(6) B. B. Newbould, *Brit. J. Pharmacol.*, **21**, 127 (1963).
(7) R. Littell and D. S. Allen, Jr., *J. Med. Chem.*, **8**, 722 (1965).
(8) E. Spaeth and G. Koller, *Chem. Ber.*, **56**, 880 (1927).
(9) M. J. Reider and R. C. Elderfield, *J. Org. Chem.*, **7**, 286 (1942).
(10) (a) E. C. Taylor and A. J. Crovetti, *J. Org. Chem.*, **19**, 1633 (1954). (b) A. Lespagnol, E. Cuingnet, and H. Beerens, *Bull. Soc. Pharm. Lille*, 60 (1955); *Chem. Abstr.*, **50**, 3438e (1956).
(11) M. Julia, H. Pinhas, and J. Igolen, *Bull. Soc. Chim. Fr.*, 2387 (1966).

Substituted Azopurines. 1. Synthesis of 8,8′-Dioxo-6,6′-azopurine

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8,8′-Dioxo-6,6′-azopurine (DOAP), a member of a new class of compounds designated as the oxoazopurines, was synthesized from adenine using sulfuric acid, potassium bromide, potassium permanganate, and hydrogen peroxide. The compound normally exists in the form of a monohydrate and the anhydrous form can be obtained by drying at 100° for 1 hr. Conversion of the anhydrous compound to the monohydrate occurs within 15 min following exposure to the atmosphere at room temperature. The nmr spectrum indicates the presence of C-H, amide N-H, and imidazole N-H protons. The compound can undergo reductive cleavage to two molecules of 8-hydroxyadenine in the presence of strong acid and granulated tin. Preliminary biological evaluation shows that 8,8′-dioxo-6,6′-azopurine is a cholinergic smooth muscle stimulant and an anticurare agent, as well as being an electron acceptor and an extremely potent inhibitor of xanthine oxidase *in vitro*.

A previous report from this laboratory has described a rapid and specific colorimetric analysis for microgram amounts of adenine compounds using sulfuric acid, potassium bromide, potassium permanganate, and hydrogen peroxide.[1] However, when large amounts of adenine, greater than 1 mg/ml of reaction mixture, were employed under the conditions of the colorimetric analysis, not only did the reaction mixture turn yellow, but a bright orange precipitate occurred. Because of the important and varied role occupied by adenine compounds and their analogs in physiological systems, the present studies were designed to investigate the chemical nature and possible biological activity of this orange precipitate.

Experimental Section

Uv spectra were determined in the solvents specified with a Beckman DB-G recording spectrophotometer and the nmr spectra were determined in DMSO-d_6 with a Varian A-60A spectrometer. Elemental analyses were performed by Schwarzkopf Microanalytical Lab., Woodside, N. Y. Where analyses are indicated only by symbols of the elements, results obtained for these elements were within ±0.4% of the theoretical values.

8,8′-Dioxo-6,6′-azopurine (I). To 2.5 l. of 1 *N* H_2SO_4 was added with stirring 15.0 g (111 mmol) of adenine.‡ The solution was filtered through Whatman No. 3 filter paper and KBr (1.8 g in 120 ml of H_2O, 15.12 mmol) added to the filtrate and stirred for 1 hr. To this solution 800 ml of 1 *N* $KMnO_4$§ (800 mmol) was added and stirred for 1 hr, resulting in a brownish purple suspension. A total of 50 ml of 30% H_2O_2 was then slowly added in 10-ml portions with stirring which decolorized the mixture and resulted in a bright orange precipitate. The reaction mixture was stirred for 1 hr and allowed to stand in the dark for 16 hr at 4°. The orange precipitate, designated as the orange adenine chromophore (abbreviated as OAC) was collected by centrifugation at 600g for 15 min employing four 150-ml capacity glass centrifuge bottles. The OAC solid present in each of the four centrifuge bottles was then successively washed 12 times with 150-ml portions of 4 *N* H_2SO_4 and collected by centrifugation at 600g for 15 min until no absorbency at 327 nm could be detected in the decanted acid wash. The OAC solid in each centrifuge bottle was then successively washed six times wth 150-ml portions of H_2O and collected by centrifugation at 600g for 1 hr,= followed by successive washing with four 150-ml portions of acetone with collection by centrifugation at 600g for 1 hr. The final washed OAC solid was combined from all four centrifuge bottles as an acetone suspension, allowed to evaporate to dryness on a large watch glass at room temperature, and then placed in a drying oven at atmospheric pressure for 16 hr at 75°: yield, 0.78 g (5.2%) of an orange microcrystalline product; mp >300°; λ max (absolute EtOH) 230 (17.1×10^3), 302 (5.6×10^3), 442 (11.5×10^3), and 490 mμ (7.4×10^3); λ max (0.1 *N* KOH) 220 (25.2×10^3), 243 (20.7×10^3), and 513 mμ (16.3×10^3). Ascending paper chromatography run on Whatman No. 1 sheets using 60:30:10 1-propanol–ammonia–H_2O, 67:20:13 absolute ethanol–pyridine–H_2O, and 5:2 absolute ethanol–0.5 *M* ammonium acetate showed one spot at R_f of 0.32, 0.12, and 0.08, respectively. *Anal.* ($C_{10}H_6N_{10}O_2 \cdot H_2O$) C, H, N, O.

In order to confirm the presence of 1 mol of water of hydration per mole of the OAC solid, the sample was dried under a stream of nitrogen for 1 hr at 100° and the elemental analyses were performed immediately after drying, keeping the sample in a sealed container to avoid picking up H_2O. *Anal.* Calcd for $C_{10}H_6N_{10}O_2$: C, 40.28; H, 2.03; N, 46.97; O, 10.73. Found: C, 39.87; H, 2.28; N, 46.53; O, 10.70. The trapped H_2O was found to be 5.41% corresponding to a theoretical value of 5.70%. Subsequent drying of the sample for 1 hr at 150 and 200° did not result in any further loss of H_2O. Conversion of the anhydrous compound to the monohydrate was found to occur within 15 min following exposure to the atmosphere at room temperature.

Inasmuch as the number of carbon and nitrogen atoms in the anhydrous empirical formula of $C_{10}H_6N_{10}O_2$ was double that of the starting material adenine, it appeared that OAC was a substituted purine dimer with two oxo groups present in the molecule. The nmr spectrum of OAC at a concentration of 20 mg/ml was taken in DMSO-d_6 (TMS) on a Varian A-60A (δ). Three peaks were found at δ 8.84, 12.05, and 12.64 ppm relative to the TMS reference. Integration of the three proton peaks revealed an integer ratio of 1:1:1, respectively, confirming the presence of six hydrogens in the OAC molecule. Peak assignments, made on the basis of D_2O exchange and ranges of known δ ppm values,[2-6] indicated the presence of two C-H protons (8.84 ppm), two amide N-H protons (12.05 ppm), and two imidazole N-H protons (12.64 ppm). Since the two protons originally present on the nitrogen attached to C-6 of adenine were found to be absent in the nmr spectrum of OAC, it appeared that the dimerization of adenine occurred through the formation of an azo linkage resulting in an oxoazopurine as shown in Figure 1, having the chemical structure of 8,8′-dioxo-6,6′-azopurine (I) abbreviated as DOAP.

Oxidation of adenine by $KMnO_4$ with KBr, followed by H_2O_2, appears most likely at the C-8 position in view of the fact that

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‡Supplied by Aldrich Chemical Co. It was found that solution of adenine in acid could be facilitated by initially homogenizing small portions of the solid in 1 *N* H_2SO_4 using a Teflon pestle homogenizer.

§A 1 *N* $KMnO_4$ solution was prepared by adding 32 g of solid $KMnO_4$ to 1 l. of H_2O and heating at 90° for 1 hr, after which time the solution was cooled to room temperature and filtered before use.

= A longer period of centrifugation is required in H_2O because of the fine microcrystalline nature of OAC.

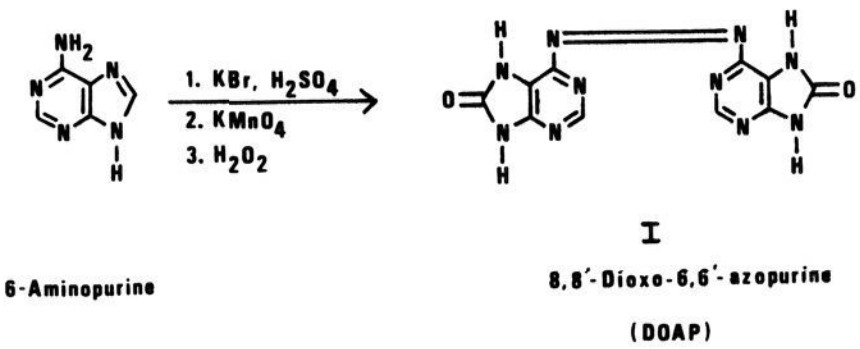

Figure 1. Synthesis of 8,8′-dioxo-6,6′-azopurine (I) from 6-aminopurine.

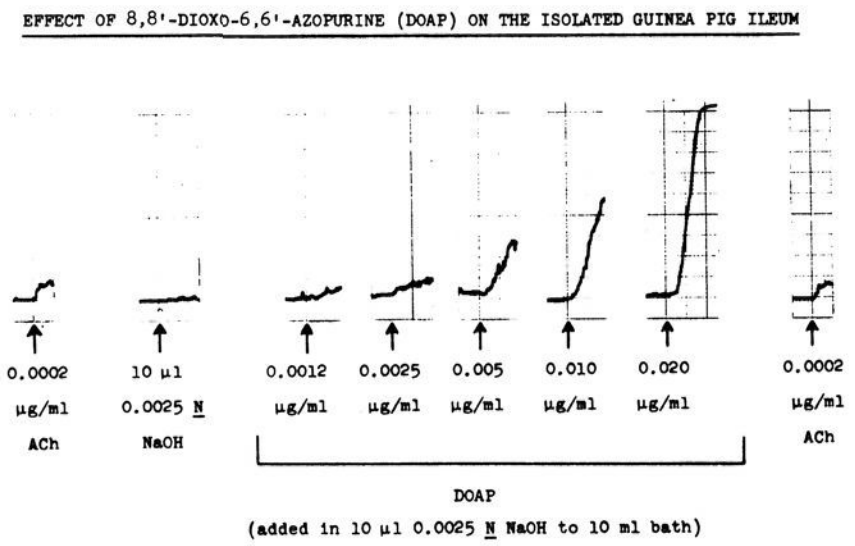

Figure 2. Effect of increasing concentrations of 8,8′-dioxo-6,6′-azopurine (DOAP) on the isolated guinea pig ileum.

purines are known to selectively form highly reactive C-8 halogen atoms that can give rise to the 8-oxo derivative *via* acid hydrolysis.[7] However, to confirm this possibility, reductive cleavage of 8,8′-dioxo-6,6′-azopurine was carried out, followed by ion-exchange chromatography of the reaction mixture. A 50-mg quantity of I was washed four times with 20-ml portions of 1 *N* HCl employing centrifugation. The washed sediment was then added to 30 ml of 4 *N* HCl containing 3.0 g of granulated tin and the mixture heated at 95° for 20 hr using an air condenser. The reaction mixture was then filtered and a 15-ml aliquot of the filtrate added to 105 ml of H_2O to make a final normality of 0.5 *N* with respect to HCl. The entire 120-ml sample was added to a 21.0 × 0.9 cm column of AG 50W-X8, 200–400 mesh resin in the hydrogen form packed with 5 psi of air pressure, following which the column was washed with 100 ml of 0.5 *N* HCl. Subsequent elution was carried out with an HCl gradient employing an apparatus previously described.[8] The mixing chamber initially contained 500 ml of 0.5 *N* HCl and the reservoir contained 5 *N* HCl. Fractions of 3 ml were collected every 2 min for a total effluent volume of 500 ml. The optical density of each tube was determined at 260, 280, and 305 nm. A single large peak of absorbance was found at an effluent volume of 159 ml (1.92 *N* HCl) with two λ max values of 280 and 267 nm having an A_{280}:A_{267} ratio of 1.04 which exactly matched the chromatographic position and spectral characteristics of an authentic sample of 8-hydroxyadenine sulfate. Inasmuch as the present chromatographic system was found to be capable of separating authentic samples of 2,8-dihydroxyadenine, 8-hydroxyadenine, 2-hydroxyadenine, and adenine in that order employing similar conditions, the position of the oxo group on C-8 and C-8′ of I as well as the symmetry of the oxoazopurine dimer was confirmed.

Discussion

Biological Results. 8,8′-Dioxo-6,6′-azopurine (DOAP) was evaluated for any possible effect on the isolated guinea pig ileum mounted in a tissue bath assembly containing 10 ml of Tyrode's solution aerated with air. Because of the limited solubility of DOAP in water, 0.0025 *N* NaOH was utilized as a suitable solvent. The middle portion of Figure 2 indicates the effect of progressively higher final bath concentrations of DOAP, each added in 10 µl of 0.0025 *N* NaOH. Beginning with a concentration of 0.0012 µg/ml, DOAP was found to produce a contraction of the isolated guinea pig ileum. The ED_{50} for DOAP in causing the isolated guinea pig ileum to contract was found to be 6 ng/ml of final bath concentration. The DOAP-induced contraction of the guinea pig ileum was found not to be blocked by the antihistaminic agent, tripelennamine. However, atropine was found to be capable of blocking the response of the ileum to DOAP.

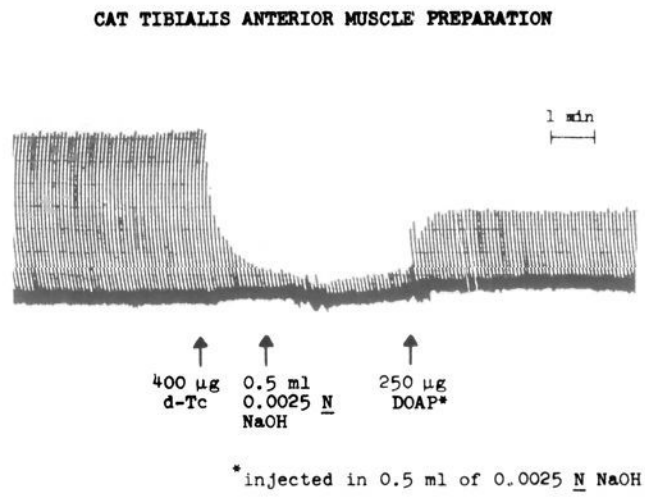

Figure 3. Effect of 8,8′-dioxo-6,6′-azopurine (DOAP) on a curare block of the indirectly elicited twitch of the cat tibialis anterior muscle preparation.

Because of the apparent cholinergic character of DOAP, experiments were initiated on its possible effect at the neuromuscular junction. Cats anesthetized with 60 mg/kg of chloralose were arranged for recording indirectly elicited twitches of the tibialis anterior muscle. Supramaximal stimuli of 0.1-msec duration at a frequency of 0.2 per sec were employed. *d*-Tubocurarine was administered intravenously to cause a 75–85% block of neuromuscular transmission. Figure 3 indicates the effect of 400 µg of *d*-tubocurarine on the height of the twitch, followed by the close-retrograde arterial injection of 0.5 ml of 0.0025 *N* NaOH. No curare reversal was observed for the dilute NaOH employed as a solvent while the close-retrograde arterial injection of 250 µg of DOAP in the same volume of dilute NaOH did cause a marked reversal of the curare block. It was found that the close-retrograde arterial injections of 25, 166, 250, and 333 µg of DOAP, each dissolved in 0.0025 *N* NaOH, resulted in 10.5, 40.0, 58.6, and 74.2% reversals of the curare block, respectively. It was also found that in the absence of *d*-tubocurarine, DOAP did not augment the indirectly elicited twitch of the cat tibialis anterior muscle.

Figure 4 demonstrates the anticurare effects of DOAP on a different skeletal muscle preparation, the rat isolated phrenic nerve–diaphragm mounted in a tissue bath assembly containing 50 ml of Krebs bicarbonate solution aerated with 95% O_2 and 5% CO_2. The top panel illustrates the effect of a final bath concentration of 1.0 µg/ml of *d*-tubocurarine on the height of the indirectly elicited twitch. The middle panel shows the lack of any effect of 5 ml of 0.005 *N* NaOH on the curare block. However, as shown in the bottom panel, a final bath concentration of 62.9 µg/ml of DOAP added in 5 ml of 0.005 *N* NaOH was found to cause a 67% reversal of the curare block of the rat diaphragm when added 5 min after the curare.

Figure 5 also presents the anticurare effects of DOAP on the isolated phrenic nerve–diaphragm preparation of the rat, only this time, it was added before the *d*-tubocurarine. The top and middle panels demonstrate the lack of any anticurare activity of the solvent employed for DOAP. The bottom panel shows that a final bath concentration of 62.9 µg/ml of DOAP added 5 min before the *d*-tubocurarine resulted in a marked reduction of the blocking action of curare on neuromuscular transmission of the rat diaphragm. It would appear that not only does DOAP have

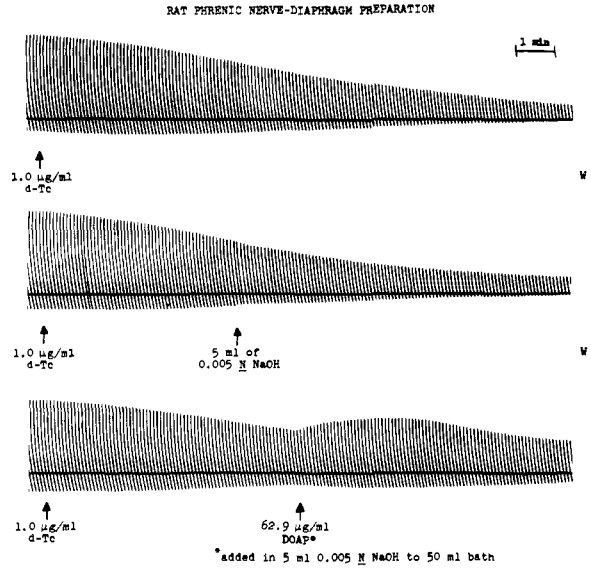

Figure 4. Anticurare effect of 8,8′-dioxo-6,6′-azopurine (DOAP) added after *d*-tubocurarine on the indirectly elicited twitch of the rat isolated phrenic nerve-diaphragm preparation.

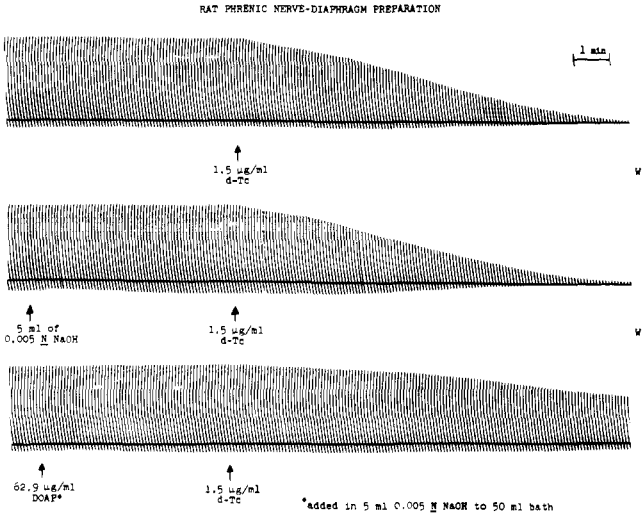

Figure 5. Anticurare effect of 8,8′-dioxo-6,6′-azopurine (DOAP) added before *d*-tubocurarine on the indirectly elicited twitch of the rat isolated phrenic nerve-diaphragm preparation.

the ability to reverse a curare block of neuromuscular transmission when added after curare, but that DOAP also has a definite protective effect against a curare block when added before curare. These data suggest that DOAP may be a direct competitive antagonist of curare and as such could be investigated as a potentially useful agent in the treatment of myasthenia gravis.

An LD_{50} study for DOAP was carried out employing adult mice. A single subcutaneous injection of the compound dissolved in 0.01 *N* NaOH was performed in the nape of the neck employing five mice each at a dose of 1, 5, 15, 25, 50, 100, 150, 200, and 250 mg/kg of body weight. The 24-hr LD_{50} value was found to be >250 mg/kg of body weight. Following observation of the same animals for an additional 5-day period, the LD_{50} value for DOAP at 5 days postinjection was determined to be 125 mg/kg of body weight.

Additional biological activities of DOAP have also been found during the course of our preliminary investigations involving any possible effects of the compound on enzymatic reactions. DOAP was initially found to cause a

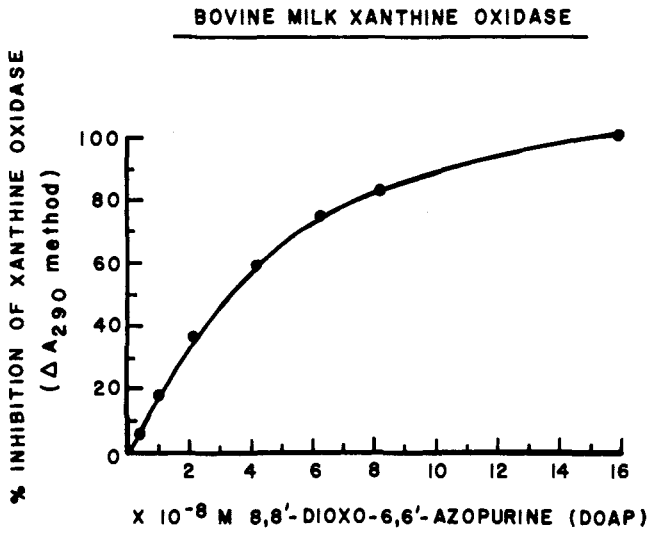

Figure 6. Inhibition of xanthine oxidase by 8,8′-dioxo-6,6′-azopurine (DOAP). Each experimental cuvette contained 1.75 ml of 0.05 *M* potassium phosphate buffer, pH 7.5, 1.0 ml of 1.6 × 10^{-5} *M* xanthine, 0.01 ml of various concentrations of DOAP in 0.005 *N* NaOH, and 0.25 ml of a 0.5 mg/ml solution of bovine milk xanthine oxidase. Xanthine oxidase activity was measured by the spectrophotometric procedure at 290 nm.

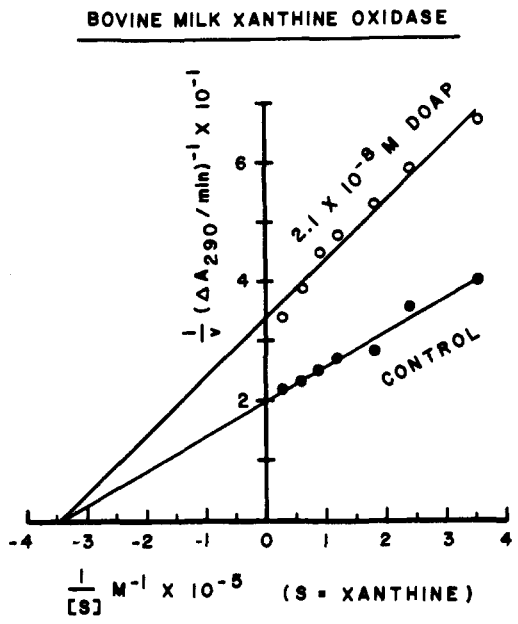

Figure 7. Lineweaver-Burk analysis of the inhibition of xanthine oxidase by 8,8′-dioxo-6,6′-azopurine (DOAP). Each cuvette contained 1.75 ml of 0.05 *M* potassium phosphate buffer, pH 7.5, 0.25 ml of a 0.5 mg/ml solution of bovine milk xanthine oxidase, and 1.0 ml of various amounts of xanthine (lower line). A duplicate set of cuvettes contained 0.01 ml of a 1.95 μg/ml solution of DOAP in 0.005 *N* NaOH for a final concentration of 2.1 × 10^{-8} *M* DOAP (upper line). Xanthine oxidase activity was measured by the spectrophotometric procedure at 290 nm.

rapid decrease in the absorbance of NADH at 340 nm. A 2.85-ml volume of 0.3 *M* Tris-HCl buffer at a desired pH containing 8.6 × 10^{-5} *M* NADH was placed in a 1-cm light path cuvette, equilibrated to 30°, and its absorbance at 340 nm monitored against water in the reference cell in a Beckman DB-G recording spectrophotometer for 5 min. A 0.15-ml aliquot of a 500 μg/ml solution of DOAP in 0.005 *N* NaOH was added to the cuvette resulting in a final concentration of 8.0 × 10^{-5} *M* DOAP and the decrease in absorbance at 340 nm recorded for an additional 5-min period. The ΔA_{340}/min was obtained from the lin-

ear portion of the curve and was observed to have a value of 0.016, 0.042, 0.112, 0.208, 0.370, and 0.067 OD units at pH 8.5, 8.0, 7.5, 7.0, 6.5, and 6.0, respectively. These data indicate that DOAP is capable of causing the rapid oxidation of NADH with a pH optimum of 6.5 and is itself apparently reduced to its hydrazo form while acting as an electron acceptor.

DOAP has also been found to be an extremely potent inhibitor of bovine milk xanthine oxidase employing a spectrophotometric method[9] which measures uric acid formation at 290 nm. Assay conditions were adjusted so that the rate of increase in absorbance using a Beckman DB-G recording spectrophotometer was linearly proportional to the amount of xanthine oxidase** present. Each experimental cuvette contained 1.75 ml of 0.05 M potassium phosphate buffer, pH 7.5, 1.0 ml of a 1.6×10^{-5} M solution of xanthine, 0.01 ml of various concentrations of DOAP in 0.005 N NaOH, and 0.25 ml of a 0.5 mg/ml solution of xanthine oxidase. After a 2-min equilibration period at 25°, the enzyme was added last, the cuvette contents were rapidly mixed, and the A_{290} was recorded with time against a reference cell from which the substrate had been omitted. The ΔA_{290}/min was obtained from the linear portion of the curve of increasing extinction in the presence and absence of DOAP. A 50% inhibition of xanthine oxidase activity was found to occur at a final concentration of 3.5×10^{-8} M DOAP in the reaction mixture (Figure 6), while allopurinol under similar conditions was found to produce a 50% inhibition of xanthine oxidase activity at 6.1×10^{-6} M. Results of both a Lineweaver and Burk plot (Figure 7) as well as a Dixon plot revealed a mean K_i of 2.8×10^{-8} M for DOAP while indicating that the nature of the inhibition of xanthine oxidase by DOAP was noncompetitive. The present finding of such a high degree of potency of DOAP as a xanthine oxidase inhibitor does suggest the further investigation of this compound as a potentially useful agent in the treatment of hyperuricemia. Experiments are now in progress in our laboratory which are designed for further studies of both the chemical and biological properties of 8,8′-dioxo-6,6′-azopurine (DOAP), a member of a new class of compounds designed as the oxoazopurines.

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References

(1) J. R. Davis and R. N. Morris, *Anal. Biochem.*, 5, 64 (1963).
(2) R. K. Robins, L. B. Townsend, F. Cassidy, J. F. Gerster, A. F. Lewis, and R. L. Miller, *J. Heterocycl. Chem.*, 3, 110 (1966).
(3) L. Twanmoh, H. B. Wood, Jr., and J. S. Driscoll, *J. Heterocycl. Chem.*, 10, 187 (1973).
(4) S. I. Chan, G. C. Y. Lee, C. F. Schmidt, and G. P. Kreishman, *Biochem. Biophys. Res. Commun.*, 46, 1536 (1972).
(5) J. R. Fox, Ph.D. Thesis, The University of Illinois, Urbana, Ill., 1965.
(6) R. R. Shoup, H. T. Miles, and E. D. Becker, *Biochem. Biophys. Res. Commun.*, 23, 194 (1966).
(7) D. J. Brown, "Fused Pyrimidines II. Purines," Wiley, New York, N. Y., 1971, pp 24, 143, 146, 175.
(8) J. R. Davis and H. Busch, *Cancer Res.*, 19, 1157 (1959).
(9) H. M. Kalckar, *J. Biol. Chem.*, 167, 429 (1947).

**Obtained from Sigma Chemical Co., St. Louis, Mo.

Synthesis and Enzymatic Activity of 1,2,4-Triazole-3-carboxamide 6′-Deoxyhomoribonucleoside-6′-phosphonic Acid and Related Compounds

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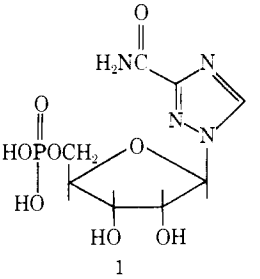
The synthesis, *via* the Wittig reaction, of the phosphonic acid corresponding to the nucleotide 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide 5′-phosphate (1) is reported. The same route is used to synthesize nucleosides with a polar substituent (carboxamide) and an ionizable group (carboxylic acid) at the position normally occupied by the phosphate group in the nucleotide. These compounds were tested as the inhibitors of the enzyme inosine 5′-phosphate dehydrogenase isolated from *Escherichia coli*. In this system, compound 1 produced 50% inhibition of this enzyme at a concentration of 6×10^{-7} M. In the same assay, the corresponding phosphonic acid 9 inhibited the dehydrogenase by 50% at 2×10^{-5} M concentration. The other compounds tested (12, 13, and 14) were not effective as inhibitors up to 3×10^{-4} M.

The conversion of certain antiviral nucleosides to the corresponding nucleotides has been shown to be a necessary process in order for these drugs to exhibit their inhibitory activity.[1,2] In our studies of the synthetic nucleoside 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide[3] (ribavirin†), which exhibits activity against both DNA and RNA viruses,[4,5] we have found evidence[6] that the corresponding 5′-phosphate 1 is the active form of this antiviral agent. This nucleotide (1) was found to be a potent competitive inhibitor of the enzyme inosine 5′-phosphate dehydrogenase.[6]



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The syntheses of several nucleoside phosphonic acids, in which the phosphate ester oxygen is replaced by a methylene group, have been reported.[7] Hampton and coworkers studied the interactions of a number of homoadenosine-6′-phosphonic acid derivatives with adenine nucleotide utilizing enzymes and found that replacement of the phosphate ester oxygen with a methylene or substituted methylene group allows these nucleotide analogs to retain varying degrees of binding to the enzyme site.[8]

We now report the synthesis of the phosphonic acid cor-

† Ribavirin is the name approved by the U. S. Adopted Names Council for this compound, previously identified as Virazole.