Irreversible Enzyme Inhibitors. XCV. 8-(*m*-Bromoacetamidobenzylthio)hypoxanthine, an Active-Site-Directed Irreversible Inhibitor of Xanthine Oxidase[1-3]

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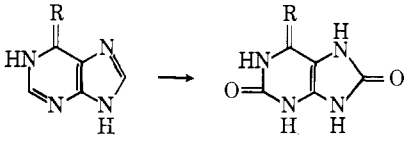
2-Benzylthiohypoxanthine and 8-benzylthiohypoxanthine with a bromoacetamido group on the *ortho* or *meta* position were synthesized as candidate active-site-directed irreversible inhibitors of xanthine oxidase; these compounds were synthesized by alkylation of the appropriate mercaptohypoxanthine (**9** or **10**) with a nitrobenzyl chloride, followed by catalytic hydrogenation and then bromoacetylation. Of the four bromoacetamidobenzylthiohypoxanthines (**7** and **8**), only 8-(*m*-bromoacetamidobenzylthio)hypoxanthine (**8b**) showed inactivation of the enzyme; at a concentration of 1.5×10^{-6} *M*, **8b** inactivated xanthine oxidase at 37° with a half-life of 50 min. That **8b** inactivated the enzyme by the active-site-directed mechanism—and not by the random bimolecular mechanism—was indicated by the failure of 1.5×10^{-6} *M* iodoacetamide to show inactivation of xanthine oxidase.

Xanthine oxidase is a catabolic enzyme that converts hypoxanthine (**1**) and xanthine to uric acid (**3**);[4] this enzyme can also detoxify 6-mercaptopurine (**2**), the antileukemic agent, by oxidation to thiouric acid (**4**).[5] The selective action of 6-mercaptopurine



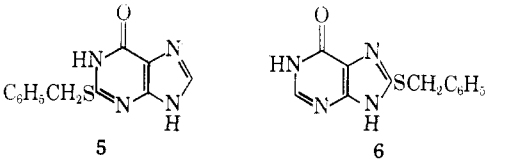
1, R=O
2, R=S

3, R=O
4, R=S

(**2**) on susceptible tumor cells has been correlated with the absence or low amount of xanthine oxidase in these tumor cell lines.[6] Conversely, tumor cell lines with high levels of xanthine oxidase would not be expected to be inhibited by 6-mercaptopurine; therefore, we proposed[7] that a selective blockade of xanthine oxidase in a tumor cell line unresponsive to 6-mercaptopurine would be a useful adjunct to 6-mercaptopurine therapy, provided the blockade had minimal effect on xanthine oxidase in normal tissues and provided the tumor cell line had sufficient inosinate pyrophosphorylase to convert 6-mercaptopurine to its lethal ribonucleotide.[8,9]

Such highly selective inhibitors of the substrate-identical enzyme from different tissues can be achieved with active-site-directed irreversible enzyme inhibitors.[10] For example, 4-(carbophenoxyamino)salicylic acid can inactivate lactic dehydrogenase from skeletal muscle with no inactivation of lactic hydrogenase from heart; conversely, 5-(carbophenoxyamino)salicylic acid can inactivate lactic dehydrogenase from heart, but not from skeletal muscle.[11] Another example is 6-(*p*-bromoacetamidophenylethyl)-5-(*p*-chlorophenyl)-2,4-diaminopyrimidine which can inactivate dihydrofolic reductase from *Escherichia coli* B, but not dihydrofolic reductase from pigeon liver.[12]

Since the design of an active-site-directed irreversible inhibitor for a given enzyme is fairly complex and unlikely to be successful by a single compound guess, a definite *modus operandi* has evolved for this design.[10] The *modus operandi* proceeds in four phases. The first phase—what groups on an inhibitor are necessary for reversible binding to the enzyme—has been achieved with xanthine oxidase.[7] The second phase—where can large groups be placed on the inhibitor without interfering with complex formation with the enzyme (bulk tolerance within the enzyme–inhibitor complex)—has also been achieved with xanthine oxidase;[7] we observed



5 **6**

that 2-benzylthio-(**5**) and 8-benzylthiohypoxanthine (**6**) were complexed tenfold and fourfold better, respectively, to the enzyme than the substrate, hypoxanthine (**1**). The third phase—placement of a leaving group on the bulk-tolerance area of the inhibitor that can form a covalent bond with the enzyme by a facile neighboring-group reaction within the enzyme–inhibitor complex—with xanthine oxidase is the subject of this paper.

Enzymatic Evaluation.—The syntheses of six candidate irreversible inhibitors (**7** and **8**) for xanthine oxidase were undertaken; all were successful except

(1) This work was generously supported by Grant CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper in this series see B. R. Baker and D. V. Santi, *J. Heterocyclic Chem.*, in press.

(3) For the previous paper on xanthine oxidase see B. R. Baker, *J. Pharm. Sci.*, in press; paper XCIII of this series.

(4) H. M. Kalckar, *J. Biol. Chem.*, **167**, 429 (1947); B. L. Horecker and L. A. Heppel, *Methods Enzymol.*, **2**, 482 (1955).

(5) G. B. Elion, S. Bieber, and G. H. Hitchings, *Ann. N. Y. Acad. Sci.*, **60**, 297 (1954); L. Hamilton and G. B. Elion, *ibid.*, **60**, 304 (1954); T. L. Loo, M. E. Michael, A. J. Garceau, and J. C. Reid, *J. Am. Chem. Soc.*, **81**, 3039 (1959).

(6) B. R. Baker, *Cancer Chemotherapy Rept.*, **4**, 1 (1959).

(7) B. R. Baker and J. L. Hendrickson, *J. Pharm. Sci.*, in press; paper XCII of this series.

(8) R. W. Brockman, *Clin. Pharmacol. Therap.*, **2**, 237 (1961); R. W. Brockman and S. Chumley, *Biochim. Biophys. Acta*, **95**, 365 (1965).

(9) For a discussion of the mechanism of action and selectivity of 6-mercaptopurine see B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley and Sons, Inc., New York, N. Y., 1967, Chapter 1.

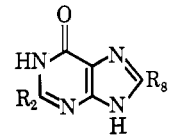
(10) (a) See ref 9, Chapters I–XII; (b) B. R. Baker, *J. Pharm. Sci.*, **53**, 347 (1964).

(11) (a) See ref 9, Chapter IX; (b) B. R. Baker and R. P. Patel, *J. Pharm. Sci.*, **53**, 714 (1964).

(12) B. R. Baker and J. H. Jordaan, *ibid.*, in press; paper LXXXVIII of this series.

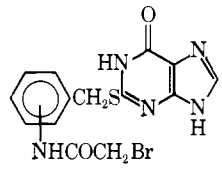
TABLE I

INHIBITION OF XANTHINE OXIDASE BY



Compd	R_2	R_8	Reversible: μM concn for 50% inhib[a,b]	Reversible: $([I]/[S])_{0.5}$[c]	Irreversible: μM concn	Irreversible: Time, min	Irreversible: % inactivatn[d]
5	$C_6H_5CH_2S$	H	0.75[e]	0.093			
6	H	$C_6H_5CH_2S$	2.8[e]	0.34			
7a	*o*-$BrCH_2CONHC_6H_4CH_2S$	H	0.024	0.0030	0.025	120	0
7b	*m*-$BrCH_2CONHC_6H_4CH_2S$	H	0.68	0.084	1.0	120	0
8a	H	*o*-$BrCH_2CONHC_6H_4CH_2S$	0.11	0.014	0.10	120	0
8b	H	*m*-$BrCH_2CONHC_6H_4CH_2S$	0.77[f]	0.095	1.5	120	90
					1.5	50[g]	50
Iodoacetamide[h]			Large		1.5	40	0
8b[h]					1.5	40	45

[a] The technical assistance of Pepper Caseria and Maureen Baker with these assays is acknowledged. [b] Xanthine oxidase from bovine milk (Nutritional Biochemical Corp.) was assayed with 8.1 μM hypoxanthine in 0.05 M Tris buffer (pH 7.4) containing 10% DMSO as previously described.[7] [c] Ratio of concentration of inhibitor to 8.1 μM hypoxanthine giving 50% inhibition. [d] See Experimental Section for assay method; per cent inactivation corrected for small amount of thermal inactivation. [e] Data from ref 7. [f] With 40.5 μM hypoxanthine as substrate, 1.24 μM **8b** was necessary for 50% inhibition. Therefore **8b** has $K_i = 3 \times 10^{-7}$ M from $K_i = K_m \times [I]/[S]$ where K_m 8.5×10^{-6} M [J. B. Wyngaarden, *J. Biol. Chem.*, **224**, 453 (1957)]; this equation is valid when $[S] > 4K_m$ [B. R. Baker, D. V. Santi, P. I. Almaula, and W. C. Werkheiser, *J. Med. Chem.*, **7**, 24 (1964)]. [g] Half-time of inactivation; see Experimental Section for assay method. [h] Run simultaneously.



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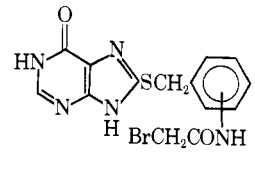

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a, *ortho*
b, *meta*
c, *para*

the *para* isomers of series c. All four compounds were excellent reversible inhibitors of xanthine oxidase, being complexed 10–340-fold more effectively than the substrate, hypoxanthine (**1**) (Table I); in fact, the *ortho* isomer (**7a**) is the best reversible inhibitor of xanthine oxidase yet observed. Introduction of the *m*-bromoacetamido group (**7b**) on 2-benzylthiohypoxanthine (**5**) gave no change in binding. Strikingly, the *o*-bromoacetamido group (**7a**) gave a 130-fold increment in binding compound to **5**. The latter result could be interpreted to indicate that the *o*-bromoacetamido group of **7a** is directly complexed to enzyme; however, this *ortho* substituent might also have an effect on the ground state of the benzylthio group with respect to the purine that allows the benzyl group to complex to xanthine oxidase by hydrophobic bonding.[3] Similarly, the *m*-bromoacetamido group (**8b**) gave a small fourfold increase in binding over 8-benzylthiopurine (**6**), but the *o*-bromoacetamido group (**8a**) gave a larger 25-fold increment.

When the four candidate irreversible inhibitors were incubated with xanthine oxidase at 37° for 2 hr, only isomer **8b** showed inactivation of the enzyme. A time study showed that **8b** at 1.5×10^{-6} M, which is sufficient to convert 95% of the enzyme to a reversible complex,[13] inactivated the enzyme with a half-life of 50 min. That **8b** did not inactivate the enzyme by a random bimolecular mechanism[13] was shown by the lack of inactivation of xanthine oxidase by 1.5×10^{-6} M iodoacetamide under identical conditions. Since the other three isomers, particularly **7b**, did not inactivate the enzyme was also indicative that **8b** did not inactivate the enzyme by the bimolecular process. Therefore, it is highly probable that **8b** inactivates the enzyme by a facile neighboring-group reaction within the enzyme–inhibitor reversible complex, the so-called active-site-directed irreversible inhibition.[10]

With this successful completion of phase III of the *modus operandi* for design of an active-site-directed irreversible inhibitor of xanthine oxidase, phase IV studies have been started on the search for a selective irreversible inhibitor effective on a tumor xanthine oxidase with minimal effect on the xanthine oxidase from other tissues.

Chemistry.—The appropriate mercapto-substituted hypoxanthine (**9** or **10**) was alkylated with the appropriate nitrobenzyl chloride in 0.1 N aqueous NaOH to give **11** or **12** in yields of 60–64% of analytically pure material (Chart I). Catalytic reduction of **11b** and **12b** in methanol suspension with a Pd–C catalyst afforded the corresponding *m*-aminobenzylthiohypoxanthines (**13b** and **14b**) in reasonable yields. With the *para* series, reductive cleavage to *p*-toluidine and the mercaptohypoxanthine occurred;[14] although reductive cleavage in the *ortho* series probably occurred also, **13a** and **14a** could be isolated in low yield. Chemical reducing agents such as $NaBH_4$ in the presence of Pd–C,[15] zinc in aqueous NaOH,[16] $FeSO_4$ in aqueous ammonia,[17] or zinc and formic acid[18] also failed to give **13c** and **14c**.

(13) (a) See ref 9, Chapter VIII; (b) B. R. Baker, W. W. Lee, and E. Tong, *J. Theoret. Biol.*, **3**, 459 (1962).

(14) C. Berse, R. Boucher, and L. Piché, *J. Org. Chem.*, **22**, 805 (1957).

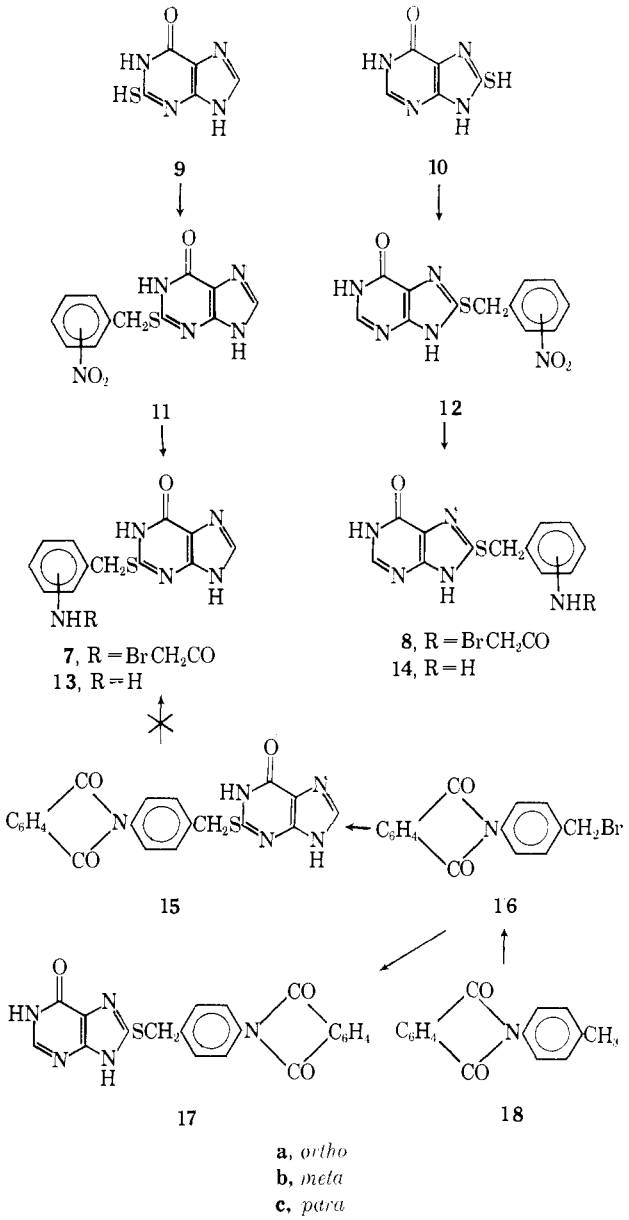
(15) T. Neilson, H. C. S. Wood, and A. G. Wylie, *J. Chem. Soc.*, 371 (1962).

(16) O. Hinsberg and F. König, *Ber.*, **28**, 2947 (1895).

(17) E. Bamberger, *ibid.*, **60**, 314 (1927).

(18) B. R. Baker, J. P. Joseph, and R. E. Schaub, *J. Org. Chem.*, **19**, 631 (1954).

CHART I



An alternate route to **13c** and **14c** not involving a catalytic hydrogenation was then investigated. N-(*p*-Tolyl)phthalimide (**18**) was halogenated on the methyl group with N-bromosuccinimide in CCl_4 catalyzed by benzoyl peroxide and ultraviolet light; *p*-phthalimidobenzyl bromide (**16**) was isolated in 76% yield. Alkylation of the mercaptohypoxanthines (**9** and **10**) with **16** in 70% aqueous DMF containing NaOH gave **15** and **17** in 72 and 35% yields, respectively, in analytical purity. Attempts to convert **15** to **13c** with hydrazine[19] or hydrazine acetate[20] were unsuccessful; a similar failure was encountered with **17**.

Bromoacetylation of the *ortho* and *meta* isomers of **13** and **14** in DMF at 0° with bromoacetic anhydride[21] gave the pure bromoacetamides (**7a** and **b**, **8a** and **b**) for study as active-site-directed irreversible inhibitors of xanthine oxidase.

(19) H. R. Ing and R. H. Manske, *J. Chem. Soc.*, 2348 (1926); J. C. Sheehan, D. W. Chappmann, and R. W. Roth, *J. Am. Chem. Soc.*, **74**, 3822 (1952).

(20) R. Schwyzer, A. Costopanagiotis, and P. Sieber, *Helv. Chim. Acta*, **46**, 870 (1963).

(21) B. R. Baker, D. V. Sanci, J. K. Coward, H. S. Shapiro, and J. H. Jordaan, *J. Heterocyclic Chem.*, **3**, 425 (1966).

Experimental Section[22]

2-(*o*-Nitrobenzylthio)hypoxanthine (11a).—To a stirred solution of 2.66 g (15.8 mmoles) of **9** in 16 ml of 1 *N* aqueous NaOH and 160 ml of water was added a solution of 2.7 g (15.8 mmoles) of *o*-nitrobenzyl chloride in 5 ml of dioxane. After being stirred for 3 hr, the mixture was acidified (AcOH). The product was collected on a filter and washed with hot water, then methanol; yield 3.8 g (79%), mp 269–275° dec. Recrystallization from DMF by addition of water gave 3.05 g (64%) of a yellow powder, mp 282–283° dec, that moved as a single spot on tlc with 2:1 methanol–benzene. The compound had ν_{max} 1580, 1550 (C=O, C=N, C=C), 1510, 1340 cm^{-1} (NO_2). See Table II for analytical data. Other compounds prepared by this method are listed in Table II under method A. Method B was similar except 3:7 water–DMF was used as reaction solvent.

2-(*m*-Aminobenzylthio)hypoxanthine (13b).—A suspension of 1.21 g (4 mmoles) of **11b** and 0.5 g of 10% Pd–C in 200 ml of methanol was shaken with hydrogen at 2–3 atm for about 8 hr when hydrogenation was complete. The filtered solution was spin evaporated *in vacuo*. Crystallization from aqueous methanol gave 0.60 g (55%) of product suitable for the next step. Recrystallization from methanol afforded the analytical sample: mp 209–211°; ν_{max} 3400, 3000 (NH_2) 1680, 1560, 1530 (NH, C=O, C=N, C=C), no NO_2 bands near 1510 and 1340 cm^{-1}. The compound gave a positive Bratton–Marshall test[23] for the aromatic amine group and moved as a single spot on tlc in 2:1 methanol–benzene. For analytical data and preparation of other compounds by this method C, see Table II.

2-(*m*-Bromoacetamidobenzylthio)hypoxanthine (7b).—To a stirred solution of 400 mg (1.47 mmoles) of **13b** in 2.5 ml of DMF cooled in an ice bath was added a solution of 380 mg of bromoacetic anhydride in 0.5 ml of DMF. After being stirred at 0° for 20 min, the mixture was diluted with 1 ml of methanol; if the product did not separate, the solution was further diluted with water to turbidity. After several hours at 0°, the mixture was filtered; yield 305 mg (53%), mp about 300° (indefinite). Recrystallization from aqueous DMF gave the analytical sample, mp about 300°. The compound gave a negative Bratton–Marshall test[23] for aromatic amines, a positive 4-(*p*-nitrobenzyl)-pyridine test[24] for active halogen, and moved as a single spot on tlc in 2:1 methanol–benzene; ν_{max} 3400–2800 (broad NH), 1680, 1650, 1610, 1580, 1550 cm^{-1} (NH, C=O, C=C, C=N). For analytical data and for other compounds prepared by this method D, see Table II.

***p*-Phthalimidobenzyl Bromide (16).**—A mixture of 8.1 g (34 mmoles) of **18** (prepared by fusion of phthalic anhydride and *p*-tolnidine,[24] mp 204–206°), 30 ml of CCl_4, 15 mg of benzoyl peroxide, and 6.05 g of N-bromosuccinimide (34 mmoles) was refluxed with stirring and with irradiation with an ultraviolet lamp for 2 hr. The hot reaction mixture was filtered and the filtrate was spin evaporated *in vacuo*. The residue and CCl_4-insoluble materials were combined and dissolved in $CHCl_3$; the insoluble succinimide was removed by filtration; then the solution was cooled to give 8.1 g (76%) of white crystals: mp 210–212°; ν_{max} 1770, 1710 cm^{-1} (phthalyl C=O).

Anal. Calcd for $C_{15}H_{10}BrNO_2$: C, 57.0; H, 3.18; N, 4.43. Found: C, 56.8; H, 3.28; N, 4.45.

Inactivation of Xanthine Oxidase.—The enzyme was purchased from Nutritional Biochemicals Corp. Reversible inhibition was measured as previously described.[7] In order to standardize the assay for inactivation, it was first determined that the velocity of the enzyme reaction using 8.1 μM hypoxanthine was propor-

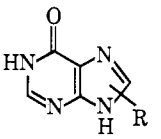
(22) Melting points were taken in capillary tubes on a Mel-Temp block and those below 250° are corrected. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B or 337 spectrophotometer. Ultraviolet spectra were determined in 10% ethanol with a Perkin-Elmer 202 spectrophotometer. Thin layer chromatograms (tlc) were run on Brinkmann silica gel GF and spots were detected by visual examination under ultraviolet light.

(23) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537 (1939).

(24) F. D. Chattaway, C. L. Cumming, and B. H. Wilsdon, *J. Chem. Soc.*, **99**, 1950 (1911).

TABLE II

PHYSICAL PROPERTIES OF



Compd[a]	R	Purine position	Method	% yield	Mp, °C dec	Calcd, % C	Calcd, % H	Calcd, % N	Found, % C	Found, % H	Found, % N	λ_{max}, mμ[b] pH 1	λ_{max}, mμ[b] pH 13
11a	*o*-$NO_2C_6H_4CH_2S$	2	A	64[c]	282–283[c]	47.6	2.92	23.1	47.7	3.10	23.3	262	270
11b	*m*-$NO_2C_6H_4CH_2S$	2	A	61[c]	254–255[c]	47.6	2.92	23.1	47.2	3.08	22.9	267	274
11c	*p*-$NO_2C_6H_4CH_2S$	2	A	61[d]	274–275[d]	47.6	2.92	23.1	47.3	3.17	22.9	273	278
12a	*o*-$NO_2C_6H_4CH_2S$	8	A	63[c]	302–303[c]	47.6	2.92	23.1	47.8	3.25	22.9	267	281
12b	*m*-$NO_2C_6H_4CH_2S$	8	A	61[c]	315–316[c]	47.6	2.92	23.1	47.7	3.15	22.9	276	282
12c	*p*-$NO_2C_6H_4CH_2S$	8	A	81	254–255[e]	47.6	2.92	23.1	47.4	3.00	23.0	278	284
13a	*o*-$NH_2C_6H_4CH_2S$	2	C	28[f]	Indef[f]	52.8	4.06	25.6	52.6	4.20	25.8	266	269
13b	*m*-$NH_2C_6H_4CH_2S$	2	C	55	209–211[e]	52.8	4.06	25.6	52.9	4.14	25.8	268	278
14a	*o*-$NH_2C_6H_4CH_2S$	8	C	41[f]	Indef[f,g]							276	283
14b	*m*-$NH_2C_6H_4CH_2S$	8	C	79	223–225[e]	52.8	4.06	25.6	52.5	4.15	25.4	278	285
7a	*o*-$BrCH_2CONHC_6H_4CH_2S$	2	D	32	277–280[c]	42.7	3.01	17.7	42.7	2.98	18.0	270	274
7b	*m*-$BrCH_2CONHC_6H_4CH_2S$	2	D	53	~300[c]	42.7	3.01	17.7	43.0	3.20	17.5	264	272
8a	*o*-$BrCH_2CONHCH_4C_6H_2S$	8	D	61	Indef[c]	42.7	3.01	17.7	42.5	3.27	17.5	276	282
8b	*m*-$BrCH_2CONHC_6H_4CH_2S$	8	D	86	~300[c]	42.7	3.01	17.7	42.5	3.27	17.6	273	283
15	*p*-$(C_6H_4(CO)_2N)C_6H_4CH_2S$	2	B	72[h]	283–287[h]	59.6	3.23	17.3	59.8	3.35	17.3	276[i]	282
17	*p*-$(C_6H_4(CO)_2N)C_6H_4CH_2S$	8	B	35[h]	276–278[h]	59.6	3.23	17.3	59.4	3.37	17.1	278[i]	283

[a] Each compound had an infrared spectrum in agreement with its assigned structure and moved as a single spot on tlc. [b] In 10% EtOH. [c] Recrystallized from aqueous DMF. [d] Recrystallized from 2-methoxyethanol–MeOH. [e] Recrystallized from MeOH. [f] Recrystallized from aqueous MeOH. [g] Not obtained analytically pure, although uniform on tlc. [h] Recrystallized from DMSO–MeOH. [i] Inflection.

tional to the enzyme concentration. The inactivation experiments were then performed as follows.

The buffer employed was 0.05 *M* Tris (pH 7.4). Bulk enzyme (0.05 ml) as purchased was diluted with 3.50 ml of buffer. In two tubes were placed 0.95 ml of enzyme solution in a 37° bath. After 5 min, 50 μl of DMSO was added to tube 1 (enzyme control) and 50 μl of DMSO containing the inhibitor was added to tube 2. The contents were mixed, the time was noted, and an 0.5-ml aliquot was withdrawn from each tube as rapidly as possible and stored at 0° until ready for assay. The aliquot from the enzyme control was labeled C_1; the aliquot from the inhibitor tube was labeled I_1. The remainders in the two tubes were then kept for 2 hr (or other time) at 37°, then cooled in an ice bath until ready for assay and labeled C_2 and I_2. The amount of enzyme remaining was assayed as follows.

In a 3-ml cuvette were placed 2.95 ml of buffer and 50 μl of 0.50 m*M* hypoxanthine.[7] The contents were vigorously shaken for about 20 sec to dissolve air; then 100 μl of C_1 (or other) aliquot was added and the rate of optical density increase at 290 mμ was followed on a Gilford recording spectrophotometer; the C_1 aliquot usually gave an OD change of about 0.025 unit/min. The C_1 and other aliquots were assayed in duplicate. The velocities in OD units/min were plotted on a log scale against the time on a linear scale.[13b] This procedure is satisfactory for a routine screen for a plus or minus answer on inactivation. As many as three inhibitor tubes can be run with one enzyme control in 1 day.

With a positive compound, a larger amount of inhibitor-enzyme mixture can be set up and a number of aliquots removed at varying times in order to obtain the half-life of the inactivation.