[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, BRANDEIS UNIVERSITY]

Synthesis of Derivatives of Glutamine as Model Substrates for Anti-tumor Agents[1]

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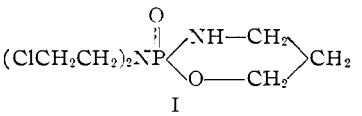
Five amide-N-substituted derivatives of DL-glutamine have been prepared for biological study by treatment with hydrazine of the products formed by reaction of N,N-phthaloylglutamic anhydride with aniline, N-methylaniline, ethanolamine, diethylamine and butylamine, respectively. The infrared absorption spectra of these compounds and the corresponding phthalimido intermediates are discussed.

In order to take advantage of amino acids as biological "carriers" by which specific toxic residues might be introduced into cellular metabolism, we synthesized a series of N-iodoacetyl- and N-fluoroacetyl-substituted amino acids. Although insignificantly cytotoxic against cell systems *in vitro*, some of the fluoroacetyl compounds have shown significant anti-tumor activity in studies in mice[2] and, in fact, two derivatives, N-fluoroacetyl-L-leucine and N-fluoroacetyl-DL-phenylalanine, of three on which results are available have passed a three sequence test for activity.[3] A number of cytotoxic derivatives of amino acids in the form of alkylating agents also have been used.[4] It is of interest that one of the most active of these as an anti-tumor agent, *p*-di-(2-chloroethyl)-aminophenylalanine, is much more potent in the form of the natural L-isomer than it is in the D-form.[4]

Other derivatives of the alkylating agents of possible interest in cancer, the phosphoroamide nitrogen mustards, depend on the action of an enzyme for activation. Administered in this way, a much higher dose of a nitrogen mustard might be delivered to the cells of a tumor with phosphamidase activity[5] than would reach these cells if the parent nitrogen mustard was administered as such.[6a-f] Recently a phosphoroamide nitrogen mustard I, the "transport" form of bis-β-chloroethylamine that would presumably be converted to the "active" form at the site of action, has been found inhibitory against a number of tumors in the rat.[7] It showed a high therapeutic index against experimental tumors in animals and positive results against some malignant tumors in humans



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are claimed,[8] although the nitrogen mustard from which this compound IV is derived, bis-β-chloroethylamine, has little cytotoxicity. Other secondary nitrogen mustards that appear better suited to this purpose have been developed.[5,6a,c,f] These transform spontaneously by intramolecular cyclization to potent tertiary nitrogen mustards that are inherently cytotoxic.

Peptide nitrogen mustards would be another class of derivatives of the alkylating agents of the same type which, in addition, are derived from possible biological carriers. N-Acylated nitrogen mustards, biologically inert as alkylating agents because of loss of basicity on nitrogen, would be activated by hydrolytic enzymes. Although it is not known to what extent the biological action of these derivatives against tumors might depend on such cellular variables as requirement for and/or active transport of amino acids, enzyme content and inherent sensitivity to nitrogen mustards, it should be noted that such dependence might result in selective inhibition and the larger the number of variables involved the greater the potential selectivity of action. Peptide nitrogen mustards would appear to be a class of compounds worth investigation for possible anti-tumor properties.

Glutamine, the γ-amide of glutamic acid, is a natural prototype for a class of peptide nitrogen mustards. It is an important constituent of protein that also plays a central role in nitrogen metabolism of mammalian tissues. Moreover, there are suggestions that this amino acid has some special function in the metabolism of malignant cells[9a-b]; and glutaminase activity has been reported to be six times as high in hepatoma as in liver of the rat.[10] Substitution of the amide-NH_2 group by appropriate nitrogen mustard residues would give analogs with latent cytotoxic action possibly selective for tumors. Little is known, however, of the metabolism of amide-nitrogen-substituted glutamines although γ-glutamylhydroxamic acid and γ-glutamyl hydrazide have been studied.[11] In order to delineate the structural requirements of substrates for glutaminase and to assess the distribution of glutaminase activity in malignant and non-malignant tissues with substrates that would serve as models for possible anti-tumor glutamyl nitrogen mustards, we have synthesized these five mono- and di-substituted-derivatives of glutamine:

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(2) To be published with G. E. Foley.

(3) Unpublished data from the Cancer Chemotherapy National Service Centre.

(4) For references see summary article by F. Bergel, *Ann. N. Y. Acad. Sci.*, **68**, 1238 (1958). See also M. I. Ishidate, Y. Sakurai and M. Izumi, *J. Am. Pharm. Assoc.*, **44**, 132 (1955); M. Izumi, *Pharm. Bull. (Japan)*, **2**, 275, 279 (1954); report by W. L. Nyalan and H. Busch, see *Chem. & Eng. News*, 46 (May 5, 1958), for studies with N,N-di-(β-chloroethyl) derivative of glycine and glutamic acid.

(5) E. Boger and O. M. Friedman, THIS JOURNAL, **80**, 2583 (1958).

(6) (a) O. M. Friedman and A. M. Seligman, *ibid.*, **70**, 3082 (1948); (b) **76**, 655 (1954); (c) **76**, 658 (1954); (d) A. M. Seligman, M. M. Nachlas, L. H. Manheimer, O. M. Friedman and G. Wolf, *Ann. Surg.*, **130**, 333 (1949); (e) A. M. Seligman, M. Milden and O. M. Friedman, *Cancer*, **2**, 701 (1949); (f) A. M. Rutenburg, L. Persky, O. M. Friedman and A. M. Seligman, *J. Pharm. Exptl. Therap.*, III, 483 (1954);

(7) H. Arnold, F. Bourseaux and N. Brock, *Naturwiss.*, **45**, 64 (1958).

(8) R. Gross and K. Lambers, *ibid.*, **45**, 66 (1958).

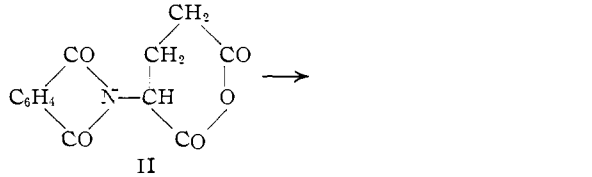
(9) (a) H. Eagle, L. Levintow and K. A. Piez, *J. Biol. Chem.*, **227**, 929 (1957), and references cited therein. (b) E. Roberts, *et al.*, *Cancer Research*, **9**, 231, 350, 645 (1949).

(10) M. Errera and J. P. Greenstein, *J. Natl. Cancer Inst.*, **7**, 285 (1947).

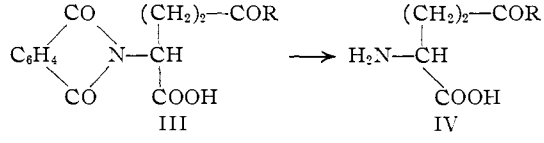
(11) A. Meister, *J. Biol. Chem.*, **210**, 17 (1954).

N-(DL-γ-glutamyl)-diethylamine (IVa), N-(DL-γ-glutamyl)-aniline (IVb), N-(DL-γ-glutamyl)-N-methylaniline (IVc), N-(DL-γ-glutamyl)-2-aminoethanol, (IVd), N-(DL-γ-glutamyl)-butylamine (IVe), and report on them here. These are being studied in microbiological and tissue culture systems and in mammalian tissues in collaboration with others elsewhere.[12]

The five derivatives were prepared by reaction of N,N-phthaloyl glutamic anhydride (II), with diethylamine, aniline, N-methylaniline, ethanolamine and *n*-butylamine, respectively, according to the method developed independently by King, *et al.*,[13a] and Sheehan, *et al.*,[13b] to give the corresponding N,N-phthaloyl-γ-glutamyl amides, IIIa to IIIe. The corresponding γ-peptides, IVa to IVe, were prepared by removal of the phthaloyl residue with hydrazine by an adaptation of the method originally developed by Ing and Manske.[14]



II



III IV

IIIa, R = $-N(C_2H_5)_2$ IVa, R = $-N(C_2H_5)_2$
IIIb, R = $-NHC_6H_5$ IVb, R = $-NHC_6H_5$
IIIc, R = $-N(CH_3)-C_6H_5$ IVc, R = $-N(CH_3)-C_6H_5$
IIId, R = $-NHCH_2CH_2OH$ IVd, R = $-NHCH_2CH_2OH$
IIIe, R = $-NH(CH_2)_3CH_3$ IVe, R = $-NH(CH_2)_3CH_3$

The phthaloyl derivatives, IIIa to IIIe, were heated with one molar equivalent of hydrazine in alcohol. The products IVa to IVe were separated in all cases from the insoluble by-product, phthaloyl hydrazide, by extraction of the residues obtained by evaporation of the reaction mixtures with water or dilute acetic acid. The procedure of King and Kidd,[13a] in which hydrazine was used in alkaline medium and the phthaloyl hydrazide that formed was precipitated with acid, was unsatisfactory for it gave impure products in poor yield.

N,N-Phthaloyl-(DL-γ-glutamyl)-aniline (IIIb) as the monohydrate, m.p. 106–107°, previously was reported[13a] from the reaction of the anhydride II with aniline; and as the monohydrate, m.p. 108–109°, from the reaction of the corresponding hydrazide with aniline,[15] when these products were recrystallized from water. Our product without water melted at 177–178°.

(12) Dr. George E. Foley, Children's Cancer Research Foundation, Boston, Mass., and Dr. Alexander M. Rutenburg, Beth Israel Hospital, Boston, Mass. Studies by Dr. Rutenburg to date with the phenylglutamine derivative IVb that will be published elsewhere have revealed that it undergoes rapid enzymatic hydrolysis by certain animal tissue homogenates, particularly by kidney and pancreas, and, in fact, appears to be an excellent reagent for the assay of glutaminases.

(13) (a) F. E. King and D. A. D. Kidd, *Nature*, **162**, 776 (1948); *J. Chem. Soc.*, 3315 (1949). (b) J. C. Sheehan and V. S. Frank, THIS JOURNAL, **71**, 1856 (1949).

(14) H. R. Ing and R. H. F. Manske, *J. Chem. Soc.*, 2348 (1926).

(15) F. E. King, B. S. Jackson and D. A. A. Kidd, *ibid.*, 243 (1951).

The results reported here confirm the observation[13] that the anhydride II opens to give γ-glutamyl derivatives essentially exclusively. N-(DL-γ-Glutamyl)-2-aminoethanol (IV) reported here, for example, proved to be identical by mixed melting point and in infrared spectrum with a sample kindly provided by Dr. N. Lichtenstein who had previously prepared it by an unambiguous synthesis from DL-pyrrolidone carboxylic acid with ethanolamine.[16] Furthermore, the five glutamyl peptides, IVa to IVe, showed absorption in the infrared at 1320–1360 cm.$^{-1}$, 1400–1420 cm.$^{-1}$, 1580–1610 cm.$^{-1}$ characteristic of ionic carboxyl group[17] and gave a positive ninhydrin reaction.[18] All our products were optically inactive as would be expected from the racemic character of the anhydride II. However, the synthesis of N-(D-γ-glutamyl)-aniline, m.p. 209°,[19] and of the L-isomer, m.p. 193–194°,[20] has been claimed although no specific proof of the structure or rotation of the L-isomer was given. The DL-racemate IVb reported here melted at 208–209°. In addition N-(L-γ-glutamyl)-butylamine had been prepared by treatment of L-pyrrolidone carboxylic acid with butylamine.[21] The infrared spectrum of a sample of the latter, kindly provided by Dr. N. Lichtenstein, in solution in solid KBr was identical with the DL-racemate IVe reported here.

The infrared spectrum[22] of the N,N-phthaloyl glutamic anhydride II showed maxima at 1815 and 1770 cm.$^{-1}$ characteristic of glutamic anhydride.[23] Strong bands at 1710–1715 and 718–720 cm.$^{-1}$ are also characteristic of the phthalimido residue.[24]

The infrared spectra of the phthalimido derivatives IIIa to IIIc showed characteristic maxima at 1710–1720 and 715–720 cm.$^{-1}$ for phthaloyl group but no band at 1815 cm.$^{-1}$, thereby indicating that opening of the anhydride ring had occurred. The removal of phthaloyl group from compounds IIIa to IIIe was readily apparent by the disappearance of the bands at 1775, 1710–1720 and 715–720 cm.$^{-1}$. The amide and the carbonyl functions in both phthalimido compounds IIIa to IIIe and in the glutamyl peptides IVa to IVe gave absorption in the expected regions.[25]

Experimental[26]

N,N-Phthaloyl-(DL-γ-glutamyl)-diethylamine (IIIa).—To the cold solution of 10.36 g. (0.04 mole) of N,N-phthal-

(16) N. Lichtenstein, H. E. Ross and P. P. Cohen, *J. Biol. Chem.*, **201**, 117 (1953).

(17) R. E. Richard and H. W. Thompson, *J. Chem. Soc.*, 1248 (1957); L. J. Bellamy, "The Infrared Spectroscopy of Organic Molecules," John Wiley and Sons, Inc., New York, N. Y., 1956, p. 139.

(18) J. M. Retinger, THIS JOURNAL, **39**, 1059 (1917).

(19) W. Voss and R. Guttmann, *Z. physiol. Chem.*, **204**, 1 (1932); *C. A.*, **26**, 2707 (1932).

(20) O. K. Behrens and M. Bergmann, *J. Biol. Chem.*, **129**, 587 (1939).

(21) N. Lichtenstein and N. Grossowiez, *ibid.*, **171**, 386 (1947).

(22) R. Stuart Tipson, *J. Org. Chem.*, **21**, 1353 (1951). The spectrum was taken in Nujol.

(23) H. H. Wasserman and H. E. Zimmerman, THIS JOURNAL, **72**, 5787 (1950), showed that glutaric anhydride gives two specific bands at 5.55 and 5.68 μ. The slight shift of 10 cm.$^{-1}$ to higher wave lengths probably is due to the phthalimido residue on the α-carbon atom.

(24) The infrared spectrum of potassium phthalimide shows strong absorption bands at 1710 and 717 cm.$^{-1}$.

(25) *Vide* Experimental.

(26) Melting points are uncorrected. Analyses were by Dr. S. M. Nagy and his associates, Microchemical Lab., M.I.T. Infrared spec-

oyl-DL-glutamic anhydride[13] (II) in 45 ml. of dry dioxane was added dropwise with shaking 4.5 ml. (0.044 mole) of freshly distilled diethylamine in 5 ml. of dry dioxane. The yellow solution was refluxed for 2 hr. After removal of the volatile material under reduced pressure, the liquid residue was dissolved in a minimum quantity of ethanol. The addition of an excess of water precipitated a semi-solid mass which on scratching with a glass rod completely solidified. The crude solid, 11.5 g., (88%), m.p. 132–133°, was crystallized from aqueous ethanol and after two recrystallizations afforded pure product, m.p. 135–136°; infrared maxima: 2.9, 3.4–3.5, 5.68, 5.80, 6.25, 7.2, 8.2, 11.1, 13.95 μ.

Anal. Calcd. for $C_{17}H_{20}N_2O_5$: N, 8.43. Found: N, 8.45.

N-(DL-γ-Glutamyl)-diethylamine (IVa).—A solution of 1.1 ml. (0.022 mole) of anhydrous hydrazine hydrate (100%) in 10 ml. of absolute ethanol was added to the solution of 6.64 g. (0.02 mole) of IIIa in 20 ml. of absolute ethanol and the mixture was refluxed for 2 hr. The solid residue, obtained after evaporation of alcohol under reduced pressure, was boiled with 100 ml. of water for 5 minutes, cooled to room temperature and filtered. The clear aqueous solution was evaporated *in vacuo*. The residue was dissolved in 10 ml. of ethanol, and then 50 ml. of acetone was added to precipitate the amino acid as a semi-solid mass which solidified in the refrigerator overnight. The solid was filtered, 3.2 g. (80%), m.p. 165–167°. Recrystallization from ethanol–acetone furnished pure compound, m.p. 167–168°; infrared maxima: 2.95, 3.38–3.44, 6.1, 6.25, 6.45, 6.7, 7.1, 7.4 μ.

Anal. Calcd. for $C_9H_{18}N_2O_3$: C, 53.49; H, 8.95; N, 13.86. Found: C, 52.89; H, 9.00; N, 13.64.

N,N-Phthaloyl-(DL-γ-glutamyl)-aniline (IIIb).—To the cold solution of 2.59 g. (0.01 mole) of II in 20 ml. of dry dioxane was added dropwise a solution of 1.0 ml. (0.011 mole) of distilled aniline in 5 ml. of dry dioxane. The solution was refluxed for 1.5 hours. Cyclohexane was added dropwise to the cold reaction mixture until the solution appeared turbid. The solution was kept for two days in the refrigerator, when buff-colored solid, 3.1 g. (88%), m.p. 165–167°, separated. Recrystallization from ethanol–cyclohexane afforded pure compound, m.p. 177–178°; infrared maxima: 3.0, 5.68, 5.8–5.85, 6.05, 6.25, 6.45, 7.2, 8.3, 11.05, 13.9 μ.

Anal. Calcd. for $C_{19}H_{16}N_2O_5$: N, 7.95. Found: N, 7.85.

N-(DL-γ-Glutamyl)-aniline (IVb).—A mixture of a solution of 14.08 g. (0.04 mole) of IIIb in 100 ml. of absolute ethanol and 2.2 ml. (0.044 mole) of anhydrous hydrazine hydrate (100%) was refluxed for 2.5 hr. After removal of the solvent under reduced pressure, 14 g. of solid, containing phthalhydrazide and the free amino compound IVb, was obtained. The solid then was boiled with 400 ml. of 30% aqueous solution of acetic acid for ten minutes, cooled to room temperature and filtered. The residue thus obtained was digested again with the same volume of 30% aqueous acetic acid for 10 minutes, cooled to room temperature and filtered. The filtrates were combined and evaporated *in vacuo* when 7.6 g. (85.4%) of compound IVb, m.p. 204–205°, was obtained. Two crystallizations from 60% aqueous ethanol afforded pure product, m.p. 208–209°. The solubilities of the glutamyl compound in hot water and hot alcohol are of the same order as those of phthalhydrazide but the removal of the latter from the former was very efficiently done with 30% aqueous acetic acid; infrared maxima: 3.05, 3.38–3.44, 6.01, 6.30, 6.47, 6.9, 7.05, 7.4 μ.

Anal. Calcd. for $C_{11}H_{14}N_2O_3$: C, 59.45; H, 6.36; N, 12.61. Found: C, 59.17; H, 6.34; N, 12.74.

N,N-Phthaloyl-(DL-γ-glutamyl)-N-methylaniline (IIIc).—To the cold solution of 5.18 g. (0.02 mole) the anhydride II in 25 ml. of dry dioxane was added a solution of 2.4 ml. (0.022 mole) of freshly distilled N-methylaniline in 10 ml. of dry dioxane with shaking. The greenish yellow reaction mixture was refluxed for 1 hr., cooled and the solvent was removed under reduced pressure. The semi-solid residue was then dissolved in a minimum quantity of ethanol and to the solution water was added dropwise until turbidity appeared. On cooling in an icebox for several hours 6.5 g. (90%) of IIIc, m.p. 180–181°, separated as needle-shaped crystals. Two crystallizations from aqueous ethanol produced pure compound, m.p. 185–186°; the infrared maxima: 2.9, 5.68, 5.85, 6.1, 6.7, 7.2, 8.3, 10.65, 13.9 μ.

Anal. Calcd. for $C_{20}H_{18}N_2O_5$: C, 65.57; H, 4.91. Found: C, 65.54; H, 5.06.

N-(DL-γ-Glutamyl)-N-methylaniline (IVc).—A solution of 1.83 g. (0.005 mole) of IIIc in 30 ml. of absolute ethanol and 5 ml. of 1 *M* hydrazine hydrate (100%) in ethanol was refluxed for 1 hr. The liquid residue obtained by removal of the solvent under reduced pressure was boiled with 50 ml. of water for five minutes, cooled to room temperature and filtered. The filtrate was evaporated *in vacuo* and dried. To the residue 5 ml. of methanol was added and kept overnight in the refrigerator when 0.78 g. (64%) of IVc, m.p. 162–163°, was obtained. Recrystallization from aqueous methanol afforded pure product, m.p. 165–166°, the infrared maxima: 2.95, 3.35–3.40, 6.07, 6.3, 6.65, 7.05, 7.15, 7.38, 7.58 μ.

Anal. Calcd. for $C_{12}H_{16}N_2O_3$: C, 61.01; H, 6.77; N, 11.86. Found: C, 61.17; H, 6.77; N, 11.80.

N-(DL-γ-Glutamyl)-2-aminoethanol (IVd).—A solution of 5.18 g. (0.02) of II and 1.3 ml. (0.022 mole) of distilled 2-aminoethanol in 30 ml. of dry dioxane was heated under reflux for 1 hr. After cooling, the solvent was removed *in vacuo*. The liquid residue was then dissolved in 40 ml. of absolute ethanol, and to the solution 1.1 ml. of anhydrous hydrazine hydrate (100%) was added. The mixture was refluxed for 1.5 hr. and after cooling, the solvent was removed under reduced pressure. The residue was boiled with 100 ml. of water for 10 minutes, cooled and filtered. The aqueous filtrate was evaporated to dryness under reduced pressure. The liquid residue was dissolved in a minimum quantity of ethanol. Enough acetone was added to the ethanol solution till turbidity appeared; allowed to stand overnight when 2.7 g. (71%) of IVd, m.p. 178–180°, was obtained. Recrystallization from aqueous ethanol yielded pure product, m.p. 193–194°; reported[16] m.p. 190–191°; the infrared maxima: 3.05, 3.4–3.45, 6.05, 6.3, 6.6, 7.05, 7.45 μ.

Anal. Calcd. for $C_7H_{14}N_2O_4$: C, 44.21; H, 7.36; N, 14.73. Found: C, 44.15; H, 7.53; N, 14.90.

N-(DL-γ-Glutamyl)-*n*-butylamine (IVe).—To the cold solution of 10.36 g. (0.04 mole) of II in 50 ml. of dry dioxane was added 4.34 ml. (0.044 mole) of distilled *n*-butylamine in 10 ml. of dioxane with shaking. The mixture was refluxed for 1 hr. and the solvent was removed under reduced pressure. The liquid residue was dissolved in 100 ml. of ethanol and to it was added 2.2 ml. of hydrazine hydrate (100%). After refluxing for 2 hr. the solvent was removed *in vacuo*. The residue was boiled with 200 ml. of water for 10 minutes, cooled and filtered. The filtrate was concentrated under reduced pressure and to it ethanol was added till the solution became turbid. On keeping overnight at room temperature 6.46 g. (80%) of IVe, m.p. 208–209°, separated which on recrystallization from aqueous ethanol furnished pure product, m.p. 213–214°; the infrared maxima: 3.05, 3.4–3.45, 6.19, 6.32, 6.7, 7.05, 7.45 μ.

Anal. Calcd. for $C_9H_{18}N_2O_3$: C, 53.49; H, 8.95; N, 13.86. Found: C, 54.00; H, 9.16; N, 14.14.

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tra were determined on a Perkin–Elmer Spectrophotometer, Model 21, in potassium bromide pellets. Optical rotation of all the products was found to be zero.