Synthesis of Fused [1,2,6]Thiadiazine 1,1-Dioxides as Potential Transition-State Analogue Inhibitors of Xanthine Oxidase and Guanase[1]

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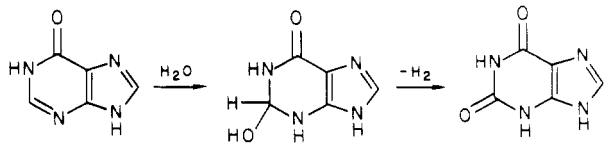
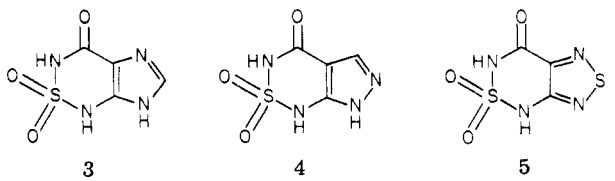
Ring closure of ethyl 3-aminopyrazole-4-carboxylate with sulfamoyl chloride gave 1,7-dihydropyrazolo[3,4-*c*][1,-2,6]thiadiazin-4(3*H*)-one 2,2-dioxide. The corresponding 4-amino analogue of this new heterocyclic ring system was similarly prepared from 3-aminopyrazole-4-carbonitrile. Treatment of 4,5,6-triamino-2*H*-1,2,6-thiadiazine 1,1-dioxide with *N*-thionylaniline gave a derivative of another new ring system, 7-amino-4*H*-[1,2,5]thiadiazolo[3,4-*c*][1,2,6]thiadiazine 5,5-dioxide. These compounds and the corresponding 4-amino- and 4-hydroxyimidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxides were examined as potential transition-state analogue inhibitors of xanthine oxidase and guanine aminohydrolase. Two of the compounds possessed K_i values of about 2×10^{-4} M with guanine aminohydrolase, but no inhibition of xanthine oxidase was observed by any at 5×10^{-4} M.

Over the last decade, evidence has accumulated that stable analogues of tetrahedral intermediates formed in the course of the enzymatic reaction can be potent inhibitors of those enzymes. A class of enzymes which is most susceptible to inhibition by these analogues are those hydrolases which catalyze an $sp^2 \rightarrow sp^3 \rightarrow sp^2$ conversion. Potent inhibition of adenosine and cytidine aminohydrolases by coformycin[2] and tetrahydrouridine,[3] respectively, represents an elegant demonstration of the "transition-state analogue" concept. Other work demonstrating that suitable aldehyde and boronic acid analogues may form tetrahedral adducts in the active site of serine and cysteine proteases has been recently reviewed.[4,5]

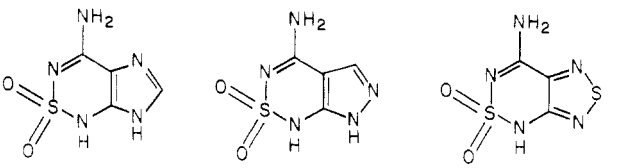
The goal of the present work is to investigate the use of tetrahedral sulfur to mimick the sp^3 intermediate in enzymatic reactions involving the type of rehybridization described above for the hydrolases. Another goal of the study is to investigate the use of tetrahedral analogues as a new class of inhibitors of certain oxidases which may function via a covalent hydrate intermediate. The only tetrahedral sulfur compounds reported to act as analogues of a tetrahedral intermediate are L-methionine (*S*)-sulfoximine, its *N*-phosphate, and L-methionine sulfoxide, all very potent inhibitors of glutamine synthetase.[6-8]

We have chosen to investigate fused [1,2,6]thiadiazine 1,1-dioxides ("2-thiapurines") as inhibitors of two enzymes which catalyze alterations to the 2 position of purines, xanthine oxidase and guanase. Xanthine oxidase catalyzes the oxidation of hypoxanthine to xanthine and then to uric acid. One recently postulated mechanism[9] for this reaction involves covalent addition of water to the position to be oxidized (and therefore formation of an sp^3 intermediate), followed by a coupled transfer of a proton and electron pair to the enzyme, which contains molybdenum in the active site. The key enzymatic intermediate formed from hypoxanthine (1) would be **2**. We therefore investigated



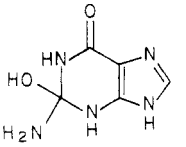


1,5-dihydroimidazo[4,5-*c*][1,2,6]thiadiazin-4(3*H*)-one 2,2-dioxide (**3**) as a potential analogue of tetrahedral intermediate **2**. Since pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (allopurinol) and [1,2,5]thiadiazolo[3,4-*d*]pyrimidin-7-(6*H*)-one are also substrates for xanthine oxidase,[10] we investigated the synthesis of 1,7-dihydropyrazolo[3,4-*c*]-[1,2,6]thiadiazin-4(3*H*)-one 2,2-dioxide (**4**) and 4*H*-[1,-2,5]thiadiazolo[3,4-*c*][1,2,6]thiadiazin-7(6*H*)-one 5,5-dioxide (**5**) as analogues of the tetrahedral intermediate presumably arising from addition of water to the respective substrates. Compound **5** could, however, not be obtained. Additionally, the amino-substituted analogues 4-amino-1,5-dihydroimidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (**6**),



4-amino-1,7-dihydropyrazolo[3,4-*c*][1,2,6]thiadiazine 2,2-dioxide (**7**), and 7-amino-4*H*-[1,2,5]thiadiazolo[3,4-*c*][1,2,6]thiadiazine 5,5-dioxide (**8**) were prepared and evaluated as inhibitors. The corresponding imidazo-, pyrazolo-, and thiadiazolopyrimidines are known to be xanthine oxidase substrates.[10]

As a model system to investigate the suitability of tetrahedral sulfur as an analogue of the sp^3 intermediate in reactions catalyzed by hydrolases, we examined compounds **3**, **4**, and **6**–**8** as inhibitors of guanine aminohydrolase (guanase). A reasonable intermediate for the guanase-assisted attack of water on the 2 position of guanine is **9**, and tetrahedral analogues **3**–**8** could con-



ceivably mimick this intermediate. Bartlett and co-workers have recently proposed analogues of guanine with tetrahedral phosphorous in the 2 position of the ring as transition-state analogues for guanase.[11]

Chemistry. After these studies were underway, the preparation of **3**[12] and **6**[13] by Stud and co-workers was accomplished by Traube ring closure of 4-hydroxy-5,6-diamino- or 4,5,6-triamino-1,2,6-thiadiazine 1,1-dioxide (**10**), respectively, with potassium dithioformate. We prepared **6** by this route, using triethyl orthoformate as the ring-closing reagent. Compound **8** was also prepared

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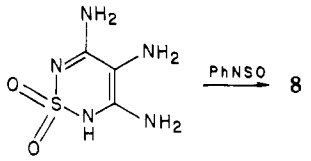
from **10** in 57% yield by treatment with *N*-thionylaniline



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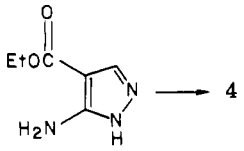
in refluxing pyridine, a method developed by Montgomery for conversion of 4,5-diaminopyrimidines to 1,2,5-thiadiazolo[3,4-*d*]pyrimidines.[14] This constitutes the first synthesis of the new heterocyclic ring system [1,2,5]-thiadiazolo[3,4-*c*][1,2,6]thiadiazine.

Fused [1,2,6]thiadiazole *S*,*S*-dioxides have previously been prepared by treating ethyl 4-amino-1,2-dimethylimidazole-5-carboxylate[15] or ethyl anthranilate[16] with sulfamoyl chloride in refluxing benzene. This method proved to be successful in the conversion of ethyl 3-aminopyrazole-4-carboxylate[17] (**11**) to **4**. This also is the



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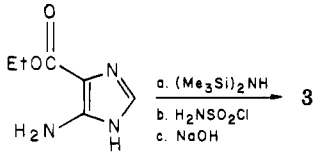
first report of a new heterocyclic ring system, pyrazolo-[3,4-*c*][1,2,6]thiadiazine. Treatment of ethyl 5(4)-aminoimidazole-4(5)-carboxylate[18] (**12**) with H_2NSO_2Cl under identical conditions, however, gave no trace of **3**.

Silylation is known to activate amino groups to acylation.[19] We have developed a new method for the synthesis of amino- and hydroxy-substituted fused [1,2,6]thiadiazole dioxides based on treatment of per(trimethylsilyl) derivatives of *o*-aminocyano- or *o*-aminoethoxycarbonyl heterocycles with sulfamoyl chloride, followed by alkali. Thus, **12** was trimethylsilylated with refluxing hexa-



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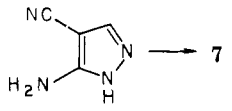

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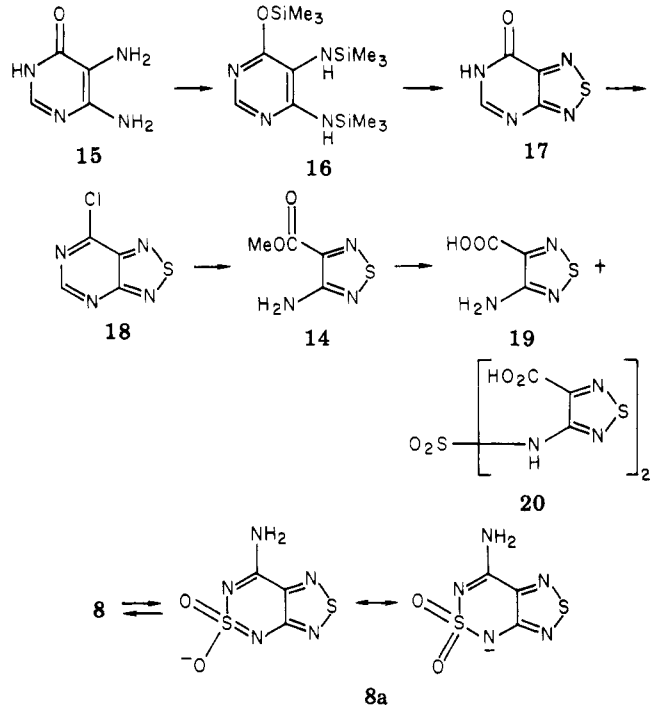
methyldisilazane and then refluxed in benzene with sulfamoyl chloride. The intermediate *N*-sulfamoyl derivative initially formed was converted to **3** by treatment with aqueous NaOH. The method was extended to the synthesis of the aminothiadiazine derivatives, as exemplified by the synthesis of **7** from 3-aminopyrazole-4-carbonitrile[20] (**13**) by the same three-step process. Compound **7** is another example of this new heterocyclic ring system, pyrazolo[3,4-*c*][1,2,6]thiadiazine.

In an attempt to prepare **5**, 4,5-diamino-6-hydroxy-1,2,6-thiadiazine 1,1-dioxide[12] was treated with *N*-thionylaniline, but only decomposition products were obtained. In order to prepare **5** by the sulfamoylation procedure described above, the preparation of methyl 3(4)-amino-1,2,5-thiadiazole-4(3)-carboxylate (**14**) was investigated.

The sulfate salt of 5,6-diaminopyrimidin-4(3*H*)-one (**15**) was treated with refluxing hexamethyldisilazane to give

Scheme I



a persilylated derivative, presumably **16** (Scheme I). Direct treatment with refluxing $SOCl_2$, followed by evaporation and treatment of the residue with refluxing methanol, gave after neutralization with aqueous buffer two major products: [1,2,5]thiadiazolo[3,4-*d*]pyrimidin-7(6*H*)-one (**17**), the product Montgomery[14] prepared from **15** and PhNSO, was obtained in 31% yield and **14** was isolated in 22% yield. When **16** was allowed to reflux for longer times with $SOCl_2$, the yield of **14** was not improved, but no **17** was found. It is logical to postulate that **17** was formed initially in the reaction, and on further exposure to refluxing $SOCl_2$ 7-chloro[1,2,5]thiadiazolo[3,4-*d*]pyrimidine (**18**) was formed. Montgomery has shown **18** to be highly susceptible to nucleophilic displacement of halogen, indicating that it has a very electron-deficient pyrimidine ring. The treatment with hot methanol, followed by water, converted **18** to the ring-opened product **14**. The hydrolytic excision of the carbon-2 of activated purines, such as 1-alkoxyadenines, is well established.[21]

Attempts to convert **14** to the thiadiazolothiadiazine **5** were unsuccessful. Compound **14** did not react directly with sulfamoyl chloride. Treatment of **14** with Me_3SiCl/Et_3N gave a silylated derivative, but subsequent treatment with sulfamoyl chloride, followed by NaOH, gave the saponification product 4(3)-amino-1,2,5-thiadiazole-3(4)-carboxylic acid (**19**). Fusion of **14** with sulfamoyl chloride gave, after NaOH treatment, *N*,*N*′-bis-[3(4)-carboxy-1,2,5-thiadiazol-4(3)-yl]sulfamide (**20**) and **15**.

The species of each compound present in solution was inferred from inspection of UV-absorption data, as recorded in Table I, and comparison of these data with reference fused pyrimidines. The λ_{max} values of **8** are qualitatively similar to the values obtained for 7-amino-[1,2,5]thiadiazolo[3,4-*d*]pyrimidine[14] (λ_{max} 340, 273, and 230 nm at pH 7). The pK_a of the fused [1,2,6]thiadiazine 1,1-dioxides is quite low (see Table I), and the anionic form **8a** present at neutral pH is isoelectronic with the thiadiazolopyrimidine, which in turn has been pointed out[14] to be isoelectronic with pteridine. Thiadiazolothiadiazine **8** exhibits bright-blue fluorescence under UV light. Compounds **4** and **7** also show strong similarities in the

Table I. Physical Properties and Enzyme Inhibition Data of the Fused [1,2,6]Thiadiazine 1,1-Dioxides

	λ_{max}, nm ($\epsilon \times 10^{-3}$)					guanase inhibn[c]	
no.	pH 1	pH 7.4	pH 12	R_f[a]	pK_a[b]	$([I]/[S])_{50}$[d]	K_i, mM[e]
4	270 (1.5)	274 (3.7)	274 (2.0)	0.27	6.5	15	0.25
	232 (4.6)	210 (1.52)	217 (13.0)				
	207 (9.2)						
7	270 (5.0)	287 (6.6)	287 (6.2)	0.50	4.1	no inhibn at 32	
	235 (14.0)	240 (5.7)	256 (5.5)				
8	310 (5.3)	357 (4.3)	357 (4.3)	0.60	3.2	11	0.19
	223 (8.5)	285 (2.7)	285 (2.7)				
		232 (9.6)	232 (9.6)				
3						no inhibn at 28	
6						no inhibn at 46	

[a] Determined on EM silica gel F-254 plates using *i*-PrOH/NH_4OH/H_2O (7:1:2) as eluant. [b] Determined titrametrically. [c] Performed as described under the Experimental Section. All values were reproducible within ±20%. [d] Concentration of substrate guanine was 13.3 μM. Calculated as described under the Experimental Section. [e] Determined from the *x* intercept ($-1/K_i$) of plots of $1/V$ vs. [*I*] at four different substrate concentrations. Both this plot (a Dixon plot) and plots of $1/V$ vs. $1/[S]$ showed linear noncompetitive kinetics (see ref 24).

UV spectrum of their monoanion to the pyrazolopyrimidine counterparts.[20] The pK_a values of the new compounds were determined titrametrically and are given in Table I.

Enzymatic Results and Discussion. Compounds **3**, **4**, and **6**–**8** were examined as inhibitors of the oxidation of hypoxanthine and xanthine. No inhibition was observed by any of these compounds at 0.5 mM under aerobic conditions. Since alloxanthine (pyrazolo[3,4-*d*]pyrimidine-4,6(5*H*,7*H*)-dione) binds very tightly to the reduced form of xanthine oxidase,[22] compounds **3**, **4**, and **6**–**8** were also incubated anaerobically with the enzyme in a closed tube with xanthine present to reduce the enzyme. No inhibition was found under these conditions either.

It is conceivable that the test compounds failed to bind to xanthine oxidase due to the negative charge they carry at neutral pH (see Table I). Since there is evidence that the substrate binds to the active-site molybdenum through a coordinate bond,[9,23] it was felt that the high electron density on one of the thiadiazine nitrogens resulting from ionization might actually increase binding potency. Alternatively, the lack of activity of these compounds is consistent with the hypothesis that an enzymatic disulfide anion is the first species to attack the carbon on the bound substrate to be oxidized.[23] Following hydride transfer (or equivalent) to the enzyme, water was postulated to attack the newly oxidized carbon and displace disulfide. Obviously, our tetrahedral analogues would be ineffective in mimicking a tetrahedral intermediate involving a covalent bond between enzyme and substrate.

The fused pyrazolothiadiazinone **4** and aminothiazolothiadiazine **8** were inhibitors of guanase, giving K_i values of about 2×10^{-4} M as determined by Dixon plots[24] (see Table I). The family of curves in the Lineweaver–Burk plots of $1/V$ vs. $1/S$ at various inhibitor concentrations indicated that the inhibition was linear noncompetitive.[24] All other compounds were ineffective as inhibitors, showing no inhibition using at least 28 times the substrate concentration. As a test of the contribution of the tetrahedral sulfur dioxide moiety to the ability of **8** to inhibit the enzyme, [1,2,5]thiadiazolo[3,4-*d*]pyrimidin-7(6*H*)-one and its 5,7-(4*H*,6*H*)-dione and 7-amino analogues were prepared by the literature method[14] and examined as inhibitors. They showed no inhibiton at 0.4 mM with a substrate concentration of 13.3 μM.

The K_i values found are not sufficiently low to allow the inhibitors to be identified as analogues of transition-state **9** at this time.[5] The noncompetitive kinetics further support this conclusion. The negative charge, however, not only does not interfere with binding but, if the thiadiazines are compared with the corresponding fused pyrimidines, it may contribute. Furthermore, we envisioned that an enzyme hydrogen-bond donor might polarize the 2,3 double bond of guanine to facilitate attack by water at position 2, and a high electron density on the corresponding nitrogen of the inhibitors might interact favorably with this H-bond donor. Finally, Lewis and Glantz have proposed that an active-site cysteine in guanase forms an initial covalent adduct (with elimination of NH_3).[25] If this is the case, then, as noted above for xanthine oxidase, the resulting covalent tetrahedral intermediates could not be mimicked by our inhibitors.

Experimental Section

All UV spectra and enzyme assays were performed on a Cary 118 or Beckman Acta III spectrophotometer. The 0.1 absorbance unit scale was used for the assays. IR spectra were taken on a Perkin-Elmer IR 457. Melting points were determined with a Thomas-Hoover apparatus and are uncorrected. Elemental analyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, and are correct within ±0.4%. All chemicals were purchased from Aldrich or Sigma Chemical Co. Enzymes were purchased from Sigma. Sulfamoyl chloride was prepared from chlorosulfonyl isocyanate (Aldrich) by the method of Graf.[26] NMR spectra were recorded on a Perkin-Elmer R-12 using tetramethylsilane as internal reference. Column chromatography was performed on Merck silica gel 60, 230–400 mesh. The mass spectrum of **14** was determined on an AEI MS 902 in the chemical-ionization mode, and **20** was determined in the field-desorption mode. The xanthine oxidase assays were adapted from those of Miller[27] and Massey.[22] The guanase assays were adapted from the procedure of Baker.[28]

1,5-Dihydroimidazo[4,5-*c*][1,2,6]thiadiazin-4(3*H*)-one 2,2-Dioxide (3). A suspension of 0.50 g (3.2 mmol) of ethyl 5(4)-aminoimidazole-4(5)-carboxylate[18] (**12**) in 10 mL of hexamethyldisilazane was refluxed for 12 h under N_2 and then evaporated to dryness. The residue was extracted with 50 mL of dry toluene, which was then filtered and evaporated. This residue was dissolved in 50 mL of dry benzene and, after the addition of 0.372 g (3.2 mmol) of sulfamoyl chloride, the mixture was refluxed for 1 h. After cooling the mixture to ambient temperature, 25 mL of 25% NaOH was added, and stirring was continued for 2 h. The aqueous phase was separated, the organic layer was washed with 2 × 15 mL of H_2O, and the combined aqueous layers were acidified with Dowex 50 × 8 (H^+). The filtered solution was evaporated to dryness. Dissolution of the residue in 10 mL of H_2O, acidification to pH 1 with concentrated HCl, and chilling gave 0.20 g (33%) of crystalline **3** with spectral properties identical with those reported.[12]

1,7-Dihydropyrazolo[3,4-*c*][1,2,6]thiadiazin-4(3*H*)-one 2,2-Dioxide (4). A stirred suspension of 1.6 g (10.3 mmol) of ethyl 3-aminopyrazole-4-carboxylate (**11**)[17] and 1.19 g (10.3 mmol) of sulfamoyl chloride in 50 mL of benzene was refluxed for 3 h; a

gum precipitated. After cooling the suspension, 25 mL of 25% NaOH was added and stirring was continued for 3 h. The aqueous layer was separated and the organic layer was washed with 2 × 15 mL of H_2O. The combined aqueous fractions were acidified with Dowex 50 × 8 (H^+). The filtered solution was evaporated to dryness in vacuo, and the residue was taken up in 15 mL of concentrated HCl. Chilling gave 1.0 g (52%) of chromatographically pure **4**. A MeOH solution of this material was treated with activated charcoal, filtered, and evaporated, and the residue was recrystallized from concentrated HCl/H_2O: mp >300 °C dec; IR 3340, 3260, 3000 (NH), 1690, 1600 (CO, CN) cm^{-1}; NMR (Me_2SO-d_6) δ 8.2 (s, CH), 8.9–7.7 (br s, NH). Anal. ($C_4H_4N_4O_3S$) C, H, N.

Methyl 3(4)-Amino-1,2,5-thiadiazole-4(3)-carboxylate (14). A stirred suspension of 4.0 g (17 mmol) of 4,5-diaminopyrimidin-4(3*H*)-one sulfate (**15**) in 20 mL of hexamethyldisilazane was refluxed for 12 h under N_2. After the addition of toluene (75 mL), the filtered solution was evaporated to dryness, giving 4.6 g of crude silylated pyrimidine. Freshly distilled $SOCl_2$ (15 mL) was added slowly and then the mixture was refluxed for 15 min. After evaporation of the $SOCl_2$ from the mixture, 100 mL of MeOH was added and the mixture was refluxed for 1 h. Aqueous 0.1 N NaOH was added to adjust the pH to 7. Final adjustment of the pH was made by the addition of 1 M phosphate buffer, pH 7.4. After the addition of 20 g of silica gel to the mixture, the solvent was evaporated to dryness under high vacuum. The residual solid was placed on top of a 5 × 75 cm column of silica gel packed in EtOAc. Elution with EtOAc gave pure **14**, 0.60 g (22%), which was recrystallized from MeOH: mp 143–144 °C; IR 1710 cm^{-1} (C=O); NMR (Me_2SO-d_6) δ 3.9 (s, 3 H, CH_3), 7.0 (s, 2 H, NH_2); MS *m/e* 159 (M^+); TLC R_f 0.9 (EtOAc). Anal. ($C_4H_5N_3O_2S$) C, H, N.

Further elution with MeOH/EtOAc (1:9) gave [1,2,5]thiadiazolo[3,4-*d*]pyrimidin-7(6*H*)-one, 0.80 g (31%), which possessed physical properties identical with those previously described.[14]

When the $SOCl_2$ mixture was refluxed for 1 h and the neutralized MeOH solution was evaporated and extracted with EtOAc, **14** was obtained directly without chromatography in 21% yield.

Reaction of 14 and Sulfamoyl Chloride. A mixture of 1.0 g (7.75 mmol) of **14** and 2.7 g (23.2 mmol) of sulfamoyl chloride was heated in an oil bath at 130 °C for 30 min. After cooling the mixture, 15 mL of 25% NaOH was added and the resulting solution was stirred for 30 min. The solution was diluted with H_2O (50 mL) and acidified to pH 1 (concentrated HCl). On standing, needles separated from the solution, which were filtered and identified as *N,N'*-bis[3(4)-carboxy-1,2,5-thiadiazol-4(3)-yl]sulfamide (**20**; 100 mg, 7%): mp 163–165 °C (evolution of gas); MS *m/e* 352. Anal. ($C_6H_4N_6O_6S_3 \cdot 2.25H_2O$) C, H, N.

The filtrate was evaporated in vacuo and the residue dried under high vacuum. This residue was extracted with 3 × 50 mL portions of hot MeOH. The combined extracts were evaporated and the residue was taken up in 20 mL of H_2O. After acidification of the solution with HCl, cooling yielded 0.50 g (44%) of crystalline 3(4)-amino-1,2,5-thiadiazole-4(3)-carboxylic acid: mp 214–216 °C dec; UV λ_{max} 330 nm at pH 1, 317 at pH 7.4, 317 at pH 12; IR 1690 cm^{-1} (CO). Anal. ($C_3H_3N_3O_2S$) C, H, N.

4-Amino-1,7-dihydropyrazolo[3,4-*c*][1,2,6]thiadiazine 2,2-Dioxide (7). A mixture of 2.0 g (18.5 mmol) of 3-aminopyrazole-4-carbonitrile (**13**),[20] 50 mL of hexamethyldisilazane, and 0.1 g of $(NH_4)_2SO_4$ was refluxed under N_2 overnight. After the addition of 100 mL of toluene, the filtered solution was evaporated to dryness and 50 mL of toluene was added. After addition of 2.50 g (21.1 mmol) of sulfamoyl chloride, the mixture was stirred for 2 h at ambient temperature, 20 mL of 25% NaOH was added, and stirring was continued for 2 h. The organic layer was separated and washed with 2 × 20 mL of H_2O, and the combined aqueous phases were diluted to 250 mL with H_2O and acidified with Dowex 50 × 8 (H^+) to pH 1. The resin was filtered and washed, and the combined filtrate and washings were concentrated in vacuo to ca. 50 mL. After cooling overnight, the filtered product was recrystallized from H_2O: yield 1.0 g (28%); mp 298–299 °C; IR 3420, 3320 (NH_2), 1660–1600 (CN) cm^{-1}. Anal. ($C_4H_5N_5O_2S$) C, H, N.

7-Amino-4*H*-[1,2,5]thiadiazolo[3,4-*c*][1,2,6]thiadiazine 5,5-Dioxide (8). A mixture of 0.30 g (1.7 mmol) of 3,4,5-triamino-2*H*-1,2,6-thiadiazine 1,1-dioxide (**10**),[13] 0.727 g (5.2 mmol) of *N*-thionylaniline, and 25 mL of pyridine was refluxed for 3 h under N_2. Evaporation in vacuo gave a dark oil, which was dissolved in 25 mL of H_2O and decolorized with carbon. The pH was adjusted to 1.0 with concentrated HCl; the product was deposited on cooling: yield 0.20 g (57%); mp >300 °C dec; IR (KBr) 3320, 3380 (NH_2), 3150 (NH), 1680, 1580, 1550 cm^{-1}. Anal. ($C_3H_3N_5O_2S_2$) C, H, N.

Enzyme Assays. Xanthine Oxidase. The assay for xanthine oxidase inhibition consisted of a 3.0-mL reaction mixture containing 4.0 μM xanthine, 22 μM EDTA, xanthine oxidase (0.02 μM with respect to flavin), and varying amounts of the compound being testing in pH 7.4 0.05 M Tris-HCl buffer. The enzyme preparation from Sigma was grade III and reputed to contain 10 units/mL. The enzyme concentration with respect to flavin was calculated from an absorbance measurement at 450 nm.[29] Enzyme activity was assayed aerobically at 25 °C by measuring the rate of uric acid formation at 295 nm. Reaction velocities with the compounds present were compared with the velocity of the uninhibited control reaction. The ratio of concentration of test compound to concentration of xanthine was increased to 125 before it was concluded that the compound lacked activity.

Incubation experiments were carried out on the reduced and oxidized form of the enzyme. For reduced enzyme incubation, 50 μL of 1.2 μM xanthine oxidase, 1.0 mL of a 5×10^{-4} M solution of the compound being tested in pH 7.4 Tris buffer, 100 μL of 6.6×10^{-4} M EDTA, and 1.5 mL of buffer were added to a culture tube. This solution was purged with nitrogen, and then 100 μL of 2.4×10^{-4} M xanthine was added, resulting in a total volume of 2.75 mL. The tube was sealed and allowed to incubate for 1 h at 25 °C. The ratio of concentration of the compound being tested to enzyme concentration was 8.3×10^3. After incubation, the tube was opened, exposed to the air, and 250 μL of 2.4×10^{-4} M xanthine added. Initial velocities were determined as before. Comparison was then made with controls without test compounds added. For incubation in the oxidized form, 50 μL of 1.2 μM xanthine oxidase, 1.0 mL of 5×10^{-4} M solution of the compound being tested in Tris-HCl buffer, 100 μL of 6.6×10^{-4} M EDTA, and 1.6 mL of buffer were added to a culture tube, giving a total volume of 2.75 mL. Incubation was carried out aerobically for 1.0 h, followed by the addition of 250 μL of 2.4×10^{-4} M xanthine and determination of the initial velocity as before. Comparisons were then made with controls without the test compounds.

Guanine Aminohydrolase. The assay for guanase inhibition consisted of a 1.0-mL reaction mixture containing 13.3 μM guanine and varying amounts of inhibitor and guanase in pH 7.4 0.05 M Tris-HCl buffer. To prepare the reaction mixture, 330 μL of 4×10^{-5} M guanine, the inhibitor solution, and buffer were added to a culture tube to give a total volume of 0.97 mL. The guanine stock was prepared fresh for a day's run by diluting 200 μL of 2.0×10^{-2} M guanine in 0.1 N NaOH to 100 mL with pH 7.4 Tris-HCl buffer. The reaction was started by the addition of 30 μL of enzyme stock solution to the reaction mixture. The enzyme stock was prepared by diluting 150 μL of rabbit liver guanase containing 10 mg of protein/mL to 2.0 mL. On addition of the enzyme, the reaction mixture was mixed and immediately transferred to a 1.0-mL cuvette. The decrease in guanine was measured at 248 nm. For a reaction mixture without inhibitor, the decrease in absorbance was 0.008 OD unit/min. The concentration of inhibitor producing 50% inhibition was determined by plotting V_0/V_I against inhibitor concentration [I], where V_0 is the velocity without inhibitor and V_I is the velocity with inhibitor concentration [I]. The I_{50} is the inhibitor concentration where $V_0/V_I = 2$.

The kinetics of guanase inhibition was evaluated using a Lineweaver–Burk reciprocal plot.[24] The substrate concentrations used were 20, 10, 6.4, and 4.8 μM. Four inhibitor concentrations were used. The K_m of rabbit liver guanase was found to be 6.8×10^{-6} M (lit. 1.25×10^{-5} M, pH 6.8[25]).

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References and Notes

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3-Phenyl-5-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]indole-2-carbonitrile, a Potent Inhibitor of Prostaglandin Synthetase and of Platelet Aggregation

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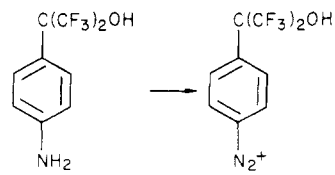
A number of indoles containing the 2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl side chain have been prepared by standard methods. Alternate, novel syntheses of indole-2-carboxamides and indole-2-carbonitriles have been developed. The title compound, **7e**, was found to be a potent inhibitor of bovine prostaglandin synthetase in vitro and to lower serum prostaglandin levels after oral or intraperitoneal administration to rats. Consistent with prostaglandin synthetase inhibition, **7e** prevented arachidonic acid induced diarrhea in mice and also collagen, ADP, or epinephrine induced platelet aggregation in human platelet-rich plasma. In contrast to many prostaglandin synthetase and platelet-aggregation inhibitors, **7e** had neither ulcerogenicity nor systemic antiinflammatory activity in rats.

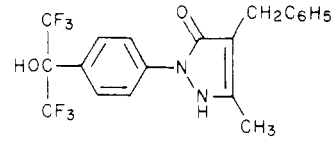
In connection with another project, we had a need to prepare the indole-2-carbonitrile **7e**. A number of 3-phenylindole-2-carbonitriles have been prepared[1-7] in the past as intermediates in the 1,4-benzodiazepine area by functional-group manipulations from the corresponding esters. These esters, in turn, have been prepared by a combination of the Japp–Klingemann reaction[8] and the Fischer indole synthesis.[9] We therefore utilized a similar sequence for the synthesis of **7e**.

Diazotization of **1** gave **2**, which was allowed to react



1 2

with **3a** as shown in Scheme I. Selective elimination of the acetyl group from the resulting **5a** and cyclization of **6a** to the indole **7a** proceeded as expected without isolation of the intermediates. The pyrazolone 8 was isolated as a byproduct of this sequence. Hydrolysis of the ester group of **7a** required at least 2 equiv of sodium hydroxide: the hydroxyl group of the $C(CF_3)_2OH$ side chain is sufficiently



8

acidic to neutralize 1 equiv of base. The acid was converted into the acid chloride **7c** on heating with phosphorus pentachloride in ether: other reagents, such as thionyl chloride, would be expected[10] to replace the hydroxyl of the side chain by chlorine. Treatment of the total reaction mixture containing **7c** with ammonia gave the amide **7d**. The ester **7a** was recovered from a number of attempts to prepare the amide from it directly with ammonia under a variety of conditions—probably due to the ionization of the $C(CF_3)_2OH$ side chain and the inability of ammonia to attack the resulting negatively charged molecule. The amide **7d** was dehydrated under a variety of conditions, preferably with polyphosphate ester[11] in chloroform, to give the nitrile **7e** in a total yield from **1** of 38%.

When **7e** was found to be a potent inhibitor of prostaglandin synthetase, alternate, more direct, synthetic routes were considered. A thorough search of the literature failed to disclose any previous synthesis of indole-2-

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