Purine N-Oxides. XV. The Synthesis of 6-Mercaptopurine 3-N-Oxide. Its Chemotherapeutic Possibilities[1]

GEORGE BOSWORTH BROWN,[2] GERSHON LEVIN,[2] SARAH MURPHY,[2] ALEX SELE,[2] H. CHRISTINE REILLY, GEORGE S. TARNOWSKI, FRANZ A. SCHMID, MORRIS N. TELLER, AND C. CHESTER STOCK

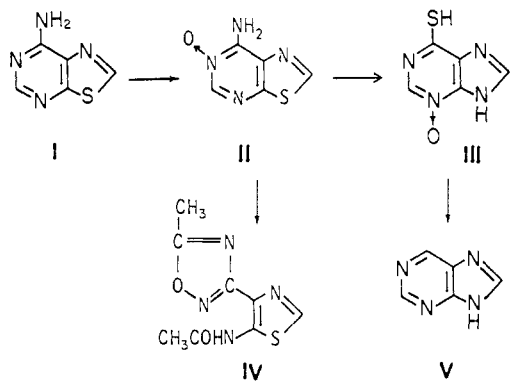
The Division of Nucleoprotein Chemistry and the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, Sloan-Kettering Division of Cornell University Medical College, New York, New York

Received November 27, 1963

The synthesis and proof of structure of 6-mercaptopurine 3-N-oxide is described. A comparison of the N-oxide with the parent 6-mercaptopurine has been made in several biological systems. The N-oxide is approximately one-tenth as toxic as the parent purine when administered intraperitoneally, but shows a similar toxicity orally. With several mouse tumors the N-oxide produces immediate responses similar to those obtained with 6-mercaptopurine, but at 10-fold the dose levels. With mouse Sarcoma 180 the complete regressions of tumors in animals held for long periods are somewhat more with the N-oxide than with 6-mercaptopurine. 6-Mercaptopurine 3-N-oxide does not depress the hemagglutinin antibody response of the host as does 6-mercaptopurine.

The 6-mercaptopurine N-oxide reported previously[3] has now been shown to be the 3-N-oxide and has been prepared in sufficient quantity for comparisons with the antitumor activity of 6-mercaptopurine. The introduction of the N-oxide function was accomplished while the sulfur was protected from oxidation in the thiazole ring of 7-aminothiazolo[5,4-*d*]pyrimidine (I) (Scheme I). In analogy to the behavior of the adenine 1-N-oxide which yielded 5-methyl-3-[5-(4-acetamido)-imidazolyl]-1,2,4-oxadiazole with acetic anhydride,[4] the pyrimidine ring in the thiazolopyrimidine N-oxide (II) has been opened in acetic anhydride to yield the 5-methyl-3-[5-(4-acetamido)thiazolyl]-1,2,4-oxadiazole (IV). II is, therefore, the 6-N-oxide and upon rearrangement gives 6-mercaptopurine 3-N-oxide (III).

SCHEME I



The 6-methylmercaptopurine 3-N-oxide was readily prepared from III with methyl iodide. Heated and sealed in 25% NH_4OH at 110–115° for 24 hr., it was largely recovered unchanged, but in refluxing morpholine it was converted to 6-morpholinopurine, with concomitant loss of the N-oxide function. Dry heat, as with some other N-oxides,[5] also causes some deoxygenation of III to 6-mercaptopurine.

Catalytic reduction of III with Raney nickel resulted both in reduction of the N-oxide and in desulfuration to yield purine (V). When Pd-C was used, the catalyst was poisoned. Photochemical decomposition did yield almost exclusively 6-mercaptopurine and a small amount of 2-hydroxy-6-mercaptopurine, as may be expected from the two pathways of photochemical alteration observed for several purine 1-N-oxides.[6] We have also observed that visible light rapidly converts solutions of a few micrograms per milliliter of the N-oxide to 6-mercaptopurine, and that the exposed surfaces of solid samples in clear glass vessels darken slightly. We therefore protected samples from light. Suspensions of 25 mg./ml., as prepared for injections, stored in dark bottles at about 5° were unchanged after 3 weeks.

The immediate tumor inhibitory effects on Sarcoma 180 of 6-mercaptopurine 3-N-oxide are similar to those obtained with 6-mercaptopurine (Table I), but with doses approximately 10-fold greater. When administered by the intraperitoneal route, the N-oxide at 1000 mg./kg./day for 1 week resulted in 100% mortality in mice bearing very small tumors, whereas 500 mg./kg./day was well tolerated by the host. In contrast, 6-mercaptopurine was lethal to the host at 100 mg./kg./day but tolerated at 50 mg./kg./day. Significant inhibition of the growth of the tumor, in the first week, was observed with the N-oxide at a dose as low as 125 mg./kg./day [treated:control diameters ($T:C$ = 0.67)] and with 6-mercaptopurine as low as 12.5 mg./kg./day ($T:C$ = 0.61). The average rate of recovery, that is, complete regression of the tumor, in mice treated with the N-oxide at doses from 500 to 62.5 mg./kg./day was 69%; in mice treated with 6-mercaptopurine at doses from 50 to 6 mg./kg./day it was 52%.

In contrast, by the oral route the two compounds exhibited similar toxicities for the host (Table II). For each compound, doses of 200 mg./kg./day or greater for 1 week were lethal, within 3 weeks, to 60% or more of the mice which bore small tumors. Effects upon the tumor were also similar. Significant inhibition of growth of the tumor occurred with doses of the N-oxide as low as 25 mg./kg./day ($T:C$ = 0.69); with 6-mercaptopurine at 12.5 mg./kg./day a $T:C$ = 0.63 was observed. For both compounds at doses from 100

(1) This investigation was supported in part by funds from the Atomic Energy Commission [Contract No. AT(30-1)-910], and from the National Cancer Institute, National Institutes of Health, Public Health Service (CA-03190-08 and -03192-08), and the Cancer Chemotherapy National Service Center (Contract SA-43ph-2445).

(2) Division of Nucleoprotein Chemistry.

(3) G. Levin and G. B. Brown, *J. Med. Chem.*, **6**, 825 (1963).

(4) M. A. Stevens, H. W. Smith, and G. B. Brown, *J. Am. Chem. Soc.*, **82**, 1148 (1960).

(5) A. R. Katritzky, *Quart. Rev.* (London), **10**, 395 (1956).

(6) G. B. Brown, G. Levin, and S. Murphy, *Biochemistry*, **3**, 880 (1964).

TABLE I

TOXICITY AND ANTITUMOR EFFECTS OF 6-MERCAPTOPURINE 3-N-OXIDE AND 6-MERCAPTOPURINE ADMINISTERED INTRAPERITONEALLY IN MICE BEARING SARCOMA 180

6-Mercaptopurine 3-N-oxide					6-Mercaptopurine				
	Effects after					Effects after			
	1 week[a]		12 weeks[b]			1 week[a]		12 weeks[b]	
Dose, mg./kg./day	A.T.D., mm.	A.W.C., g.	Mortality[c]	Recovery	Dose, mg./kg./day	A.T.D., mm.	A.W.C., g.	Mortality[c]	Recovery
1000	3.7	−4.5	6/6[d]		100	4.2	−1.0	6/6[d]	
500	5.9	+1.5	5/18	13/18	50	5.2	+1.0	9/24	15/24
250	6.4	+2.0	10/30	20/30	25	7.3	+1.5	8/18	9/18
125	8.4	+2.0	5/24	18/24	12.5	7.7	+3.0	10/18	8/18
62.5	10.7	+3.0	9/24	15/24	6	10.1	+2.5	5/11	5/11
25	10.8	+4.0	1/6	2/6	3	9.5	+2.5	2/6	4/6
12.5	11.1	+3.0	6/6	0/6	1.5	11.1	+3.0	5/6	1/6
Control	12.6	+3.0	24/24	0/24	Control	12.6	+3.0	24/24	0/24

[a] Results after 1 week of treatment: A.T.D., average diameter of tumors; A.W.C., average change in host body weight. [b] Results 12 weeks after initiation of treatment: mortality, number of deaths/total number of mice in group; recovery, number of mice without tumor/total number of mice in group. [c] Deaths due to progressively growing tumors. [d] Deaths due to drug toxicity. These occurred within the first 2 weeks; tumors were small.

to 12.5 mg./kg./day, the average rate of recovery from tumor was approximately 30%.

By the oral route, single high doses of the N-oxide (Table II) were well tolerated by the host and were possibly more effective in promoting recovery from the tumor (approximately 40%) than were repeated daily doses. Single doses of 6-mercaptopurine of 500 to 125 mg./kg. produced recovery from the tumor in only 3 of 18 mice (17%).

In a series of solid tumors in mice (Table III), those inhibited by 6-mercaptopurine generally responded similarly to treatment with the N-oxide. The effective dose of the latter, by the intraperitoneal route, is 10-fold or more greater than that necessary for 6-mercaptopurine itself, and with no observable toxicities at doses of the N-oxide of 500 mg./kg./day, except in C57BL/6 mice bearing the E0771 and B16 tumors. With Ehrlich ascites and Mecca lymphosarcoma, there was no inhibition, as measured by total packed cell volume or mean survival time, respectively.

The 7-aminothiazolo[5,4-*d*]pyrimidine 6-N-oxide, a new compound in this work and an analog of adenine, was also assayed against the series of tumors. With Ridgway osteogenic sarcoma, it produced a significant retardation of tumor growth, in one out of two tests, both at the end of therapy and in the second week. The degree of growth retardation was small but statistically significant. The active dose, 500 mg./kg./day, produced no toxic effects in any of the strains of mice in which these tumors are carried. With mouse Sarcoma 180, melanoma B16, carcinoma E0771, carcinoma C1025, sarcoma T241, Mecca lymphosarcoma, Ehrlich ascites tumor, and Walker rat carcinosarcoma 256, no inhibition was observed at the highest dose of the drug, 500 mg./kg./day. The parent 7-aminothiazolopyrimidine was toxic at 250 mg./kg./day in tumor bearing C57BL/6 male mice, while tolerated at that dose in $CHKRF_1$ and ICR female mice. It was also inactive toward all of the tumors except Ridgway osteogenic sarcoma.

In view of the immuno-depressant activities of 6-mercaptopurine and several of its analogs,[7] this biological characteristic was determined for 6-mercaptopurine 3-N-oxide. The effect on heterohemagglutinin antibody was assayed, with doses of 500 and 1000 mg./kg./day compared with 6-mercaptopurine at 75 mg./kg./day, and with a control group injected with diluent (0.5% carboxymethylcellulose in physiological saline) only. Ten mice were used per group. The results of a representative test (Table IV) show that 6-mercaptopurine had some inhibitory effect, but the N-oxide had no significant activity. Drug toxicities were approximately equivalent at the dosages selected.

Discussion

The 6-mercaptopurine 3-N-oxide, administered either intraperitoneally or orally, exerts antitumor effects similar to those produced by 6-mercaptopurine. Several mouse tumors that respond to treatment with 6-mercaptopurine are also inhibited by its 3-N-oxide. The extent of recovery, which is defined as subsequent regressions of the tumors and indefinite survival of the animals, from Sarcoma 180 is about 35% greater with the N-oxide ($0.01 < P < 0.05$).

The outstanding difference between the N-oxide and the parent 6-mercaptopurine lies in the greatly reduced toxicity of the former when given intraperitoneally. This is accompanied by a corresponding increase in the dose necessary for tumor inhibition.

By the oral route, the maximum tolerated dose of the N-oxide, on repeated administration, was only about one fifth of that tolerated intraperitoneally. Since twice as much 6-mercaptopurine is tolerated orally as is tolerated intraperitoneally, the effective oral doses for the two compounds become similar.

The data suggest that the biological activities of the 6-mercaptopurine 3-N-oxide result from an alteration of the compound, possibly reduction to 6-mercaptopurine or a derivative of it, as can also be inferred from the known metabolic results with adenine 1-N-oxide.[8,9] The large difference between the doses of N-oxide effective by the intraperitoneal and oral routes, and the similarity between the orally effective doses of the N-oxide and 6-mercaptopurine suggest that the N-oxide is rapidly altered in the gastrointestinal tract.

We once proposed[10] that one theoretical advantage of an N-oxide of a chemotherapeutically effective purine

(7) H. C. Nathan, S. Bieber, G. B. Elion, and G. H. Hitchings, *Proc. Soc. Exptl. Biol. Med.*, **107**, 796 (1961).

(8) G. B. Brown, D. A. Clarke, J. J. Biesele, L. Kaplan, and M. A. Stevens, *J. Biol. Chem.*, **233**, 1509 (1958).

(9) D. Dunn, M. H. Maguire, and G. B. Brown, *ibid.*, **234**, 620 (1959).

(10) G. B. Brown, IVth International Congress of Biochemistry, Colloquia, Vol. XIII, Pergamon Press, London, 1958, p. 111.

TABLE II

TOXICITY AND ANTITUMOR EFFECTS OF 6-MERCAPTOPURINE 3-N-OXIDE AND 6-MERCAPTOPURINE ADMINISTERED ORALLY IN MICE BEARING SARCOMA 180

6-Mercaptopurine 3-N-oxide						6-Mercaptopurine					6-Mercaptopurine 3-N-oxide				
Effects after						Effects after					Effects after				
1 week[a]		5 weeks[b]				1 week[a]		5 weeks[b]			1 week[a]		12 weeks[b]		
Dose × 7, mg./kg./day	A.T.D., mm.	A.W.C., g.	Mortality[d]	Recovery		A.T.D., mm.	A.W.C., g.	Mortality[d]	Recovery		Dose × 4, mg./kg.	A.T.D., mm.	A.W.C., g.	Mortality[d]	Recovery
400	3.6	−2.5	6/6[e]			4.0	−4.0	6/6[e]			1000	5.9	+1.0	2/6	4/6
200	4.8	−0.5	4/6[e]			6.2	−1.0	5/6	0/6		500	5.5	+3.0	8/12	4/12
100	5.2	+1.5	2/5	0/6		5.5	+2.5	3/6	1/6		250	5.3	+2.0	4/12	8/12
50	6.2	+2.5	0/6	4/6		8.1	+3.5	2/6	4/6		125	5.8	+4.0	9/12	3/12
25	7.6	+1.5	3/6	1/6		8.4	+3.5	3/6	2/6		62.5	6.7	+2.0	4/6	2/6
12.5	11.2	+4.0	2/6	1/6		10.2	+3.5	3/6	0/6		31.3	9.7	+2.0	5/6	1/6
6.25						12.1	+2.0	3/6	1/6						
Control	11.0	+3.5	1/6	0/6		16.1	+3.0	6/6	0/6		Control	10.1	+2.0	12/12	0/12

[a] See footnote Table I. [b] Results 5 weeks after initiation of treatment; see footnote Table I. [c] Results 12 weeks after initiation of treatment; see footnote Table I. [d] Deaths due to progressively growing tumors. [e] Deaths due to drug toxicity. These occurred within the first 3 weeks; tumors were small.

lay in the possibility that it, like adenine 1-N-oxide,[9] might undergo reduction *in vivo* to release slowly the parent purine and alter the "dosage schedule." Encouraging results were first obtained with 6-methylpurine 1-N-oxide, which was[11] about 200-fold less toxic to tissues in culture and about 30-fold less toxic in mice *in vivo*, and the therapeutic index toward Ca-755 was improved. The hypothesis of a lesser toxicity of 6-mercaptopurine slowly released from its N-oxide may not appear to be in agreement with the data of Regelson, *et al.*,[12] who found, in man, with both 6-mercaptopurine and 9-ribosyl-6-mercaptopurine that prolonged dosage at minimal levels, including intravenous drip, produced maximal toxicity per unit of drug. However, their drugs were administered extracellularly, and if 6-mercaptopurine 3-N-oxide, like some adenine 1-N-oxide derivatives,[13-15] had to be both converted to a nucleotide and reduced intracellularly, direct comparison may not be valid.

The possible N-oxides of purines include 1-, 3-, 7-, and 9-N-oxides, and of these we can draw on several chemical and metabolic experiences with purine 1-N-oxides. The vagaries of sulfur and heterocyclic N-oxide chemistry have permitted us to obtain the 3-N-oxide of 6-mercaptopurine, and comparisons with 1-N-oxides of other purines may be inappropriate. We do have a xanthine 3-N-oxide which, like the 6-mercaptopurine 3-N-oxide, is also not toxic to mice at 500 mg./kg. for 1 week.[16]

The importance of immunity in the therapy of various diseases, including cancer, has been increasingly stressed,[17-19] and the immuno-depressant activity of 6-mercaptopurine has been emphasized.[17] Whereas 6-mercaptopurine has some activity with respect to hemagglutinin antibody inhibition, the N-oxide was found to have no such activity in this system (Table IV). The relative activities remain the same when the Antibody Index of Nathan, *et al.*,[3] is calculated. The index for 6-mercaptopurine was 0.40 (active) in comparison with 0.68 (inactive) for the N-oxide at both doses. The test performed was only one of several that might have been used[18] and was measured by one of a number of possible immunological parameters. A 6-mercaptopurine derivative that does not reduce antibody production, and thus host immunity, should be of definite advantage.

Explanations of the reduced toxicity of this 6-mercaptopurine 3-N-oxide, and of its mode of action, must await considerable experimentation. Only clinical testing will determine whether it possesses advantages over 6-mercaptopurine in the treatment of leukemia in man.

(11) M. A. Stevens, A. Giner-Sorolla, H. W. Smith, and G. B. Brown, *J. Org. Chem.*, **27**, 567 (1962).

(12) W. Regelson, J. F. Holland, E. Fee, III, G. L. Gold, T. Hall, M. Krant, S. O. Miller, and B. I. Shnider, *Cancer Chemotherapy Rept.*, **36**, 41 (1964).

(13) S. Frederiksen and H. Klenow, *Cancer Res.*, **22**, 125 (1962).

(14) A. Coddington and M. Bagger-Sørensen, *Biochim. Biophys. Acta*, **72**, 598 (1963).

(15) S. Frederiksen, *ibid.*, **76**, 366 (1963).

(16) R. M. Cresswell, H. K. Mader, T. Strauss, and G. B. Brown, *J. Org. Chem.*, in press.

(17) G. H. Hitchings and G. B. Elion, *Pharmacol. Rev.*, **15**, 365 (1963).

(18) J. L. Amiel, G. Madie, M. Matsukara, A. M. Mery, G. Dagade, R. Tebenbaum, S. Garottini, and V. Palma, *Immunology*, **7**, 511 (1964).

(19) R. T. Prehn, *J. Natl. Cancer Inst.*, **31**, 791 (1963).

TABLE III

ACTIVITY OF 6-MERCAPTOPURINE 3-N-OXIDE AND 6-MERCAPTOPURINE AGAINST A SERIES OF TUMORS

	6-Mercaptopurine 3-N-oxide					6-Mercaptopurine				
Tumor system	Dose, mg./kg./day	T:C[a]	Effect[b]	A.W.C.[c] T/C	Mortality[d]	Dose, mg./kg./day	T:C[a]	Effect[b]	A.W.C.[c] T/C	Mortality[d]
Adenocarcinoma E0771	500	0.29	+	−5.0/+1.9	1/5	30	0.21	+	−0.5/−0.5	1/5
	250	0.54	+	−0.3/+0.8	0/5	15	0.35	+	−3.8/+1.9	1/5
	125	0.57	+	−0.1/+1.9	0/5	7.5	0.55	+	−2.1/+1.9	0/5
Carcinoma 1025	500	0.42	+	+0.4/+1.9	0/5	30	0.33	+	−0.4/+2.3	0/10
	250	0.45	+	−2.5/+2.7	1/4	15	0.45	+	−1.1/+1.8	0/10
	125	0.70	−	+1.8/+1.9	0/5	7.5	0.55	+	0.0/+2.3	0/10
Sarcoma T241	250	0.80	−	−3.9/+0.9	0/5	15[e]	0.72	−	−4.6/+0.9	0/5
Ridgway osteogenic	500	0.43	+	+1.0/4.2	0/5	30	0.36	+	+1.3/4.0	0/9
sarcoma	250	0.45	+	+0.7/+3.9	0/5	15	0.32	+	0.0/+4.0	0/10
	125	0.91	−	+3.3/+4.2	0/5	7.5	0.52	+	+0.9/+4.0	0/10
Melanoma B16	250		Toxic		6/10	31.3		Toxic		9/10
	125	0.66	+	−2.9/−0.3	1/5	15.6	0.60	+	−2.8/−0.3	1/10
						7.8	0.78	−	−1.0/−0.3	0/10

[a] Ratio of average diameter of tumors in treated animals to that of tumors in controls. [b] +, significant inhibition of tumor; −, no inhibition of tumor. [c] Average weight change (g.) in surviving mice; treated/controls. [d] Deaths/total number of mice treated. [e] 30 mg./kg. was toxic to the host.

TABLE IV

EFFECTS OF 6-MERCAPTOPURINE AND 6-MERCAPTOPURINE 3-N-OXIDE ON HETEROHEMAGGLUTININ ANTIBODY PRODUCTION IN SWISS ICR/HA MICE[a]

	Hemagglutinin antibody titer			
			6-MP N-Oxide	
Dilution	Control	6-MP, 75 mg./kg.	500 mg./kg.	1000 mg./kg.
	Av. host wt. change, g.			
	+1.1	−0.8	+1.4	0
4	4+	4+	2+	4+
8	4+	4+	3+	4+
16	4+	3+	3+	4+
32	4+	3+	3+	4+
64	4+	3+	3+	3+
128	4+	2+	3+	3+
256	3+	1+	2+	2+
512	3+	±	2+	2+
1,024	2+	±	2+	1+
2,048	2+	0	1+	1+
4,096	1+		1+	±
8,192	1+		±	±
16,000	±		0	0
32,000	±			
64,000	0			

[a] Ten mice/group; nine mice (1 moribund mouse not used) for the N-oxide group treated at 1000 mg./kg. There were no deaths in any group. [b] Reciprocal of serum dilution.

Experimental

Chemical Methods.—The procedures described have proven optimal from the standpoints of convenience and yield. The oxidation of I has been carried out in 2 days, rather than in 3 weeks, with 70 to 80 g. of I in 200 ml. of acetic acid and 150 ml. of 30% H_2O_2. Initial cooling was needed because of the exothermic character of the reaction, and the yields were only 50–60%. Crude I, not fully freed of formic acid, can be used in the oxidation when it is prepared from 4,5-diamino-6-mercaptopyrimidine[20] by refluxing in 98% formic acid and evaporation *in vacuo*. The rearrangement of II to III has also been carried out in 1 equiv. of aqueous NaOH at temperatures up to reflux, but with lower yields, 20 to 30%, and the product required additional recrystallizations to free it of some 6-mercaptopurine arising by deoxygenation through heating, and hypoxanthine arising from the action of hot alkali.

Melting points were determined in capillary tubes in a calibrated apparatus. Chromatograms were developed, ascending on Whatman No. 1 paper with the solvents: A, 2-propanol–1% $(NH_4)_2SO_4$ (2:1) with ammonium sulfate soaked paper[21]; B, butanol–water–acetic acid (4:1:1); C, 3% ammonium chloride; D, 1-propanol–water (3:1). The R_f values are in Table V. The ultraviolet absorbtion spectra (Table VI) were determined with a Cary Model 15 spectrophotometer.

TABLE V

R_f VALUES ON CHROMATOGRAPHY IN VARIOUS SOLVENTS

	Solvent system[a]			
Compound	A	B	C	D
4,5-Diamino-6-mercaptopyrimidine	0.65	0.41	0.52	0.52
7-Aminothiazolo[5,4-*d*]pyrimidine	0.83	0.70	0.42	0.74
7-Aminothiazolo[5,4-*d*]pyrimidine 6-N-oxide[b]	0.67	0.56	0.66	0.57
6-Mercaptopurine 3-N-oxide[c]	0.50	0.26	0.56	0.22
6-Mercaptopurine[d]	0.67	0.45	0.42	0.60
5-Methyl-3-[5-(4-acetamido)-thiazolyl]-1,2,4-oxadiazole	0.95	0.79	0.70	0.85
6-Methylmercaptopurine 3-N-oxide	0.67	0.51	0.40	0.46
2-Hydroxy-6-mercaptopurine	0.62	0.50	0.27	0.43
2-Hydroxy-6-methylmercaptopurine	0.71	0.50	0.52	0.59
Purine	0.87	0.62	0.72	0.69
Hypoxanthine	0.69	0.40	0.61	0.52
6-Morpholinopurine	0.95	0.71	0.50	0.72
6-Methylmercaptopurine	0.93	0.72	0.46	0.81
Unknown product of oxidation	0.55	0.25	0.73	0.30

[a] See Experimental. [b] Bluish. [c] Reddish. [d] White fluorescence under ultraviolet light.

7-Aminothiazolo[5,4-*d*]pyrimidine 6-N-Oxide.—A suspension of 50 g. of 7-aminothiazolo[5,4-*d*]pyrimidine[22,23] in 100 ml. of glacial acetic acid in a 500-ml. beaker was stirred with a rod, and 50 ml. of 30% H_2O_2 was added. The beaker was covered with a watch glass. Much of the starting material dissolved in a few hours and as the N-oxide began to precipitate the suspension became yellow. The evolution of oxygen stirred the solution gently and resulted in the accumulation of solids up the sides of the beaker. After 1 week, an additional 25 ml. of 30% H_2O_2 was added, and the solids were stirred into the liquid. The progress of the reaction can be followed by chromatography in solvent D. After 3 weeks, the product was collected in a large sintered-glass Büchner funnel and was washed by suspension in 200 ml. of water. The unreacted 7-aminothiazolopyrimidine was largely separated from the N-oxide by washing the mixture by suspension, on the funnel, in 200 ml. of warm tetrahydrofuran. Complete removal of the starting material was accomplished by further extraction of the solids with tetrahydrofuran in a Soxhlet extractor overnight. The solids were again suspended, on a funnel, in 100 ml. of water which removed traces of an unidentified material, the R_f values of which are given in Table V.

(20) G. B. Elion and G. H. Hitchings, *J. Am. Chem. Soc.*, **76**, 4027 (1954).

(21) N. Anand, V. M. Clark, R. H. Hall, and A. R. Todd, *J. Chem. Soc.*, 3665 (1952).

(22) G. B. Elion, W. H. Lange, and G. H. Hitchings, *J. Am. Chem. Soc.*, **78**, 2858 (1956); Burroughs-Wellcome & Co., Inc., U. S. Patent 2,933,498 (April 19, 1960).

(23) Purchased from Burroughs-Wellcome & Co., Inc., Tuckahoe, N. Y.

TABLE VI

SPECTRAL DATA

Compound	pH	$\lambda_{max}^{H_2O}$ mμ	ε × 10⁻³	$\lambda_{min}^{H_2O}$ mμ	ε × 10⁻³
7-Aminothiazolo-[5,4-*d*]pyrimidine 6-N-oxide[a]	1.2	224	24.8	252	6.8
		256	7.0	261	6.7
		264	7.0		
		285[b]	3.4		
	6	230	24.4		
		265[b]	6.9	295	1.9
		315	2.6		
6-Mercaptopurine 3-N-oxide	1.0	250	4.5	240	4.3
		268	4.7	256	4.4
		335	11.9	292	3.4
	6.9	256	6.3	250	6.0
		343	21.5	284	1.9
	12.6[c]	248	11.8	230	7.9
		334	21.9	278	2.8
5-Methyl-3-[5-(4-acetamido)-thiazolyl]-1,2,4-oxadiazole	1.2	281	13.4	240	4.3
	7.6	283	13.4	240	3.6
	11.0	225	9.8	245	3.9
		310	11.0		
6-Methylmercaptopurine 3-N-oxide	1.5	250[b]	6.7	268	3.4
		318	20.5		
	6.9	245	15.0	228	7.6
		312	17.2	268	2.9
	12.6	245	16.3		
		312	17.0		

[a] Unstable in base. [b] Shoulder. [c] Shoulder of ε 13,000–14,000 at 215 mμ. No strong absorption maximum, as characteristic of most purine 1-N-oxides, is observed in this region.

The air-dried product, slightly yellow, 45 g., 80–82% yield (90% on unrecovered starting material), darkening from 235° dec., was used in the next step.

A sample was recrystallized from water, with charcoal, to yield white plates, m.p. *ca.* 278° dec. (capillary) when placed in the bath at 260–270°.

Anal. Calcd. for $C_5H_4N_4OS$: C, 35.71; H, 2.37; N, 33.33; S, 19.05. Found: C, 35.94; H, 2.48; N, 33.50; S, 19.04.

The tetrahydrofuran extracts were cooled and approximately 5 g. of the starting material was recovered for addition to subsequent batches.

Ten or 20 batches of the oxidation mixture were run simultaneously in a hood with a surrounding temperature of 22°, and with a temperature rise in the beakers of 3–4°. Ten or more batches were then combined in a 24-cm. Büchner funnel for collection and washing.

6-Mercaptopurine 3-N-Oxide.—A three-necked flask, in a bath thermostated at 60°, was equipped with a nitrogen inlet tube, stirrer, and funnel. A suspension of 50 g. of 7-aminothiazolo-[5,4-*d*]pyrimidine 6-N-oxide in 1 l. of methanol was heated to 60° under an atmosphere of nitrogen. The flask was wrapped in aluminum foil to exclude excessive light and 600 ml. of 1 *N* NaOH was then added dropwise over a period of 1 hr. After 22 hr. at 60°, a little charcoal was added, the solution was filtered while warm to remove traces of solids, and 500 ml. of 10% acetic acid was added. The solution was cooled in the dark, and the yellow product was collected and washed by suspension in water and alcohol. The yield was 21 g., 42%, of a product which contained no more than traces of 6-mercaptopurine by chromatographic assay. This was dissolved in about 130 ml. of 1 *N* NaOH, or 1 equiv., and 0.5 g. of charcoal was added. The solution was filtered and acidified with 10% acetic acid. The yellow to tan product collected (20 g., m.p. 230° dec.) was washed with water and alcohol, dried *in vacuo* at 40°, and stored in dark bottles. It was free of 6-mercaptopurine and was an analytically pure monohydrate. Drying at 100° resulted in incomplete removal of the water of hydration and in significant decomposition. Five such batches have been combined for the recrystallization step.

Anal. Calcd. for $C_5H_4N_4OS \cdot H_2O$: C, 32.26; H, 3.22; N, 30.11; S, 17.20. Found: C, 32.57; H, 2.92; N, 30.60; S, 17.57.

A small sample was still lemon yellow after repeated recrystallizations.

Anal. Found: C, 32.29; H, 3.00; N, 30.26; S, 17.40.

Samples of 7-aminothiazolopyrimidine 6-N-oxide not free of 7-aminothiazolopyrimidine may result in 6-mercaptopurine as an impurity in the 6-mercaptopurine 3-N-oxide. That may be removed by further recrystallizations from aqueous solutions, but it is preferable to avoid this by adequate purification of the thiazolopyrimidine N-oxide.

Purine from 6-Mercaptopurine 3-N-Oxide.—Raney nickel was added to a solution of 25 mg. of 6-mercaptopurine 3-N-oxide in 10 ml. of 5% NH_4OH. The spectrum of the supernatant changed rapidly toward a 262-mμ maximum,[24] and after 1 hr. at room temperature chromatographic analysis showed that the conversion to purine was essentially complete.

6-Mercaptopurine from its 3-N-Oxide.—A solution of 20 γ/ml. of 6-mercaptopurine 3-N-oxide in a 50-ml. quartz flask was irradiated[25] for 30 min. at 15 cm. from a Hanovia lamp, Type S-100, with a Corning filter, No. 9863, transmitting strongly at about 253.7 mμ. The solution was concentrated *in vacuo* and chromatographic and spectral analysis showed that the 6-mercaptopurine 3-N-oxide was all destroyed and that 6-mercaptopurine[25] was the main product. A very small amount of material was present at the R_f of 2-hydroxy-6-mercaptopurine.[26]

5-Methyl-3-[5-(4-acetamido)thiazolyl]-1,2,4-oxadiazole.—A solution of 500 mg. of 7-aminothiazolo[5,4-*d*]pyrimidine 6-N-oxide in 25 ml. of acetic anhydride was refluxed for 30 min., cooled, and allowed to stand 18 hr. after the addition of methanol. The excess solvent was removed *in vacuo*, and the residual oil solidified on refrigeration. The product was recrystallized from ethanol to yield 300 mg. (45%), m.p. 171–172°, of a product with the following analysis.

Anal. Calcd. for $C_8H_8N_4O_2S$: C, 42.90; H, 3.57; N, 25.00; S, 14.28. Found: C, 42.69; H, 3.42; N, 25.22; S, 14.28.

6-Methylmercaptopurine 3-N-Oxide.—To a mixture of 100 mg. of 6-mercaptopurine N-oxide suspended in 0.8 ml. of 1 *N* NaOH and 0.2 ml. of water, 0.10 ml. of CH_3I was added. The mixture was stirred at room temperature for 80 min., and the pH was then adjusted to 5 with concentrated acetic acid. The product was collected and recrystallized from 50% ethanol to yield 50 mg., 47%, of white crystals of m.p. 246–249°.

Anal. Calcd. for $C_6H_6N_4OS$: C, 39.55; H, 3.29; N, 30.77; S, 17.65. Found: C, 39.39; H, 3.40; N, 30.55; S, 17.71.

Reaction of 6-Methylmercaptopurine 3-N-Oxide with Morpholine.—A solution of 100 mg. of 6-methylmercaptopurine N-oxide in 1 ml. of morpholine was heated for 24 hr. at 130°. Excess morpholine was removed *in vacuo*, and 3 ml. of water was added. The product was precipitated with acetic acid, collected, and recrystallized from water to yield 50 mg. (44%), m.p. 203–205°. The product was identical with 6-morpholinopurine[22] in spectral and chromatographic properties.

Anal. Calcd. for $C_9H_{11}N_5O$: C, 52.69; H, 5.38; N, 34.10. Found: C, 53.34; H, 5.73; N, 33.50.

Biological Methods

Both 6-mercaptopurine and the N-oxide were prepared for injection by grinding gently in 0.5% carboxymethylcellulose in 0.85% aqueous NaCl. Suspensions were prepared weekly and were stored between injections at 5° in the dark.

A. Sarcoma 180.—The biological effects of 6-mercaptopurine 3-N-oxide were compared with those of 6-mercaptopurine. Tests for inhibition of the growth of Sarcoma 180 and for recovery from the tumor were conducted as described in detail elsewhere.[27,28] Small fragments of tumor were implanted subcutaneously in the right axillary region of ICR/Ha female mice (Millerton Farms, 18–22 g.). Treatment was begun 24 hr. later and continued for 7 consecutive days. Mice received 0.5 ml. twice daily by the intraperitoneal route, or 0.5 ml. once daily by oral intubation. Controls

(24) A. Bendich, P. J. Russell, Jr., and J. J. Fox, *J. Am. Chem. Soc.*, **76**, 6073 (1954).

(25) G. B. Elion, E. Burgi, and G. H. Hitchings, *ibid.*, **74**, 411 (1952).

(26) A. G. Beaman, *ibid.*, **76**, 5633 (1954).

(27) C. C. Stock, *Am. J. Med.*, **8**, 658 (1950).

(28) D. A. Clarke, F. S. Philips, S. S. Sternberg, C. C. Stock, G. B. Elion, and G. H. Hitchings, *Cancer Res.*, **13**, 593 (1953).

bearing implants of the same tumor were treated similarly with 0.5% carboxymethylcellulose in 0.85% aqueous NaCl. Beginning 1 day after cessation of treatment, weekly measurements of two perpendicular axes of each tumor in surviving mice were made with vernier calipers. From these values, an average diameter for each experimental group was calculated. Evaluation of inhibitory effect is based on the calculated ratio between the average diameter of tumors in treated mice and that of tumors in control animals. A ratio of 0.75 or less is considered to be indicative of antitumor activity in the Sarcoma 180 system. The mice were considered to have recovered when the tumor was no longer palpable.

The mice were weighed at the times of implantation and of measurement of tumors, and the average change in body weight for each experimental group was calculated. Deaths were recorded daily. Unless otherwise noted deaths may be attributed to the growth of the tumor.

B. Tumor Spectrum.—Tests with other transplanted solid mouse tumor systems were conducted similarly. With the mammary carcinoma E0771, sarcoma T241, and melanoma B16, treatment was begun 1 day after implantation of tumor; with Carcinoma 1025 and the Ridgway osteogenic sarcoma (ROS), treatment was begun 5 days after implanation. In all systems, the mice received 0.5 ml. by the intraperitoneal route, once daily, for 7 days. Inhibition of the growth of the tumors was maximal 1 week after the end of therapy, and evaluations at that time are in Table III. A $T:C$ of 0.7 or less, at a well tolerated dose, for E0771, T241, B16, or ROS, and 0.6 or less for C1025 are considered to be statistically valid indications of significant inhibition of the respective solid tumors.[29] Methods for Ehrlich ascites tumor and Mecca lymphosarcoma were those described.[29]

C. Immuno-Depressant Effect.—The test system used was essentially that of Nathan, *et al.*[7] The tests were carried out as follows: Groups of 10 young adult female Swiss ICR/Ha mice were given a single intraperitoneal injection of tanned sheep erythrocytes, followed several hours later by an intraperitoneal injection of a suspension of the test compound (day 0). Four additional daily injections of the compound were given for a total of five doses. On day 12, all survivors were bled from the retro-orbital venous plexus using heparinized capillary pipettes. Equal amounts of blood from each mouse in the group were pooled and the serum was collected. Twofold serial dilutions of the serum in physiological saline were made, and the heterohemagglutinin antibody titers were measured against sheep erythrocytes. Minimal response (±) readings were confirmed microscopically.

Acknowledgment.—The authors wish to thank Miss Valentina Fetzer, Mr. Raul Pena, Mrs. Miyono Schmid, Miss Barbara Smol, Mrs. Beverly Stern, and Mrs. Marion Eilbert for their technical assistance in conducting the *in vivo* experiments.

(29) F. A. Schmid, J. G. Cappucino, P. C. Merker, and G. S. Tarnowski, *Cancer Res.* (Supplement), in press.

Pyrimidinecarbamates and Thiolcarbamates Derived from Amino- and Oxopyrimidines. II[1,2]

ELIZABETH DYER AND HENRY RICHMOND

Department of Chemistry, University of Delaware, Newark, Delaware

Received July 11, 1964

By reaction of aminopyrimidines with various chlorothiolformates the following new thiolcarbamates were prepared: from 2-aminopyrimidine, the S-methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, *t*-butyl, *n*-octyl, and *p*-chlorophenyl 2-pyrimidinethiolcarbamates; from 5-aminouracil, the S-ethyl, *n*-propyl, isopropyl, *n*-butyl, *t*-butyl, and *n*-octyl 2,4-dioxo-1,2,3,4-tetrahydro-5-pyrimidinethiolcarbamates; from 2,4-diaminopyrimidine, the di-S-ethyl and di-S-*n*-butyl 2,4-pyrimidinebis(thiolcarbamates); from 4,6-diaminopyrimidine, di-S-ethyl 4,6-pyrimidinebis(thiolcarbamate). The reaction of diethyl pyrocarbonate with aminopyrimidines gave new carbamates in good yields: ethyl 6-amino-4-pyrimidinecarbamate, diethyl 4,6-pyrimidinedicarbamate, diethyl 2,4-pyrimidinedicarbamate, and ethyl 2,6-dimethyl-4-pyrimidinecarbamate. The thiolcarbamates were converted to oxygen analogs by treatment with mercuric chloride and triethylamine in the presence of an alcohol. Uracil reacted with ethyl chlorothiolformate at the 1-position to give S-ethyl 3,4-dihydro-2,4-dioxo-1(2H)-pyrimidinecarbothioate. Attack by chloroformates occurred at the same position. None of these substances showed significant activity as an anticancer agent.

The present study is a continuation of an investigation of the preparation and properties of pyrimidinecarbamates and related compounds, which are possible anticancer agents. In the previous work[2] carbamates were obtained by the interaction of alkyl chloroformates with aminopyrimidines. In the current work thiolcarbamates were prepared by the reaction of chlorothiolformates[3] with both mono- and diaminopyrimidines. It was hoped that replacing an oxygen atom by sulfur would increase physiological activity. Another purpose of this work was to determine with certainty the location of the substituting groups.

By treating 2-aminopyrimidine, 2,4-diaminopyrimidine, and 4,6-diaminopyrimidine with various chlorothiolformates in the presence of a base, thiolcarbamates of the following structures were obtained. The

(1) (a) Supported by Public Health Service Research Grant No. CA-03477 from the National Cancer Institute; (b) from the Ph.D. Thesis of Henry Richmond, University of Delaware, 1964.

(2) Part I: E. Dyer, M. L. Gluntz, and E. J. Tanck, *J. Org. Chem.*, **27**, 982 (1962).

(3) The alkyl chlorothiolformates were kindly supplied by the Stauffer Chemical Co.