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

# Potential Anticancer Agents.[1] XXXVIII. Alkylating Agents Related to Phenylalanine Mustard. II

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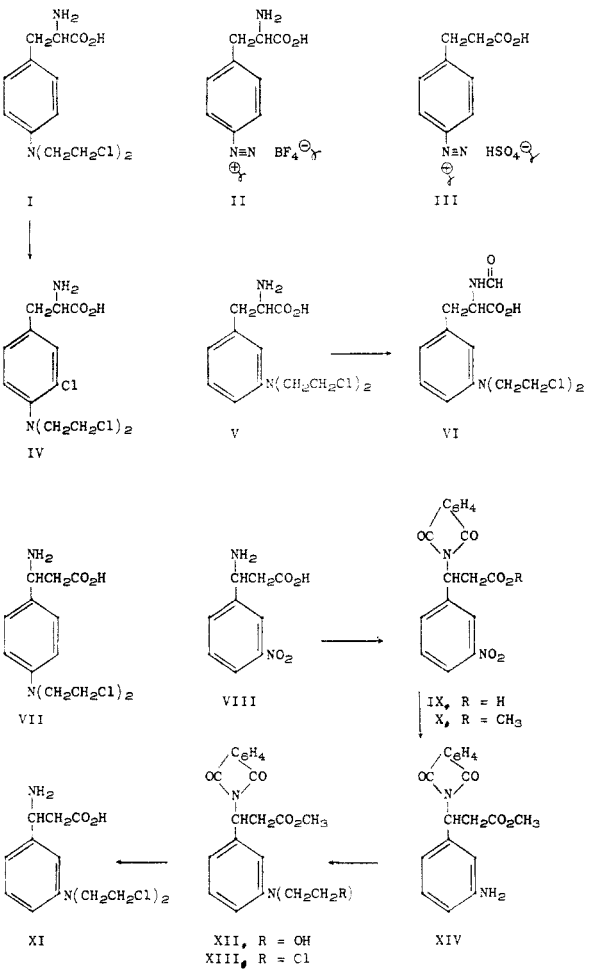
Three alkylating agents related to phenylalanine mustard have been synthesized, namely, 3-{*m*-[bis(2-chloroethyl)amino]-phenyl}-β-alanine (XI), 3-{4-[bis(2-chloroethyl)amino]-3-chlorophenyl}-DL-alanine (IV), and 3-{*m*-[bis(2-chloroethyl)-amino]phenyl}-*N*-formyl-DL-alanine (VI).

The increased selectivity of antitumor activity of phenylalanine mustard[2,3] (I) over that of some of the other nitrogen mustards ($HN_2$, Chlorambucil, Nitromin) would indicate that the phenylalanine moiety is a good carrier for alkylating groups. Replacement of the nitrogen mustard group of phenylalanine mustard with the diazonium group[4] gave a compound (II) effective against Ehrlich ascites and Leukemia L-1210 whereas 3-(*p*-diazoniumphenyl)propionic acid bisulfate III,[5] an analog of chlorambucil, was found to be inactive against Leukemia L-1210 and much less effective against Ehrlich ascites than was the diazonium compound derived from phenylalanine.

Biological test results[6] on the *meta*-isomer of phenylalanine mustard (V)[4] show that it has a more favorable chemotherapeutic index on Carcinoma-755, Ehrlich ascites, and Cloudman melanoma S-91 than does phenylalanine mustard (I), that it is equally effective against Sarcoma 180, and Dunning rat leukemia,[13] but that it is less effective against Leukemia L-1210.

Recently, the synthesis of 3-{*p*-[bis(2-chloroethyl)amino]phenyl}-β-alanine (VII), the β-amino acid isomer of phenylalanine mustard has been reported.[7,8] This compound was reported[8] to inhibit completely the growth of the Walker-256 tumor in rats when given at a dose of 20 mg./kg. of animal weight. It also inhibited Sarcoma 180 at a dose of 50 mg./kg. of animal weight.




This paper reports the synthesis of 3-{*m*-[bis(2-chloroethyl)amino]phenyl}-β-alanine (XI) by a synthetic route analogous to that used in this Laboratory for the synthesis of *meta*-phenylalanine mustard[4] (V); however, as is usually encountered with the synthesis of nitrogen mustards, the sequence had its individual idiosyncrasies to be overcome. In addition to XI, two other nitrogen mustards related to phenylalanine mustard were synthesized, namely, 3-{4-[bis(2-chloroethyl)-amino]-3-chlorophenyl}-DL-alanine (IV) and 3-

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{*m*-[bis(2-chloroethyl)amino]phenyl}-*N*-formyl-DL-alanine (VI).

The synthesis of 3-(*m*-nitrophenyl)-β-alanine (VIII) from *m*-nitrobenzaldehyde, ammonium acetate, and malonic acid according to the method used for the synthesis of β-phenyl-β-alanine[9] gave VIII in 67% yield. Earlier attempts to prepare VIII by the method of Rodionov, *et al.*,[10] using *m*-nitrobenzaldehyde, aqueous ammonia, and malonic acid resulted in lower yields of VIII contaminated with *m*-nitrocinnamic acid. Paper chromatography[11] proved to be useful in detecting whether samples of VIII were free of *m*-nitrocinnamic acid. Compound VIII traveled with an $R_f$ of 0.68 in solvent A and was ninhydrin-positive while *m*-nitrocinnamic acid had an $R_f$ of 0.91 in the same system when detected by its ultraviolet absorption.

3-(*m*-Nitrophenyl)-β-alanine (VIII) was converted to crystalline IX in quantitative yield using phthalic anhydride in pyridine followed by treatment with acetic anhydride. Esterification of IX with methanol saturated with hydrogen chloride afforded crystalline methyl β-(*m*-nitrophenyl)-1,3-dioxo-2-isoindolinepropionate (X) in 58% yield. Catalytic hydrogenation of X using 5% palladium on charcoal gave a quantitative yield of methyl β-(*m*-aminophenyl)-1,3-dioxo-2-isoindolinepropionate (VIV) as a crystalline solid.

Hydroxyethylation of XIV with ethylene oxide in aqueous acetic acid afforded methyl β-{*m*-[bis(2-hydroxyethyl)amino]phenyl}-1,3-dioxo-2-isoindolinepropionate (XII) in 85% yield as an analytically pure oil. This material traveled as a single spot ($R_f$ 0.74) when chromatographed in solvent D[11] while the starting compound (XIV) had an $R_f$ of 0.59 in the same system. Chlorination of XII using thionyl chloride in refluxing chloroform solution gave methyl β-{*m*-[bis(2-chloroethyl)amino]phenyl}-1,3-dioxo-2-isoindolinepropionate (XIII) in 49% yield. This compound traveled as a single spot ($R_f$ 0.51) in solvent D on paper. The technique[4] of dissociation of the hydrochloride of XIII, produced upon chlorination of XII with thionyl chloride—namely, evaporation of a methanolic solution of the hydrochloride—allowed the crystalline free base of XIII to be obtained.

Previous experience in this laboratory with acid hydrolysis of the *N*-phthalyl blocked methyl ester of *m*-phenylalanine mustard was extremely useful in the hydrolysis of XIII to XI. When XIII was refluxed for three hours in concentrated hydrochloric acid, the solution chilled and filtered to remove phthalic acid, then neutralized with sodium acetate to *p*H5, a yellow gum separated which could be extracted with chloroform. Concentration of the chloroform extracts gave a sirup which on crystallization from acetone afforded 3-{*m*[bis(2-chloroethyl)amino]phenyl}-β-alanine (XI) as a white solid, m.p. 178–182°, in 45% yield. An analytical sample of XI, m.p. 185–188°, was obtained by solution of the crude solid in 20% hydrochloric acid followed by neutralization with sodium acetate to reprecipitate XI.

Ring chlorination of aromatic compounds often has a profound effect on their biological activities; hence, it was of interest to modify the structure of phenylalanine mustard by ring chlorination and determine what effect this had on its antitumor activity. In order to prevent over-chlorination and avoid the problem of separation of mixtures of mono- and dichlorinated mustard, I was chlorinated with one equivalent of sulfuryl chloride in acetic acid. From this reaction, a monochloro derivative of phenylalanine mustard, m.p. 166–173°, was obtained in 41% yield. As the amine group of I could be expected to control the orientation of the incoming chlorine, the structure of the compound is probably 3-{4-[bis(2-chloroethyl)amino]-3-chlorophenyl}-DL-alanine (IV). The elementary analysis of IV would not allow one to distinguish it from the hydrochloride of phenylalanine mustard (I). That the compound isolated was not the hydrochloride was shown by the low ionic chloride value (0.45%). The absence of free carboxyl absorption in the infrared also supports structure IV as a zwitterion rather than the mustard (I) hydrochloride with an unionized carboxyl.

Knunyants, Kil'disheva, and Golubeva[12] reported the synthesis of *N*-formylphenylalanine mustard in 1956. The *N*-formyl derivative of L-phenylalanine mustard has been reported to be more effective on Dunning Leukemia than was phenylalanine mustard.[13] Peptides of *N*-formylphenylalanine mustard have been synthesized and are reported to be less toxic then the parent mustard yet still show decided antitumor activity.[14]

This paper reports the synthesis of 3-{*m*-[bis(2-chloroethyl)amino]phenyl}-*N*-formyl-DL-alanine (VI) in 91% yield by treatment of *m*-phenyl-

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(11) Paper chromatograms were run by the descending technique on Whatman No. 1 paper in solvent A, *n*-butyl alcohol-acetic acid–water (5:2:3); solvent B, *n*-butyl alcohol saturated with water; or solvent C, ammonium sulfate–isopropyl alcohol–water (2:28:70); and on Schleicher and Schuell No. 2495 acetylated paper in solvent D, benzene-methanol-water (2:6:1). The compounds were detected by their ultraviolet absorption, and in the case of the amino acids, by the use of ninhydrin reagent.

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alanine mustard (V)[4] with formic acid in acetic anhydride. The *N*-formyl derivative (VI) was obtained as an analytically pure solid, m.p. 151–152°. The absence of any appreciable amount of *m*-phenylalanine mustard (V) as contaminant was shown by movement of VI as a single ninhydrin-negative spot ($R_f$ 0.93) in solvent system A when spotted at 200 $\gamma$ whereas V could be detected at 5 $\gamma$ using ninhydrin reagent and had an $R_f$ of 0.85 in the same solvent system.[11]

## EXPERIMENTAL[15]

*3-(m-Nitrophenyl)-β-alanine* (VIII). A mixture of 30.2 g. (0.20 mole) of *m*-nitrobenzaldehyde, 20.8 g. (0.20 mole) of malonic acid, and 30.8 g. (0.40 mole) of ammonium acetate in 50 ml. of 95% ethanol was heated on a steam bath under reflux for 5 hr. The mixture was cooled to room temperature and filtered. The yellow solid was washed with 20 ml. of 95% ethanol and the washings discarded. The remaining solid was partially dissolved in 150 ml. of 2*N* hydrochloric acid at room temperature and the undissolved solid (*m*-nitrocinnamic acid) removed by filtration and washed with 10 ml. of water. The combined filtrate and washings were neutralized with 20 ml. of 15*N* ammonium hydroxide solution. As the solution was cooled, a precipitate formed; yield, 28.0 g. (67%), m.p. 224–226° (lit.,[10] m.p. 226–227°); $\lambda_{max(\mu)}^{Nujol}$ 4.75 ($NH_3^+$); 6.20 (aryl); 6.32, 7.12 ($CO_2^-$); 6.50, 7.35 ($NO_2$); 12.1 (*m*-disubstituted benzene). The compound traveled as a single spot ($R_f$ 0.68) in solvent system A[11] and was ninhydrin-positive. The acid insoluble by-product, *m*-nitrocinnamic acid, had an $R_f$ of 0.91 in the same system when detected by its ultraviolet absorption.

The method of Radionov and Malewinskaja[10] gave 20–30% yields.

*β-(m-Nitrophenyl)-1,3-dioxo-2-isoindolinepropionic acid* (IX). A mixture of 9.42 g. (0.045 mole) of 3-(*m*-nitrophenyl)-β-alanine (VIII) and 6.62 g. (0.045 mole) of phthalic anhydride in 100 ml. of pyridine was refluxed until all the solid had dissolved (4.5 hr.). The solution was concentrated *in vacuo* to a yellow oil which was dissolved in 30 ml. of acetic anhydride and refluxed for 10 min. The resulting solution was poured onto ice and acidified to *p*H 2 with concd. hydrochloric acid. The gum which formed was triturated with the acid solution until solidification occurred; yield, 15.3 g. (100%), m.p. 108–115°, of solvated crystals that were pure enough for the next step. Several recrystallizations from chloroform-petroleum ether (b.p. 30–60°) gave an analytical sample, m.p. 158°; $\lambda_{max(\mu)}^{Nujol}$ 5.63, 5.83 (phthaloyl and carboxyl C=O); 6.53, 7.42 ($NO_2$); 13.85 (phthaloyl). The compound traveled as one spot ($R_f$ 0.95) in solvent system A[11] (ninhydrin negative). In solvent D, IX traveled as a single spot ($R_f$ 0.56). The starting material VIII had an $R_f$ of 0.66 in the same system (ninhydrin positive).

*Anal.* Calcd. for $C_{17}H_{12}N_2O_6$: C, 60.0; H, 3.55; N, 8.23; Found: C, 60.0; H, 3.44; N, 8.28.

*Methyl β-(m-nitrophenyl)-1,3-dioxo-2-isoindolinepropionate* (X). To 20 ml. of methanol saturated with hydrogen chloride was added 3.05 g. (9.0 mmoles) of β-(*m*-nitrophenyl)-1,3-dioxo-2-isoindolinepropionic acid (IX). The mixture was refluxed for 2 hr., then cooled to room temperature whereupon white crystals separated. The mixture was concentrated *in vacuo* to about 10 ml., then chilled in ice to yield 1.86 g. (58%) of white solid, m.p. 117–121°, suitable for the next step.

An analytical sample was prepared by recrystallization from absolute ethanol, m.p. 116.5–118°; $\lambda_{max(\mu)}^{Nujol}$ 5.75, 5.84 (phthaloyl and ester C=O); 6.50, 7.38 ($NO_2$); 8.28 (ester C—O—C); 13.78 (phthaloyl). The compound traveled as a single spot ($R_f$ 0.94) in solvent system A[11] and as a single spot ($R_f$ 0.26) in solvent system D.

*Anal.* Calcd. for $C_{18}H_{14}N_2O_6$: C, 61.0; H, 3.98; N, 7.91. Found: C, 61.0; H, 4.15; N, 8.02.

*Methyl β-(m-aminophenyl)-1,3-dioxo-2-isoindolinepropionate* (XIV). A mixture of 14.2 g. (0.040 mole) of methyl β-(*m*-nitrophenyl)-1,3-dioxo-2-isoindolinepropionate (X) and 1.5 g. of 5% palladium on charcoal (moistened with about 10 ml. of methyl cellosolve) was suspended in 200 ml. of methanol and hydrogenated at 53 psig at room temperature until no more hydrogen was absorbed (2 hr.). The catalyst was filtered from the reaction mixture and washed well with methanol. The filtrate was concentrated *in vacuo* to a yellow solid; yield 13.0 g. (100%), m.p. 130–132°, suitable for the next step.

An analytical sample, m.p. 131–133°, was obtained by recrystallization from methanol, then from chloroform–petroleum ether (b.p. 30–60°); $\lambda_{max(\mu)}^{Nujol}$ 2.93, 2.99 (NH); 5.63, 5.73, 5.82 (phthaloyl and ester C=O); 6.21, 6.67 (aryl); 8.27 (ester C—O—C); 13.8 (phthaloyl). The compound traveled as a single spot ($R_f$ 0.59) in solvent system D[11] (ninhydrin negative).

*Anal.* Calcd. for $C_{18}H_{16}N_2O_4$: C, 66.7; H, 4.97; N, 8.64. Found: C, 66.5; H, 4.92; N, 8.80.

*Methyl β-{m-[bis(2-hydroxyethyl)amino]phenyl}-1,3-dioxo-2-isoindolinepropionate* (XII). To a solution of 13.0 g. (0.040 mole) of methyl β-(*m*-aminophenyl)-1,3-dioxo-2-isoindolinepropionate (XIV) in 80 ml. of glacial acetic acid were added 60 ml. of water and 22 ml. (0.44 mole) of ethylene oxide. The flask was stoppered and allowed to stand at room temperature for 24 hr. The solution was poured into 200 ml. of water and neutralized with solid sodium hydrogen carbonate. The oil which separated was extracted with ethyl acetate, the extracts dried over anhydrous magnesium sulfate, then concentrated *in vacuo* to a red-brown oil; yield, 14 g. (85%); $\lambda_{max(\mu)}^{film}$ 2.95 (OH); 5.62, 5.72, 5.82 (phthaloyl and ester C=O); 6.23, 6.63 (aryl); 8.50 (ester C—O—C); 9.30, 9.85 (C—OH); 12.81 (*m*-disubstituted benzene); 13.83 (phthaloyl). The compound traveled as a single spot ($R_f$ 0.74) in solvent system D.[11]

*Anal.* Calcd. for $C_{22}H_{24}N_2O_6$: C, 64.1; H, 5.87; N, 6.79. Found: C, 64.0; H, 5.94; N, 6.63.

*Methyl β-{m-[bis(2-chloroethyl)amino]phenyl}-1,3-dioxo-2-isoindolinepropionate* (XIII). To a solution of 19.0 g. (0.047 mole) of methyl β-{*m*-[bis(2-hydroxyethyl)amino]-phenyl}-1,3-dioxo-2-isoindolinepropionate (XII) in 65 ml. of chloroform was added 9.0 ml. (0.124 mole) of thionyl chloride in 20 ml. of chloroform. This solution was refluxed for 2 hr. and then concentrated *in vacuo* to a dark sirup. Four 50-ml. portions of methanol were added and the solution evaporated after each addition. After standing overnight under methanol, the sirup had solidified and was recrystallized from ethanol to give 10.3 g. (49%) of a white solid, m.p. 105–111°, that was suitable for the next step.

A small portion was recrystallized three times from ethanol to give a constant melting solid, m.p. 115–116°; $\lambda_{max(\mu)}^{Nujol}$ 5.64, 5.74, 5.82 (phthaloyl and ester C=O); 6.22, 6.67 (aryl); 8.32, 8.51 (ester C—O—C); 12.18 (*m*-disubstituted benzene); 13.80 (phthaloyl); and no OH near 3 or 9–10 $\mu$. The compound moved as a single spot ($R_f$ 0.51) in solvent system D.[11]

*Anal.* Calcd. for $C_{22}H_{22}Cl_2N_2O_4$: C, 58.7; H, 4.90; Cl, 15.8; N, 6.23. Found: C, 59.0; H, 5.15; Cl, 15.6; N, 6.43.

*3-{m-[Bis(2-chloroethyl)amino]phenyl}-β-alanine* (XI). A solution of 7.5 g. (0.021 mole) of methyl β-{*m*-[bis(2-chloroethyl)amino]phenyl} - 1,3 - dioxo - 2 - isoindolinepropionate (XIII) in 75 ml. of concd. hydrochloric acid was refluxed for 3 hr. The solution was chilled in ice and the phthalic acid removed by filtration. The filtrate was neutralized with a saturated aqueous sodium acetate solution to *p*H 5. The yellow gum which formed was extracted with three 25-ml. portions of chloroform. The combined extracts were

(15) Melting points were taken on a Fisher-Johns block and are uncorrected.

dried over anhydrous magnesium sulfate, then concentrated *in vacuo* to a brown sirup. This sirup was dissolved in 50 ml. of hot acetone and the solution allowed to stand overnight at room temperature. The white, powdery solid which had separated was collected and washed with acetone; yield 2.3 g. (45%); m.p. 178–182°. A portion of this solid was dissolved in 20% hydrochloric acid and neutralized with a saturated aqueous solution of sodium acetate causing a white solid to separate. The filtrate upon partial concentration *in vacuo* yielded an analytical sample of VII, m.p. 185–188°; $\lambda_{max(\mu)}^{Nujol}$ 6.21, 6.30, 6.40 ($NH_3^+$, $CO_2^-$, aryl); 6.63 (aryl, $NH_3^+$); 7.06 ($CO_2^-$); 12.8 (*m*-disubstituted benzene); 13.5 (C—Cl). The compound (XI) traveled as a single ninhydrin and ultraviolet absorbing positive spot ($R_f$ 0.84) in solvent system A.[11]

*Anal.* Calcd. for $C_{13}H_{18}Cl_2N_2O_2$: C, 51.2; H, 5.92, Cl, 23.2; N, 9.18. Found: C 51.1; H, 6.06, Cl, 23.3; N, 9.20.

*3-{4-[Bis(2-chloroethyl)amino]-3-chlorophenyl}*-DL-*alanine* (IV). To a stirred suspension of 3.05 g. (0.010 mole) of 3-{*p*-[bis-(2-chloroethyl)amino]phenyl}-DL-alanine (I) in 30 ml. of glacial acetic acid heated to 50° was added dropwise in about 2–3 min. 0.85 ml. (0.010 mole) of sulfuryl chloride, maintaining the temperature between 50–55° by external cooling. Ten minutes after all the sulfuryl chloride had been added, the solution was concentrated *in vacuo* to 5 ml., diluted with 10 ml. of water, and neutralized with saturated sodium acetate solution. The gum which separated was triturated with water until it solidified. Solution of the solid in 25 ml. of methanol and addition of water caused a dark gum to separate. After removal of this gum, further addition of water yielded a granular solid upon chilling; yield 1.4 g. (41%), m.p. 166–173°; $\lambda_{max(\mu)}^{Nujol}$ 4.80 ($NH_3^+$); 6.09 (amino acid I); 6.65 (amino acid II); 6.31, 7.13 ($CO_2^-$). The compound (IV) traveled as a single ninhydrin positive spot ($R_f$ 0.72) in solvent system C.[11] The starting material (I) traveled nearly the same ($R_f$ 0.68) in that system.

*Anal.* Calcd. for $C_{13}H_{17}Cl_3N_2O_2$: C, 45.9; H, 5.04; Cl, 31.3. Found: C, 45.5; H, 5.18, Cl, 31.3. An analysis for ionic chloride, carried out at 0°, gave 0.45%.

*3-{m-[Bis(2-chloroethyl)amino]phenyl}-N-formyl-*DL-*alanine* (VI). To a solution of 0.60 g (2.0 mmole) of 3-{*m*-[bis-(2-chloroethyl)amino]phenyl}-DL-alanine (V) in 4 ml. of 90% formic acid was added 1.2 ml. of acetic anhydride. The red colored solution was warmed at 50–55° for 30 min. After cooling, the reaction mixture was diluted with 20 ml. of water and the product slowly crystallized; yield; 0.61 g. (91%), m.p. 145–150°. A sample for analysis was prepared by recrystallization from absolute ethanol, m.p. 151–152°; $\lambda_{max(\mu)}^{Nujol}$ 2.99 (NH); 5.80 (acid C=O); 6.19 (amide C=O); 6.64 (amide NH); 12.9 (*m*-disubstituted benzene). This compound (VI) traveled as a single spot ($R_f$ 0.93) in solvent system A[11] and had an $R_f$ of 0.71 in solvent system B. The chromatograms were ninhydrin negative at 200 $\gamma$ while the starting amino acid (V) was detectable at 5 $\gamma$ using ninhydrin reagent, thus showing less than 3% of (V) could have been present. Compound V had an $R_f$ of 0.85 in solvent system A and $R_f$ of 0.48 in solvent system B.[11]

*Anal.* Calcd. for $C_{14}H_{18}Cl_2N_2O_3$: C, 50.4; H, 5.42; Cl, 21.3; N, 8.40. Found: C, 50.5; H, 5.44; Cl, 21.0; N, 8.34.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACOLOGY, SCHOOL OF MEDICINE, YALE UNIVERSITY]

# Synthesis of Some 8-Purinyl Nitrogen Mustards[1]

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As part of a program concerned with the preparation of nitrogen mustards intended to exhibit biological specificity, the 8-bis(β-chloroethyl)amino derivatives of xanthine, hypoxanthine, and adenine were synthesized.

In recent years large numbers of nitrogen mustards, *i.e.*, compounds containing the bis-(β-chloroethyl)amino grouping, have been synthesized as potential antitumor agents. In the hope of increasing the biological specificity of these compounds the mustard group has been attached to various carrier molecules such as antimalarial drugs,[3] amino acids[4,5] steroids,[6] and carbohydrates,[7] to name only a few.

In view of the hypothesis that double armed mustards exert their carcinostatic effects by reacting with the phosphate groups of nucleic acids, thus causing cross-linking between adjoining double helices,[8] it seemed of interest to synthesize compounds which would facilitate the likelihood of such cross-linking occurring.

This problem was approached in two ways, (a) by synthesizing 8,8′-bispurines,[9] and (b) by synthesizing the 8-purinyl nitrogen mustards described in this communication. Incorporation of 8,8′-bispurines (or their deoxyribonucleosides) in neighboring deoxyribonucleic acid double helices might result in direct crosslinking, while incorporation of

(1) This work was supported, in part, by grants CY-3937 and CY-2817 from the National Institutes of Health, Public Health Service.

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