and an antagonist (*dextro*-isomer). The results obtained with (+)-isoproterenol and compounds IX, XI, XII, and XIII strongly suggest that the same explanation applies to all the compounds in the sympathomimetic amine series reported as partial agonists by Ariëns and Simonis (11).

CONCLUSIONS

Phentolamine, trifluoperazine, promazine, phenindamine, benztropine, and other competitive sympathetic α-receptor blockers injected intravenously with phenoxybenzamine in rats blocked the long-acting adrenolytic effect of the latter. A highly significant correlation was found between the sympathetic α-receptor protective and adrenolytic activities of 17 compounds.

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Irreversible Enzyme Inhibitors XCII

Inhibition of Xanthine Oxidase by Some Purines and Pyr midines

By B. R. BAKER and J. L. HENDRICKSON

Twenty-six selected compounds were investigated as inhibitors of xanthine oxidase, the enzyme that can also detoxify 6-mercaptopurine; these compounds consisted of 19 purines, four pyrimidines, two 8-azapurines, and one imidazole. Among the inhibitors that complexed to xanthine oxidase as well or better than the substrate, hypoxanthine, were thioguanine, adenine, and 6,8-dihydroxy-2-methylthiopurine (XXVI). The larger 2-benzylthio-6,8-dihydroxypurine (XXVII) was synthesized and found to inhibit equally as well as XXVI. Then 2-benzylthiohypoxanthine (XXVIII) and 8-benzylthiohypoxanthine (XXIX) were synthesized; these two compounds were complexed elevenfold and threefold better, respectively, to the enzyme than the substrate. Thus the enzyme showed bulk tolerance for the benzylthio group of XXVII–XXIX; the benzylthio group is a logical group for placement of electrophilic groups to give candidate active-site-directed irreversible inhibitors of xanthine oxidase.

XANTHINE OXIDASE is a catabolic enzyme involved in degradation of purines in a cell; the enzyme normally oxidizes hypoxanthine (I) and xanthine (II) to uric acid (III) (1, 2); the latter is then either excreted or further catabolized depending upon the species. The enzyme can also oxidize 2-hydroxypurine (IV) or 8-hydroxypurine to uric acid (III) (3) and adenine (V) to 6-amino-2,8-dihydroxypurine (VI) (4). The enzyme slowly oxidizes 4-hydroxypyrazolo-[3,4-*d*]pyrimidine (IX) to the 4,6-dihydroxy derivative (X), both of which are good inhibitors of xanthine oxidase (5, 6); in fact, IX is now marketed[1] for the treatment of gout since IX slows formation of uric acid. Other known inhibitors of xanthine oxidase are an assortment of N^6-substituted 2-hydroxyadenines (XI) and S^6-substituted 2-hydroxy-6-mercaptopurines (XII) (7).

Of interest to cancer chemotherapy is the oxidation of the anticancer agent, 6-mercaptopurine (VII), to thiouric acid (VIII) (8–10) by xanthine oxidase in mammals, thus detoxifying the drug. It has been proposed (11) that the selective action on cells by 6-mercaptopurine (VII) may be due in part to selective detoxification; if a cell can activate 6-mercaptopurine (VII) to its lethal ribonucleotide (12–14), but due to a lack of sufficient xanthine oxidase cannot detoxify VII by oxidation to the nontoxic thiouric acid, then the cell will be killed. Those normal and cancer cell lines that can oxidize VII to VIII would be less affected by VII; thus some cancer cell lines that can activate 6-mercaptopurine (VII) to the ribonucleotide may not respond to the drug due to the rapid detoxification of VII. (Scheme I.)

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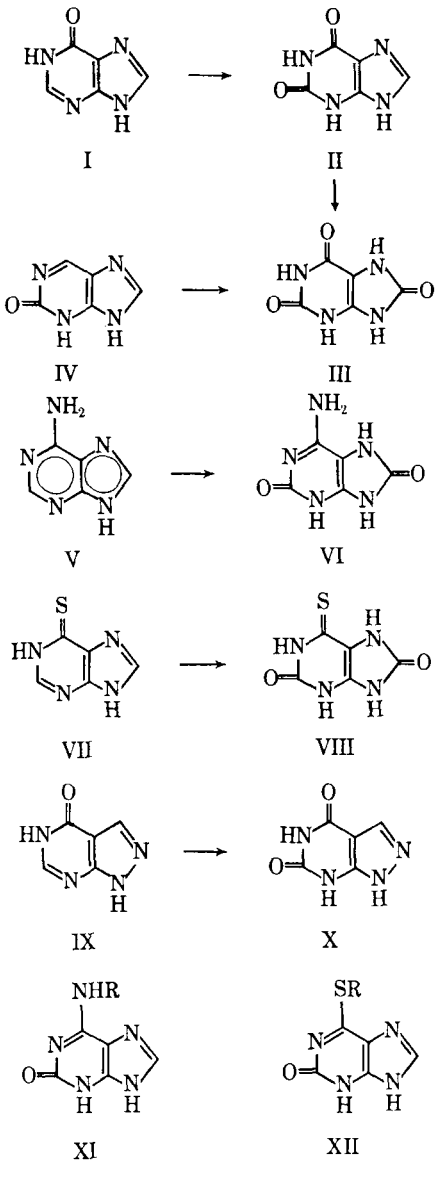
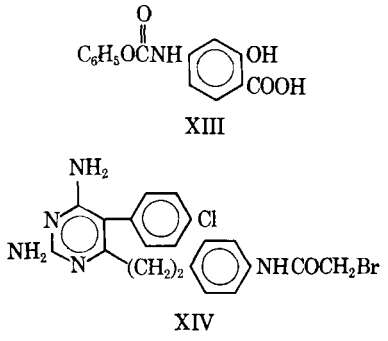
Taken from the senior thesis submitted by J. L. Hendrickson.
The technical assistance of Maureen Baker and Pepper Caseria with some of the assays in Tables I and II is acknowledged. See under *Experimental* for assay method.
Previous paper: Baker, B. R., and Coward, J. K., *J. Heterocyclic Chem.*, **4**, 202(1967).
[1] Trademarked as Allopurinol by Burroughs Wellcome Co.



Scheme I

An inhibitor that could block xanthine oxidase in a cancer cell line with minimal blockade of the xanthine oxidase in normal cells could be a useful adjunct to treatment with 6-mercaptopurine. Reversible inhibitors rarely show sufficient tissue specificity to be of direct chemotherapeutic use without relying on unpredictable secondary considerations for selectivity such as an impaired active-transport system (15) or a lowered amount of detoxification enzyme (11, 16). In contrast, active-site-directed irreversible enzyme inhibitors can show tissue specificity since this type of inhibitor has an extra dimension of specificity not present in reversible inhibitors (14, 17, 18). For example, XIII can inactivate the lactic dehydrogenase from skeletal muscle, but not the lactic dehydrogenase from heart, and *vice versa* with the 5-isomer of XII (17, 19, 20). Another example is XIV, which will inactivate dihydrofolic reductase from *E. coli* B, but not pigeon liver (21).



In order to design an active-site-directed irreversible inhibitor of an enzyme, the following *modus operandi* has been developed (17, 19).

Phase I—The binding points on a reversible inhibitor should be determined, at least to the extent that substrate properties are destroyed, but binding is not; some binding points can be eliminated if compensated by a structural change that will give stronger binding at some other point in the molecule.

Phase II—The determination of areas on the inhibitor where bulky groups can be placed without interfering with the ability of the inhibitor to complex with the enzyme, known as either bulk tolerance within the enzyme–inhibitor complex or as a noncontact area between enzyme and inhibitor within the complex.

Phase III—A covalent bond-forming group should then be placed on a noncontact area of the inhibitor; if the dimension between the reversible complexing region on the enzyme and an enzymic nucleophilic group are correct, then a facile neighboring group reaction will occur between the inhibitor and enzyme that leads to selective inactivation of the enzyme by formation of a covalent bond.

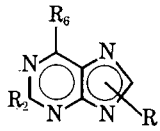
Phase IV—The irreversible inhibitor from phase III is then modified to take advantage of the bridge principle of specificity (18–20) until the desired tissue specificity for the given enzyme is achieved.

Initial studies on phases I and II are the subjects of this paper.

DISCUSSION

Since xanthine oxidase can oxidize purines at the 2-, 6-, or 8-position, either several catalytic sites are present on the enzyme with one mode of purine binding or the purines can bind in one of several different

TABLE I—INHIBITION OF XANTHINE OXIDASE BY



Compd.	R_2	R_6	R	μM Concn. for 50% Inhibition	$[I/S]_{0.5}$[a]	Source
XV	SH	OH	8-OH	110	14	Aldrich
XVI	SH	OH	8-SH	12	1.5	NBC
XVII	SH	SH	8-SH	20	2.5	Aldrich
XVIII	NH_2	OH	8-OH	31	3.8	Aldrich
XIX	NH_2	NH_2	H	34[c]	4.2	NBC
XX	NH_2	OH	H	40[d]	4.9	NBC
XXI	NH_2	SH	H	7.7	0.95	NBC
XXII	H	Cl	H	100	12	BW
XXIII	H	n-C_4H_9S-	H	260[e]	32	Aldrich
XXIV	NH_2	OH	9-Me	58	7.1	Robins
XXV	NH_2	OH	7-Me	~1000[f]	120	Robins
XXVI	CH_3S	OH	8-OH	11	1.4	Aldrich
XXVII	$C_6H_5CH_2S$-	OH	8-OH	10	1.2	Exptl.
XXVIII	$C_6H_5CH_2S$-	OH	H	0.75[g]	0.093	Exptl.
XXIX	H	OH	8-$SCH_2C_6H_5$	2.8[g]	0.34	Exptl.
XXX	H	NH_2	H	5.6[h]	0.69	NBC
XXXI	NH_2	SCH_3	H	330	41	Sigma
XXXII	NH_2	NHC_6H_5	H	25	3.1	Robins
XXXIII	.....8-Azaguanine.....			7.5	0.93	NBC
XXXIV	.....8-Azaxanthine.....			84	10	NBC

[a] Ratio of concentration of inhibitor to 8.1 μM hypoxanthine giving 50% inhibition; $K_m = 8.5 \times 10^{-6}$ M has been reported (22). [b] Sources: Aldrich, Aldrich Chemical Co.; NBC, Nutritional Biochemical Corp.; BW, Burroughs Wellcome Co.; Robins, a gift from Professor Roland K. Robins; Exptl., see under *Experimental*; Sigma, Sigma Chemical Co. [c] Previously reported (22) as a substrate with 5% of the rate of hypoxanthine and $K_m = 7.8 \times 10^{-6}$ M. [d] Previously reported (23) as an inhibitor, but the relative amount of binding to the enzyme was not recorded. [e] Estimated from 20% inhibition at a concentration of 60 μM, the limit of solubility. [f] Estimated from 11% inhibition at a concentration of 200 μM. [g] No substrate properties at 100 μM. [h] Previously reported (22) as a substrate with 1% of the rate of hypoxanthine ($K_m = 12 \times 10^{-6}$ M) and as an inhibitor of hypoxanthine with $K_i = 11 \times 10^{-6}$ M.

rotomeric conformations and the enzyme has only one catalytic site; the latter seems a more plausible concept and has been previously invoked to explain the oxidation of aminopurines by xanthine oxidase (3). The concept of several rotomeric binding configurations has also been invoked for (*a*) binding to chymotrypsin (24, 25), (*b*) the binding of adenosine and adenylic acid to certain enzymes (26), and (*c*) the binding of pyrimidines to dihydrofolic reductase (27-31). When different rotomers of a molecule can bind to an enzyme which depends upon the substituents on the inhibitor, the exact groups of the inhibitor complexing to the enzyme are difficult to ascertain with reasonable probability (30, 31). Therefore, with xanthine oxidase it is possible to make some generalizations about binding, but what specific groups of the inhibitor are complexed to the enzyme is fraught with uncertainty at this time. However, these generalizations are sufficient to lead to phase II for construction of candidate active-site-directed irreversible inhibitors of xanthine oxidase. The following generalizations from the data in Table I seem reasonable.

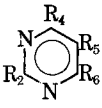
A—If no hydroxyl, thiol, or amino group is present on the purine, binding is poor; note that 6-chloropurine (XXII) and 6-butylmercaptopurine (XXIII) are complexed poorly to the enzyme, but hypoxanthine (the substrate), and adenine (XXX) are complexed well; 6-mercaptopurine (VII) is a known substrate (8-10).

B—A thiol group gives better binding than a hydroxyl group in some cases; note that thioguanine (XXI) binds fivefold better than guanine (XX), and XVI is a tenfold better inhibitor than XV. However, 2,6,8-trimercaptopurine (XVII) is not a better inhibitor than 2,8-dimercaptohypoxanthine (XVI). *S*-Methylation of thioguanine (XXI) to XXXI is extremely detrimental to binding, indicating that NHC=S moiety of thioguanine (XXI) is involved in binding to the enzyme.

C—More than one hydroxyl group can be detrimental to binding. Note that 2-benzylthiohypoxanthine (XXVIII) is a thirteenfold better inhibitor than its 8-hydroxy derivative (XXVII). If two hydroxyl groups are already present, an additional thiol can be detrimental to binding; note that 2-methylthio-6,8-dihydroxypurine (XXVI) is a tenfold better inhibitor than 2-thio-6,8-dihydroxypurine (XV). An exception occurs with aminopurines; 8-hydroxyguanine (XVIII) is a slightly better inhibitor than guanine (XX) and adenine (XXX) is a considerably better inhibitor than guanine (XX).

D—Guanine (XX) is a reasonably good inhibitor of xanthine oxidase, being complexed one-fifth as well as the substrate, hypoxanthine. Introduction of the 8-aza group, as in 8-azaguanine (XXXIII) gives a fivefold increment in binding. Replacement of the 6-hydroxyl group of guanine (XX) by amino (XIX) or introduction of a 9-methyl (XXIV) gives little change in binding; contrariwise, introduction of the 7-methyl group (XXV) is severely detrimental to binding.

TABLE II—INHIBITION OF XANTHINE OXIDASE BY



Compd.	R_2	R_4	R_5	R_6	μM Concn. for 50% Inhibition	$[I/S]_{0.5}$[a]	Source[b]
XXXV	NH_2	OH	H	CH_3	130	16	Aldrich
XXXVI	OH	OH	H	CH_3	>5000	>620	NBC
XXXVII	NH_2	OH	H	NH_2	460	57	Aldrich
XXXVIII	OH	OH	NH_2	H	>2000	>250	EKC
XXXIX	..5-Aminoimidazole-4-carboxamide..				950	120	Aldrich

[a] Ratio of concentration of inhibitor to 8.1 μM hypoxanthine giving 50% inhibition; see under *Experimental*. [b] Aldrich Aldrich Chemical Co.; NBC, Nutritional Biochemicals Corp.; EKC, Eastman Kodak Co.

Since 2-methylthio-6,8-dihydroxypurine (XXVI) was complexed to the enzyme nearly as well as the substrate, hypoxanthine, bulk tolerance at the 2-position was investigated. 2-Benzylthio-6,8-dihydroxypurine (XXVII) was complexed as effectively as the 2-methylthiopurine (XXVI) thus showing tolerance for the bulk of the *S*-benzyl group within the enzyme–inhibitor complex. Removal of the 8-hydroxyl group of (XXVII) to give 2-benzylthiohypoxanthine (XXVIII) gave the most effective inhibitor in Table I, XXVIII being complexed elevenfold better than the substrate hypoxanthine. 8-Benzylthiohypoxanthine (XXIX) was not so good an inhibitor as its 2-isomer (XXVIII), but XXIX was still complexed threefold better than hypoxanthine; thus, there is also bulk tolerance for an 8-benzylthio group within the enzyme–inhibitor complex. Introduction of an N^6-phenyl group (XXXII) on 2,6-diaminopurine (XIX) did not decrease binding, indicating bulk tolerance for this phenyl group.

Compounds XXVII, XXVIII, and XXIX are good candidates for phase III studies on the construction of active-site-directed irreversible inhibitors of xanthine oxidase; attachment of covalent forming groups to these three compounds for phase III studies is underway.

Table II lists the inhibition results with four pyrimidines and one imidazole; none were sufficiently good inhibitors to warrant further pursuit. However, an interesting contrast can be noted on the relative binding of 5-aminoimidazole-4-carboxamide (XXXIX) and guanine (XX) to xanthine oxidase and guanase (32). Guanine (XX) complexes twice as well as XXXIX to guanase, but 24-fold better than XXXIX to xanthine oxidase; thus, the mode of binding of guanine to the two enzymes is clearly different.

EXPERIMENTAL

Methods

Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected. Ultraviolet spectra were determined in water with a Perkin-Elmer 202 spectrophotometer. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B spectrophotometer. Thin-layer chromatograms were run on Brinkmann Silica Gel GF in 3:1 chloroform–ethanol and spots were detected by visual examination under ultraviolet light. The analytical samples moved as a single spot and had infrared spectra in agreement with their assigned structures.

2 - Benzylmercapto - 6, 8 - dihydroxypurine (XXVII)—To a solution of 553 mg. (3 mmoles) of 6,8-dihydroxy-2-mercaptopurine (XV) in 3 ml. of 1 *N* aqueous sodium hydroxide was added 30 ml. of water and 0.35 ml. (3 mmoles) of benzyl chloride. After being stirred at ambient temperature for 5 hr., the mixture was acidified with acetic acid. The product was collected on a filter, then washed successively with hot water, acetone, and hot ethanol. The crude product was dissolved in dilute aqueous sodium hydroxide, then the solution was clarified with decolorizing carbon and acidified with acetic acid to give 406 mg. (49%) of product. Recrystallization from *N,N*-dimethylformamide gave 224 mg. (27%) of a white solid, m.p. over 300°. $\lambda_{max.}$ (pH 1): 278 mμ; (pH 13): 284 mμ.

Anal.—Calcd. for $C_{12}H_{10}N_4O_2S$: C, 52.5; H, 3.68; N, 20.4. Found: C, 52.7; H, 3.80; N, 20.3.

2-Benzylmercapto-6-hydroxypurine (XXVIII)—Reaction of 505 mg. (3 mmoles) of 6-hydroxy-2-mercaptopurine with benzyl chloride, as described for the preparation of XXVII, gave after recrystallization from aqueous 2-methoxyethanol 195 mg. (26%) of white powder, m.p. 263–265°. $\lambda_{max.}$ (pH 1): 272 mμ; (pH 13): 280 mμ.

Anal.—Calcd. for $C_{12}H_{10}N_4OS$: C, 55.8; H, 3.91; N, 21.7. Found: C, 55.9; H, 3.99; N, 21.9.

8-Benzylmercapto-6-hydroxypurine (XXIX)—This was prepared from 6-hydroxy-8-mercaptopurine hydrate in the same fashion as XXVIII; yield, 295 mg. (40%), m.p. 273–274° $\lambda_{max.}$ (pH 1): 280 mμ; (pH 13): 284 mμ.

Anal.—Calcd. for $C_{12}H_{10}N_4OS$: C, 55.8; H, 3.91; N, 21.7. Found: C, 55.9; H, 3.92; N, 21.8.

Inhibition of Xanthine Oxidase (2)—Xanthine oxidase from milk was obtained as a 5–10 unit suspension per ml. of 60% saturated ammonium sulfat from Nutritional Biochemicals Corp.; 1 unit supposedly converts 1 μmole of xanthine to uric acid per minute. For 1 day's assay, 0.05 ml. of enzyme was diluted with 2.95 ml. of buffer. Tris buffer (0.05 *M*), pH 7.4, was employed. Hypoxanthine was stocked as a 50 m*M* solution in 1 *N* aqueous sodium hydroxide; for 1 day's assays the stock solution was diluted 1:100 with buffer to give an 0.5 m*M* solution. Inhibitors XXIII–XXV, XXVII–XXIX, and XXXII were dissolved in dimethylsulfoxide and di-

luted with the same; the remainder of the inhibitors were dissolved in 10 mM aqueous sodium hydroxide and diluted with water.

In a 3-ml. cell were placed 2.70 ml. of buffer, 0.30 ml. of dimethylsulfoxide, and 50 μl. of 0.5 mM hypoxanthine. The solution was vigorously shaken for about 20 sec. to absorb air. Then 50 μl. of diluted enzyme solution was added and the rate of increase in absorbance at 290 mμ was noted with a Gilford recording spectrophotometer; sufficient enzyme was employed to give about 0.01 absorbance units change per minute. If the inhibitor was dissolved in water, up to 0.3 ml. could be used in the cell by decreasing the buffer by the corresponding amount. If the inhibitor was dissolved in dimethylsulfoxide, up to 0.30 ml. could be used, keeping the total volume of dimethylsulfoxide at 0.30 ml. Sufficient inhibitor was used to give 30–70% inhibition; the μM concentration of inhibitor necessary for 50% inhibition ($V_0/V_I = 2$) was determined by plotting V_0/V_I against I, where V_0 = velocity without inhibitor, V_I = velocity with inhibitor, and I = inhibitor concentration (33).

The cell concentration of hypoxanthine was 8.1 μM; the velocity was the same in the presence or absence of 10% dimethylsulfoxide.

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Irreversible Enzyme Inhibitors XCIII

Hydrophobic Bonding to Xanthine Oxidase by Some Phenylpurines

By B. R. BAKER

Nine derivatives of guanine with alkyl, aryl, or aralkyl groups at the 8- or 9-position were investigated as inhibitors of xanthine oxidase. Maximum hydrocarbon interaction with the enzyme was observed with the in-plane 9-phenyl substituent, a 100-fold increment in binding being observed. The "2"-position of purines was also investigated with phenyl substituents on the 6-position of 4-mercaptopyrazolo[3,4-*d*]pyrimidine; a thirtyfold increment in binding was observed with either a *p*-nitrophenyl or a *p*-methoxyphenyl substituent at the 6-position of the pyrazolopyrimidine.

THE PREVIOUS PAPER of this series discussed the possible chemotherapeutic use for a blockade of xanthine oxidase (1): since 6-mercaptopurine can be detoxified by oxidation to the nontoxic 6-thiouric, an active-site-directed irreversible inhibitor that is sufficiently tissue specific on a given tumor line would be a useful adjunct to 6-mercaptopurine therapy. The normal substrates of xanthine oxidase, hypoxanthine and xanthine, are highly polar molecules; therefore, if a hydrophobic region could be detected by purines substituted by hydrocarbon groups, this hydrophobic region would most probably be just adjacent to the active site, but not part of

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