thione (VIc) (500 mg., 1.65 mmoles), potassium carbonate (228 mg., 1.65 mmoles), α-chlorotoluene (0.21 ml., 1.8 mmoles), and 5 ml. of DMF was stirred at 60–65° for 3 hr. The reaction mixture was cooled and poured into 20 ml. of cold water, a yellowish cheese-like solid being formed. After overnight refrigeration, the aqueous solution was decanted from the solid, which was then recrystallized from methyl alcohol to give pale yellow crystals, which were washed with ether and air-dried; yield 540 mg. (83%); m.p. 109–110°; λ_{max} in mμ ($\epsilon \times 10^{-3}$): *p*H 1—247 (11.8), 333 (13.2); *p*H 7—247 (14.6), 335 (14.1); *p*H 13—246 (15.5), 337 (14.1). A mixed melting point with an authentic sample of the 7-isomer was not depressed.

Anal. Calcd. for $C_{21}H_{20}N_4S_2 \cdot {}^1/_4\ H_2O$: C, 63.60; H, 5.19; S, 16.15. Found: C, 63.77; H, 5.12; S, 16.24.

7-Methyl-1-[2-(p-tolylthio)ethyl]-7H-purine-6(1H)-thione (IXb). Iodomethane (0.063 ml., 1.0 mmole) was added dropwise to a stirred mixture of 1-[2-(*p*-tolylthio)ethyl]-purine-6(1*H*)-thione (VIc) (280 mg., 0.92 mmole), potassium carbonate (127 mg., 0.92 mmole), and 3 ml. of DMF. The mixture was heated at 40–50° for 4 hr., and then poured into 20 ml. of cold water. The thick red oil that formed was separated by decantation and crystallized from aqueous methyl alcohol; yield 29 mg. (10%); m.p. 144°; λ_{max} in mμ ($\epsilon \times 10^{-3}$): *p*H 1—247 (9.4), 330 (14.0); *p*H 7—244 (11.4), 330 (16.0); *p*H 13—244 (11.9), 330 (15.7).

Anal. Calcd. for $C_{15}H_{16}N_4S_2$: C, 56.90; H, 5.10; N, 17.73; S, 20.20. Found: C, 56.89; H, 5.49; N, 17.65; S, 19.90.

1-[2-(p-Tolylthio)ethyl]hypoxanthine (XII). To a stirred solution of inosine (1.00 g., 3.76 mmoles) in warm dimethyl sulfoxide (10 ml.) was added potassium carbonate (569 mg.) and 2-chloroethyl *p*-tolyl sulfide[12] (700 mg.). The mixture was heated for 18 hr. at 80–100°, and then cooled to room temperature and poured into 50 ml. of cold water. The volatiles were removed under reduced pressure and the brown residue was refluxed for 2 hr. with a mixture of 5 ml. of concentrated hydrochloric acid and 95 ml. of ethyl alcohol. Again the volatiles were removed under reduced pressure, and the brown syrup that remained dissolved in water. This aqueous solution was treated with charcoal, filtered, adjusted to pH 5 with sodium hydroxide solution, and refrigerated overnight. The brown solid that was deposited was recrystallized from methyl alcohol to give a white solid which was dried at 60° for 4 hr. over phosphorus pentoxide, *in vacuo;* yield 210 mg. (20%); m.p. 168°; λ_{max} in mμ ($\epsilon \times 10^{-3}$): *p*H 1—249.5 (13.4); *p*H 7—253 (12.5); *p*H 13—258 (12.8).

Anal. Calcd. for $C_{14}H_{14}N_4OS$: C, 58.72; H, 4.93; N, 19.57. Found: C, 58.62; H, 4.90; N, 19.36.

Acknowledgment. The authors are indebted to Dr. W. J. Barrett and the members of the Analytical Section of Southern Research Institute who performed the spectral and most of the microanalytical determinations reported, and to Mrs. Dale Carruthers and Miss Mary Broadaway for technical assistance. Some of the analyses reported were performed by the Galbraith Microanalytical Laboratories, Knoxville, Tenn.

BIRMINGHAM, ALA.

(12) D. Klamann and H. Bertsch, *Chem. Ber.*, **88**, 201 (1955).

[CONTRIBUTION FROM THE CLAYTON FOUNDATION BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS]

Nitrogen Mustard Analog of Thiocytosine

HAYIM SEGAL[1] AND CHARLES G. SKINNER

Received July 27, 1961

2,4-Dimercaptopyrimidine was interacted with diethanolamine in the presence of pyridine to yield *N,N*-bis-(2-hydroxyethyl)thiocytosine, which was subsequently treated with thionylchloride to produce the nitrogen mustard analog. Some preliminary biological studies on the latter compound are indicated.

A number of purine and pyrimidine derivatives which are structurally related to basic chemical structures that are normally present in the cell are known to be effective antitumor agents. The replacement of hydroxy groups has been successful in the production of antitumor agents; for example, the hydroxy group of hypoxanthine has been replaced by the thiol grouping to yield 6-mercaptopurine,[2] an efficient antitumor agent in a number of systems.[3] Alkylating agents containing the nitrogen mustard radical (bis-(2-chloroethyl)-amino-) have also been widely used on a large number of different types of chemical structures.[4]

In the present study an attempt was made to place both of these types of groupings in a single compound, and the structure chosen for the "carrier molecule"[5] was the natural pyrimidine metabolite, cytosine. 4-[Bis-(2-chloroethyl)amino]-2-pyrimidinethiol was accordingly synthesized, and the antitumor activity on an implanted RC mammary adenocarcinoma, as well as some antimicrobial activities, was determined.

The syntheses of only a few pyrimidines containing the nitrogen mustard grouping attached directly to the ring have been described—e.g.,

(1) Rosalie B. Hite Postdoctoral Fellow 1960–1961; on leave from The Weizmann Institute of Science, Rehovoth, Israel.

(2) G. B. Elion, E. Burgi, and G. H. Hitchings, *J. Am. Chem. Soc.*, **74**, 411 (1952).

(3) G. H. Hitchings *et al.*, *Ann. N. Y. Acad. Sci.*, **60**, 195 (1954).

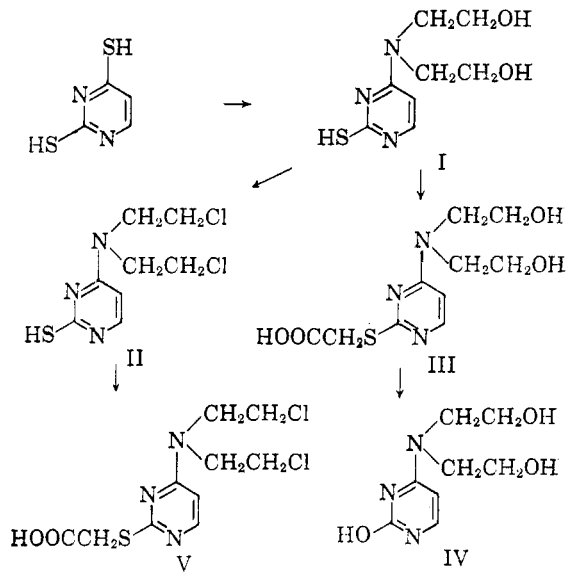
(4) R. B. Ross and P. E. Swartzentruber, *Literature Survey of Nitrogen Mustards*, 1959 Cancer Chemotherapy National Service Center, National Institutes of Health, Bethesda 14, Md.

(5) F. Bergel and J. A. Stock, *J. Chem. Soc.*, 2409 (1954).

5-[bis(2-chloroethyl)amino]uracil[6] and its 1-methyl analog,[7] 4-[bis-(2-chloroethyl)amino]-2-chloro-6-methyl-5-nitropyrimidine,[3] and 5-[bis-(2-chloroethyl)amino]-6-methyluracil.[8] In contrast to the synthetic procedures utilized to prepare these compounds, the direct reaction of diethanolamine with an appropriate mercaptopyrimidine derivative would appear to be a potential synthetic route to nitrogen mustard analogs. However, it has been reported that amination of the 4-thiol group of 2,4-dimercaptopyrimidine with secondary amines higher than dimethylamine does not yield the anticipated condensation product.[9] The use of pyridine to facilitate cyclo-dehydrosulfidation reactions has been utilized in the conversion of 4-(substituted)-amino-5-thioformamidopyrimidines to the corresponding purines.[10] Because in the latter instance, the effect of pyridine might be as an alkaline reagent to effect the removal of the acidic hydrogen sulfide formed in the reaction, comparable conditions were used in an attempt to carry out a dehydrosulfidation utilizing a heterocyclic thiol grouping and diethanolamine. In the presence of pyridine the interaction of diethanolamine with 2,4-dimercaptopyrimidine produced the desired intermediate, *N,N*-bis(2-hydroxyethyl)thiocytosine. The optimum reaction conditions herein described involve a thermal condensation at reflux temperature of 1 part of 2,4-dimercaptopyrimidine with 4 parts each of diethanolamine and pyridine over a period of about 6 hours. Increasing the reaction time or the ratio of pyridine in the reaction mixture decreased the yield. The direct amination of 2,4-dimercaptopyrimidine with diethanolamine was also successful in the presence of water when the reaction period was extended; however, the ultimate isolation of the reaction product proved to be difficult and the yield was lower.

The formation of the desired nitrogen mustard analog, 4-[bis-(2-chloroethyl)amino]-2-pyrimidinethiol, from the corresponding bis-2-hydroxyethyl derivative was accomplished through direct chlorination using thionyl chloride in bis-(2-methoxy)-ethyl ether ("Diglyme") containing a trace of water. This nitrogen mustard is highly hygroscopic, soluble in most polar solvents, and relatively insoluble in nonpolar solvents such as ether and ethyl acetate. Treatment of this derivative with chloroacetic acid produced the anticipated S-carboxymethylthio analog. The ethylenimonium form[11] of this mustard was also isolated as a picrate salt. The synthetic sequence is summarized in the accompanying equations.

Treatment of *N,N*-bis(2-hydroxyethyl)thiocytosine with chloroacetic acid produced the anticipated 2-carboxymethylthio derivative and acid hydrolysis of the latter compound yielded *N,N*-bis-(2-hydroxyethyl)cytosine.



These substituted-pyrimidines were subsequently examined for antimicrobial activity as well as their ability to inhibit growth of an implanted mouse tumor. At concentrations of 200 γ/ml., none of these derivatives were inhibitory to growth of either *Escherichia coli* 9723 in an inorganic salts-glucose medium[12] or to *Streptococcus lactis* 8039 as tested under assay conditions previously described for this organism.[13] Using *Lactobacillus arabinosus* 17-5 and *Leuconostoc dextranicum* 8086 grown in a previously described medium,[13] only the *N,N*-bis-(2-chloroethyl)thiocytosine (II) was inhibitory to microbial growth under the assay conditions. The toxic level for complete inhibition of growth by II in the latter two organisms was about 200 γ/ml. and 60 γ/ml., respectively. The toxicity in *L. dextranicum* was not appreciably reversed by high levels of either a liver extract or an enzymatic digest of casein.

A preliminary examination of the antitumor properties of these analogs using a RC mammary adenocarcinoma implanted in dba mice by a previously reported assay technique[14] indicated only slight inhibitory properties. The LD_{50} of II appeared to be about 2 mg./20 g. animal; however,

(6) D. A. Lyttle and H. G. Petering, *J. Am. Chem. Soc.*, **80**, 6459 (1958).

(7) A. Benitez, L. O. Ross, L. Goodman, and B. R. Baker, *J. Am. Chem. Soc.*, **82**, 4585 (1960).

(8) L. F. Larionova and G. N. Platonova, *Voprosy Onkologii*, **1**, 36 (1955); *Chem. Abstr.*, **51**, 6862 (1957).

(9) P. B. Russell, G. B. Elion, E. A. Falco, and G. H. Hitchings, *J. Am. Chem. Soc.*, **71**, 2279 (1949).

(10) J. Baddiley, B. Lythgoe, D. McNeil, and A. R. Todd, *J. Chem. Soc.*, **383** (1943).

(11) A. Gilman and F. S. Phillips, *Science*, **103**, 409 (1946).

(12) E. H. Anderson, *Proc. Natl. Acad. Sci.*, **32**, 120 (1946).

(13) J. M. Ravel, L. Woods, B. Felsing, and W. Shive, *J. Biol. Chem.*, **206**, 391 (1954).

(14) A. Taylor, G. F. McKenna, and H. M. Burlage, *Tex. Repts. Biol. Med.*, **14**, 538 (1956).

seven daily injections of one-half this dosage decreased the tumor growth by about one-third as compared to untreated control animals. I and III were about equally as effective as the nitrogen mustard analog II at a dosage of 1 mg./20 g. animal. As tumor specificities have been observed with a number of cytotoxic agents, additional studies of these derivatives in other systems may be warranted.

EXPERIMENTAL[15]

4-[Bis-(2-hydroxyethyl)amino]-2-pyrimidinethiol (N,N-Bis(2-hydroxyethyl)thiocytosine). (I) A mixture of 5.76 g. of 2,4-dimercaptopyrimidine,[16] 16.8 g. of diethanolamine, and 10 ml. of pyridine was heated under reflux for about 6 hours. After cooling, methanol was added to the reaction mixture and it was placed in a refrigerator overnight. There was obtained 5.28 g. of product which was recrystallized from ethanol for elemental analysis, m.p. 204–208° dec.

Anal. Calcd. for $C_8H_{13}N_4SO_2$: C, 44.64; H, 6.09; N, 19.52; S, 14.89. Found: C, 44.73; H, 5.89; N, 19.43; S, 14.59.

This condensation was also carried out in aqueous medium by heating under reflux for about 24 hours a mixture of 2.88 g. of 2,4-dimercaptopyrimidine and 2.31 g. of diethanolamine in 10 ml. of water. After cooling, 0.35 g. of unreacted pyrimidine was recovered, and the product was precipitated by the addition of 5.4 g. of mercuric chloride in 35 ml. of ethanol to yield 5.3 g. of light-yellow solid, m.p. 204–210° dec. The mercuric chloride complex was decomposed by heating with concentrated hydrochloric acid, filtered, and hydrogen sulfide was bubbled through the solution. After filtration, the solution was reduced to dryness *in vacuo* to yield 1 g. of product which was identical with the compound described above as determined by the lack of depression of a mixture melting point, and comparable R_f values in solvent systems A, B, and C of 0.32, 0.31, and 0.62, respectively.

4-[Bis-(2-hydroxyethyl)amino]-2-carboxymethylthiopyrimidine Hydrochloride (III). A solution of 4.3 g. of 4-[bis-(2-hydroxyethyl)-amino]-2-pyrimidinethiol and 1.89 g. of chloroacetic acid in 10 ml. of water was warmed with stirring for about 1 hour. The solvent was removed and the residue was crystallized from methanol–ethyl acetate to yield 4.58 g. of material, m.p. 153–156° dec.

Anal. Calcd. for $C_{10}H_{15}N_3SO_4 \cdot HCl$: C, 38.74; H, 4.88; N, 13.56. Found: C, 38.91; H, 5.12; N, 13.83.

4-[Bis-(2-hydroxyethyl)amino]-2-pyrimidinol Hydrochloride [N,N-Bis-(2-hydroxyethyl)cytosine Hydrochloride] (IV). A sample of 1.43 g. of 4-[bis-[2-hydroxyethyl)amino]-2-carboxymethylthiopyrimidine hydrochloride was heated under reflux for about 4 hours in the presence of 20 ml. of concentrated hydrochloric acid. The solvent was removed *in vacuo*, the residue was washed with ether, and finally dissolved in methanol and treated with Darco G-60. After standing at room temperature there was recovered 0.4 g. of product, m.p. 214–215° dec. R_f values in solvent systems B and C were 0.11 and 0.65, respectively.

Anal. Calcd. for $C_8H_{13}N_3O_3 \cdot HCl$: N, 17.83. Found: N, 17.77.

4-[Bis-(2-chloroethyl)amino]-2-pyrimidinethiol (N,N-bis-(2-chloroethyl)thiocytosine) (II). To a solution containing 1.2 ml. of thionyl chloride and 1 ml. of ethanol in 33 ml. of bis-(2-methoxy)ethyl ether ("Diglyme") was added 3.23 g. of 4-[bis-(2-hydroxy-ethyl)amino]-2-pyrimidinethiol followed by the slow addition of 5.75 ml. more of thionyl chloride. After the exothermic reaction had subsided, the reaction mixture was stirred at room temperature for about 20 hours. The solvent was removed *in vacuo*, and the dry residue was taken up in 200 ml. of hot ethanol. After treatment with Darco G-60, the solution was again reduced to dryness *in vacuo* to yield 3.33 g. of a tan amorphous solid, m.p. (sealed capillary) 150–151° dec. The sample was extremely hygroscopic and failed to crystallize satisfactorily from the usual solvent systems; a sample was dried for elemental analysis *in vacuo* over phosphorous pentoxide. R_f data include B and C, 0.48 and 0.77, respectively.

Anal. Calcd. for $C_8H_{11}N_3SCl_2 \cdot H_2O$: C, 35.56; H, 4.85; N, 15.55; S, 11.86; Cl, 26.24; O, 5.94. Found: C, 35.50; H, 4.57; N, 15.78; S, 11.63; Cl, 25.82; O, 5.69.

4-[Bis-(2-chloroethyl)amino]-2-carboxymethylthiopyrimidine (V). To a solution of 0.5 g. of 4-[bis-(2-chloroethyl)-amino]-2-pyrimidinethiol in 5 ml. of water was added 0.2 g. of chloroacetic acid, and the reaction mixture was heated under reflux with stirring for about 5 minutes. After stirring an additional 3 hours, the solvent was removed *in vacuo*. The residue was washed with ether, then dissolved in ethanol, and a few drops of ether were added to induce precipitation. Recrystallization from ethanol–ethyl acetate yielded 0.5 g. of highly hygroscopic material, m.p. 142–145° dec. R_f values in solvents B and C were 0.52 and 0.73, respectively.

Anal. Calcd. for $C_{10}H_{13}N_3SCL_2O_2$: C, 38.71; H, 4.22; N, 13.54; S, 10.34. Found: C, 38.28; H, 4.01; N, 13.73; S, 11.00.

N-(2-Chloroethyl)-N-(2-mercapto-4-pyrimidinyl)-N-ethyleneimonium picrate. A sample of 250 mg. of 4-[bis-(2-chloroethyl)-amino]-2-pyrimidinethiol in 5 ml. of ethanol was heated under reflux for about 2.5 hours, cooled, and 1 g. of picric acid was added. After heating an additional hour, 1 ml. of water was added and the reaction mixture was placed in the refrigerator overnight. The resulting brownish-red crystals were dissolved in methanol and treated with Darco G-60. Upon cooling, there was recovered 170 mg. of product, which was recrystallized from methanol, m.p. 198–200° dec. It gave a negative test for ionic chloride, and had R_f values in solvents A, B, and C of 0.89, 0.61, and 0.97, respectively.

Anal. Calcd. for $C_{14}H_{13}N_6O_7SCl \cdot H_2O$: C, 36.35; H, 3.27; N, 18.17; S, 6.93. Found: C, 36.13; H, 3.45; N, 18.20; S, 6.62.

Acknowledgment. The authors wish to express their deep appreciation to Professor W. Shive for his very valuable advice and counsel during the course of this investigation.

AUSTIN, TEX.

(15) All melting or decomposition points are uncorrected. The authors are indebted to G. F. McKenna of The Clayton Foundation Biochemical Institute for a preliminary report of the antitumor properties of these compounds, and to Dr. J. M. Ravel and her group for the microbial assays. The paper chromatographic data were determined by the ascending technique, and the spots were observed by fluorescence (or quenching) under an ultraviolet lamp emitting at either 254 or 360 mμ, using the following solvent systems: "A"—*n*-butyl alcohol–water (85:15); "B"—*n*-butyl alcohol–acetic acid–water (4:1:1); "C"—*iso*-propyl alcohol–hydrochloric acid:water (4:1:1). Carbon, hydrogen, and nitrogen analyses were carried out in the authors' laboratory by C. Hedgcoth and J. D. Glass; the other elemental analyses were conducted by Alfred Bernhardt, Munich, Germany.

(16) D. J. Brown, *J. Soc. Chem. Ind.*, 69, 353 (1950).