Synthesis of *lin*-Benzoallopurinol

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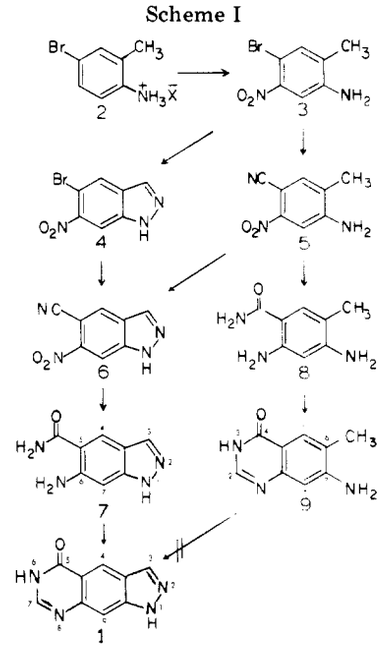
The synthesis of pyrazolo[4,3-*g*]quinazolin-5-one (*lin*-benzoallopurinol, **1**), a fluorescent analogue of 4-hydroxypyrazolo[3,4-*d*]pyrimidine (allopurinol), by a route commencing with a trisubstituted benzene is described. The title compound was found to be a substrate for but not an inhibitor of xanthine oxidase.

Recent work in this laboratory has centered on the synthesis of "stretched out" versions of biologically active purine compounds.[1] Encouraged by the fluorescence properties and biological activity of laterally extended analogues in this series,[2] we have initiated an investigation of similarly extended analogues of drugs and antibiotics. One goal has been to synthesize pyrazolo[4,3-*g*]-quinazolin-5-one (*lin*-benzoallopurinol, **1**), a 2.4 Å wider version of allopurinol (**1**, shaded area only),[3] which is presently the drug of choice for the treatment of hyperurecemia (Zyloprim, Burroughs-Wellcome)[4] and a potent inhibitor of xanthine oxidase.[5,6]

Two paths to the title compound were envisioned. Both are based on the flexible starting material 4-bromo-2-methyl-5-nitroaniline (**3**) which has been elaborated successfully to *lin*-benzoallopurinol (**1**) as shown in Scheme I. 4-Bromo-2-methylaniline was converted to its sulfate salt (**2**) in order to direct nitration to the C-5 position and thus provide the precursor for a linear substitution pattern. Compound **3** underwent cyclization efficiently to 5-bromo-6-nitroindazole (**4**) on treatment with sodium nitrite in glacial acetic acid at room temperature.[7,8] The next step was the modification of the phenyl ring to give an amide or amide equivalent at the C–Br site. This was best accomplished by treating either compound **3** or **4** with cuprous cyanide[9] in anhydrous pyridine, and the conversion **3** → **5** proved easier and more economical than **4** → **6**. Formation of 5-cyano-6-nitroindazole (**6**) from 4-amino-5-methyl-2-nitrobenzonitrile (**5**) was highly efficient.[7,8] The combination of reduction and partial hydrolysis by means of Raney nickel and hydrazine hydrate

Scheme I



in methanol[10] converted **5** to 4-amino-5-methylanthranilamide (**8**), which was sensitive to oxidation. The immediate heating of **8** in formic acid produced 7-amino-6-methyl-4-quinazolone (**9**), but this compound resisted ring closure with nitrous acid to *lin*-benzoallopurinol (**1**), probably due to insufficient activation of the methyl group.[7] The successful route to *lin*-benzoallopurinol lay through the combination of reduction and partial hydrolysis[10] of compound **6** to 6-aminoindazole-5-carboxamide (**7**) and direct cyclization with formic acid to pyrazolo[4,3-*g*]quinazolin-5-one (**1**). The preferred route from 4-bromo-2-methyl-5-nitroaniline to *lin*-benzoallopurinol is **3** → **5** → **6** → **7** → **1**.

The *lin*-benzoallopurinol was found to be fluorescent (Figure 1), as were the *lin*-benzoadenine derivatives that we have described previously.[2] In ethanol solution, the fluorescence quantum yield for **1** was 0.19, the fluorescence lifetime was 4 ns, and the fluorescence emission maximum was 380 nm upon excitation at 315 nm. We have investigated the properties of *lin*-benzoallopurinol as both inhibitor and substrate with xanthine oxidase. Using conventional assay methods,[11] we compared the rate of uric acid formation (as an indication of the rate of consumption of hypoxanthine) with that of the disappearance of *lin*-benzoallopurinol (by UV methods, Figure 1) and found them to be nearly identical. The apparent Michaelis constants for the two substrates, similarly measured, were

(1) (a) Leonard, N. J.; Morrice, A. G.; Sprecker, M. A. *J. Org. Chem.* **1975**, *40*, 356. (b) Morrice, A. G.; Sprecker, M. A.; Leonard, N. J. *Ibid.* **1975**, *40*, 363. (c) Keyser, G. E.; Leonard, N. J. *Ibid.* **1976**, *41*, 3529. (d) Leonard, N. J.; Sprecker, M. A.; Morrice, A. G. *J. Am. Chem. Soc.* **1976**, *98*, 3987.

(2) (a) Scopes, D. I. C.; Barrio, J. R.; Leonard, N. J. *Science* **1977**, *195*, 296. (b) Schmidt, M. J.; Truex, L. L.; Leonard, N. J.; Scopes, D. I.; Barrio, J. R. *J. Cyclic Nucleotide Res.* **1978**, *4*, 201. (c) Leonard, N. J.; Scopes, D. I. C.; VanDerLijn, P.; Barrio, J. R. *Biochemistry* **1978**, *17*, 3677. (d) Kauffman, R. F.; Lardy, H. A.; Barrio, J. R.; Barrio, M. del C. G.; Leonard, N. J. *Ibid.* **1978**, *17*, 3686. (e) VanDerLijn, P.; Barrio, J. R.; Leonard, N. J. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 4204. (f) VanDerLijn, P.; Barrio, J. R.; Leonard, N. J. *J. Biol. Chem.* **1978**, *253*, 8694. (g) Leonard, N. J. *Heterocycles* **1979**, *12*, 129. (h) See also: Leonard, N. J.; Keyser, G. E. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 4262.

(3) Synthesis: Schmidt, P.; Druey, J. *Helv. Chim. Acta* **1956**, *39*, 986. Robins, R. K. *J. Am. Chem. Soc.* **1956**, *78*, 784.

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(8) This is essentially the route of: Noelting, E. *Chem. Ber.* **1904**, *37*, 2556.

(9) For other examples of CN substitutions see: Friedman, L.; Shechter, H. *J. Org. Chem.* **1961**, *26*, 2522; Ikan, R.; Rapaport, E. *Tetrahedron* **1967**, *23*, 3823.

(10) Butler, K.; Partridge, M. W. *J. Chem. Soc.* **1959**, 2396.

(11) "Biochemica Information"; Boehringer Mannheim GmbH: Mannheim, Germany, 1973; Vol. I, p 179.

0022-3263/79/1944-4609$01.00/0

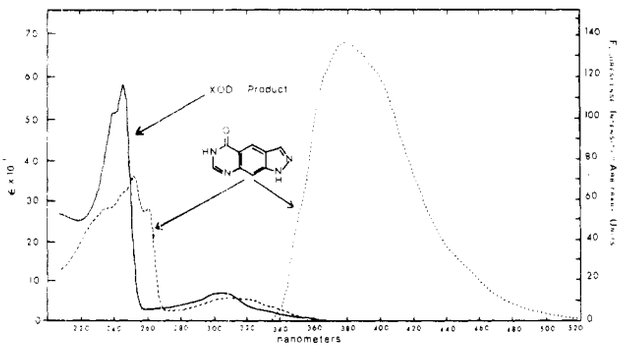

Figure 1. Ultraviolet absorption spectra of *lin*-benzoallopurinol (1) (---) and its xanthine oxidase (XOD) product (—) in phosphate buffer at pH 7.7; corrected fluorescence emission spectrum of *lin*-benzoallopurinol in ethanol (···).

found to be nearly identical, $K_M = 1.6 \times 10^{-6}$ M, and in the range of the values reported for hypoxanthine.[12]

Because of the close similarity in the behavior of hypoxanthine and *lin*-benzoallopurinol (1) with xanthine oxidase, we calculated a theoretical amount of inhibition that would be observed in an assay for xanthine oxidase activity in which the concentration of substrate (hypoxanthine) was varied while the potential inhibitor (*lin*-benzoallopurinol) concentration was kept constant. For the purpose of these kinetic calculations, it was assumed that the total substrate concentration was the simple sum of the concentrations of inhibitor and substrate. This calculated result was identical with that actually observed, suggesting that our compound may be a substrate essentially indistinguishable from hypoxanthine by xanthine oxidase.

In a second experiment, samples of xanthine oxidase were preincubated[13] with allopurinol or *lin*-benzoallopurinol to compare their behavior as inhibitors with an unincubated sample. Consistent with the results described above, it was observed that the *lin*-benzoallopurinol had no significant inhibitory effect, whereas the allopurinol, as previously known, reduced the rate of uric acid formation, in our experiment by a factor of 500. Thus far, the linear benzologues of purine substrates[1d] (e.g., hypoxanthine and xanthine) and the pyrazolopyrimidine inhibitors[12a] of xanthine oxidase (e.g., allopurinol), which we have synthesized and tested, are substrates for xanthine oxidase.[2h] The defined dimensional change in a potent xanthine oxidase inhibitor negates the inhibitory properties of B-ring alteration, as in allopurinol, while allowing those of the compound as a substrate to remain.

Experimental Section

All thin-layer chromatographic separations were performed on Merck precoated silica gel F-254 plates with fluorescent backing. Melting points were determined on a Büchi melting point apparatus and are uncorrected. The NMR spectra were recorded on a Varian Associates EM-390 or HA-220 spectrometer using tetramethylsilane as an internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), integration, coupling constants, and assignment. Mass spectra were obtained on a Varian MAT CH-5 low-resolution or a Varian MAT-731 high-resolution spectrometer coupled with a 620i computer and a STATOS recorder by Mr. J. Carter Cook and his staff. Ultraviolet absorption spectra were obtained on a Beckman Acta MVI spectrometer, and the maxima are reported as follows: solvent, wavelength, absorption coefficient, and description (sh = shoulder). Molecular fluorescence emission and excitation spectra were measured on a Spex Fluorolog spectrofluorometer using *lin*-benzo-ATP in aqueous solution, $\Phi = 0.40$,[2f] as a reference. A cross-correlation subnanosecond fluorometer, previously described,[14,15] interfaced with a Monroe 1880 programmable calculator was used by Mr. Joseph B. Holtwick to determine fluorescence lifetimes. Microanalyses were performed by Mr. Josef Nemeth and his staff or by Midwest Microlab, Ltd., Indianapolis, IN. Raney nickel (no. 28) was used as purchased from the Grace Chemical Co., Pittsburg, TN. Buttermilk xanthine oxidase (xanthine:oxygen oxidoreductase; EC No. 1.2.3.2) was purchased from Sigma Chemical Co., St. Louis, MO.

4-Bromo-2-methyl-5-nitroaniline (3). A solution of 4-bromo-2-methylaniline sulfate (2; made from 0.93 g (5 mmol) of 4-bromo-2-methylaniline in 25 mL of diethyl ether and enough concentrated sulfuric acid to precipitate the aniline sulfate and isolate the salt by filtration) in 2 mL of concentrated sulfuric acid was added in one portion to an ice-cold solution of 500 mg (5 mmol) of potassium nitrate in 10 mL of concentrated sulfuric acid. The resulting solution was kept overnight at room temperature before being poured over 125 mL of crushed ice and neutralized with concentrated ammonium hydroxide. A yellow solid separated which was washed with water and dried, giving 770 mg (66%) of yellow needles: mp 108 °C; NMR ($(CD_3)_2SO$) δ 2.12 (s, 3, CH_3), 5.65 (s, 2, NH_2), 7.25 (s, 1, C_{ar}–H), 7.38 (s, 1, C_{ar}–H); mass spectrum, *m/e* 230, 232 (M^+).

Anal. Calcd for $C_7H_7BrN_2O_2$: C, 36.36; H, 3.03; N, 12.12. Found: C, 36.02; H, 3.03; N, 12.08.

5-Bromo-6-nitroindazole (4). A solution of 1.15 g (5 mmol) of 4-bromo-2-methyl-5-nitroaniline (3) in 125 mL of glacial acetic acid was treated with a solution of 345 mg (5 mmol) of sodium nitrite in 1.0 mL of water, stirred for 15 min, and allowed to stand at room temperature for 3 days. The acetic acid was removed in vacuo, leaving an oily residue to which 25 mL of water was added. The solid that precipitated was collected by filtration to yield 1.14 g (94%) of orange prisms: mp 192–195 °C; NMR ($(CD_3)_2SO$) δ 8.20 (s, 1), 8.28 (s, 1), 8.33 (s, 1); mass spectrum, *m/e* 241, 243 (M^+).

Anal. Calcd for $C_7H_4BrN_3O_2$: C, 34.71; H, 1.65; N, 17.36. Found: C, 35.14; H, 1.62; N, 17.56.

4-Amino-5-methyl-2-nitrobenzonitrile (5). A solution of 2.0 g (8.66 mmol) of 4-bromo-2-methyl-5-nitroaniline (3) in 50 mL of dry pyridine (stored over KOH) was treated with 2.0 g (22.5 mmol) of cuprous cyanide, and the suspension was heated at reflux overnight. The reaction solution was allowed to cool to room temperature, filtered to remove excess solid cuprous cyanide, and poured into 350 mL of dilute ammonium hydroxide; the solid precipitate was collected. The filtrate was extracted with benzene until the organic layer was colorless, and the combined organic extracts were dried (Na_2SO_4) and evaporated in vacuo. The solid thus obtained was combined with the precipitate to give 1.1 g (72%) of the benzonitrile (5) as orange needles: mp 216–216.5 °C; NMR ($(CD_3)_2SO$) δ 2.13 (s, 3, CH_3), 6.50 (s, 2, NH_2), 7.43 (s, 1, C_{ar}–H), 7.52 (s, 1, C_{ar}–H); mass spectrum, *m/e* 177 (M^+).

Anal. Calcd for $C_8H_7N_3O_2$: C, 54.23; H, 3.98; N, 23.72. Found: C, 54.09; H, 4.01; N, 23.93.

5-Cyano-6-nitroindazole (6). A. From 4-Amino-5-methyl-2-nitrobenzonitrile (5). A solution of 250 mg (1.41 mmol) of 4-amino-5-methyl-2-nitrobenzonitrile (5) in 25 mL of glacial acetic acid was treated with a solution of 97 mg (1.4 mmol) of sodium nitrite in 1 mL of water. The combined solution was stirred vigorously for 15 min and kept at room temperature for 3 days. The solvent was removed in vacuo (water pump), and the oil that remained was treated with 10 mL of water, which gave a precipitate. This solid was isolated by filtration and dried to give 260 mg (98%) of a tan solid: mp 198 °C dec; NMR ((C-

(12) The literature values of K_M were obtained by extrapolation of a double reciprocal plot to the *x* axis: (a) Krenitsky, T. A.; Neil, S. M.; Elion, G. B.; Hitchings, G. *Arch. Biochem. Biophys.* **1972**, *150*, 585; $K_M < 10^{-6}$, value determined at pH 6.8 with ferricyanide as electron acceptor. (b) Fridovich, I.; Handler, P. *J. Biol. Chem.* **1958**, *233*, 1578; $K_M = 1.13 \times 10^{-6}$, value determined at pH 7.8 with oxygen as electron acceptor. (c) Greenlee, L.; Handler, P. *J. Biol. Chem.* **1964**, *239*, 1090; $K_M = 1.3 \times 10^{-6}$ M, value quoted from preceding reference.

(13) Spector, T.; Johns, D. G. *J. Biol. Chem.* **1970**, *245*, 5079.

(14) Spencer, R. D.; Weber, G. *Ann. N.Y. Acad. Sci.* **1969**, *158*, 361.

(15) Spencer, R. D.; Vaughan, W. M.; Weber, G. *Mol. Lumin., Int. Conf.* **1969**, 607.

$D_3)_2SO$) δ 8.43 (s, 1, C_{ar}–H), 8.55 (s, 1, C_{ar}–H), 8.63 (s, 1, C_{ar}–H); mass spectrum, *m/e* 188 (M^+).

B. From 5-Bromo-6-nitroindazole (4). A solution of 1.0 g (4.1 mmol) of 5-bromo-6-nitroindazole (**4**) in 50 mL of dry pyridine (dried over KOH) was treated with 1 g (11 mmol) of cuprous cyanide, and the suspension was heated at reflux overnight. The reaction mixture was allowed to cool to room temperature, filtered to remove excess cuprous cyanide, and concentrated to 15 mL in vacuo. This solution was poured into 100 mL of dilute ammonium hydroxide, and the solid was isolated by filtration. The filtrate was extracted exhaustively with ethyl acetate, and the combined extracts were dried over Na_2SO_4 and evaporated in vacuo. The two solids were combined to give 350 mg (45%) of a tan solid identical in all respects with that prepared by route A.

6-Aminoindazole-5-carboxamide (7). A solution of 2.88 g (15 mmol) of 5-cyano-6-nitroindazole (**6**) in 300 mL of methanol was treated with 3 mL of 98% hydrazine hydrate and warmed to 45 °C. Raney nickel was added with vigorous stirring. When gas evolution had ceased and further addition of the catalyst produced no more effervescence, the suspension was treated with activated charcoal and filtered. Evaporation of the methanol in vacuo afforded 2.0 g (11.4 mmol, 76%) of a white solid that was used in the next step without further purification: mp 265 °C dec; NMR ($(CD_3)_2SO$) δ 6.32 (s, NH_2), 6.57 (s, 1, C_{ar}–H), 7.81 (s, 1, C_{ar}–H), 7.95 (s, 1, C_{ar}–H); mass spectrum, *m/e* 176 (M^+).

Anal. Calcd for $C_8H_8N_4O$: C, 54.54; H, 4.58; N, 31.80. Found: C, 54.44; H, 4.54; N, 31.65.

4-Amino-5-methylanthranilamide (8). The same procedure as used for **7** was performed under nitrogen from **5**. After evaporation of the methanol, a single recrystallization from anhydrous methanol gave 200 mg (72%) of a colorless solid: mp 165 °C; NMR ($(CD_3)_2SO$) δ 1.93 (s, 3, CH_3), 4.80 (br, NH_2), 5.80 (s, 1, C_{ar}–H), 7.12 (s, 1, C_{ar}–H); mass spectrum, *m/e* 165 (M^+).

7-Amino-6-methyl-4-quinazolone (9). A solution of 4-amino-5-methylanthranilamide (**8**; 100 mg, 0.65 mmol) in 50 mL of 98% formic acid was heated to reflux for 8 h. Removal of the solvent in vacuo (azeotropic removal with toluene) gave 110 mg (97%) of tan solid: mp 212 °C dec; NMR ($(CD_3)_2SO$) δ 2.45 (s, 3, CH_3), 7.95 (s, 1, C_{ar}–H), 8.03 (s, 1, C_{ar}–H), 8.17 (s, 1, C_{ar}–H); mass spectrum, *m/e* 175 (M^+).

Pyrazolo[4,3-*g*]quinazolin-5-one (*lin*-Benzoallopurinol, 1). A solution of 2.0 g (11.4 mmol) of 6-aminoindazole-5-carboxamide (**7**) in 100 mL of 98% formic acid was heated at reflux for 8 h. The solvent was removed in vacuo, and the remaining traces of formic acid were removed by azeotropic evaporation with toluene to give 1.73 g (82%) of a solid which was recrystallized from dimethylformamide as a white powder: mp >300 °C dec; NMR ($(CD_3)_2SO$) δ 7.71, 8.03, 8.39, 8.69 (4 s, 1 each, C_{ar}–H), 13.41 (br, NH); UV λ_{max} (EtOH) 232 nm (ε 28 200), 247 (33 000), 254 (36 300), 263 (27 500), 312 (5700).

Anal. Calcd for $C_9H_6N_4O$: C, 58.06; H, 3.25; N, 30.19; mass spectrum, *m/e* 186.0541 (M^+). Found: C, 58.00; H, 3.32; N, 30.04; mass spectrum, *m/e* 186.0527 (M^+).

Oxidation with Xanthine Oxidase and Oxygen.[16] The procedures used were modifications of the method of xanthine oxidase assay described by Boehringer Mannheim.[11] Final assay mixtures had a total volume of 3.05 mL when *lin*-benzoallopurinol was the substrate, 3.2 mL for the inhibition study, or 2.0 mL for the kinetics study determined with preincubated enzyme, in a cuvette with a 1.0-cm light path. The assay mixtures contained oxygen as the final electron acceptor, which did not have to be bubbled in before the assays,[17] sodium EDTA at 0.10 mM, potassium phosphate buffer (pH 7.8) at 0.1 M, substrate to be oxidized at the specified concentrations, and 50 μL of an enzyme solution prepared by diluting 0.5 mL of Sigma buttermilk xanthine oxidase (20 mg/mL of suspension) with 10.0 mL of an ice-cold solution of 2 M ammonium sulfate. The oxidation of the hypoxanthine was monitored as the formation of uric acid at 292 nm,[18] and the oxidation of *lin*-benzoallopurinol was monitored at 260 nm (ε 26 000). Similar values of K_M and V_{max} were observed for both substrates: $K_M = 1.6 \times 10^{-6}$ M and $V_{max} = 8.9 \times 10^{-1}$ μm min^{-1} for 1.

In a study of the possible inhibitory effect of *lin*-benzoallopurinol (1) on uric acid formation from hypoxanthine, the concentration of inhibitor (allopurinol or 1) was 1.06×10^{-6} M.

In the preincubation study, three 100-μL aliquots of an enzyme solution prepared by diluting Sigma xanthine oxidase (vide supra) by a factor of 10 with ice-cold 2 M ammonium sulfate were incubated with a 1.9-mL aliquot of phosphate buffer (vide supra), 1.7×10^{-5} M *lin*-benzoallopurinol in phosphate buffer, or 1.7×10^{-5} M allopurinol in phosphate buffer, respectively, at room temperature for 2 h. For each of the three assays, 1.0 mL of the incubation mixture was added to 1.0 mL of a solution of 2.6×10^{-6} M hypoxanthine in phosphate buffer, and the formation of uric acid was monitored as a function of time.

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Registry No. 1, 71785-46-1; 2, 71785-47-2; 3, 71785-48-3; 4, 71785-49-4; 5, 71785-50-7; 6, 71785-51-8; 7, 71785-52-9; 8, 71785-53-0; 9, 71785-54-1; 4-bromo-2-methylaniline, 583-75-5.

(16) Bray, R. C. In "The Enzymes", 3rd ed.; Boyer, P., Ed.; Academic Press: New York, 1975; Vol. XII, p 299.

(17) We found that the assay results were the same whether oxygen was bubbled into the assay mix before addition of enzyme or not.

(18) The value of ε for uric acid was arrived at by allowing a solution of known hypoxanthine concentration to be oxidized to completion by xanthine oxidase.