[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL SCIENCES, STANFORD RESEARCH INSTITUTE]

Potential Anticancer Agents.[1] XVII. Alkylating Agents to Phenylalanine Mustard. I.

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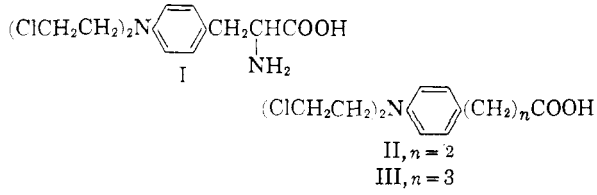
A biological rationale for synthesis of two phenylalanine mustard analogs (XVII and XX) is presented. 3-{*m*-[Bis-(2-chloroethyl)-amino]-phenyl}-DL-alanine (XVII) was synthesized from α-chloro-*m*-nitrotoluene *via* the key intermediate, methyl 3-(*m*-aminophenyl)-N-phthaloyl-DL-alanate (XIV). 3-(*p*-Diazoniumphenyl)-DL-alanine fluoborate (XX) was synthesized by the selective action of nitrous acid on 3-(*p*-aminophenyl)-DL-alanine (XIX). Both phenylalanine mustard analogs (XVII and XX) showed activity against certain selected tumors.

The report in 1946 by Gilman and Philips[2] on the effect of nitrogen mustards on tumors has given great impetus to the search for effective cancer chemotherapeutics in general and nitrogen mustards in particular. By late 1957[3] almost 500 different nitrogen mustards had been synthesized and presumably evaluated against experimental tumors; of these, about 30 have been brought to clinical trial. A recent symposium[4] assessed the accomplishments of these efforts on clinical and biological effects of alkylating agents and also projected some of the possible future accomplishments in this area. At this conference, Bergel[5] summarized the relationship of structure to activity of nitrogen mustards; he gave the evidence available to support the hypothesis that alkylating agents consist of a carrier group and the alkylating group and that the differences in effects and side effects on tumors might be related to the differences in the carrier group.[6] Although these alkylating agents can alkylate cellular proteins or nucelic acids,[7] the question of their exact mode of action and the reason they selectively affect certain tumors more than normal cells or other tumors remains to be answered.[8]

Biological Rationale.—Among the more interesting nitrogen mustards from the standpoint of attempts to rationalize their mode of action are phenylalanine mustard[9] (sarcolysine)[10] (I) and chlorambucil (III).[11] These clinically interesting compounds[4] have been shown to be capable of blocking protein synthesis in a bacterial system.[12]



I

II, $n = 2$
III, $n = 3$

The nitrogen mustards that proved to be most effective in reducing the amount of incorporation of radioactive glycine or phenylalanine into the bacterial protein were also, in general, those most effective in inhibiting the growth of the Walker rat sarcoma.[12] The most active compounds were L-phenylalanine mustard (I), chlorambucil (III) and its lower homolog (II), and 2,2′,2″-trichlorotriethylamine; a number of other mustards exhibited lesser degrees of activity, some being inactive.

As a working hypothesis it is interesting to speculate that I, II and III can fit the site normally occupied by L-phenylalanine during protein synthesis, particularly since D-phenylalanine mustard is considerably less active than its L-isomer against the Walker sarcoma and in inhibiting bacterial protein synthesis. Since I, II and III are all effective, it is suggestive that in the protein synthesizing system the phenylalanine is attached to a site (enzymatic or template) only by the carboxyl and not by the α-amino group, else II and III would be inactive and the α-amino group of L-phenylalanine would not be available for peptide formation. Once these mustards I–III have occupied the site for L-phenylalanine, their alkylating groups can then combine irreversibly by alkylation of some active group near the site.

Luck[13] has studied the effect of L-phenylalanine mustard (I) and its two next higher homologs on the Cloudman malignant melanoma (S-91) and has found these three compounds to be effective. In contrast, chlorambucil (III) has no effect. One can conclude that for activity against this melanoma, it is necessary for the mustard to fit an enzyme site normally occupied by L-phenylalanine by attachment through its amino group and probably through its carboxyl group. Thus, L-phenylalanine mustard (I) fits this site, then alkylates some spatially available active group to give an irreversible combination, hence blocking some enzymatic conversion on L-phenylalanine, perhaps to L-tyrosine and 3-(3,4-dihydroxyphenyl)-L-alanine.

(1) This program is under the auspices of the Cancer Chemotherapy National Service Center, National Cancer Institute, Contract SA-43-ph-1892. For the preceding paper of this series see C. D. Anderson, L. Goodman and B. R. Baker, THIS JOURNAL, **81**, 898 (1959).

(2) A. Gilman and F. S. Philips, *Science*, **103**, 409 (1946).

(3) "Literature Survey of Nitrogen Mustards," R. B. Ross, E. W. Foltz, P. E. Swartzentruber, R. B. Ing and J. Clapp, Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md., 1957.

(4) A symposium sponsored jointly by the New York Academy of Sciences and the Cancer Chemotherapy National Service Center, March 28–30, 1957, L. H. Schmidt, conference chairman; *cf. N. Y. Acad. Sci.*, **68**, 657 (1958).

(5) F. Bergel, *ibid.*, **68**, 1238 (1958).

(6) The carrier hypothesis for other types of active drugs has been proposed by H. R. Ing, *Trans. Faraday Soc.*, **39**, 372 (1943).

(7) P. Alexander, *Advances Cancer Res.*, **2**, 1 (1954); W. C. J. Ross, *ibid.*, **1**, 397 (1952).

(8) A. Haddow, *N. Y. Acad. Sci.*, **68**, 1258 (1958).

(9) F. Bergel, V. C. E. Burnop and J. A. Stock, *J. Chem. Soc.*, 1233 (1955); F. Bergel and J. A. Stock, *ibid.*, 2409 (1954).

(10) L. F. Larionov, A. S. Khokhlov, E. N. Shkodinskaia, O. S. Vasina, V. I. Trusheikina and A. M. Novikova, *Lancet*, **269**, 169 (1955).

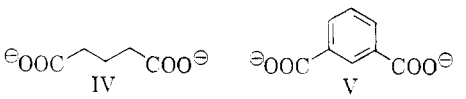
(11) J. L. Everett, J. J. Roberts and W. C. J. Ross, *J. Chem. Soc.*, 2386 (1953).

(12) A. R. Crathorn and G. D. Hunter, *Biochem. J.*, **67**, 37 (1957).

(13) J. M. Luck, *Cancer Res.*, **17**, 1071 (1957).

The carrier concept[5] for nitrogen mustards can thus be fitted nicely into the antimetabolite concept.[14,15] The carrier, for example L-phenylalanine, fits the site for the metabolite (L-phenylalanine); then the alkylating group combines irreversibly with some active group near the site.[16]

This concept of irreversible combination of a metabolite bearing a properly oriented alkylating group should have considerable chemotherapeutic application. Although Woolley[18] has stated that an antimetabolite should be as close as possible in structure to the corresponding metabolite, his concept should be modified to state that *an antimetabolite should be as close as possible in structure to that part of the molecule where the stereospecific requirements of the enzyme surface must be met.* Certainly there can be space on the metabolite that is away from the enzyme surface where considerable change in size can be made without influencing adsorption on the enzyme surface. Caughey, Smiley and Hellerman[19] observed that glutarate (IV) and isophthalate (V) competitively inhibited glutamic acid dehydrogenase to the same extent but that phthalate and terephthalate were much less effective. With the reasonable assumption that bonding to the enzyme occurs through the



IV V

two carboxylate groups, they stated, "it is apparent that the portion of the molecule orientated toward the enzyme surface is structurally much the same in both these compounds (glutarate and isophthalate)"; it is obvious that the remainder of the benzene ring constitutes a large change in gross measurements compared with the metabolite. Inhibitors of the enzymatic oxidation of succinate to fumarate can vary considerably in gross measurements, provided the change is only at one α-position. Thus, α-alkylsuccinic acids, even with large alkyl groups, competitively inhibit succinic dehydrogenase.[20]

In accordance with this concept that metabolites properly substituted with an alkylating group could have considerable utility in chemotherapy by blocking specific enzyme systems, syntheses of a variety of alkylating groups attached to substrates in several areas of metabolism have been initiated in this Laboratory in order to provide compounds to test this hypothesis.

Biological Results.—One of the areas of metabolism that lends itself to further study is the synthesis of alkylating agents derived from phenylalanine, since a considerable number of these phenylalanine analogs have already been synthesized and evaluated; the phenylalanine system is also ideal for the variability in synthetics that can be made. There are two questions that can theoretically be answered, (1) what are the spatial limitations for the alkylating group to be able to combine with an active function on the enzyme (or template) after the compound has entered the site of the metabolite and (2) what types of alkylating groups can be employed?

In order to help answer the first question, the *meta* isomer of DL-phenylalanine nitrogen mustard (XVII) has now been synthesized. This DL-compound[21] shows marked inhibition of the Cloudman S-91 transplanted melanoma in preliminary trials, being about ten times as active as the L-isomer of *p*-phenylalanine mustard (I) and only two to three times as toxic, thus showing a more favorable chemotherapeutic index. The *m*-phenylalanine mustard (XVII) also effectively inhibited Leukemia L-1210, Sarcoma 180 and Adenocarcinoma 755. In fact, against the latter tumor XVII was greater than four times as active and had at least twice the chemotherapeutic index as DL-sarcolysine.[22] Further evaluation in tumor and enzyme systems should provide additional data needed to answer the first question.

To throw some light on the second question, 3-(*p*-diazoniumphenyl)-DL-alanine, isolated as the fluoborate XX, was synthesized. This compound showed borderline activity against Sarcoma 180 and a considerably higher order of activity against Leukemia L-1210.[22] The activity of this compound shows that an alkylating group other than a mustard can be attached to the benzene ring of DL-phenylalanine and still impart activity. Although the *meta* isomer of 3-(diazoniumphenyl)-DL-alanine was synthesized and isolated as its difluoborate XXII, it was too unstable in aqueous solution to warrant testing.

Synthesis.—The synthesis of the *meta*-isomer XVII of 3-{*p*-[bis-(2-chloroethyl)-amino]-phenyl}-DL-alanine (I) was undertaken by the first of several processes described by Bergel, Burnop and Stock[9] for the *para*-isomer I. Condensation of α-chloro-*m*-nitrotoluene (VI) with ethyl acetamidomalonate afforded crystalline VII in 73% yield. Catalytic reduction of the nitro group of VII in ethanol with a palladium–charcoal catalyst gave a quantitative yield of the amino derivative VIII. Hydroxyethylation of the amino group of VIII with ethylene oxide in dilute acetic acid resulted in a quantitative yield of the diethanolamine XII, which was obtained in crystalline form. Attempts to convert XII to the blocked mustard XV with phosphoryl chloride in benzene[9] did not lead to the

(14) D. W. Woolley, "A Study of Antimetabolites," John Wiley and Sons, Inc., New York, N. Y., 1952.

(15) R. O. Roblin, Jr., *Chem. Revs.*, **38**, 255 (1946).

(16) O-Diazoacetyl-L-serine (azaserine) and 6-diazo-5-oxo-L-norleucine (DON) probably function in a similar manner. The observation has been made that these two compounds fit an enzyme site normally occupied by L-glutamine during the amination of formylglycinamide ribotide to formylglycinamidine ribotide, then these alkylating agents combine irreversibly with the enzyme.[17]

(17) B. Levenberg, I. Melnick and J. M. Buchanan, *J. Biol. Chem.*, **225**, 163 (1957).

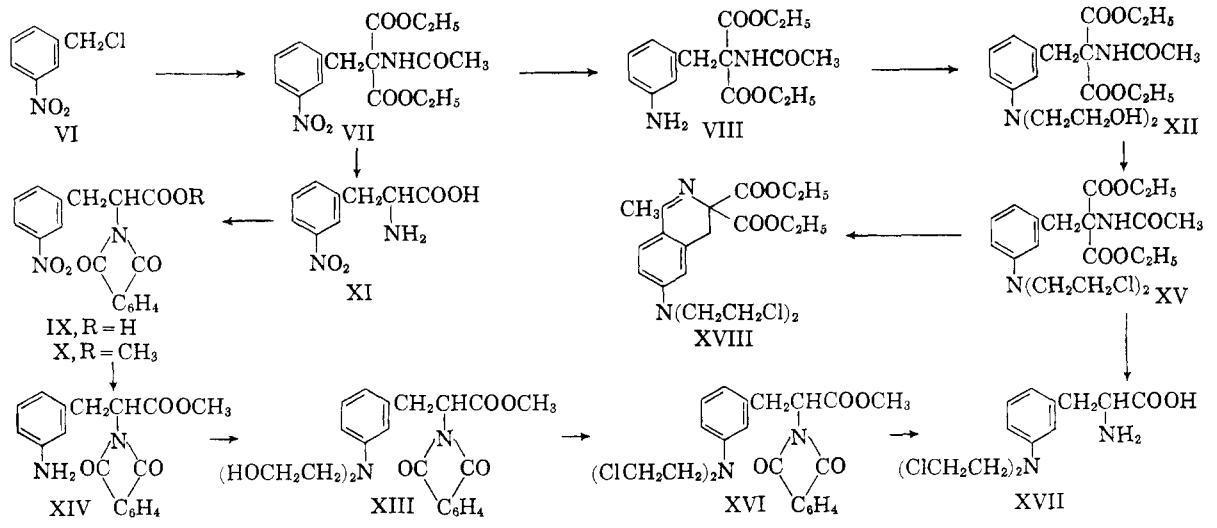
(18) Ref. 14, p. 227.

(19) W. S. Caughey, J. D. Smiley and L. Hellerman, *J. Biol. Chem.*, **224**, 591 (1957).

(20) W. Franke, E. Holz and G. Toschen, *Ann.*, **608**, 168 (1957).

(21) This compound has also been synthesized by a different route by T. S. Osdene, D. N. Ward, W. H. Chapman and H. Rakoff, THIS JOURNAL, **81**, 3100 (1959). An exchange of samples showed both materials to be identical. We wish to thank Dr. Osdene for the melanoma test data.

(22) We wish to thank Dr. Joseph Greenberg and his staff of this Department for these assays, performed under a contract with the Cancer Chemotherapy National Service Center, National Cancer Institute.



expected product; instead, Bischler–Napieralski[23] ring closure to the dihydroisoquinoline XVIII appeared to take place. Although this compound could be crystallized, it was not fully purified. That the isoquinoline XVIII had been formed was indicated by lack of amide NH infrared absorption near 6.6 μ and by an ultraviolet absorption typical of the dihydroisoquinoline system.[25]

The use of thionyl chloride to convert XII to the blocked mustard XV was also investigated. When the reaction was run long enough to convert all the hydroxyl groups to chloro, as shown by infrared absorption spectra and paper chromatography, the resultant reaction mixture was a dark tar that appeared unpromising.

Since the N-acetyl blocking group of XII was inadequate for protection during conversion to the blocked mustard, the more stable N-phthaloyl blocking group[9] was investigated. Hydrolysis of VII with concentrated hydrochloric acid afforded a 94% yield of 3-(*m*-nitrophenyl)-DL-alanine (XI) hydrochloride. This hydrochloride was smoothly converted to 3-(*m*-nitrophenyl)-N-phthaloyl-DL-alanine (IX) in 96% yield when treated with phthalic anhydride in pyridine followed by acetic anhydride. Esterification of IX with methanolic hydrogen chloride afforded a 76% yield of crystalline methyl ester X. Catalytic hydrogenation of X gave a quantitative yield of methyl 3-(*m*-aminophenyl)-N-phthaloy-DL-alanate (XIV) as an oil that could not be crystallized but gave only a single spot (R_f 0.61) when chromatographed on acetylated paper with solvent A.[26] The starting material had R_f 0.45. Hydroxyethylation of XIV with ethylene oxide in aqueous acetic acid afforded the crystalline diethanolamine XIII in 86% yield.

Considerable study was required to find optimum conditions for conversion of XIII to the blocked mustard XVI with thionyl chloride. Paper chromatography on acetylated paper with solvent A[26] was of great aid in this study, since the starting material XIII had R_f 0.77 and the product XVI had R_f 0.30. In the first run, the reaction of XIII with thionyl chloride in chloroform gave a mixture of hydrochlorides as the product; this mixture obviously readily dissociated back to the free bases, since paper chromatography with solvent A gave considerable streaking (due to dissociation of the hydrochlorides) as well as two spots for starting material and product. Thus, the hydrochlorides could be readily converted to the free bases by evaporation of their methanolic solution; the product XVI was then easily crystallized from ethanol. Optimum conditions were found to be the use of a 20% excess of thionyl chloride in boiling chloroform for 3 hr., a 73% yield of the crystalline blocked mustard XVI being obtained.

The hydrolysis of the blocked mustard XVI to the final *m*-phenylalanine mustard XVII also proved to be a difficult step. Again, paper chromatography aided a great deal in solution of this problem. The desired product was assumed to be the component in the hydrolysis mixture giving R_f 0.48 in solvent B and R_f 0.85 in solvent C,[26] since it was the only spot near that given by authentic *p*-phenylalanine mustard (I), which had R_f 0.43 in solvent B and R_f 0.87 in solvent C. One of the difficulties during the hydrolysis of XVI to XVII with hydrochloric acid was that evaporation of the acid solutions *in vacuo*[9] seemed to cause considerable conversion of XVII to components that moved slower on paper, presumably by conversion of the chloroethyl groups of XVII to hydroxyethyl groups. This difficulty in the work-up was avoided by hydrolysis with a higher ratio of compound to hydrochloric acid than is normally used,[9] then neutralization with sodium acetate without evaporation.

(23) The Bischler–Napieralski ring closure of an acylated phenethylamine to a dihydroisoquinoline[24] takes place much more readily *para* to a ring activating group, as in XV in XVIII, than does ring closure *meta* to the activating group, as in the *p*-phenylalanine mustard synthesis.[9]

(24) W. M. Whaley and T. R. Govindachari in R. Adams, "Organic Reactions," Vol. VI, John Wiley and Sons, Inc., New York, N. Y., 1951.

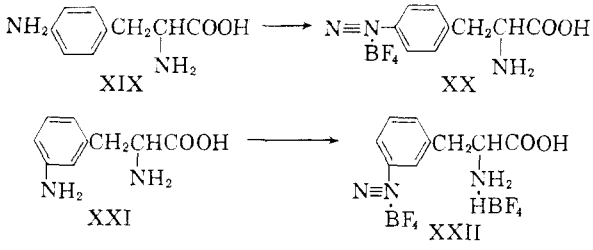
(25) J. L. Bills and C. R. Noller, THIS JOURNAL, **70**, 958 (1948).

(26) Paper chromatograms were run by the descending technique on Schleicher and Schuell No. 2043B acetylated paper with benzene/methanol/water (6/2/1)[27] (solvent A), or on Whatman No. 1 paper with water-saturated butanol (solvent B), or with butanol/acetic acid/water (5/2/3) (solvent C). Spots were detected by observation under an ultraviolet light; ninhydrin spray was also used for the free α-amino acids.

(27) T. Wieland and W. Kracht, *Angew. Chem.*, **69**, 172 (1957).

Since the product did not separate in crystalline form on neutralization of an acid solution,[9] the relatively high R_f value in solvent B[26] suggested that the product XVII could be isolated by butanol extraction from an aqueous system, thus separating XVII from its overhydrolyzed products. Unfortunately, good separation from pigmented material proved infeasible by this method and the red, amorphous product could not be crystallized. However, extraction of XVII from the aqueous mixture with chloroform[28] gave a light-colored, amorphous solid that was readily crystallized from acetone. The resultant XVII was analytically pure and was homogeneous on paper with solvents B and C.[26]

The selective diazotization of 3-(*p*-aminophenyl)-DL-alanine[29] with nitrous acid to 3-(*p*-diazoniumphenyl)-DL-alanine presented the problem that



the α-amino group could also react with nitrous acid to form an α-hydroxyl group. The study of the formation of the diazonium group of XX was aided by coupling with the Bratton–Marshall reagent, N-1-naphthylethylenediamine, then measurement of the optical density of the absorption maximum at 484 mμ. Treatment of XIX with nitrous acid caused immediate formation of the diazonium group of XX, and the diazonium group was stable for at least 0.5 hr. at 0°. The optimum conditions for diazotization to XX were treatment of a solution of XIX in 2.4 equivalents of 4 *N* hydrochloric acid with 1 equivalent of sodium nitrite. Addition of sodium fluoborate gave a 73% yield of crystalline fluoborate XX. Apparently, the selective action of nitrous acid is based on its much more rapid reaction with the *p*-amino group than the α-amino group. The fluoborate XX was stable for at least eight days at room temperature.

When 3-(*m*-aminophenyl)-DL-alanine (XXI), prepared by catalytic hydrogenation of 3-(*m*-nitrophenyl)-DL-alanine (XI), was treated with nitrous acid under the same conditions as those used for the *para*-isomer XIX, no insoluble fluoborate could be isolated. However, diazotization in 48% fluoboric acid[30] with sodium nitrite allowed isolation of 3-(*m*-diazoniumphenyl)-DL-alanine as its difluoborate XXII. Unfortunately, this compound slowly decomposed in the solid state at 20°. Its decomposition, with evolution of nitrogen, was fairly rapid in aqueous solution at 0°, thus precluding any attempts at biological testing.

(28) The use of chloroform for this work-up was suggested by Dr. T. S. Osdene.

(29) E. D. Bergmann, THIS JOURNAL, **74**, 4947 (1952).

(30) The use of 48% fluoboric acid for preparation of water-soluble diazonium fluoborates was discovered by A. Roe and C. E. Teague, Jr., *ibid.*, **73**, 687 (1951); *cf.* also B. R. Baker, R. E. Schaub, J. P. Joseph, F. J. McEvoy and J. H. Williams, *J. Org. Chem.*, **17**, 164 (1952).

Experimental

Ethyl Acetamido-(*m*-nitrobenzyl)-malonate (VII).—To a solution of 1.15 g. (0.05 mole) of sodium in 80 ml. of absolute ethanol was added 10.8 g. (0.05 mole) of ethyl acetamidomalonate followed by a solution of 9.2 g. (0.054 mole) of α-chloro-*m*-nitrotoluene (VI) in 50 ml. of absolute ethanol. After being refluxed with stirring for 18 hr., the neutral reaction mixture was cooled in an ice-bath. The product was collected on a filter and washed with water; yield 12.9 g. (73%), m.p. 153–155°; this material was suitable for the next step. Two recrystallizations of a 1.0-g. sample from 20 ml. of absolute ethanol afforded an analytical sample, m.p. 159–160°; λ_{max}^{KBr} 3.09 μ (NH), 5.74 (ester C=O), 6.10 (amide C=O), 6.56, 7.38 (NO_2), 9.80 (ester C–O–C). This compound traveled as a single spot (R_f 0.94) on paper with solvent C.[26]

Anal. Calcd. for $C_{16}H_{20}N_2O_7$: C, 54.5; H, 5.72; N, 7.97. Found: C, 54.7; H, 5.77; N, 7.89.

Ethyl Acetamido-(*m*-aminobenzyl)-malonate (VIII).—A mixture of 4.0 g. (0.011 mole) of VII, 25 ml. of ethyl acetate, 10 ml. of absolute ethanol and 1.0 g. of 5% palladium–charcoal catalyst was shaken with hydrogen (1 atm.) for 2 hr., when reduction was complete. Evaporation of the filtered solution to dryness *in vacuo* left 3.5 g. (99%) of white solid, m.p. 148–153°, that was suitable for the next step. Two recrystallizations of a sample (0.4 g.) from 2 ml. of ethanol gave white crystals, m.p. 158–159°; λ_{max}^{KBr} 2.99, 6.25 μ (NH), 5.76 (ester C=O), 6.21 (amide C=O), 8.27 (ester C–O–C), no NO_2 near 7.38.

Anal. Calcd. for $C_{16}H_{22}N_2O_5$: C, 59.6; H, 6.88; N, 8.69. Found: C, 59.4; H, 6.78; N, 8.80.

This compound traveled on paper[26] as a single spot with R_f 0.74 in solvent A and R_f 0.86 in solvent C.

Ethyl Acetamido-{*m*-[bis-(2-hydroxyethyl)-amino]-benzyl}-malonate (XII).—To a solution of 4.0 g. (0.012 mole) of VIII in 25 ml. of water and 16 ml. of glacial acetic acid was added 5 ml. of ethylene oxide. After 24 hr. at room temperature, the solution was concentrated to a sirup *in vacuo*, then dissolved in 20 ml. of ethyl acetate. The resultant solution was washed with excess 5% aqueous sodium bicarbonate, dried with magnesium sulfate, then evaporated *in vacuo*, leaving 5.0 g. of a sirup that traveled as a single spot (R_f 0.80) on acetylated paper with solvent A.[26] Solvent A or B on Whatman No. 1 paper did not separate product XII from starting material VIII.

The sirup crystallized after several weeks at room temperature, then had m.p. 92–96°. Two recrystallizations from ethanol (1 g./ml.) gave white crystals, m.p. 101–102°; λ_{max}^{KBr} 2.91 μ (OH), 5.70 (ester C=O), 5.98 (amide C=O), 6.60 (amide NH); λ_{max}^{EtOH} 260 mμ (ε 13,100), 308 (ε 2750).

Anal. Calcd. for $C_{20}H_{30}N_2O_7$: C, 58.5; H, 7.36; N, 6.82. Found: C, 58.8; H, 7.31; N, 6.81.

Several attempts were made to convert XII to the blocked mustard XV with thionyl chloride under a variety of conditions. With short reaction times[1] considerable OH remained at 2.9 μ in the infrared spectrum of the crude product. Reaction conditions strenuous enough to remove most of the hydroxyl groups (as evidenced by lack of absorption near 2.9 μ) gave dark, unpromising tars that had complex paper chromatograms. With phosphoryl chloride in boiling benzene[9] for 25 minutes, XII was converted to a yellow, crystalline product which was not fully purified, but had m.p. 85–95°. The lack of amide NH absorption in the 6.6 μ region of its infrared spectrum, as well as its ultraviolet spectrum of λ_{max}^{EtOH} 327 mμ (ε 21,200), 397 (ε 4400) and $\lambda_{max}^{pH\,1}$ 397 mμ (ε 36,800), indicates that ring closure to the dihydroisoquinoline XVIII had taken place. Similar spectral shifts have been observed with 3,4-dihydro-1-methyl-6,7-methylenedioxyisoquinoline.[25]

3-(*m*-Nitrophenyl)-DL-alanine (XI) and Hydrochloride.—A mixture of 100 g. (0.30 mole) of VII and 1500 ml. of 12 *N* hydrochloric acid was refluxed for 6 hr., then kept at 3° overnight. The crystals of XI hydrochloride which formed were collected on a filter and washed with cold 1 *N* hydrochloric acid, then ether; yield 70 g. (94%), m.p. 207–227° dec.; this material was suitable for the next step.

An analytical sample of XI, m.p. 209–212°, was obtained by suspending a sample of its hydrochloride in water, neutralizing with ammonia water, then recrystallizing the resultant solid twice from water; λ_{max}^{KBr} 3.30, 6.12, 6.64 μ (NH_3^+), 6.30, 7.12 (carboxylate), 6.52, 7.35 (NO_2).

Anal. Calcd. for $C_9H_{10}N_2O_4$: C, 51.4; H, 4.74; N, 13.3. Found: C, 51.0; H, 5.02; N, 13.3.

Both the pure amino acid and its crude hydrochloride traveled on paper as a single spot (R_f 0.57) in solvent C.[26]

3-(*m*-Nitrophenyl)-N-phthaloyl-DL-alanine (IX).—A mixture of 70 g. (0.28 mole) of XI hydrochloride, 450 ml. of reagent pyridine and 41.5 g. (0.28 mole) of phthalic anhydride was refluxed for 90 minutes. The resultant solution was concentrated to a sirup *in vacuo*. The sirup was refluxed in 150 ml. of acetic anhydride for 5 minutes, then poured into 1100 ml. of cold water and acidified to *p*H 3 with hydrochloric acid. The granular precipitate which formed was triturated with water, collected on a filter and washed with water; yield 93 g. (96%), m.p. 194–200°. This material was suitable for the next step. Two recrystallizations from chloroform–Skellysolve C (3:2) gave white crystals, m.p. 201–202°; λ_{max}^{KBr} 5.65, 5.80 μ (phthalyl C=O and carboxy C=O); R_f 0.74 (solvent A).[26]

Anal. Calcd. for $C_{17}H_{12}N_2O_6$: C, 60.0; H, 3.56; N, 8.23. Found: C, 59.9; H, 3.61; N, 7.91.

Methyl 3-(*m*-Nitrophenyl)-N-phthaloyl-DL-alanate (X).—To 500 ml. of methanol saturated with hydrogen chloride at 0–5° was added 83 g. (0.24 mole) of IX. The mixture was refluxed for 90 minutes, during which time the starting material dissolved and the product separated. The mixture was concentrated to about 200 ml., then cooled to 0°. The solid that separated was collected on a filter, washed with cold methanol, then recrystallized from 750 ml. of ethanol; yield 66.2 g. (76%) of product, m.p. 124–126°, that was suitable for the next step. No attempt was made to obtain further crops. An analytical sample was prepared by recrystallization from ethanol; it had m.p. 126–127°; λ_{max}^{KBr} 5.62, 5.81 μ (phthalyl C=O), 5.72 (ester C=O), 7.96 (ester C–O–C), 6.52, 7.40 (NO_2); R_f 0.45 (solvent A).[26]

Anal. Calcd. for $C_{18}H_{14}N_2O_6$: C, 61.0; H, 3.98; N, 7.90. Found: C, 61.3; H, 4.06; N, 7.72.

Methyl 3-(*m*-Aminophenyl)-N-phthaloyl-DL-alanate (XIV).—A mixture of 60.6 g. (0.17 mole) of X, 250 ml. of ethanol and 6 g. of 5% palladium–charcoal was shaken with hydrogen at an initial pressure of 50 p.s.i. for 90 minutes, when hydrogenation was complete. The filtered solution was evaporated to dryness *in vacuo*, leaving 55 g. (100%) of a sirup which could not be induced to crystallize; it was free of X (R_f 0.45) and had R_f 0.61 (solvent A)[26]; λ_{max}^{KBr} 2.90, 2.98 μ (NH_2), 5.62, 5.81 (phthalyl C=O), 5.72 (ester C=O), 8.00 (ester C–O–C), no NO_2 near 6.5 or 7.4.

Methyl 3-{*m*-[Bis-(2-hydroxyethyl)-amino]-phenyl}-N-phthaloyl-DL-alanate (XIII).—To a solution of 6.75 g. of crude XIV in 15 ml. of acetic acid was added 15 ml. of water, then 10 ml. of ethylene oxide. After 24 hr. at room temperature, the solution was poured into 100 ml. of water, then neutralized with solid sodium bicarbonate and extracted with ethyl acetate. The combined extracts, dried with magnesium sulfate, were evaporated to dryness *in vacuo*. Trituration of the residue with about 5 ml. of water caused crystallization. The solid was suspended in ether and collected on a filter; yield 7.4 g. (86%) of product, m.p. 88–93°, suitable for the next step since it traveled as a single spot (R_f 0.77) on paper in solvent A.[26] Recrystallization of a sample from ethanol–ether gave pale yellow crystals, m.p. 87–90°; λ_{max}^{KBr} 2.93, 9.55 μ (O–H), 5.65, 5.80 (phthalyl C=O), 5.71, 8.00 (ester).

Anal. Calcd. for $C_{22}H_{24}N_2O_6$: C, 64.3; H, 5.86. Found: C, 64.2; H, 6.11.

Methyl 3-{*m*-[Bis-(2-chloroethyl)-amino]-phenyl}-N-phthaloyl-DL-alanate (XVI).—To a solution of 12.0 g. (0.028 mole) of XIII in 40 ml. of chloroform was added 4.8 ml. (0.068 mole) of thionyl chloride in 12 ml. of chloroform. After being refluxed for 3 hr., the solution was evaporated to a sirup *in vacuo*. The residual hydrochloride was dissolved in 20 ml. of methanol and the evaporation repeated. This evaporation with 20-ml. portions was repeated twice more, leaving XVI as a solid cake. Recrystallization from 400 ml. of ethanol gave 9.2 g. (73%) of product, m.p. 118–125°, that was suitable for the next step. For analysis a sample was recrystallized twice more from ethanol, giving pale yellow crystals of constant m.p. 119–124°; λ_{max}^{KBr} 5.65, 5.81 μ (phthalyl C=O), 5.72, 8.00 (ester), no OH near 3.0 or 9.5.

Anal. Calcd. for $C_{22}H_{22}Cl_2N_2O_4$: C, 58.8; H, 4.93; N, 6.23; Cl, 15.8. Found: C, 58.9; H, 5.07; N, 6.15; Cl, 15.3.

Both the crude product (m.p. 118–123°) and the analytical sample moved on acetylated paper with solvent A[26] as a single spot with R_f 0.30.

3-{*m*-[Bis-(2-chloroethyl)amino]-phenyl}-DL-alanine (XVII).—A mixture of 4.0 g. (9.0 mmoles) of XVI and 40 ml. of 12 *N* hydrochloric acid was refluxed for 3 hr. The resultant red solution was cooled to 0°, filtered from phthalic acid and then neutralized with 70 ml. of saturated aqueous sodium acetate, which caused a red gum to separate. The mixture was extracted with chloroform (3 × 20 ml.). The combined extracts were washed four times with 10-ml. portions of water, when the washings became colorless. Dried with magnesium sulfate, the organic solution was evaporated to dryness *in vacuo*, leaving 2.5 g. of cream-colored, amorphous solid. This solid was heated with 40 ml. of acetone, which caused crystallization. The nearly colorless crystals were collected on a filter and washed with acetone; yield 1.0 g., m.p. 165–168°. The acetone filtrate deposited an additional 0.20 g. (total 44%) of crystals, m.p. 165–168°, which were analyzed, since no suitable solvent combination for recrystallization could be found.

Anal. Calcd. for $C_{13}H_{15}Cl_2N_2O_2$: C, 51.2; N, 5.92; N, 9.18; Cl, 23.2. Found: C, 51.1; H, 6.05; N, 8.86, 8.97; Cl, 22.8.

Both crystalline fractions were homogeneous when chromatographed on paper,[26] giving R_f 0.48 with solvent B and R_f 0.85 with solvent C. Both fractions had λ_{max}^{KBr} 3.30, 3.90 μ (NH_3^+), 6.21, 6.62 (NH_3^+, aryl), 7.12 (COO^-).

3-(*p*-Diazoniumphenyl)-DL-alanine Fluoborate (XX).—3-(*p*-Nitrophenyl)-DL-alanine[9] was reduced catalytically to 3-(*p*-aminophenyl)-DL-alanine (XIX)[29] in quantitative yield. The latter had R_f 0.28 on paper with solvent C.[26] A solution of 9.0 g. (0.05 mole) of XIX in 30 ml. (0.12 mole) of 4 *N* hydrochloric acid was cooled to 6° in an ice-bath. A solution of 3.5 g. (0.05 mole) of sodium nitrite in 7 ml. of water was added dropwise with stirring over a period of 20 minutes, the temperature being maintained at 0–5°. Five minutes after the addition was complete, the solution was treated with 5.5 g. (0.05 mole) of sodium fluoborate. To this solution was added 50 ml. of 95% ethanol. After 15 minutes, the product was collected on a filter and washed with cold absolute ethanol (3 × 25 ml.), then with ether (4 × 25 ml.); yield 7.0 g.

The combined filtrate and washings were cooled in an ice-bath until the supernatant cleared. The supernatant liquid was decanted from the oil; the latter was stirred with 50 ml. of cold ethanol, which effected crystallization. The solid was collected on a filter and washed with 50 ml. of cold ethanol and 75 ml. of cold ether; total yield 10.2 g. (73%). An analytical sample was obtained by solution of 0.7 g. in 6 ml. of ice-water followed by addition of 10 ml. of absolute ethanol and 3 ml. of ether. The white crystals melted at 132° dec. and had λ_{max}^{KBr} 3.44, 6.15 μ (NH_3^+), 4.31 ($N{\equiv}N^+$), 6.33, 7.10 (COO^-), 9.25–9.65 (BF_4^-).

Anal. Calcd. for $C_9H_{10}BF_4N_3O_2$: C, 38.7; H, 3.61; N, 15.0. Found: C, 38.7; H, 3.83; N, 14.6.

The compound traveled as a single spot (R_f 0.25) on Whatman No. 1 paper with isopropyl alcohol/6 *N* HCl (17:8); the spot was detected both by ultraviolet absorption and by ninhydrin spray. This solvent may well have rapidly converted XX to some other compound.

Nitrogen analyses on a sample that had stood for 8 days at room temperature (about 20°) showed that XX was stable for at least this period. At higher temperatures (60–100°), the compound darkened.

3-(*m*-Aminophenyl)-DL-alanine (XXI).—A mixture of 19.5 g. (0.079 mole) of XI hydrochloride, 200 ml. of water and 2 g. of 5% palladium–charcoal was shaken with hydrogen at an initial pressure of 40 p.s.i. Reduction was complete in 2 hours. The filtered solution was evaporated to dryness *in vacuo*, leaving 16.0 g. (94%) of XXI hydrochloride as a white solid, m.p. 238–248° dec.[31]

(31) The dihydriodide salt has been prepared by the action of hydriodic acid and phosphorus on 3,6-bis-(*m*-nitrobenzylidene)-2,5-piperazinedione by H. Veda, *Ber.*, **61B**, 146 (1928). The dihydrochloride salt has been synthesized by tin and hydrochloric acid reduction of 5-(*m*-nitrobenzylidene)-hydantoin followed by barium hy-

A suspension of 9.5 g. of the above hydrochloride in 50 ml. of water was adjusted to *p*H 7 with concentrated ammonia water. The resulting solution was diluted with 150 ml. of absolute ethanol. When the walls of the flask were scratched, the product began to crystallize; yield 4.75 g. (60%), m.p. 238–255° dec. No attempt was made to obtain a second crop. Several recrystallizations of a sample from 50% ethanol gave white crystals, m.p. 242–257° dec.[31]; λ_{max}^{KBr} 2.99 μ (NH_2), 3.40, 6.66 (NH_3^+), 7.10 (COO^-).

Anal. Calcd. for $C_9H_{12}N_2O_2$: C, 60.0; H, 6.71; N, 15.5. Found: C, 60.4; H, 6.77; N, 15.5.

Both the hydrochloride and the free base traveled on paper as a single spot (R_f 0.34) in solvent C.[26]

3-(*m*-Diazoniumphenyl)-DL-alanine Difluoborate (XXII).—A solution of 0.35 g. (2 mmoles) of XXI in 3 ml. of 48% fluoboric acid was cooled to 4° in an ice-bath, causing separation of a salt. The mixture was treated dropwise with stirring with a solution of 0.16 g. (2 mmoles) of sodium nitrite in 0.3 ml. of water. The solution was treated with 6 ml. of ice-cold 1:1 absolute ethanol–ether. The product which separated was collected on a filter and washed with 40 ml. of cold absolute ethanol in portions, then 40 ml. of cold ether in portions. The white solid, m.p. 99° dec., gradually turned pink on standing at room temperature; yield 0.45 g. (81%). For analysis a sample was quickly dissolved in the minimum of ice-water, then diluted with ice-cold 1:1 absolute alcohol–ether until turbid. The sample was collected on a filter and dried for 30 minutes at 0° and 4 mm., then immediately analyzed. The analysis indicated contamination with some monofluoborate since the C:H:N ratio was in close agreement for a salt of 3-(*m*-diazoniumphenyl)-DL-alanine.

Anal. Calcd. for $C_9H_{11}B_2F_8N_3O_2$: C, 29.5; H, 3.02; N, 11.5. Found: C, 30.8; H, 3.29; N, 12.5.

After three weeks at 20°, the sample had only 9.63% nitrogen, thus showing its instability even in the solid state. When dissolved in water at 0°, it evolved nitrogen at a noticeable rate. The freshly prepared analytical sample showed COOH absorption at 3.84 and 5.80 μ, $-N\equiv N$ at 4.36 μ, NH_3^+ at 6.14 and 6.58 μ and broad BF_4^- absorption centering at 9.50 μ in the infrared.

droxide hydrolysis; *cf.* H. R. Henze, W. B. Whitney and M. A. Eppright, THIS JOURNAL, **62**, 565 (1940).

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL SCIENCES, STANFORD RESEARCH INSTITUTE]

Potential Anticancer Agents.[1] XVIII. Synthesis of Substituted 4,5-Trimethylenepyrimidines

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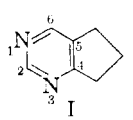
The condensations of 2-carbethoxycyclopentanone (III) with guanidine, thiourea and 2-methyl-2-thiopseudourea led to 2-amino-4-hydroxy- (IV), 4-hydroxy-2-mercapto- (XX) and 4-hydroxy-2-(methylthio)-5,6-trimethylenepyrimidine (XXIX), respectively. Compounds IV, XX and XXIX were converted to a variety of substituted 4,5-trimethylenepyrimidines, several of which are closely related structurally to biologically important purines.

Purines and pyrimidines are associated with a wide variety of biologically important systems and, accordingly, much work has been concerned with the synthesis of antimetabolites of these classes of compounds. The success of such compounds as 6-purinethiol[2] and 5-fluorouracil[3] as chemotherapeutic agents is justification for further investigations in these areas. The synthesis of 4,5-trimethylenepyrimidine (6,7-dihydro-5H-cyclopentapyrimidine) (I) and of a number of substitution products then leads to potential natural



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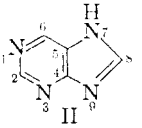

II

purine (II) antagonists as well as natural pyrimidine antagonists.

Condensation of 2-carbethoxycyclopentanone (III) with excess guanidine carbonate in ethanol gave 2-amino-4-hydroxy-5,6-trimethylenepyrimidine (IV), the analog of guanine, in about 50% yield. Attempts to prepare IV with guanidine hydrochloride by the procedure described by Hull, *et al.*,[4] gave yields of about 20% in contrast to the reported yield of 62.5%; a competing cleavage of III to methyl ethyl adipate seemed to be responsible for the poor yields of IV encountered. Compound IV has also been prepared (in unstated yield) by Braker, *et al.*,[5] by the fusion of III and guanidine carbonate.

The hydroxy compound IV was converted in high yield to the chloro compound V by careful treatment with phosphoryl chloride. Extended heating in the chlorination medium led to extensive decomposition and to low yields of V, a compound which has been reported previously.[4,5] Compound V was a versatile intermediate and it was converted to a variety of products (VI through XVIII).

Treatment of V with ethanolic ammonia at 150° gave a good yield of the diamino compound VI; the reaction was impractically slow at 110°. The 4-methylamino- VII 4-benzylamino- IX and 4-diethylamino-5,6-trimethylenepyrimidine X were similarly prepared. It is interesting that compounds VI and VII were directly isolable from

(1) This work was carried out under the auspices of the Cancer Chemotherapy National Service Center, National Cancer Institute, Contract No. SA-43-ph-1892. For the preceding paper of this series, *cf.* Helen F. Gram, Carol W. Mosher and B. R. Baker, THIS JOURNAL, **81**, 3103 (1959).

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