as tetrahydrofolate. N^{α}-Tetrahydropteroyl-N^{ϵ}-(di-α-lysyl)lysine was the most active thymidylate synthetase substrate of the polylysine derivatives, being 67% as active as tetrahydrofolate. Addition or deletion of lysyl residues resulted in diminished activity. It is noteworthy that substrate activity is retained in spite of the positively charged poly(amino acid) side chain. None of the enzymatically prepared tetrahydrohomopteroyl derivatives tested was as active as N^{α}-tetrahydrohomopteroyl glutamate (tetrahydrohomofolate) as an inhibitor of *E. coli* thymidylate synthetase.

Since our report a number of years ago[3] on polyglutamates prepared from N^{α}-pteroyllysine, several laboratories[4,5] have turned their attention to syntheses in an area of the folic acid field that had been inactive since the earliest preparations of pteroylpolyglutamates.[6] The solid-phase technique for synthesis of these compounds initiated in the laboratories of Krumdieck and Baugh has made possible recent studies of pteroylpolyglutamates in our own[7] as well as in other laboratories.[8,9]

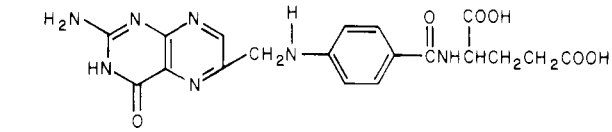
We have continued our earlier studies to include polylysyl derivatives of N^{α}-pteroyllysine, making available for the first time analogues of folic acid containing a positively charged side chain in place of the negatively charged polyglutamate chain found in nature. This report describes the synthesis and enzymatic behavior of both polyglutamyl and polylysyl compounds prepared from N^{α}-pteroyllysine and N^{α}-homopteroyllysine with murine leukemia L1210 dihydrofolate reductase and thymidylate synthetase of *Escherichia coli*.

Results and Discussion

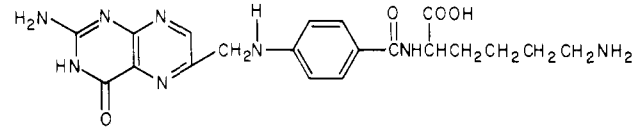
Chemistry. In Chart I are shown the structures of folic acid (*N*-pteroylglutamic acid) and N^{α}-pteroyllysine. The two compounds are closely related, but the latter contains a primary aminoethyl group in place of the terminal carboxyl group of folic acid. The N^{ϵ} primary amino group reacted readily with acyl anhydrides, with *O*-methylisourea, and with amino acid *N*-carboxyanhydrides. Each compound so prepared traveled as a single ultraviolet absorbing spot on paper chromatography and showed absorption spectra nearly identical with that of folic acid (or homofolic acid in the case of those compounds prepared from N^{α}-homopteroyllysine), indicating that reaction at the N^{10} (or N^{11}) amino group did not take place.

The polymerization of amino acid *N*-carboxyanhydrides by primary amines has been reviewed in detail.[10] The reaction of N^{α}-pteroyllysine with γ-ethyl glutamate *N*-carboxyanhydride is depicted in Scheme I. The length of the peptide chain attached to the N^{ϵ}-amino group of N^{α}-pteroyllysine was determined by the conditions of the synthetic procedure. Polymerization of amino acid *N*-carboxyanhydrides by the N^{ϵ}-amino group of N^{α}-pteroyllysine (or N^{α}-homopteroyllysine) in aqueous solution consistently gave no more than three amino acid residues attached to the N^{ϵ}-amino group of lysine although

Chart I



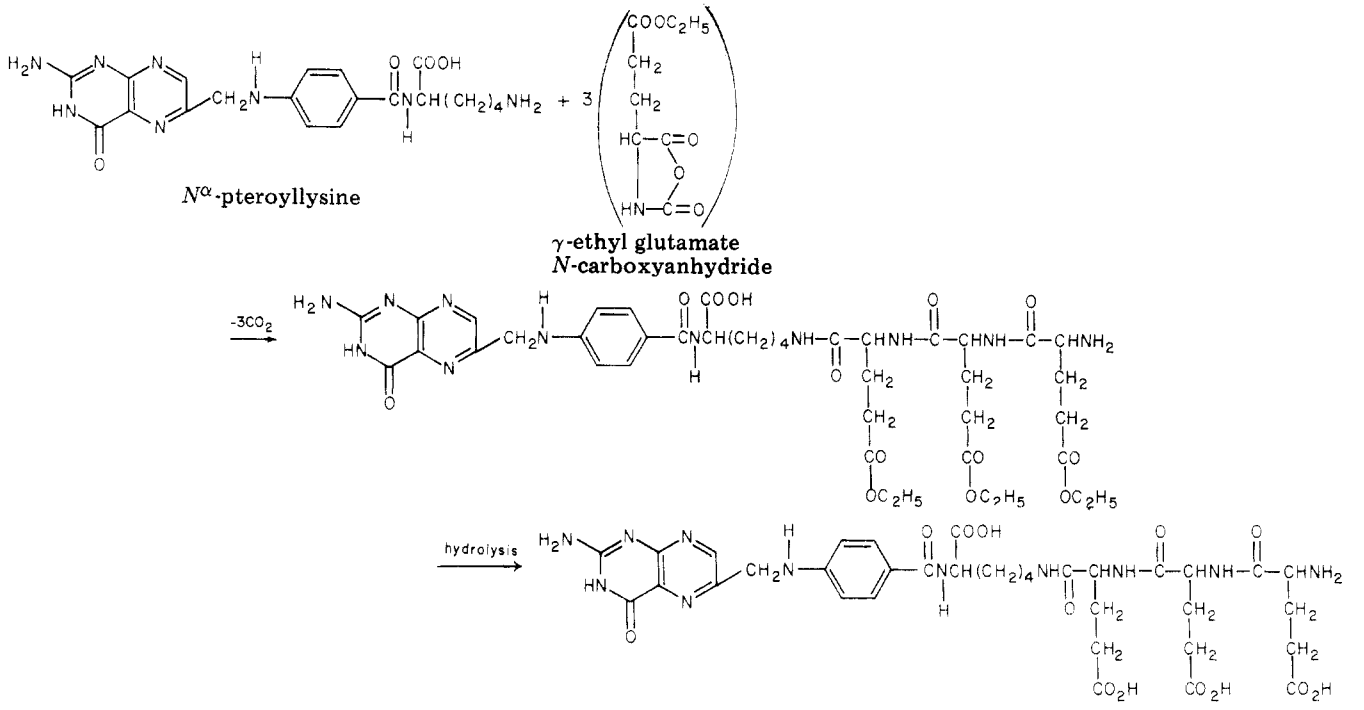
N-pteroylglutamic acid (folic acid)



N^{α}-pteroyllysine

the anhydrides were used at a ratio up to five times that of the initiator. Thus γ-ethyl glutamate *N*-carboxyanhydride gave mono-, di-, and triglutamyl derivatives. A longer glutamate chain was obtained by polymerization in anhydrous dimethylformamide; however, compounds containing more than a pentaglutamate were not separated by paper chromatography since the mixtures ran at the solvent front. A satisfactory method for separation using different solvent systems or by column chromatography was not found. With N^{ϵ}-(*tert*-butyloxycarbonyl)lysine N^{α}-carboxyanhydride only a single lysine residue was added to the N^{ϵ}-amino group of N^{α}-pteroyllysine. Apparently more than one lysine residue was added by polymerization in anhydrous dimethylformamide, but the resultant compounds were found difficult to manipulate. A superior reagent for the synthesis of polylysines attached to the N^{ϵ}-amino group of N^{α}-pteroyllysine was N^{ϵ}-carbethoxylysine N^{α}-carboxyanhydride. With this reagent five or more lysine residues were attached to the initiator. These compounds separated well up to the pentapeptide level, but as with the polyglutamates, a good method of separation for the longer polypeptide chains was not found. Alanine *N*-carboxyanhydride did not react readily with the N^{ϵ}-amino group of N^{α}-pteroyllysine (or N^{α}-homopteroyllysine) under the conditions that gave satisfactory results with the carboxy anhydrides of substituted glutamic acid or lysine. Thus only a few compounds containing alanine were prepared. We observed that elongation of an existing peptide chain, or preparation of chains containing a different amino acid from that first added to

Scheme I

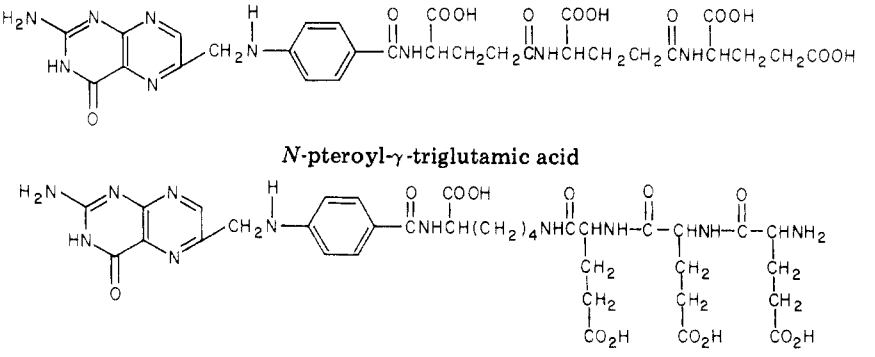


N^{α}-pteroyllysine

γ-ethyl glutamate *N*-carboxyanhydride

N^{α}-pteroyl-N^{ϵ}-(α-triglutamyl)lysine

Chart II



N-pteroyl-γ-triglutamic acid

N^{α}-pteroyl-N^{ϵ}-(α-triglutamyl)lysine

the N^{ϵ}-amino group, was not possible, since the primary amino group α to the peptide bond [as in N^{α}-pteroyl-N^{ϵ}-(α-glutamyl)lysine] did not initiate polymerization of amino acid *N*-carboxyanhydrides.

In Chart II is shown the structure of a naturally occurring polyglutamate (conjugate) of folic acid, pteroyl-γ-triglutamic acid (teropterin), and the structure of N^{α}-pteroyl-N^{ϵ}-(α-triglutamyl)lysine, a typical compound prepared in the present study. The natural conjugates of pteroic acid consist solely of glutamic acid linked exclusively as a γ-peptide. Synthetic conjugates of pteroic acid have been prepared in which the peptide chain is the same as that of the natural compounds[4,5] and in which the terminal amino acid varies.[11] Our synthetic conjugates are α-peptides and the sense of the peptide chain is reversed as compared to the natural substances. These compounds all possess a terminal amino group in contrast to the terminal carboxyl group found in nature. The synthetic polylysyl conjugates of N^{α}-pteroyllysine represent the first group of compounds related to folic acid that carries a net positive charge.

Biology. All of the derivatives of N^{α}-pteroyllysine and N^{α}-homopteroyllysine were reduced to their 7,8-dihydro forms by dithionite.[12] The dihydro form of each compound showed an absorbance spectrum very similar to that of 7,8-dihydrofolate (λ_{max} 282 nm, λ_{sh} 304 nm). The relative rates of reduction of these dihydro compounds by L1210 dihydrofolate reductase are shown in Table I, column 1. In our earlier study with the L1210 enzyme,[13] little selectivity was observed. The ready reduction of the natural γ-peptides (polyglutamates, negative side chains) had been previously reported with this[13] and other [8,14,15] dihydrofolate reductases, making it appear likely that the polyglutamate compounds prepared in this study would serve as good substrates for the L1210 enzyme. However, the good relative rates of reduction observed with the polylysyl compounds (positive side chains) were not anticipated. The charge reversal in the side chain does not prevent productive binding to the enzyme. This implies that the side chain extends into solution and shows little interaction with the protein.

The tetrahydro compounds (λ_{max} 300 nm) prepared by enzymatic reduction with dihydrofolate reductase were assayed for cofactor or inhibitor activity with *E. coli* thymidylate synthetase. The relative rates of tetrahydropteroyl compounds as cofactors of thymidylate synthetase are listed in Table I, column 2. The good cofactor activity of the smaller acylated tetrahydro-

Table I. Enzymatic Behavior of Compounds Prepared from N^{α}-Pteroyllysine and N^{α}-Homopteroyllysine

N^{α}-Pteroyl-	Rel rate[a]	Rel rate[b]	Rel inhibn[c]
Glutamate	1.0[a]	1.00[b]	
Lysine	1.1	0.40	
N^{ϵ}-(*tert*-Butyloxycarbonyl)-lysine	1.5	0.50	
Homoarginine	1.5	0.56	
N^{ϵ}-Acetyllysine	1.4	0.68	
N^{ϵ}-Chloroacetyllysine	1.5	0.70	
N^{ϵ}-(α-Glutamyl)lysine	1.5	0.51	
N^{ϵ}-(Di-α-glutamyl)lysine	1.5	0.55	
N^{ϵ}-(Tri-α-glutamyl)lysine	1.8	0.63	
N^{ϵ}-(Tetra-α-glutamyl)lysine	1.3	0.78	
N^{ϵ}-(Penta-α-glutamyl)lysine	1.0	0.88	
N^{ϵ}-Alanyllysine	1.1	0.44	
N^{ϵ}-(Dialanyl)lysine	1.1	0.36	
N^{ϵ}-Lysyllysine	1.2	0.32	
N^{ϵ}-(Di-α-lysyl)lysine	1.2	0.67	
N^{ϵ}-(Tri-α-lysyl)lysine	0.27	0.32	
N^{ϵ}-(Tetra-α-lysyl)lysine	0.57	0.15	
N^{ϵ}-[$N^{\epsilon'}$-(*tert*-Butyloxycarbonyl)-lysyl]lysine	1.6		
N^{α}-Homopteroyl-			
Glutamate	1.0[a]		1.00[c]
Lysine	0.52		0.05
N^{ϵ}-(*tert*-Butyloxycarbonyl)lysine	3.5		0.18
Homoarginine	1.0		0.08
N^{ϵ}-(α-Glutamyl)lysine	1.9		0.10
N^{ϵ}-(Di-α-glutamyl)lysine	2.4		0.23
N^{ϵ}-(Tri-α-glutamyl)lysine			0.26
N^{ϵ}-(Tetra-α-glutamyl)lysine	1.5		0.47
N^{ϵ}-Alanyllysine	1.1		0.08

[a] Reduction of dihydro compounds by murine leukemia L1210 dihydrofolate reductase. [b] Cofactor activity of tetrahydro compound with *E. coli* thymidylate synthetase. [c] Inhibition of *E. coli* thymidylate synthetase by tetrahydro compounds.

pteroyllysines was anticipated from results reported in the earlier study, in which the most stringent specificity requirements appeared to be directed to the vicinity of the α-carbon of the first amino acid bound in peptide linkage to tetrahydropteroic acid. We had expected that the tetrahydropteroyllysine polyglutamates might well mimic the naturally occurring γ-polyglutamates in their activity with thymidylate synthetase.[8,13] However, the good cofactor activity shown by tetrahydropteroyllysyl polylysines was not anticipated. As in the case of the L1210 reductase, a side chain carrying an opposite charge does not interfere with productive binding to thymidylate synthetase. The increase in substrate activity upon addition of polyglutamyl residues is similar to that observed with *Lactobacillus casei* thymidylate synthetase[8] although the side chains in the present study are "reversed sense α" compared to the natural γ-polyglutamates.

Since a limited amount of homopteroic acid was available for this study, a smaller number of peptides of N^{α}-homopteroyllysine were prepared. The data on relative inhibition of thymidylate synthetase are recorded in Table I, column 3. The low level of inhibition of this enzyme by tetrahydrohomopteroyllysine was improved by substitution on the N^{ϵ}-amino group. The N^{ϵ}-tetraglutamyl derivative was about one-half as inhibitory as tetrahydrohomofolate. The K_i for tetrahydrohomofolate is 2.8×10^{-7} M for *E. coli* thymidylate synthetase.[13] In a related study we have shown that the mono-, di-, and triglutamyl derivatives of N^{α}-tetrahydropteroyllysine serve as good cofactors for porcine liver transformylase.[7] The N^{5}-formyl-N^{α}-tetrahydropteroyllysine mono-, di-, and triglutamates so prepared were only slightly more inhibitory to *E. coli* thymidylate synthetase than 5-formyltetrahydrofolate, in contrast to 5-formyltetrahydropteroyl γ-hexaglutamate which was at least a 200-fold more potent inhibitor of *E. coli* thymidylate synthetase than was the monoglutamate.

Conclusions

The polyglutamyl or polylysyl derivatives of N^{α}-pteroyllysine or N^{α}-homopteroylysine may be prepared by polymerization of amino acid *N*-carboxyanhydrides. The peptides obtained are attached to the N^{ϵ}-amino group of lysine. The 7,8-dihydro forms of these compounds are substrates for murine leukemia L1210 dihydrofolate reductase. The tetrahydro compounds prepared by enzymatic reduction are substrates or inhibitors of *E. coli* thymidylate synthetase. The change in polarity from the negative polyglutamyl to the positive polylysyl side chain does not result in large differences in cofactor activity with either enzyme.

Experimental Section

Synthesis of N^{α}-Pteroyllysine and N^{α}-Homopteroyllysine. N^{α}-pteroyl-N^{ϵ}-(*tert*-butyloxycarbonyl)lysine was prepared as previously described.[13] The *tert*-butyloxycarbonyl group was removed with trifluoroacetic acid. Anhydrous trifluoroacetic acid (25 ml) was added to 250 mg of N^{α}-pteroyl-N^{ϵ}-(*tert*-butyloxycarbonyl)lysine. The solid dissolved and was stirred at 25–30 °C for 1 h. The solvent was evaporated in vacuo and the residue triturated with anhydrous ether. The solid was washed with 25–50-ml portions of ether while protected from bright light. Finally the solid was filtered and dried to give 247 mg of N^{α}-pteroyllysine which still contained traces of trifluoroacetic acid. In a similar manner, N^{α}-homopteroyllysine was prepared from its corresponding N^{ϵ}-*tert*-butyloxycarbonyl derivative. Each compound traveled as a single uv-absorbing spot on paper chromatography.

Synthesis of N^{α}-Pteroylhomoarginine and N^{α}-Homopteroylhomoarginine. Water (5 ml), 20 mg of N^{α}-pteroyllysine, and 172 mg of *O*-methylisourea sulfate were adjusted to pH 10 with 1 N sodium hydroxide solution. The mixture, after standing overnight at ambient temperature, was centrifuged to remove a white solid. The yellow supernatant was adjusted to pH 3 with 12 N hydrochloric acid. The solid that separated was washed twice by suspension and centrifugation with 10-ml portions of water. The solid, which traveled as a single uv-absorbing spot on paper chromatography, gave a positive Sakaguchi test for monosubstituted guanidines. The test was negative with N^{α}-pteroyllysine. In a similar manner, N^{α}-homopteroyllysine was converted to N^{α}-homopteroylhomoarginine. The product, which traveled as a single uv-absorbing spot on paper chromatography, gave a positive Sakaguchi test, while N^{α}-homopteroyllysine did not.

Synthesis of N^{α}-Pteroyl-N^{ϵ}-acetyllysine and N^{α}-Pteroyl-N^{ϵ}-chloroacetyllysine. Sodium hydroxide solution (10 ml, 0.1 N) was added to 50 mg of N^{α}-pteroyllysine and the solution cooled to 0 °C. Sodium hydroxide solution (1 ml, 10 N) and 0.60 ml of acetic anhydride were added. The mixture was stirred at 0 °C for 30 min. The solution was adjusted to pH 3 with 12 N hydrochloric acid and the solid was separated by centrifugation with two 10-ml portions of water. Similarly, N^{α}-pteroyl-N^{ϵ}-chloroacetyllysine was prepared using 975 mg of chloroacetic anhydride in place of acetic anhydride. Each compound traveled as a single uv-absorbing spot on paper chromatography.

Preparation of *N*-Carboxyanhydrides. The method of Fuchs–Farthing[16] was used to prepare these compounds. The appropriate amino acid (5 g) was suspended in 150 ml of dioxane. Phosgene was passed through the mixture at ice bath temperature for 30 min. The suspension was stirred at ambient temperature for 1 h and evaporated. The residue was extracted with ethyl acetate and the *N*-carboxyanhydride was precipitated by addition of petroleum ether (bp 30–60 °C). The solid was filtered, washed with petroleum ether, and dried. A yield of 70% was obtained for γ-ethyl glutamate *N*-carboxyanhydride (mp 73 °C),[10] 42% for N^{ϵ}-carbobenzoxylysine N^{α}-carboxyanhydride (mp 98 °C),[10] and 34% for N^{ϵ}-(*tert*-butyloxycarbonyl)lysine N^{α}-carboxyanhydride (mp 134 °C). The *N*-carboxyanhydride of alanine[10] and the N^{α}-carboxyanhydride of N^{ϵ}-carbethoxylysine were

Table II. Analytical Data Obtained for Compounds Prepared from N^{α}-Pteroyllysine and N^{α}-Homopteroyllysine

N^{α}-Pteroyl-	R_{FA}[a]	Amino acid analyses		
		Glu[b]	Ala[b]	Lys[c]
Glutamate	1.00			
Lysine	0.50			
N^{ϵ}-(*tert*-Butyloxycarbonyl)lysine	0.92			1.5
Homoarginine	0.32			
N^{ϵ}-Acetyllysine	0.84			
N^{ϵ}-Chloroacetyllysine	0.79			
N^{ϵ}-(α-Glutamyl)lysine	0.79	1.0		
N^{ϵ}-(Di-α-glutamyl)lysine	1.04	2.0		
N^{ϵ}-(Tri-α-glutamyl)lysine	1.24	3.1		
N^{ϵ}-(Tetra-α-glutamyl)lysine	1.44	4.3		
N^{ϵ}-(Penta-α-glutamyl)lysine	1.45	5.6		
N^{ϵ}-Alanyllysine	0.83		1.4	
N^{ϵ}-(Dialanyl)lysine	0.99		2.6	
N^{ϵ}-Lysyllysine	0.77			1.9
N^{ϵ}-(Di-α-lysyl)lysine	0.94			
N^{ϵ}-(Tri-α-lysyl)lysine	1.07			
N^{ϵ}-(Tetra-α-lysyl)lysine	1.20			
N^{ϵ}-[$N^{\epsilon'}$-(*tert*-Butyloxycarbonyl)lysyl]lysine	0.95			
N^{α}-Homopteroyl-				
Glutamate	1.27			
Lysine	0.83			
N^{ϵ}-(*tert*-Butyloxycarbonyl)lysine	1.10			
Homoarginine	0.61			
N^{ϵ}-(α-Glutamyl)lysine	1.05	1.2		
N^{ϵ}-(Di-α-glutamyl)lysine	1.26	1.7		
N^{ϵ}-(Tri-α-glutamyl)lysine	1.34	3.0		
N^{ϵ}-(Tetra-α-glutamyl)lysine	1.38	4.4		
N^{ϵ}-Alanyllysine	1.16		1.1	

[a] Paper chromatography, Whatman No. 1 paper, 0.10 N ammonium bicarbonate, ascending; ratio of distance traveled by compound to that traveled by folic acid. [b] Value obtained for amino acid other than lysine which served as internal standard. [c] Value obtained from comparable OD_{280} absorption using N^{α}-pteroyllysine standard.

obtained as noncrystalline solids and were not further purified. As noted[10] a high degree of purity of the *N*-carboxyanhydrides is not necessary for the preparation of short peptide chains.

Reaction of N^{α}-Pteroyllysine and N^{α}-Homopteroyllysine with Amino Acid *N*-Carboxyanhydrides in Aqueous Dimethylformamide. DMF (20 ml), 5 ml of water, and 100 mg of N^{α}-pteroyllysine were mixed and adjusted to pH 11 with 1 N sodium hydroxide solution. Then a fivefold excess of the appropriate amino acid *N*-carboxyanhydride in 1–2 ml of DMF was added. The solution was stirred overnight at 25–30 °C. Sodium hydroxide solution (100 ml, 0.2 N) was added and the mixture heated at 100 °C (under nitrogen) for 30 min. The solution was cooled to 20 °C, filtered, and adjusted to pH 3–5.5 as required (see below). The solid that separated was centrifuged and washed twice by suspension and centrifugation with 10 ml of water. Several drops of ammonia were added to the gelatinous solid, giving a solution which was applied to Whatman No. 1 paper (about 1 drop/cm) and chromatographed in 0.1 N ammonium bicarbonate solution. The papers were dried and the strips cut out and eluted with 3 N aqueous ammonia. The solutions were evaporated and the free compounds precipitated by careful adjustment of pH. For the polyglutamyl and polyalanyl compounds, the optimum value was 3–4; for the polylysyl compounds precipitation was usually better at about 4–5.5. In every case it was important that the volume of water in which the ammonium salt was dissolved was kept at a minimum.

Polymerization in Anhydrous Dimethylformamide. Trifluoroacetic acid (10 ml) and 100 mg of N^{α}-(N^{2}-acetyl-N^{10}-trifluoroacetylpteroyl)-N^{ϵ}-(*tert*-butyloxycarbonyl)lysine methyl ester[13] were stirred at ambient temperature 1 h. The solution was evaporated to dryness in vacuo. The solid was washed thoroughly with ether, filtered, and dried. The solid was then dissolved in 10 ml of DMF and poured through a column (1.3 × 20 cm) containing 2 g of DEAE-cellulose in DMF. The compound was eluted with DMF and the eluate evaporated. The residue was taken up in 10 ml of DMF and a fivefold excess of the appropriate amino acid *N*-carboxyanhydride in 2 ml of DMF was added. The solution was stirred at ambient temperature overnight. The DMF was removed in vacuo and 50 ml (0.2 N) of sodium hydroxide solution was added to the residue. The mixture was heated at 100 °C (under nitrogen) for 30 min, cooled to 20 °C, and adjusted to the appropriate pH with 12 N hydrochloric acid. The solid that separated was centrifuged and washed twice by suspension and centrifugation with 10-ml portions of water. The peptides were separated as previously described. The chromatographic behavior of the compounds prepared by these procedures is described in Table II, column 1.

Amino Acid Analyses. Each compound was analyzed for amino acid content. In a small (6 × 50 mm) test tube was placed 50 μl of a 10 mg/ml solution of compound to be analyzed, followed by 50 μl of 12 N hydrochloric acid. Controls containing the appropriate amino acids and a water blank were carried through the same procedure. The tubes were sealed and autoclaved at 110 °C for 4 h. The tubes were opened and the mixture was evaporated in vacuo; the residue was neutralized with 0.1 N sodium bicarbonate solution and evaporated to dryness. Water (50 μl) was added to each tube, and after mixing and centrifugation, 2 μl of each supernatant was spotted on a strip of Whatman No. 1 paper which had previously been dipped in 0.02 N Tris buffer, pH 7.4. Electrophoresis at 1000 V for 30 min separated the amino acids. The paper was dried and dipped in 0.5% ninhydrin in ethanol. The electrophoretogram was developed at 60 °C for 30 min in a moist atmosphere. The spots were eluted with 1.5 ml of 75% ethanol containing 7.5 mg of copper sulfate. The quantity of each amino acid was determined from the optical density of the eluate at 520 nm. The values obtained by this procedure are listed in Table II, column 2.

Enzymes and Enzyme Assays. All derivatives of N^{α}-pteroyllysine and N^{α}-homopteroyllysine were reduced to the dihydro form with sodium dithionite in aqueous mercaptoethanol.[13] Each dihydro compound showed λ_{max} 282 nm, λ_{sh} 304 nm. The enzymatic reductions to tetrahydro compounds by TPNH were carried out with murine leukemia L1210 dihydrofolate reductase as previously described.[13] Each tetrahydro compound (λ_{max} 300 nm) was then tested for cofactor or inhibitor activity with *E. coli* thymidylate synthetase. The determination of relative rates with both enzymes and of relative inhibition of thymidylate synthetase by tetrahydrohomopteroyl compounds is described in detail in an earlier paper.[13]

Acknowledgment. We thank Dr. R. L. Kisliuk for his interest and encouragement during the course of this work.

References and Notes

(1) Presented in part at the 152nd National Meeting of the American Chemical Society, New York, N.Y., Sept 13, 1966.
(2) Supported by U.S. Public Health Service Grants CA 05997 and CA 11449.
(3) L. T. Plante, E. J. Crawford, and M. Friedkin, Abstracts of the 152nd National Meeting of the American Chemical Society, New York, N.Y., 1966, CO56.
(4) C. L. Krumdieck and C. M. Baugh, *Biochemistry*, 8, 1568 (1969).
(5) H. A. Godwin, I. H. Rosenberg, C. R. Ferenz, P. M. Jacobs, and J. Meienhofer, *J. Biol. Chem.*, **247**, 2266 (1972).
(6) J. H. Boothe, J. H. Mowat, B. L. Hutchings, R. B. Angier, C. W. Waller, E. L. R. Stokstad, J. Semb, A. L. Gazzola, and Y. Subbarow, *J. Am. Chem. Soc.*, **70**, 1099 (1948).
(7) M. Friedkin, L. T. Plante, E. J. Crawford, and M. Crumm, *J. Biol. Chem.*, **250**, 5614 (1975).
(8) R. L. Kisliuk, Y. Gaumont, and C. M. Baugh, *J. Biol. Chem.*, **249**, 4100 (1974).
(9) L. M. Kozloff, M. Lute, and C. M. Baugh, *J. Virol.*, **11**, 637 (1973).
(10) E. Katachalski and M. Sela, *Adv. Protein Chem.*, **13**, 243 (1958).
(11) C. M. Baugh, J. C. Stevens, and C. L. Krumdieck, *Biochim.*

Biophys. Acta, **212**, 116 (1970).
(12) M. Friedkin, E. J. Crawford, and D. K. Misra, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **21**, 176 (1962).
(13) L. T. Plante, E. J. Crawford, and M. Friedkin, *J. Biol. Chem.*, **242**, 1466 (1967).
(14) J. R. Bertino, J. P. Perkins, and D. G. Johns, *Biochemistry*, **4**, 839 (1965).
(15) J. K. Coward, K. N. Parameswaran, A. R. Cashmore, and J. R. Bertino, *Biochemistry*, **13**, 3899 (1974).
(16) A. C. Farthing, *J. Chem. Soc.*, 3213 (1950).

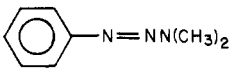
Use of Cluster Analysis in the Development of Structure–Activity Relations for Antitumor Triazenes

W. J. Dunn, III,* M. J. Greenberg, and Soledad S. Callejas

Department of Medicinal Chemistry, College of Pharmacy, University of Illinois at the Medical Center, Chicago, Illinois 60680. *Received January 12, 1976*

A series of antitumor triazenes in which the members of the series are physicochemically distinct was designed using the cluster analysis approach as proposed by Hansch and his co-workers. The series that resulted was tested against Sarcoma 180 in the mouse and the antitumor activities were analyzed using regression techniques. The structure–activity relations that resulted are discussed in terms of proposed mechanisms of action.

1-Phenyl-3,3-dimethyltriazene (I) was reported in 1955 to be active against Sarcoma 180 in the mouse.[1] More recent studies by Lin et al.[2] showed that the 2-, 3-, and 4-$CONH_2$ and 2-, 3-, and 4-CO_2CH_3 analogues of I were active against L1210 mouse leukemia. Also, a number of analogues of I have been reported by Shealy et al.[3] to be active against L1210.



I

While a number of reports of the antitumor activity of analogues of I have appeared, there have been no reports of the systematic design of a series of I on which quantitative structure–activity studies have been done.

We recently undertook such a study which led to the preparation and testing of seven analogues (compounds 1–4, 6, 7, and 14, Table I). Attempts to derive quantitative relationships from these data yielded ambiguous results due to considerable interrelationships between the physicochemically derived independent variables considered. At this time, we became aware of the work of Hansch et al.[4] in which they proposed that cluster analysis could be used in the design of series such as ours. The result is a series in which the physicochemical properties of each analogue are unique and the chance of collinearity between these variables is minimized. This report deals with our results of the use of cluster analysis in the design of antitumor derivatives of the lead substance I.

Cluster Analysis and Its Application. Hansch et al.[4] selected 90 substituent groups and applied to their physicochemical constants, heirarchial clustering to factor the groups according to their properties. These properties are in terms of π, the Hansch constant, F and R, the field and resonance constants, respectively, molar refractivity (MR), and molecular weight (MW). Four sets (sets 1–4 in their report) were treated in this way with their set 1 factored according to the parameters π^2, π, F, R, MR, and MW, set 2 according to the parameters π^2, π, F, and R, set 3 according to π, F, R, and MR, and set 4 according to π, F, and R. Within each set four levels of factorization were carried out. In set 2, for example, the lowest level produced from the 90 substituents five clusters, the next highest gave 10 clusters, the next 20, and the last 60 clusters. The higher the level of factorization the less forcing occurs on clustering so the more distinction is obtained.

For this study we have selected for investigation the dependence of activity on π^2, π, F, R, MR, and MW. This requires set 1 of Hansch's clusters. We selected the 10 level of this set and from this 14 analogues resulted. We considered only monosubstituted 3- and 4-substituted triazenes and assumed the 3-substituted and 4-substituted analogues to be a single series. This assumption is based on the data reported by Lin et al.[2] These workers found that the placing of the same substituent in the 3 or 4 position had little, if any, effect on the level of antitumor activity.

The analogues designed and prepared are given in Table I. Also included in this table are the biological data and physicochemical substituent constants. Table II summarizes the physical data of the triazenes synthesized in this study and Table III is a correlation matrix of the groups and their variables.

Experimental Section

Synthesis. The majority of the triazenes in this study were prepared by coupling the appropriate aryldiazonium cation with dimethylamine according to the procedure of Lin et al.[2] This procedure could not be used for the preparation of *p*-(3,3-dimethyl-1-triazeno)cinnamic acid. In this case the aryldiazonium cation was isolated as the stable fluoborate salt and this species was coupled with dimethylamine according to the procedure of Kolar.[5] The results are given in Table III and the melting points reported are uncorrected. Structures and purity were verified by thin-layer chromatography, mass spectra, and infrared and ^1H NMR spectroscopy.

Biological Testing. The triazenes were tested for activity against Sarcoma 180 ascitic tumor in the mouse using a modification of the procedure of Sartorelli.[6] Donor mice were sacrificed by asphyxiation with chloroform, followed by removal of the top layer of skin of the intraperitoneal cavity, and fluid was removed. A threefold dilution of this fluid was made with sterile saline and 0.1 ml (approximately 2×10^6 cells) was injected into 15–18-g female albino Swiss mice. Each test group was composed of six mice and a control of six mice was determined each time a test was made.

The triazenes were suspended in sterile saline with Tween 20 and administered on alternate sides of the intraperitoneal cavity 24 h after implantation. This was repeated daily for 3 days. Initial dose levels were set at 0.25, 0.38, and 0.50 of the LD_{50} for that particular drug. Other dosage levels were set depending on initial activity and toxicity as determined by weight loss. The test and control groups were observed daily and the day of death was recorded. A dose–response curve was generated from which the log dose (mg/kg) required to give T/C (%) = 130 was obtained. The error in such a determination can be as high as 22% as