[1-β-Mpa,7-Sar]arginine-vasopressin (XVI). The protected peptide XIV (128 mg, 0.087 mmol) was deblocked, reoxidized, desalted, and purified as for IV: yield 48 mg (53%); $[\alpha]^{22}_D$ −58.7° (c 0.43, 1 N AcOH); TLC R_f 0.23 (BAW), 0.17 (BAWP). M_r calcd 1058.2; found, 1058. Amino acid analysis: Asp, 1.01; Glu, 1.08; Gly, 1.00; Tyr, 0.96; Phe, 0.98; Arg, 1.01; NH_3, 3.06. Analysis following performic acid oxidation[40] gave a Cys(O_3H)/Gly ratio of 1.02:1.00.

Boc-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-MeAla-Arg(Tos)-Gly-resin (XVII). Part of the 2-peptide-resin XI (4.54 g, 2 mmol) was converted to protected 8-peptide-resin (6.36 g, 96%) in six cycles of solid-phase peptide synthesis.

Z-Cys(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-MeAla-Arg(Tos)-Gly-NH_2 (XVIII). A single cycle of deprotection, neutralization, and coupling with Z-Cys(Bzl)-OH converted 8-peptide-resin XVII (1.59 g, 0.5 mmol) to the protected 9-peptide-resin (1.70 g). This material was ammonolyzed, and the amide of 9-peptide (701 mg, 86% based on substituted Gly) was purified by precipitation as detailed in the preparation of protected [7-Sar]oxytocin: mp 214–216 °C; $[\alpha]^{22}_D$ −35.7° (c 1, DMF); TLF R_f 0.68 (BAW), 0.74 (BAWP). Anal. ($C_{81}H_{97}N_{15}O_{16}S_3$) C, H, N. Amino acid analysis: Asp, 1.00; Glu, 1.03; Gly, 1.00; Tyr, 0.97; Phe, 0.97; Cys(Bzl), 1.97; Arg, 0.96, NH_3, 3.1.

β-Mpa(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-MeAla-Arg(Tos)-Gly-NH_2 (XIX). Boc-8-peptide-resin XVIII (1.59 g, ~0.5 mmol) was subjected to one cycle of solid-phase peptide synthesis to yield the protected peptide-resin (1.63 g). Material was ammonolyzed and the product purified as described for the preparation of analogue II: yield of 597 mg (80% based on substituted Gly); mp 213–215 °C; $[\alpha]^{22}_D$ −32.2° (c 1, DMF); TLC R_f 0.58 (BAW), 0.48 (CM). Anal. ($C_{73}H_{90}N_{14}O_{14}S_3$) C, H, N.

[7-*N*-MeAla]arginine-vasopressin (XX). The protected 9-peptide XVIII (180 mg, 0.11 mmol) was deblocked, reoxidized, and purified as for IV: yield 97 mg (82%); $[\alpha]^{22}_D$ −13.1° (c 0.5, 1 N AcOH); TLC R_f 0.10 (BAW), 0.22 (BAWP). M_r calcd, 1087.2; found, 1087. Amino acid analysis: Asp, 1.01; Glu, 1.00; Gly, 1.00; $^1/_2$-Cys, 1.99; Tyr, 0.99; Phe, 1.02; Arg, 1.01; NH_3, 3.07.

[1-β-Mpa,7-*N*-MeAla]arginine-vasopressin (XXI). Acyl peptide XIX (190 mg, 0.128 mmol) was reduced by sodium in liquid ammonia as detailed above for IV: yield 102 mg (78%); $[\alpha]^{23}_D$ −68.8° (c 0.33, 1 N AcOH); TLC R_f 0.27 (BAW), 0.31 (BAWP). M_r calcd 1072.2; found, 1072. Amino acid analysis: Asp, 1.02; Glu, 1.04; Gly, 1.00; Tyr, 0.99; Phe, 1.01; Arg, 1.00; NH_3, 3.04. Analysis following performic acid oxidation gave a Cys(O_3H)/Gly ratio of 1.02:1.00.

Acknowledgment. This work was supported by research grants from the Polish Academy of Sciences (MR.I.12.1.6.3), the U.S. National Institute of Arthritis, Metabolic and Digestive Diseases (Grant AM 10080), the Life Sciences Foundation Inc., and the Stella and Charles Gutmann Foundation Inc. We express our appreciation to Dr. M. Manning, Medical College of Ohio, for his interest and for use of his laboratory for reduction–oxidation and purification steps of two analogues. We also thank Nocif Yamin for conscientious technical assistance.

Registry No. II, 84558-67-8; III, 84558-68-9; IV, 77225-24-2; V, 84558-69-0; VII, 84558-71-4; VIII, 84558-72-5; IX, 84558-73-6; X, 84558-74-7; XIII, 84582-76-3; XIV, 84558-76-9; XV, 84558-77-0; XVI, 84558-78-1; XVIII, 84558-79-2; XIX, 84558-80-5; XX, 84558-81-6; XXI, 84558-82-7; Boc-Asn-ONp, 4587-33-1; Boc-Gln-ONp, 15387-45-8; Z-Cys(Bzl)-OH, 3257-18-9; β-Mpa(Bzl)-OH, 2899-66-3.

(40) Li, C. H. *J. Biol. Chem.* **1957**, *229*, 157.
(41) Chan, W. Y.; du Vigneaud, V. *Endocrinology* **1962**, *71*, 977.
(42) Manning, M.; Coy, E. J.; Sawyer, W. H.; Acosta, M. *J. Med. Chem.* **1973**, *16*, 463.
(43) Manning, M.; Balaspiri, L.; Moehring, J.; Haldar, J.; Sawyer, W. H. *J. Med. Chem.* **1976**, *19*, 842.

Preparation of 2-Amino-4(3*H*)-oxopyrimido[5,4-*b*][1,4]thiazines (5-Thiapterins[1]) and Their Evaluation as Cofactors for Phenylalanine Hydroxylase

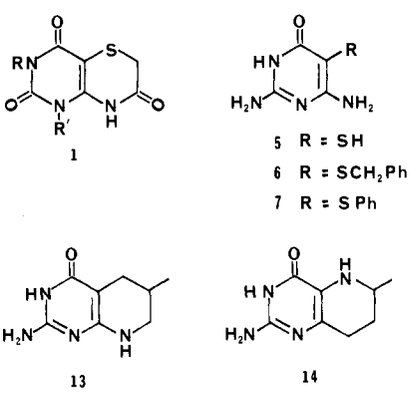
Robert N. Henrie II, Robert A. Lazarus, and Stephen J. Benkovic*

Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802. *Received July 6, 1982*

Reaction of diethyl chloromalonate with β-mercapto amines, **9**, gave 1,4-thiazin-3-ones, **10**, which were alkylated exclusively at the lactam oxygen with triethyloxonium tetrafluoroborate and subsequently condensed with guanidine to give the first reported 5-thiapterins, **8**. Oxidation of **8** with *m*-chloroperoxybenzoic acid gave the *S*-oxides, **12**. Both **8** and **12** were found to be good inhibitors of rat liver phenylalanine hydroxylase competitive with 6-methyltetrahydropterin, with **8** exhibiting lower K_i's than the corresponding **12**. The 8-thiapterin **4** was a much poorer inhibitor.

L-Phenylalanine hydroxylase (phenylalanine 4-monooxygenase, EC 1.14.16.1) is a mammalian, pterin-dependent monooxygenase that catalyzes the para hydroxylation of L-phenylalanine to form L-tyrosine by using dioxygen as cosubstrate.[2] As such, PAH[3] serves as the initiator for the metabolism of phenylalanine, as well as the initiating enzyme in the biosynthesis of the catecholamines.[4] A tetrahydropterin cofactor is required for hydroxylation: L-*erythro*-tetrahydrobiopterin is the natural cofactor, although many simple tetrahydropterins

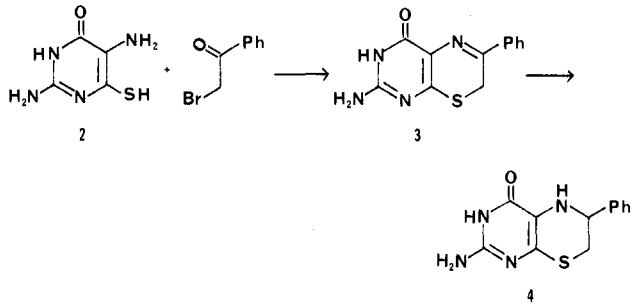
Chart I



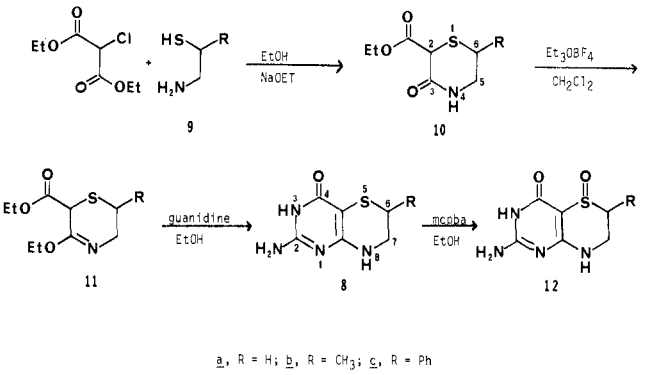
(1) The less cumbersome name 5-thiapterin will be used in place of the systematically correct 2-amino-4(3*H*)-oxopyrimido[5,4-*b*][1,4]thiazine throughout this paper.
(2) Goodwin, B. L. In "Aromatic Amino Acid Hydroxylases and Mental Disease", M. B. H. Youdim, Ed., Wiley, New York, 1979, Chapter 2.
(3) Abbreviations used: PAH, L-phenylalanine hydroxylase; mcpba, *m*-chloroperoxybenzoic acid; 6-MePH$_4$, 6-methyl-5,6,7,8-tetrahydropterin.
(4) Blau, K. In ref 2, Chapter 3.

0022-2623/83/1826-0559$01.50/0

Scheme I



Scheme II



a, R = H; b, R = CH_3; c, R = Ph

show high cofactor activity.[5] Also, PAH has been shown to be an iron protein containing one iron per subunit of active enzyme, with the iron content and specific activity being directly correlated.[6]

The precise mechanism of the hydroxylation reaction catalyzed by PAH remains undefined. A presumptive pterin 4a-hydroperoxide intermediate would be consistent with recent findings from this laboratory.[7] In addition, the obligatory role of iron in the catalytic reaction invites speculation that a high valent iron–oxygen species serves as the actual hydroxylating agent.[8]

In an endeavor to trap either the pterin 4a-hydroperoxide or the iron–oxygen reactive species, we have prepared cofactor analogues in which the 5-nitrogen of the tetrahydropterin has been replaced with a nucleophilic sulfur atom. The synthesis and enzymology of the resulting 5-thiapterins and their *S*-oxides are described in this report.

Synthesis and Characterization. Only a few representatives of the pyrimido[5,4-*b*][1,4]thiazine class have been reported, all of which possess either 1,3-dialkyl-2,4,7-trioxo (**1**, Chart I) or 1,3-dialkyl-2,4,6,7-tetraoxo substitution patterns.[9,10] In general, a 1,3-dialkyl-6-amino-5-chlorouracil has been reacted with a mercaptoacetic acid derivative, followed by ring closure to the lactam using acetic anhydride.[9] Nucleophilic displacement of chloride from the 5-chlorouracil is successful in this case because the lactam moieties of the uracil have been protected from ionization by alkylation. Preparation of a potential PAH cofactor with 2-amino-4-oxo substitution would require a different strategy.

Two synthetic strategies can be proposed for the construction of a 5-thiapterin: (1) annelation of the 1,4-thiazine ring onto a suitably functionalized pyrimidine and (2) annelation of the pyrimidine ring onto a 1,4-thiazine. Since 2-amino-4-oxo substitution is required for cofactor (or inhibitor) activity in the pteridine series,[5,11] we wished to maintain the same substitution pattern in the pyrimido[5,4-*b*][1,4]thiazine series. The first synthetic strategy, therefore, was chosen, since the common pyrimidine precursor would maintain the substitution in that ring while allowing maximum flexibility for substitution in the 1,4-thiazine ring.

Just prior to the initiation of our work, Nair had published a synthetic route to 8-thiapterins [2-amino-4-(3*H*)-oxopyrimido[4,5-*b*][1,4]thiazines] that utilized the desired strategy.[12] Thus, reaction of 2,5-diamino-6-mercaptopyrimidone (**2**) with an α-bromo ketone afforded the 8-thiapterins (Scheme I). We were able to reproduce this procedure and reduce the resulting 6-phenyl-8-thiapterin, **3**, to the 5,6-dihydro compound, **4**, using an excess of sodium borohydride in trifluoroacetic acid.[13] In order to adapt this route to the preparation of 5-thiapterins, the unknown 5-mercapto-6-aminopyrimidone (**5**) was required, which presumably could be made from the 5-(benzylthio)pyrimidone (**6**) by proteolysis or hydrogenolysis. Thus, preparation of **6** became the immediate synthetic goal. Attempted condensation of guanidine with ethyl α-cyano-*S*-benzylmercaptoacetate[14] in refluxing ethanol in a classical pyrimidine synthesis resulted only in fragmentation of the ester. Alternatively, high-temperature reaction (200 °C) of ethyl *S*-benzylmercaptoacetate with cyanoguanidine, with or without high-boiling cosolvents,[15] gave none of the desired pyrimidine **6**. Substitution of *S*-benzylmercaptoacetonitrile for the ethyl ester (to prepare the corresponding 2,4,6-triaminopyrimidine) also yielded only black tarry mixtures. When the analogous reaction of ethyl phenylthioacetate with cyanoguanidine was shown by TLC to afford none of the 5-(phenylthio)pyrimidone

(5) (a) Bailey, S. W.; Ayling, J. E. *Biochem. Biophys. Res. Commun.* **1978**, *85*, 1614. (b) Ayling, J. E.; Boehm, G. R.; Textor, S. C.; Pirson, R. A. *Biochemistry* **1973**, *12*, 2045.

(6) Gottschall, D. W.; Dietrich, R. F.; Benkovic, S. J.; Shiman, R. *J. Biol. Chem.* **1982**, *257*, 845.

(7) An 8a-hydroperoxide is now rigorously excluded: Lazarus, R. A.; de Brosse, C. W.; Benkovic, S. J. *J. Am. Chem. Soc.* **1982**, *104*, 6869.

(8) Various high valent iron–oxygen enzyme model compounds have recently been prepared and shown to account for some of the observed chemical and spectral properties of the peroxidases and P-450 enzymes: (a) Groves, J. T.; Nemo, T. E., Myers, R. S. *J. Am. Chem. Soc.* **1979**, *101*, 1032. (b) Chang, C. K.; Kuo, M.-S. *Ibid.* **1979**, *101*, 3413. (c) Hanson, L. K.; Chang, C. K.; Davis, M. S.; Fajer, J. *Ibid.* **1981**, *103*, 663. (d) Groves, J. T.; Haushalter, R. C.; Nakamura, M.; Nemo, T. E.; Evans, B. J. *Ibid.* **1981**, *103*, 2884. (e) Welborn, C. H.; Dolphin, D.; James, B. R. *Ibid.* **1981**, *103*, 2869. (f) Chevrier, B.; Weiss, R.; Lange, M.; Chottard, J.-C. Mansuy, D. *Ibid.* **1981**, *103*, 2899.

(9) Schroeder, E. F.; Dodson, R. M. *J. Am. Chem. Soc.* **1962**, *84*, 1904.

(10) Schroeder, E. F. U.S. Patent 3 080 364, 1963; *Chem. Abstr.* **1963**, *59*, 6420*h*. U.S. Patent 3 155 665, 1964; *Chem. Abstr.* **1965**, *62*, 4036*e*. U.S. Patent 3 270 011, 1966; *Chem. Abstr.* **1966**, *65*, 15397*g*. U.S. Patent 3 277 093, 1966; *Chem. Abstr.* **1966**, *65*, 20149*a*. U.S. Patent 3 303 192, 1967; *Chem. Abstr.* **1967**, *66*, 95072*z*.

(11) Kaufman, S.; Fisher, D. B. in "Molecular Mechanisms of Oxygen Activation"; O. Hayaishi, Ed.; Academic Press: New York, 1974, Chapter 8.

(12) Nair, M. G.; Boyce, L. H.; Berry, M. A. *J. Org. Chem.* **1981**, *46*, 3354.

(13) Cf. Gribble, G. W.; Leese, R. M.; Evans, B. E. *Synthesis* **1977**, *172*. Gribble, G. W.; Kelly, W. J.; Emery, S. E. *Ibid.* **1978**, *763*, for borohydride reductions in trifluoroacetic acid.

(14) Hayashi, S.; Furukawa, M.; Yamamoto, J.; Niigata, K. *Chem. Pharm. Bull.* **1967**, *15*, 1188.

(15) Cf. Modest, E. J.; Chatterjee, S.; Kangur, H. *J. Org. Chem.* **1962**, *27*, 2708; Modest, E. J.; Chatterjee, S.; Protopapa, H. K. *J. Org. Chem.* **1965**, *30*, 1837, for similar pyrimidine syntheses with cyanoguanidine.

Table I. Physical Data for 5-Thiapterins and Precrusors

no.	R	purified yield, %	recrystn solvent	mp, °C	formula	analyses
10a	H	66	CCl_4	94-96	$C_7H_{11}NO_3S$	C, H, N
10b	CH_3	74		oil	$C_8H_{13}NO_3S$	M^+ calcd, 203.0616; found, 203.0607
10c	C_6H_5	52	CCl_4	120-123	$C_{13}H_{15}NO_3S$	C, H, N; M^+ calcd, 265.0773; found, 265.0776
11a	H	*a*		oil	$C_9H_{15}NO_3S$	M^+ calcd, 217.0773; found, 217.0774
11b	CH_3	*a*		oil	$C_{10}H_{17}NO_3S$	M^+ calcd, 231.0929; found, 231.0931
11c	C_6H_5	*a*		oil	$C_{15}H_{19}NO_3S$	M^+ calcd, 293.1086; found, 293.1076
8a	H	48	H_2O	288-293 dec	$C_6H_8N_4OS$	C, H, N
8b	CH_3	51	EtOH	265-269 dec	$C_7H_{10}N_4OS \cdot 0.5H_2O$	C, H, N
8c	C_6H_5	34	MeOH	*b*	$C_{12}H_{12}N_4OS \cdot MeOH$	C, H, N
12a	H	92	EtOH	>300 dec	$C_6H_8N_4O_2S \cdot 0.5H_2O$	C, H, N
12b	CH_3	95	EtOH-acetone	220-230 dec	$C_7H_{10}N_4O_2S$[c]	C, N; H[d]
12c	C_6H_5	95	MeOH-acetone	246-250 dec	$C_{12}H_{12}N_4O_2S \cdot 0.75H_2O$	C, H, N

[a] Essentially quantitative: crude products were used directly in the next reaction. [b] mp ~180-185 partial (MeOH), 195 resolidify, 270-280 dec. [c] Crystallized as partial ethanolates/partial hydrates of various stoichiometry. The sample used for analysis contained 0.6EtOH and 1.0H_2O. [d] H: calcd, 6.05; found, 4.86.

Table II. UV Spectra of 5-Thiapterins and 5-Thiapterin *S*-Oxides

no.	λ_{max} (0.1 N HCl)	ϵ	λ_{max} (pH 6.8)[a]	ϵ	λ_{max} (0.1 N NaOH)	ϵ
8a	304	9 910	302	8 210	289	7 390
	277 (infl)	6 250	266	5 580	270	6 820
			223	21 200		
8b	305	9 740	302	8 540	290	7 300
	277 (infl)	6 290	267	5 950	270	6 480
			223	21 300		
8c	303	10 600	300	8 870	287	8 910
	280	8 560	277 (infl)	7 190		
			222	26 900		
12a	259	12 300	260	12 200	260	9 990
	224	17 100	224	33 900		
12b	259	12 600	261	11 600	261	10 200
	225	18 400	225	33 100		
12c	260	11 500	261	11 100	261	9 910
	225	25 800	226	37 300		

[a] 0.1 M KPO_4, pH 6.8.

(7), an authentic sample of which was available,[16] this route to **6** was abandoned.

Ultimately, the 1,4-thiazine to 5-thiapterin strategy was adopted for the preparation of **8** (Scheme II). Condensation of diethyl chloromalonate with β-mercapto amines **9** in absolute ethanol afforded the 1,4-thiazin-3-ones **10** in good yield with regiospecific placement of the 6-R group (Table I). Although similar condensations of β-mercapto amines with α-chlorocarbonyl compounds have been reported,[17,18] this appears to be the first use of diethyl chloromalonate as a condensation partner. Both ester and lactam CO stretches were observed in the IR spectra of **10** at 1725–1740 and 1635–1680 cm^{-1}, respectively. Attempted fusion reaction of **10** with guanidine carbonate at ca. 160 °C to give **8** directly[19] resulted in extensive resinification, and a UV spectrum of the product indicated that no pyrimidine chromophore had been formed (vide infra).

Exclusive alkylation of the lactam oxygen occurred with 1.2 equiv of triethyloxonium tetrafluoroborate in methylene chloride at room temperature, affording the lactim ethers **11** in essentially quantitative yield.[20] Similar regiospecific lactam alkylations have been accomplished with 3-thiomorpholone[21] and 2-carbethoxy-2,3-dihydro-1,4-benzothiazin-3-one[22] with triethyloxonium tetrafluoroborate. In the IR spectra of lactim ethers **11**, the lactam CO stretch at 1635–1680 cm^{-1} has been replaced by a C=N stretch of weaker intensity at 1690 cm^{-1}. All lactim ethers **11** were colorless oils; therefore, the exact masses of the molecular ions were determined in place of elemental analysis, and the crude lactim ethers **11** were used directly in the condensation with guanidine to form 5-thiapterins, **8** (Table I).

Condensation of lactim ethers **11** with 2 equiv of guanidine in refluxing ethanol afforded the 5-thiapterins **8** in moderate yield as tan crystalline solids.[23] The UV spectra of **8** indicated that the pyrimidine nucleus had been formed (Table II). At pH 6.8, a long-wavelength absorbance at ca. 302 nm (ϵ 8210–8870 M^{-1}) was observed. Schroeder and Dodson[9] had reported that pyrimido[5,4-*b*][1,4]thiazinetriones of the type **1** had a single UV absorbance in methanol at 317–333 nm with extinctions of 6110–8210 M^{-1}. They also noted that a UV λ_{max} (MeOH) greater than 300 nm with ϵ 6000–8750 M^{-1} was charac-

(16) Lazarus, R. A.; Dietrich, R. F.; Wallick, D. E., Benkovic, S. J. *Biochemistry* **1981**, *20*, 6834.
(17) Stoodley, R. J. *Adv. Heterocycl. Chem.* **1979**, *24*, 293.
(18) (a) Dunn, A. R.; McMillan, I.; Stoodley, R. J. *Tetrahedron* **1968**, *24*, 2985. (b) Kitchin, J.; Stoodley, R. J. *Tetrahedron* **1973**, *29*, 3023.
(19) Cf. DeGraw, J.; Goodman, L. *Can. J. Chem.* **1963**, *41*, 3137.
(20) Glushkov, R. G.; Granik, V. G. *Russ. Chem. Rev.* **1969**, *38*, 913. *Adv. Heterocycl. Chem.* **1970**, *12*, 185.
(21) Glushkov, R. G.; Todd, A. R. *Khim. Geterotsikl. Soedin.* **1968**, *4* (3), 433.
(22) Granik, V. G.; Glushkov, R. G. *Khim.-Farm. Zh.* **1971**, *5* (5), 10.
(23) Russian workers had utilized a similar reaction sequence for the synthesis of a 5-thioalloxazine.[22]

teristic of the pyrimido[5,4-*b*][1,4]thiazine ring system; the 5-(alkylthio)uracils, which serve as precursors to **1**, show λ_{max} (MeOH) ≤272 nm with ϵ 12000–15000 M^{-1}. Similarly, we have observed that the 5-(phenylthio)pyrimidone (**7**) has no long-wavelength UV absorbance: λ_{max} (pH 6.8) 267 nm (ϵ 14500), 246 (16100).

Oxidation of **8** with a slight excess of purified *m*-chloroperoxybenzoic acid in absolute ethanol afforded the 5-thiapterin *S*-oxides, **12**, in excellent yield. Oxidation could be monitored easily by UV spectroscopy: the characteristic absorbance at >300 nm disappeared rapidly and was replaced by absorbances at ca. 260 (ϵ 11000–12200 M^{-1}) and 225 nm (ϵ 33100–37300 M^{-1}) at pH 6.8. Schroeder and Dodson[9] had performed similar oxidations on **1** with peroxybenzoic acid to obtain the corresponding sulfoxide, which had a UV λ_{max} (MeOH) at 316 nm, a hypsochromic shift of 17 nm compared to **1**. This contrasts with the more than 40-nm hypsochromic shift observed upon S-oxidation of 5-thiapterins, **8**. As confirmatory evidence for the sulfoxide structure of **12**, IR spectra (in KBr) were recorded, and strong bands at 915–990 cm^{-1} were observed that were absent in the spectra of **8**. This rather low S–O stretching frequency implies that the sulfoxide is hydrogen bonded to the hydroxy tautomer of the 4-oxo group in the solid state.[24]

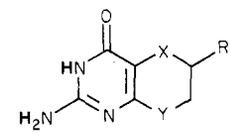
The 360-MHz ^1H NMR spectra of **8a** and **12a** were obtained in order to observe the effect of sulfur oxidation on the 6-protons. The complex multiplet at δ 2.718 for the 6-protons of **8a** was observed to split into a doublet (δ 2.806) and a doublet of triplets (δ 2.328) upon S-oxidation, indicating that only one conformer of **12a** was present. The upfield signal is assigned to the 6-axial proton by analogy with related dihydro-1,4-thiazine 1-oxides[17,18] in which the resonance of the axial proton α to sulfur is shifted upfield by as much as 0.75 ppm with respect to the equatorial proton. All the coupling constants of the ABCDX system of **12a** could be resolved (Table V; see paragraph at the end of paper concerning Supplementary Material), and their magnitudes were in agreement with model compounds.[17,18]

It should be noted that *S*-oxides **12** are stable compounds, recrystallizable from alcohols or water, in contrast to the Schroeder and Dodson *S*-oxides. The propensity for Pummerer rearrangements in the latter must be due to the high acidity of the 6-hydrogen, caused by tandem electronic effects of the 5-sulfoxide and 7-oxo groups.[25] Also, both the 5-thiapterins **8** and their *S*-oxides **12** are completely air stable in acidic, neutral, or alkaline solution, in contrast to their tetrahydropterin isosteres, which oxidize rapidly in air at neutral or alkaline pH.[26] Thus, we have generated a new class of stable compounds isosteric with the cofactors of PAH and other pterin-dependent oxygenases. The enzymology of these compounds as potential cofactors for PAH will now be discussed.

Enzymology. When assayed for cofactor activity by Shiman's method,[27] neither 5-thiapterins, **8**, or their S-oxides, **12**, nor the 8-thiapterin, **4**, was observed to function as cofactors for rat liver PAH. Also, no changes in the UV spectra of the PAH assay mixtures during incubation which could be attributed to either S-oxidation of **8** or deoxygenation of **12** were observed. However, both **8** and **12** were found to be potent inhibitors of PAH, competitive with the synthetic cofactor 6-methyltetrahydropterin (6-$MePH_4$) used in our assays (Table III, Figure 1). In general, the K_i's of **8** correlated well with the K_m's of the corresponding tetrahydropterins, being of the same order of magnitude, with the exception of the 6-phenyl compound **8c**, which binds 15-fold tighter than 6-phenyltetrahhydropterin. This is in stark contrast to the 6-phenyl-8-thiapterin, **4**, which binds quite poorly (K_i = 500 μM). The 2500-fold difference in binding of **8c** and **4** indicates that N-8 is important for proper binding to PAH; changing the heteroatom from nitrogen to sulfur greatly decreases cofactor (inhibitor) binding. N-5, however, evidently can be substituted by S, since 5-thiapterins, **8**, and their tetrahydropterin isosteres have K_i's and K_m's of the same order of magnitude. This phenomenon has been observed previously in the deazapterin series.[16] Thus, 6-methyl-5-deazatetrahydropterin (**13**) is a good inhibitor of PAH, competitive with 6,7-dimethyltetrahydropterin, with a K_i of 50 μM. In contrast, 6-methyl-8-deazatetrahydropterin (**14**) is neither a substrate nor an inhibitor: it apparently does not bind to the enzyme, since it lacks an 8-nitrogen or related heteroatom.

The 5-thiapterin *S*-oxides, **12**, are poorer inhibitors than their parent 5-thiapterins, **8**, by factors of 4–5 (R = H, CH_3) to 35 (R = C_6H_5). Presumably this is due to PAH

Table III. Kinetic Constants for Compounds 8 and 12



no.	R	X	Y	K_m, μM	K_i, μM	inhibition
	H	NH	NH	130[a]		
8a		S	NH		85	competitive
12a		SO	NH		400	competitive
	CH_3	NH	NH	40		
8b		S	NH		36	competitive
12b		SO	NH		125	competitive
	C_6H_5	NH	NH	3[a]		
8c		S	NH		0.2	competitive
12c		SO	NH		7	competitive
4		NH	S		500	competitive

[a] Data from ref 5a.

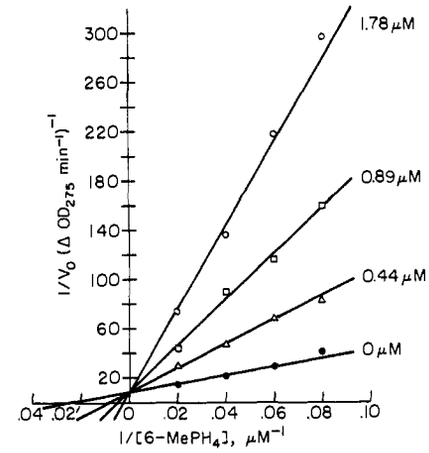


Figure 1. Lineweaver–Burk plot of PAH inhibition using 8c, vs. 6-$MePH_4$.

(24) (a) Silverstein, R. M.; Bassler, G. C.; Morrill, T. C. "Spectrometric Identification of Organic Compounds", 3rd ed.; Wiley: New York, 1974, Chapter 5. (b) Bellamy, L. J. "The Infrared Spectra of Complex Molecules", 2nd ed., Chapman and Hall: London, 1980; Vol. 2, pp 195–200.
(25) Cf. Bobek, M. *J. Heterocyl. Chem.* **1982**, *19*, 131.
(26) (a) Armarego, W. L. F.; Schou, H. *Aust. J. Chem.* **1978**, *31*, 1081. (b) Mager, H. I. X. in "Chemistry and Biology of Pteridines"; Pfleiderer, W., Ed.; Walter de Gruyter: Berlin, 1975; pp 753–773.
(27) Shiman, R.; Gray, D. W.; Pater, A. *J. Biol. Chem.* **1979**, *254*, 11300.

being intolerant of steric congestion at the 5 position. Thus, it has been shown that alkylation of N-5 of a tetrahydropterin reduces binding affinity.[16] For example, 5,6,7-trimethyltetrahydropterin has a K_i (competitive) of 330 μM, whereas 6,7-dimethyltetrahydropterin has a K_m of 90–100 μM.[16] Also, none of the isolable 4a-adducts of **13** bind to PAH.[28] These adducts are congested at the 4a–5 positions.

The air-stable 6-(phenylthio)pyrimidone (**7**) also serves as an excellent inhibitor of PAH, with a K_i of 4.7 μM.[16] This inhibitor differs from **8c** in that it lacks the 6,7 bridge of the pyrazine ring. In general, tetrahydropterins are better cofactors and/or inhibitors than their corresponding pyrimidines lacking the 6,7 bridge.[5,16] 5-Thiapterin **8c** is the best competitive inhibitor of PAH presently known.

In conclusion, structural requirements for successful hydroxylation cofactors are rather inflexible. Our work indicates that N-8 is necessary for binding to PAH, and N-5 is required for the appropriate redox chemistry with oxygen to occur. Substitution of sulfur for N-5 provides air-stable isosteres of tetrahydropterins, which are excellent competitive inhibitors of PAH but which do not intercept a hydroxylating species.

Experimental Section

IR spectra were measured on a Perkin-Elmer 735 spectrophotometer. UV spectra and inhibition studies were performed on a Cary 118 or 219 spectrophotometer. ^1H NMR spectra were recorded on a Varian EM360 (60 MHz) or Bruker 360 MHz instrument with Me_4Si as internal standard. Melting points were obtained with a Fisher-Johns melting point apparatus and are uncorrected. Mass spectra were measured by the PSU Mass Spectrometry Facility with a Kratos MS9/50. Microanalyses were performed by MHW Laboratories, Phoenix, AZ.

Reagents were obtained from the following sources: guanidine hydrochloride, (99%) and diethyl chloromalonate (96%), Aldrich Chemical Co.; β-mercaptoethylamine hydrochloride (**9a**), Sigma Chemical Co.; triethyloxonium tetrafluoroborate, Alfa-Ventron Corp. MCPBA (Aldrich Chemical Co.) was purified by washing with a pH 7.5 phosphate buffer.[29] β-Mercaptopropylamine hydrochloride (**9b**) and β-mercaptophenethylamine hydrochloride (**9c**) were prepared from the amino alcohols via thiazolidine-2-thiones.[30] EtOH was degassed by purging with dry nitrogen for at least 0.5 h.

PAH was purified from rat liver by the method of Shiman et al.[27] 6-MePH_4 was prepared as previously described.[16]

2-Amino-4(3*H*)-oxo-6-phenylpyrimido[4,5-*b*][1,4]thiazine (3). A solution of 110 mg (0.60 mmol) of 2,5-diamino-6-mercaptopyrimidone (**2**)[12] and 140 mg (0.70 mmol) of freshly prepared α-bromoacetophenone[31] in 100 mL of 20% H_2O–EtOH was refluxed for 4.5 h. The yellow solution was concentrated to ca. 15 mL by rotary evaporation and cooled, providing orange crystals of **3**, which were collected and washed with EtOH and ether: yield 156 mg (94%); mp 281–283 °C dec; UV λ_{max} (0.1 N NaOH) 378, 266, 235 nm; NMR (F_3AcOH) δ 4.75 (s, 2, H-7), 7.4–8.3 (m, 5, C_6H_5).

2-Amino-4(3*H*)-oxo-6-phenyl-5,6-dihydropyrimido[4,5-*b*][1,4]thiazine (4). To a solution of 62 mg (0.24 mmol) of **3** in 2 mL of trifluoroacetic acid was added 50 mg (1.3 mmol) of sodium borohydride in several portions. The pale yellow solution was concentrated to ca. 1 mL with a stream of nitrogen, and 20 mL of ether was added. The precipitate was washed with ether to give 73 mg of off-white powder, mp 250–258 °C dec. Recrystallization from aqueous acetone afforded 32 mg (51%) of the pale yellow free base: mp 255 °C dec; UV λ_{max} (0.1 N HCl) 326, 282, 230 nm; UV λ_{max} (0.5 N KOH) 309, 227 nm; UV λ_{max} (0.2 M KPO_4, pH 6.8) 326, 238 nm. Anal. ($C_{12}H_{12}N_4OS$) C, H, N.

2-(Ethoxycarbonyl)-5,6-dihydro-2*H*-[1,4]thiazin-3-ones (10a–c). Under a dry nitrogen atmosphere, 3.90 g (20 mmol) of diethyl chloromalonate was added to an ice-cold solution of 41 mmol of sodium ethoxide (from 0.94 g of Na) in 100 mL of degassed EtOH; 20 mmol of the desired β-mercapto amine hydrochloride, **9a–c**, in 50 mL of degassed EtOH was added dropwise with stirring over 0.5 h. The mixture was allowed to stir overnight at room temperature (20 h) and neutralized with 1 N HCl. The ethanol was evaporated, and the residue was suspended in water and brine. Extracting with $CHCl_3$, drying the extracts over anhydrous Na_2SO_4, and evaporating afforded the crude 1,4-thiazines. Purification was achieved by recrystallization from CCl_4 or by medium-pressure silica gel column chromatography[32] with EtOAc–CH_2Cl_2 mixtures.

2-(Ethoxycarbonyl)-3-ethoxy-5,6-dihydro-2*H*-[1,4]thiazines (11a-c). Under a dry nitrogen atmosphere a solution of 10 mmol of the 1,4-thiazin-3-one, **10**, and 12 mmol of triethyloxonium tetrafluoroborate in 60 mL of dry CH_2Cl_2 was stirred at room temperature overnight (20 h). The mixture was cooled in ice, and 50% aqueous K_2CO_3 was added until the pH remained alkaline. Rapid stirring was continued for 15 min in ice, the mixture was filtered to remove KBF_4, and the layers were separated. The aqueous layer was extracted with CH_2Cl_2, and the combined organic layers were dried over anhydrous Na_2SO_4. Evaporation of the solvent afforded the lactim ethers as oils, which were used directly in the next reaction. Purification could be achieved by medium-pressure silica gel column chromatography[32] with 7.5% EtOAc–CH_2Cl_2.

2-Amino-4(3*H*)-oxopyrimido[5,4-*b*][1,4]thiazines (8a–c). To 20 mmol of sodium ethoxide (from 0.46 g of Na) in 50 mL of degassed absolute EtOH was added 2.0 g (21 mmol) of dry guanidine hydrochloride and 10 mmol of the crude 2-(ethoxycarbonyl)-3-ethoxy[1,4]thiazine, **11**. The mixture was refluxed under dry nitrogen overnight (20 h). After neutralization with HCl and cooling, the NaCl was removed by filtration and the EtOH was evaporated. The residue was triturated with water (R = H, CH_3) or acetone (R = Ph) and filtered, and the tan solids were recrystallized from water or methanol (Table I).

2-Amino-4(3*H*)-oxopyrimido[5,4-*b*][1,4]thiazine *S*-Oxides (12a–c). The 5-thiapterin **8** (20 mg) was dissolved in 15–20 mL of absolute EtOH with warming. The solution was cooled to room temperature, and 20 mg of purified[29] *m*-chloroperoxybenzoic acid was added. After 2–3 h at room temperature, the white product was collected by filtration. If the product did not precipitate, 2 vol of acetone was added. Recrystallization was from EtOH or EtOH–acetone (Table I).

Inhibition Studies with PAH. The enzyme assay utilized for inhibition studies measured the initial rate of tyrosine formation at 25 °C by following the increase in absorbance at 275 nm.[16,27] 6-MePH_4 (12.5–50 μM) was used as the cofactor vs. the following inhibitor concentrations (μM): **8a**, 0–213; **8b**, 0–100; **8c**, 0–1.78; **12a**, 0–306; **12b**, 0–258; **12c**, 0–38. Lineweaver–Burk plots showed inhibition competitive with 6-MePH_4 in all cases (cf. Figure 1). The derived K_i's are presented in Table III.

Acknowledgment. We gratefully acknowledge the assistance of Dr. Robert Minard and Greg Hancock of the Penn State University Mass Spectrometry Facility for obtaining the mass spectra and the National Institutes of Health for a postdoctoral fellowship to R.N.H.

Registry No. **2**, 37489-38-6; **3**, 84099-69-4; **4**, 84099-70-7; **8a**, 84099-71-8; **8b**, 84099-72-9; **8c**, 84099-73-0; **9a**·HCl, 156-57-0; **9b**·HCl, 4146-16-1; **9c**·HCl, 3852-66-2; **10a**, 84099-74-1; **10b**, 84099-75-2; **10c**, 84099-76-3; **11a**, 84099-77-4; **11b**, 84099-78-5; **11c**, 84099-79-6; **12a**, 84099-80-9; **12b**, 84099-81-0; **12c**, 84099-82-1; α-bromoacetophenone, 70-11-1; diethyl chloromalonate, 14064-10-9; guanidine hydrochloride, 50-01-1; triethyloxonium tetrafluoroborate, 368-39-8; L-phenylalanine hydroxylase, 9029-73-6.

Supplementary Material Available: Tables IV and V containing IR and NMR data for selected new compounds (2 pages). Ordering information is given on any current masthead page.

(28) Moad, G.; Luthy, C.; Benkovic, P. A.; Benkovic, S. J. *J. Am. Chem. Soc.* **1979**, *101*, 6068.
(29) Schwartz, N. N.; Blumbergs, J. H. *J. Org. Chem.* **1964**, *29*, 1976.
(30) (a) Dewey, C. S.; Bafford, R. A. *J. Org. Chem.* **1965**, *30*, 491. (b) Owen, T. C. *J. Chem. Soc. C* **1967**, 1373.
(31) Rather, J. B.; Reid, E. E. *J. Am. Chem. Soc.* **1919**, *41*, 75–83.
(32) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.