[CONTRIBUTION FROM THE KETTERING-MEYER LABORATORY,[1] SOUTHERN RESEARCH INSTITUTE]

Synthesis of Potential Anticancer Agents. XXVII. The Ribonucleotides of 6-Mercaptopurine and 8-Azaguanine[2]

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The ribonucleotides of 6-mercaptopurine and 8-azaguanine were prepared by the reaction of 2-cyanoethyl dihydrogen phosphate with the 2′,3′-*O*-isopropylidene derivatives of the corresponding ribonucleosides followed by hydrolytic removal of the blocking groups. Some preliminary biological observations are discussed.

There is much evidence to support the view that, to inhibit growth, two important anticancer agents, 6-mercaptopurine [purine-6(1H)-thione] and 8-azaguanine [5-amino-*v*-triazolo[4,5-*d*]pyrimidin-7-(6H)-one], must be metabolized *in vivo* to their ribonucleotides (Va and Vb), which may then inhibit growth by interfering with normal nucleotide metabolism.[3-7] Resistance of certain microbiological systems and of certain mouse neoplasms to inhibition by 6-mercaptopurine or 8-azaguanine has been interpreted as resulting from the inability of the resistant mutants to metabolize these "fraudulent purines" to their corresponding ribonucleotides (Va and Vb).[3-5] One method of circumventing this resistance might be to treat these mutants with the nucleotides Va and Vb prepared synthetically, since Tomizawa and Aronow have found that, in tissue culture, mouse fibroblasts that have become resistant to 6-mercaptopurine cannot utilize hypoxanthine or inosine but can utilize inosinic acid.[8]

To investigate this possibility, we have developed a chemical synthesis based on the elegant method of Gilham and Tener[9] for the preparation of both ribonucleotides in relatively large amounts not readily prepared by published enzymatic[6,7,10] or chemical-enzymatic methods.[11] The same steps were employed in the synthesis of both compounds (Va and Vb), but it was necessary to vary the conditions used in each case. For example, 6-mercaptopurine ribonucleoside [9-β-D-ribofuranosyl-9H-purine-6(1H)-thione, Ia][12,13] was best converted into its 2′,3′-*O*-isopropylidene derivative IIa by stirring a suspension of it (Ia) in acetone at room temperature for four hours in the presence of copper sulfate and ethanesulfonic acid.[14] This procedure failed completely in the case of 8-azaguanosine [5-amino-β-D-ribofuranosyl-3H-*v*-triazolo[4,5-*d*]pyrimidin-7-(6H)-one, Ib],[15] presumably because of its insolubility. The addition of a minimun of three equivalents of *p*-toluenesulfonic acid[16] to a suspension of Ib in acetone caused gradual solution and conversion of Ib to its 2′,3′-*O*-isopropylidene derivative (IIb). The failure of IIb to give a positive Schiff's test and the presence of bands attributable to primary N—H bonds in its infrared spectrum was considered proof that the isopropylidene group was actually on the sugar moiety rather than the 5-amino group. There was no evidence that any diisopropylidene derivative was formed unless the reaction was run for a longer period of time.

Reaction of 9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-9H-purine-6(1H)-thione (IIa) with 2-cyanoethyl dihydrogen phosphate, prepared by a modification of the method of Cherbuliez,[17] in pyridine in the presence of dicyclohexylcarbodiimide gave 9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl) - 9H - purine - 6(1H) - thione 5′ - (2-cyanoethyl)phosphate (IIIa). Although this material traveled about the same as IIa on paper

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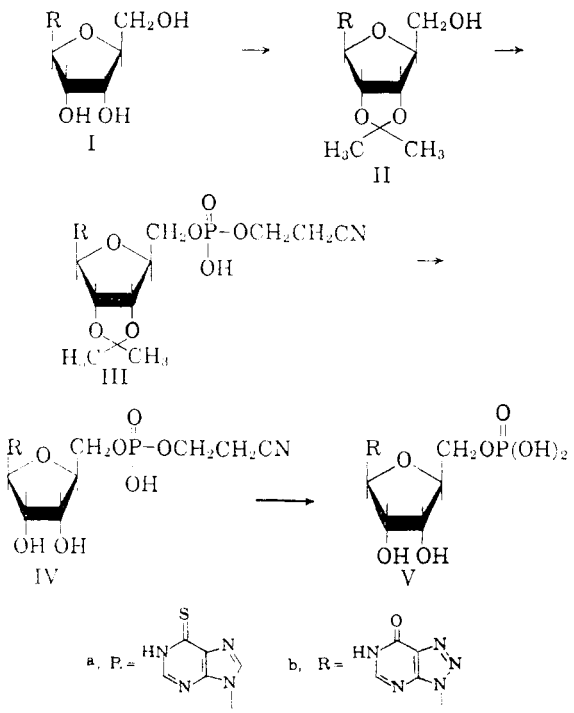
chromatograms in four solvent systems, the spots gave a positive phosphate test. In addition, IIIa travels when subjected to electrophoresis, whereas IIa does not. The isopropylidene group of IIIa was removed by hydrolysis in 0.3*N* hydrochloric acid at room temperature for twenty-four hours. The resulting 9-β-D-ribofuranosyl-9H-purine-6(1H)-thione 5′-(2-cyanoethyl)phosphate (IVb) was isolated but not obtained analytically pure. It traveled as a single spot chromatographically and electrophoretically and gave a positive Schiff's test and a positive phosphate test.

The cyanoethyl ester (IVa) was hydrolyzed to 6-mercaptopurine ribonucleotide [9-β-D-ribofuranosyl-9H-purine-6(1H)-thione 5′-phosphate, Va] by heating it (IVa) in 3*N* aqueous lithium hydroxide at 100° for fifteen minutes. The resulting ribonucleotide was chromatographically and electrophoretically homogeneous and identical in all respects with enzymatically prepared material.[18] It was isolated analytically pure as its barium salt.



Treatment of 5-amino-3-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-3H-*v*-triazolo[4,5-*d*]pyrimidin-7(6H)-one (IIb) with 2-cyanoethyl dihydrogen phosphate and dicyclohexylcarbodiimide in pyridine gave 5-amino-3-(2′,3′-*O*-isopropylidene)ribofuranosyl-3H-*v*-triazolo[4,5-*d*]pyrimidin-7(6H)-one 5′-(2-cyanoethyl)phosphate (IIIb). This material was hydrolyzed with acid to give 5-amino-3-β-D-ribofuranosyl-3H-*v*-triazolo[4,5-*d*]pyrimidin-7(6H)-one 5′-(2-cyanoethyl)phosphate (IVb), which, in turn, was hydrolyzed with base to give 8-azaguanylic acid [5-amino-3-β-D-ribofuranosyl-3H-*v*-triazolo[4,5-*d*]-pyrimidin-7(6H)-one 5′-phosphate, Vb]. This material was chromatographically and electrophoretically pure and identical in all respects with an enzymatically prepared sample[7]; it was obtained analytically pure in the form of its barium salt. Again, the intermediates were isolated and characterized chromatographically, but they were not obtained analytically pure.

Preliminary tests have shown that KB cells grown in Eagle's medium are inhibited by both 6-mercaptopurine ribonucleotide and by 8-azaguanylic acid at 1.9 μmole/l., whereas the lowest concentrations at which 6-mercaptopurine and 8-azaguanine are inhibitory are 0.8 μmoles/l. and 3.4 μmoles/l., respectively.

EXPERIMENTAL

The ultraviolet absorption spectra were determined in aqueous solution with a Beckman DK-2 spectrophotometer, but the optical densities at the maxima were determined with a Beckman DU. The infrared spectra were determined in pressed potassium bromide disks with a Perkin-Elmer model 21 spectrophotometer. Melting points were determined on a Kofler-Heizbank and are corrected.

The paper chromatograms were run by the descending technique on Whatman No. 1 paper in the following solvent systems: A, water-saturated butyl alcohol[19]; B, butyl alcohol–acetic acid–water (5/2/3)[20]; C, isopropyl alcohol–ammonium hydroxide–water (14/1/15)[21]; D, 0.1*M* phosphate buffer, *p*H 6.5.[12] Adenine was used as a standard on all chromatograms and the distance it traveled was assigned a value of 1.00; all other compounds are expressed relative to this value (R_{Ad}). Paper electrophoresis was carried out on Whatman 3MM paper in (E) 0.05*M* ammonium formate buffer (*p*H 3.5) at a potential gradient of approximately 20 volts/cm. for 2 hr. or in (F) 0.05*M* sodium tetraborate (*p*H 9) at a potential gradient of 15 volts/cm. for 1.5 hr.[22] Inosinic acid was used as a standard on all electrophoresis strips and the distance it migrated was assigned a value of 1.00; all other compounds are expressed relative to this value (M_{In}). The chromatographic and electrophoretic data are listed in Table I.

TABLE I

Compound	R_{Ad} Values, Solvent System A	B	C	D	M_{In} Values, Solvent System E	F
Ia	0.47	0.73	0.75	1.68	—	—
b	0.38	0.70	0.70	1.87	—	—
IIa	1.62	1.24	1.40	1.61	—	—
b	1.50	1.25	1.29	1.36	—	—
IIIa	0.26	0.92	1.04	2.28	0.80	—
b	0.42	0.78	1.05	2.00	—	—
IVa	0	0.42	0.40	2.05	—	—
b	0	0.39	0.56	2.24	—	—
Va	0	0.26	0.12	2.07	0.97	1.08
b	0	0.23	0	2.26	0.75	1.00

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2-Cyanoethyl dihydrogen phosphate. Some difficulty was encountered in the preparation of 2-cyanoethyl dihydrogen phosphate by the method of Cherbuliez,[17] since under the conditions of the reaction the cyano group is easily hydrolyzed to the amide. Infrared studies showed that heating the reaction mixture promoted hydrolysis. A modification of this method consistently gave an 11% yield of the barium salt of 2-cyanoethyl dihydrogen phosphate.

To 80 g. of polyphosphoric acid[23] was slowly added 27 g. of orthophosphoric acid. The mixture was stirred for 15 min. and left stoppered 16 hr. at room temperature. To the mixture was then slowly added 27 g. of hydracrylonitrile at such a rate as to keep the reaction temperature below 40°. The mixture was stirred for 1.25 hr. at room temperature, then chilled, and diluted carefully with 200 ml. of cold water. To the resulting solution, which had been washed twice with 200 ml. of ether, was added slowly solid barium carbonate until the precipitate which formed no longer redissolved; this required about 116 g. The solution was then placed in an ice bath and the *p*H raised to 8 with warm concentrated barium hydroxide solution. The barium phosphate which formed was filtered off and washed thoroughly with water. The combined filtrate and washings were evaporated to about 150 ml. and then diluted with four volumes of ethanol. The precipitate which formed was collected and dried for 8 hr. over phosphorus pentoxide at 110°/0.07 mm.; yield, 11.56 g. (11%).

To a suspension of 6.00 g. (21.0 mmoles) of the barium salt of 2-cyanoethyl dihydrogen phosphate in 100 ml. of water was slowly added, with constant stirring, 42.0 ml. of 1*N* sulfuric acid. After stirring the resulting suspension for 15 min. at room temperature, the precipitate of barium sulfate was filtered off and the filtrate evaporated to dryness *in vacuo*. To remove the last traces of water, the residue was dissolved in 50 ml. of pyridine and evaporated to dryness; this process was repeated. The product remained as a colorless syrup.

9-(2′,3′-O-Isopropylidene-β-D-ribofuranosyl-9H-purine-6(1H)-thione (IIa).[24] To a suspension of 5.50 g. (19.3 mmoles) of 9-β-D-ribofuranosyl-9H-purine-6(1H)-thione in 275 ml. of acetone was added 11.0 g. of anhydrous copper sulfate, and then 5.50 ml. of ethanesulfonic acid. The resulting reaction mixture, protected by a calcium chloride tube, was stirred at room temperature for 4 hr. and then filtered; the filtrate was evaporated *in vacuo* at room temperature to 137 ml. and the residue slowly poured into 732 ml. of 10% sodium carbonate. The resulting yellow-orange solution was washed with chloroform (4 × 275 ml.) until the color was removed from the aqueous layer. Upon neutralization of the aqueous layer with glacial acetic acid, a precipitate formed which was collected as a white powder: yield, 3.74 g. (50%); m.p., 275° dec. The analytical sample was obtained by recrystallization from water. It was dried at 110°/0.07 mm. over phosphorus pentoxide for 48 hr.: m.p., 275° dec.

Spectral data. λ max in mμ ($\epsilon \times 10^{-3}$): *p*H 1–223–224 (10.3), 322 (23.5); *p*H 7–226–227 (10.6), 319 (22.0); *p*H 13–231–232 (15.7), 311 (23.2). ν in cm.$^{-1}$: 3400 (OH); 2970, 2925, and 2850 (CH); 1605, 1595, and 1550 (C=C, C=N); 1090 (C—O—C), and 1060 (C—O of CH_2OH).

Anal. Calcd. for $C_{13}H_{16}N_4O_4S$: C, 48.13; H, 4.97; N, 17.27. Found: C, 47.72; H, 5.01; N, 17.07.

6-Mercaptopurine ribonucleotide [9-β-D-ribofuranosyl-9H-purine-6(1H)-thione 5′-phosphate, Va]. To a solution of 21.0 mmoles of 2-cyanoethyl dihydrogen phosphate (from 6.00 g. of its barium salt) in 150 ml. of dry pyridine was added 17.3 g. (83.8 mmoles) of dicyclohexylcarbodiimide followed by 3.39 g. (10.5 mmoles) of 9-(2′-3′-*O*-isopropylidene-β-D-ribofuranosyl)-9H-purine-6(1H)-thione. After about 5 min. a precipitate began to form in the resulting solution, which was kept in a tightly sealed flask at room temperature for 2 days. The reaction solution was diluted with 21 ml. of water and left at room temperature for 1 hr. before the precipitate of dicyclohexylurea which formed was removed by filtration: yield, 12.1 g. (64%). The filtrate was evaporated to dryness *in vacuo* at 30°, and the residue dissolved in 200 ml. of water. The aqueous solution was washed with 100 ml. of chloroform. The aqueous solution of 9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-9H-purine-6(1H)-thione 5′-(2-cyanoethyl)phosphate (IIIa) was diluted with hydrochloric acid to give 199 ml. of a 3*N* solution, which was kept at room temperature for 24 hr. before the hydrochloric acid was neutralized with 9.95 ml. of 6*N* sodium hydroxide. The aqueous solution of 9-β-D-ribofuranosyl-9H-purine-6(1H)-thione 5′-(2-cyanoethyl)phosphate (IVa) was diluted with lithium hydroxide to give 292 ml. of a 0.5*N* solution, which was heated in a 100° oil bath for 15 min. A slight precipitate of lithium phosphate which formed was filtered off, and the solution stirred for 15 min. with 133 ml. of Amberlite IR-120(H) ion-exchange resin added in batches. The resin was removed by filtration, and the *p*H of the filtrate was carefully raised to 7.5 with barium hydroxide solution. The resulting cloudy solution was filtered through a Celite pad and the filtrate was evaporated to 260 ml. and diluted with twice this volume of absolute ethanol. The precipitate which resulted was collected and washed with ethanol and then with ether: yield, 2.24 g. (45%). The analytical sample was obtained by precipitation of this material from an aqueous ethanol solution. It was dried over phosphorus pentoxide at 110°/0.07 mm. for 16 hr.

Spectral data. λ max in mμ: ($\epsilon \times 10^{-3}$); *p*H 1–324 (21.5); *p*H 7–321 (22.6); *p*H 13–311 (22.2). ν in cm.$^{-1}$: 3440 (broad) (OH); 1595, 1570 (shoulder), and 1540 (shoulder) (C=C, C=N); 1075 (broad) (P—O—C).

Anal. Calcd. for $C_{10}H_{11}BaN_4O_7PS \cdot 2H_2O$: C, 22.44; H, 2.82; N, 10.46; P, 5.78. Found: C, 22.20; H, 2.76; N, 10.27; P, 5.20.

5-Amino-3-(2′,3′-O-isopropylidene-β-D-ribofuranosyl)-3H-v-triazolo[4,5-d]pyrimidine-7(6H)-one (IIb). To a stirring suspension of 3.62 g. (12.8 mmoles) of 8-azaguanosine in 550 ml. of anhydrous acetone[7] was quickly added 7.30 g. (38.4 mmoles) of *p*-toluenesulfonic acid. After complete solution, which required about 4 hr., the solution was stirred 1 hr. at room temperature. The solution was then concentrated to 100 ml. and poured into 300 ml. of cold water containing 70 g. of Amberlite IR-120(OH) ion-exchange resin and additional resin was added until *p*H 7 was obtained (total—240 g. of resin). The resin was then filtered off and washed thoroughly with water. The combined filtrate and washings, 700 ml., were evaporated *in vacuo* at 35° to dryness. The white, solid residue was crystallized from 75 ml. of water after charcoal treatment: yield, 2.74 g. The solid was recrystallized from water: yield, 2.26 g. (54%); m.p., 232° dec. This material was found by chromatographic analysis to contain less than 5% of 8-azaguanosine. The analytical sample was obtained by recrystallizing once more from water. It was dried over phosphorus pentoxide at 110°/0.07 mm. for 18 hr.: m.p., 247° dec.

Spectral data. λ max in mμ ($\epsilon \times 10^{-3}$): *p*H 1–256 (13.5); *p*H 7–256 (12.8); *p*H 13–222 (22.7) and 279 (11.9). ν in cm.$^{-1}$: 3430 (OH); 3330 and 3190 (NH), 2990 and 2930 (CH), 1705 (C=O), 1640 (NH), 1585 and 1535 (C=C, C=N), 1090 (C—O—C), and 1060 (C—O of CH_2OH).

Anal. Calcd. for $C_{12}H_{16}N_6O_5$: C, 44.44; H, 4.97; N, 25.92. Found: C, 44.16; H, 5.02; N, 26.22.

8-Azaguanylic acid [5-amino-3-β-D-ribofuranosyl-3H-v-triazolo[4,5-d]pyrimidin-7(6H)-one 5′-phosphate, Vb]. To a solution of 17.5 mmoles of 2-cyanoethyl phosphate (from 5.00 g. of its barium salt) in 130 ml. of anhydrous pyridine was added quickly 7.20 g. (35.0 mmoles) of dicyclohexylcarbodiimide followed by 2.84 g. (8.75 mmoles) of 5-amino-3-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-3H-*v*-triazolo-

(23) We wish to thank the Victor Chemical Works, 155 N. Wacker Dr., Chicago, Ill., for a generous supply of their polyphosphoric acid.

(24) The original synthesis of this compound using mixed alkanesulfonic acid was carried out under the direction of Dr. H. J. Schaeffer.

[4,5-*d*]pyrimidin-7(6H)-one. Swirling gave a solution and after about 5 min. a precipitate began to form. After standing in a tightly sealed flask at room temperature for 2 days, the reaction mixture was diluted with 17.5 ml. of water and left at room temperature for 1 hr. The precipitate of 1,3-dicyclohexylurea (5.88 g.) was removed by filtration. The filtrate was evaporated to dryness *in vacuo*, the residue dissolved in 100 ml. of water, and the resulting solution washed with chloroform (5 × 100 ml.). The aqueous solution of 5-amino-3-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-3H-*v*-triazolo[4,5-*d*]pyrimidin-7(6H)-one 5′-(2-cyanoethyl)phosphate (IIIb) was diluted with enough 1*N* sulfuric acid to give 170 ml. of a 0.1*N* solution. The resulting solution was left at room temperature for 2 days and then neutralized by the addition of 1.46 g. (8.5 mmoles) of barium hydroxide in 150 ml. of water. The precipitate of barium sulfate was removed by filtration. The aqueous solution of 5-amino-3-β-D-ribofuranosyl-3H-*v*-triazolo[4,5-*d*]pyrimidin-7(6H)-one 5′-(2-cyanoethyl)phosphate (IVb) was diluted with 66.7 ml. of 3*N* lithium hydroxide and enough water to give 400 ml. of a 0.5*N* solution, which was heated for 15 min. in a 100° oil bath. After removal of the precipitate which formed, the solution was stirred for 30 min. with 315 ml. of Amberlite IR-120(H) ion-exchange resin. The resin was filtered off and washed thoroughly. The combined filtrate and washings (800 ml.) were diluted with an equal volume of ethanol, and the precipitate which formed was collected by filtration; yield, 3.19 g. (73%). To prepare the analytical sample, this material was washed with boiling water and dried at 110°/0.07 mm. over phosphorus pentoxide for 8 hr.

Spectral data. λ max in mμ (ε × 10^{-3}): *p*H 1–255 (12.9); *p*H 7–255 (12.6); *p*H 13–222 (23.8) and 279 (11.7). ν in $cm.^{-1}$: 3410 (OH); 3300–3100 (NH); 2930 (CH); 1700 (C=O); 1640 (NH); 1600, 1530 (shoulder), and 1540 (C=C, C=N); 1090 (P—O—C).

Anal. Calcd. for $C_9H_{11}BaN_6O_8P$: C, 21.64; H, 2.22; N, 16.82; P, 6.20. Found: C, 21.40; H, 2.56; N, 16.47; P, 6.06.

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[CONTRIBUTION FROM THE KETTERING-MEYER LABORATORY,[1] SOUTHERN RESEARCH INSTITUTE]

Synthesis of Potential Anticancer Agents. XXVIII. Simple Esters of 6-Mercaptopurine Ribonucleotide[2]

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6-Mercaptopurine ribonucleotide and six simple ester derivatives have been prepared from 9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-9H-purine-6(1H)-thione by reaction with diphenyl, dibutyl, and diethyl phosphorochloridates followed by appropriate hydrolysis reactions.

It has now been firmly established that neoplasms susceptible to the action of either 6-mercaptopurine (purine-6(1H)-thione) or 8-azaguanine (5-amino-*v*-triazolo[4,5-*d*]pyrimidin-7(6H)-one) convert these compounds to their respective ribonucleotides. Neoplasms that are resistant to these two compounds (whether the resistance is natural or acquired) do not have the pyrophosphorylase necessary to carry out this conversion.[3,4] It is questionable whether this resistance can be overcome by treatment with synthetically prepared ribonucleotides since it is well known that the nucleotides of the naturally occurring purines are poorly incorporated into cell nucleic acids[5] and, indeed, it has been shown that they are not incorporated intact.[6,7] These findings raise serious doubts that nucleotides, as such, can penetrate the cell membrane. This difficulty might be overcome if one could prepare an ester of a nucleotide which could penetrate the cell wall and then be metabolized to the nucleotide itself.[8] Toward this end, some simple esters of 6-

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(7) H. Weinfeld, P. M. Roll, E. Carroll, G. B. Brown, and C. P. Rhoads, *Cancer Research*, **17**, 122 (1957).

(8) O. C. Touster found that, whereas sodium D-glucuronate is not metabolized by humans, the nonionized glucuronic acid lactone is. He postulated that this difference may be due to the ability of the lactone to penetrate the cell membrane and then be hydrolyzed to the acid which is metabolized.[9]

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