of solid were combined and suspended in H_2O, the pH was adjusted to 2 with 3 N HCl, and the aqueous phase was washed with Et_2O. The aqueous phase was then adjusted to pH 8 with concentrated NH_4OH and extracted several times with EtOAc. The combined extracts were extracted with saturated brine, dried over $MgSO_4$, and treated with activated charcoal (Darco), and a slight excess of ethereal HCl was added which caused a precipitate to form. This was filtered and dried and recrystallized twice from EtOH–Et_2O to give 309 mg (10%) of **18**·HCl as an off-white crystalline solid, mp 229–230 °C. Anal. ($C_{17}H_{19}NO_3$·HCl·0.5H_2O) C, H, N. A ^1H NMR was consistent with structure **18**·HCl.

Biological Test Procedures. Isolated Perfused Rabbit Ear Artery. This procedure was thoroughly described[10] and is based on methodology reported by Hieble and Pendleton[17] and Steinsland and Hieble[18] using an apparatus developed by Steinsland et al.[25] Results are reported as the nanomolar concentration of compound necessary to inhibit 50% of the constrictor response (increase in perfusion pressure) to electrical stimulation.

Adenylate Cyclase. This method was also thoroughly described[10] and is based on methodology reported by Setler et al.[6] using cAMP measurement procedures of Kebabian et al.[26] and Carenzi et al.[27] Results are reported as the nanomolar concentration of compound necessary to cause a 50% increase in cAMP formation relative to the maximum increase of this compound over the range of concentrations tested.

Acknowledgment. We thank Caleb Jervay and James Foley for expert technical assistance in running the rabbit ear artery assay and adenylate cyclase assay, respectively. We also thank E. Reich for performing the elemental analyses. Appreciation is also expressed to Carl Kaiser for advice and counsel on manuscript preparation.

Registry No. **1**, 77386-12-0; **1**·CH_3SO_3H, 77386-13-1; **2**, 72912-39-1; **2** (acid), 63856-83-7; **2** (acid chloride), 104421-97-8; **3**, 71157-96-5; **4**, 104421-89-8; **5**, 104421-90-1; **5**·CH_3SO_3H, 104421-91-2; **6**, 7537-08-8; **6** (acid), 78495-65-5; **6** (acid chloride), 103346-89-0; **7**, 72912-25-5; **8**, 104421-92-3; **9**, 104422-02-8; **9**·CH_3SO_3H, 104438-47-3; **9**·HBr, 104422-00-6; **10**, 93-40-3; **10** (acid chloride), 10313-60-7; **11**, 104421-93-4; **12**, 104421-94-5; **13**, 104422-03-9; **13**·HBr, 104421-95-6; **14**, 120-20-7; **15**·HBr, 20012-10-6; **15**·HCl, 62717-42-4; **16**, 104422-04-0; **16**·HCl, 62751-58-0; **17**·CH_3SO_3H, 104113-88-4; **18**, 104422-05-1; **18**·HCl, 104421-96-7; (*p*-methoxyphenyl)ethanolamine acetate, 93981-57-8; *N*-[2-hydroxy-2-(4-methoxyphenyl)ethyl]-2-(2-bromo-3,4-dimethoxyphenyl)acetamide, 104421-98-9; *N*-[2-hydroxy-2-(4-methoxyphenyl)ethyl]-2-(2-bromo-3,4-dimethoxyphenyl)ethylamine, 104113-97-5; allyl bromide, 106-95-6; (*p*-methoxyphenyl)-ethanolamine, 55275-61-1; *N*-[2-(*p*-methoxyphenyl)-2-hydroxyethyl]-2-(3,4-dimethoxy-2-fluorophenyl)acetamide, 104421-99-0; *N*-[2-(*p*-methoxyphenyl)-2-hydroxyethyl]-2-(3,4-dimethoxy-2-fluorophenyl)ethylamine, 95413-95-9; *N*-[2-hydroxy-2-(*p*-methoxyphenyl)ethyl]-2-(3,4-dimethoxyphenyl)acetamide, 104422-01-7; *N*-[2-hydroxy-2-(4-methoxyphenyl)ethyl]-2-(3,4-dimethoxyphenyl)ethylamine, 62717-74-2; styrene oxide, 96-09-3; *N*-(2-hydroxy-2-phenylethyl)homoveratrylamine, 20011-97-6; *p*-methoxymandelonitrile, 33646-40-1.

(25) Steinsland, O. S.; Furchgott, R. F.; Kirkepas, S. M. *J. Pharmacol. Exp. Ther.* **1973**, *184*, 346–356.
(26) Kebabian, J. W.; Petzold, G. L.; Greengard, P. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 2145.
(27) Carenzi, A.; Gillin, J. C.; Guidotti, A.; Schwartz, M. A.; Trabucchi, M.; Wyatt, R. J. *Arch. Gen. Psychiatr.* **1975**, *32*, 1056.
(28) Guidotti, A.; Weiss, B.; Costa, E. *Mol. Pharmacol.* **1972**, *8*, 521.

Synthetic Analogues of Tetrahydrobiopterin with Cofactor Activity for Aromatic Amino Acid Hydroxylases

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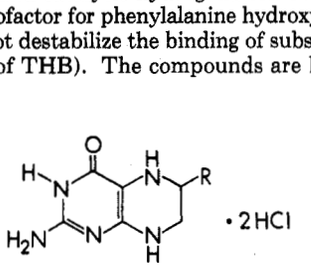
Tetrahydrobiopterin (THB) analogues with 6-alkoxymethyl substituents, **3a–j**, where the substituents were straight- and branched-chain alkyl ranging from methyl to octyl, have been synthesized by the Taylor method from pyrazine ortho amino nitriles by guanidine cyclization, hydrolysis in aqueous NaOH, and catalytic hydrogenation over Pt in trifluoroacetic acid (TFA). The best of these compounds, **3b**, is an excellent cofactor for phenylalanine hydroxylase, tyrosine hydroxylase (V = 154% of THB), and tryptophan hydroxylase, does not destabilize the binding of substrate (K_m^{tyr} = 23 μM), and is recycled by dihydropteridine reductase (V = 419% of THB). The compounds are being evaluated as cofactor replacements in biopterin-deficiency diseases.

(6*R*)-L-*erythro*-Tetrahydrobiopterin (THB, 1) is known to be the cofactor for the monooxygenases that hydroxylate phenylalanine, tyrosine, and tryptophan (see Scheme I).[1,2] For a complete description of the biosynthesis and biochemistry of THB, see the reports by Smith, Duch, and Nichol.[2,3] The critical role that THB plays in the rate-limiting step in the biosynthesis of neurotransmitter amines and the metabolism of phenylalanine forms the basis for its involvement in diseases in which cofactor availability or synthesis may by inadequate. With the recognition of the deficiency of THB in various diseases, especially Parkinson's disease (PD), came the attempt to treat patients with exogenous cofactor.

Abnormal THB levels in blood, cerebrospinal fluid (CSF), and urine have been observed in several diseases with use of different assay procedures.[1a,2] Of particular interest are reports associating decreases in THB content



1, R = -CH(OH)-CH(OH)-CH_3

2, R = Alkyl

3, R = -CH_2OR

in blood, cerebrospinal fluid, and urine with neurological and psychiatric diseases.[4a–e] CSF THB levels measured

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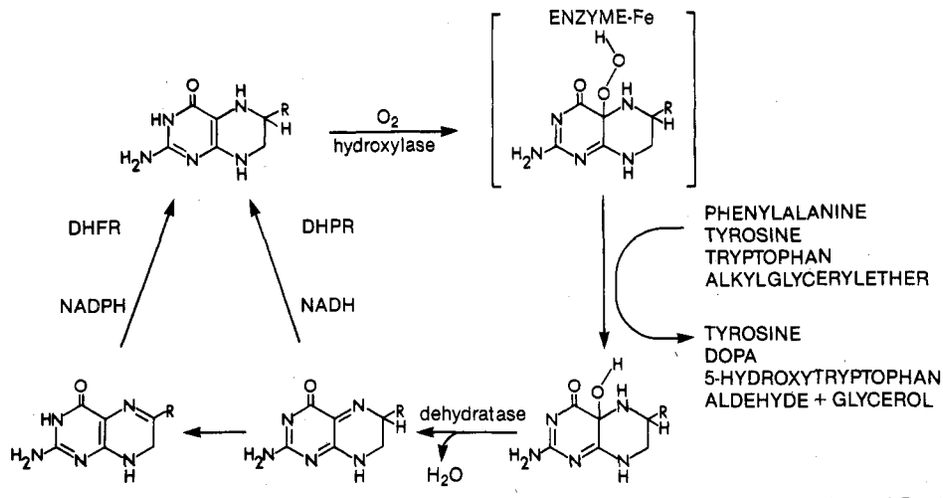
(1) (a) Kaufman, S. J. *J. Biol. Chem.* **1958**, *230*, 931; *Adv. Hum. Gene.* **1983**, *13*, 217. (b) Nagatsu, T.; Mitzutani, K.; Nagatsu, I.; Matuura, S.; Sugimoto, T. *Biochem. Pharmacol.* **1972**, *21*, 1945. (c) Hosoda, S.; Glick, D. *J. Biol. Chem.* **1966**, *241*, 192.
(2) Nichol, C.; Smith, G.; Duch, D. *Annu. Rev. Biochem.* **1985**, *54* 729.

0022-2623/87/1830-0040$01.50/0

Scheme I[a]



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by HPLC may indicate changes in biopterin metabolism in brain, but the relationship of serum and urinary THB levels to CNS diseases is hard to interpret because of dilution by THB from peripheral tissue.

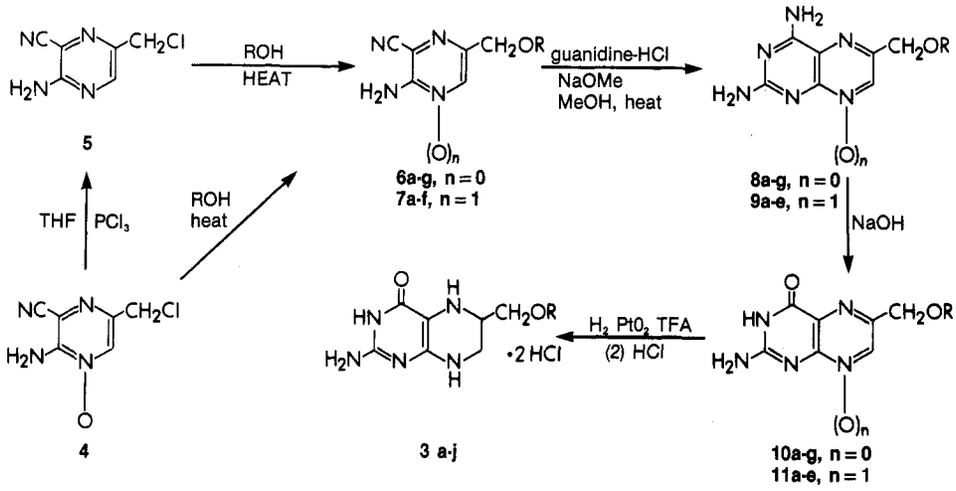
A THB deficiency has been found in atypical phenylketonuria (PKU), which is characterized by metabolic defects in the synthesis or regeneration of THB.[5] Blair et al. found decreased levels of biopterin in the urine (but see above) of patients with bipolar, but not unipolar, affective disorders.[4c] Conversely, Van Kammen et al. found normal levels of total biopterin in CSF of patients with schizophrenia and affective disorders.[4d] Levels of THB in CSF of PD patients are about half those of normal subjects.[6-9] In some cases of torsional dystonia, CSF THB levels were normal,[8] whereas total biopterin was lower than normal in other patients with general dystonia[10] and drastically lower in patients with inherited dystonia.[11] Also, a THB deficiency in CSF has been observed in a number of other diseases including senile dementia, Alzheimer's disease, Jakob–Creutzfeldt disease, Shy–Drager syndrome, Steel–Richardson syndrome, and lead or aluminum poisoning.[12-15]

Of the various diseases, the greatest opportunity for cofactor replacement therapy is in early Parkinson's disease when the degree of neuronal loss is still associated with retention of tyrosine hydroxylase and some capacity for dopamine synthesis in the striatum. First, the number of PD patients is increasing as the average population age increases. Currently, the disease strikes roughly 1% of people over the age of 60. Present therapy is limited in many ways.[16] The efficacy of THB in Parkinson's disease has been studied in a few patients receiving single doses of THB resulting in improvement in two cases[4a] and no change in another.[17] The limitations in the use of THB for therapy are difficulty of synthesis, expense, poor oral absorption, and poor entry into the brain.[18] However, the temporary responses of patients treated with THB encourage the search for a better compound for cofactor replacement therapy. Second, any information that is obtained on the activity of cofactor analogues in PD may be applicable to other, less understood neurological conditions.

Responses to cofactor replacement therapy in other diseases have been variable. Kaufman et al. reported improvement in one atypical PKU patient given THB, but another showed no persistent improvement with THB or the analogue 6-MTHP (**2**, R = Me).[19a] McInnes et al. found that 6-MTHP controlled the hyperphenylalaninemia but not the neurological problems of an atypical PKU patient.[19b] In contrast, Niederwieser et al. found that THB at doses of 22 mg/kg relieved all symptoms for 4 days in one patient, for 1 day in a second patient, and not at all in a third patient.[19c] The treatment of some dystonias with THB resulted in improvements in motor function in several patients. Three patients with foot dystonias showed improvement within 45 min of an iv dose of THB at 1.5–5

(3) Smith, G.; Nichol, C. *Biochem. Biophys. Res. Commun.* **1984**, *120*, 761.
(4) (a) Curtius, H.-Ch.; Müldner, H.; Niederwieser, A. *J. Neural Transm.* **1982**, *55*, 301. (b) Curtius, H.-Ch.; Niederwieser, A.; Levine, R.; Lovenberg, W.; Woggon, B.; Angst, J. *Lancet* **1983**, (i), 657. (c) Blair, J.; Morar, C.; Hamon, C.; Barford, P.; Pheasant, A.; Whitburn, S.; Leeming, R.; Ynolds, G.; Coppen, A. *Lancet* **1984**, (ii), 163. (d) Van Kammen, D.; Levine, R.; Sternberg, D. *Psychopharmacol. Bull.* **1978**, *14*(4), 51. (e) Levine, R.; Lovenberg, W. *Lancet* **1984**, (i), 283.
(5) (a) Dhondt, J.-L. *J. Pediatr. (St. Louis)* **1984**, *104*, 501. (b) Danks, D., et al. *J. Inherited* Metab. *Dis.* **1978**, *1*, 49.
(6) Lovenberg, W.; Levine, R.; Robinson, D.; Ebert, M.; Williams, A.; Calne, D. *Science (Washington, D.C.)* **1979**, *204*, 624.
(7) (a) Nagatsu, T., et al. *Adv. Neurol.* **1984**, *40*, 407. (b) Nagatsu, T.; Yamaguchi, T.; Kato, T.; Sugimoto, T.; Matsuura, S.; Akino, M.; Nagatsu, I.; Iizuka, R.; Narabayashi, H. *Clin. Chim. Acta* **1981**, *109*, 305.
(8) Robinson, D.; Levine, R.; Williams, A.; Statham, N. *Psychopharmacol. Bull.* **1978**, *14*(4), 49.
(9) Williams, A.; Ballenger, J.; Levine, R.; Lovenberg, W.; Calne, D. *Neurology* **1980**, *30*, 1244.
(10) LeWitt, P.; Newman, R.; Miller, L.; Lovenberg, W.; Eldridge, R. *N. Engl. J. Med.* **1983**, *308*, 157.
(11) Williams, A.; Eldridge, R.; Levine, R.; Lovenberg, W.; Paulson, G. *Lancet* **1979**, (ii), 410.
(12) Leeming, R. *J. Ment. Defic. Res.* **1981**, *25* (Part 4), 231.
(13) Dhondt, J.; Farriaux, J. *Path Biol.* **1980**, *28*, 397.
(14) Williams, A.; Levine, R.; Chase, T.; Lovenberg, W.; Caine, D. *J. Neurol., Neurosurg. Psychiatry* **1980**, *43*, 735.
(15) Leeming, R.; Blair, J.; Melikian, V. *Lancet* **1979**, (i), 215.
(16) Muenter, M. *Current Therapy* **1984**, 766–773. Caine, D. *Clin. Neuropharmacol.* **1982**, *5* (Suppl. 1), S38.
(17) LeWitt, P. *Int. Symp. Parkinson's Disease, 7th* **1982**, 83.
(18) Kapatos, G.; Kaufman, S. *Science (Washington, D.C.)* **1981**, *212*, 655.
(19) (a) Kaufman, S.; Kapatos, G.; McInnes, R.; Schulman, J.; Rizzo, W. *Pediatrics* **1982**, *70*, 376. (b) McInnes, R.; Kaufman, S.; Warsh, J.; Van Loon, G.; Milstien, S.; Kapatos, G.; Soldin, S.; Walsh, P.; MacGregor, D.; Hanley, W. *J. Clin. Invest.* **1984**, *73*, 458. (c) Niederwieser, A.; Curtius, H.; Wang, M.; Leupoid, D. *Eur. J. Pediatr.* **1982**, *138*, 110.

Scheme II



mg/kg.[10] The relationship between THB and depression has been studied. Curtius et al. obtained improvement in two patients with inhibited endogenous depression given a single 1-g dose of THB orally, but a patient with agitated endogenous depression did not respond.[4a] In a later study, a patient whose symptoms were controlled by 500 mg/day of THB relapsed at 100 mg/day.[4b]

With this background, we sought to prepare synthetic cofactor analogues that overcome the limitations of THB. We now report that compounds of structure **3** have interesting activity as biopterin cofactor analogues.[20]

Chemistry

Previous work on the synthesis of biopterin and analogues has been reported by Pfleiderer,[21] Bailey and Ayling,[22] Nagatsu,[23] Armarego et al.,[24] Viscontini and Bieri,[25] and Taylor and Jacobi.[26] For this work, we relied primarily on the general method of Taylor et al.[27] This sequence offers good versatility while providing regiochemical control (Scheme II). Initially, the *N*-oxide **4** was deoxygenated by treatment with PCl_3 to obtain pyrazine **5**. However, we discovered that the *N*-oxide could be carried through the entire Taylor sequence and reduced in the last step along with the pyrazine ring. This alteration shortened the sequence by one step and gave more easily handled intermediates. The ethers **6** and **7** were prepared by heating the chloromethyl compounds **4** and **5** in the appropriate alcohol until the reaction was complete.[28] Reaction times were long for hindered alcohols, but yields were still acceptable. The addition of bases caused decomposition. Yields ranged from 41% to 83% after recrystallization (hexanes, CH_2Cl_2, or CCl_4) or chromatography (see Table I).

The pyrazine *N*-oxides **7** are characterized in the NMR spectra (Me_2SO-d_6) by singlets at δ 4.35 for Ar CH_2O and δ 8.35 for the ring hydrogen. This latter value compared to δ 8.25 for the pyrazines **6**. The IR spectra contain nitrile absorptions at 2220–2240 cm^{-1} and strong bands in the 1650-, 1525-, 1350-, 1140-, 1095-, and 805-cm^{-1} regions. The UV maxima in 0.1 N HCl are at 225, 250, and 380 nm.

Cyclization of the ortho amino nitriles to diaminopteridines **8** and **9** was carried out under the exact conditions of Taylor.[28] In some cases the products precipitated from the reaction mixture and were filtered off, but in other cases the solvent was evaporated, and the crude product was washed with water. The crude products were recrystallized from methanol (pteridines **8**) or 2-methoxyethanol (*N*-oxides **9**), and the average yield was 68%. The infrared spectra of diaminopteridines generally contain peaks at 1630, 1540, 1450, 1350, 1180, 1050, and 990 cm^{-1}. The NMR spectra (Me_2SO-d_6) of *N*-oxides **9** have characteristic peaks at δ 4.50 for Ar CH_2O and δ 8.35 for the aromatic ring proton, while the shifts for pteridines

Table I. 3-Aminopyrazine-2-carbonitriles **6** and 4-Oxides **7**

no.	R	rctn time, h	yield, %	mp, °C	anal.
6a	Me	24	70	amorph	ref 27
6b	Et	25.5	73	108–109	C, H, N
6c	*n*-Pr	96	68	79–80.5	C, H, N
6d	2-Pr	128	53	92–94	C, H, N
6e	*n*-Bu	143	82	82–83	C, H, N
6f	*t*-Bu	825	53	125–126	C, H, N
6g	*n*-octyl	86	46	87–88	C, H, N
7a	Me	18	80	133–135	C, H, N
7b	Et	168	78	117–118	C, H, N
7c	*n*-Bu	55[a]	58	77–79	C, H, N
7d	*i*-Bu	47	41	94–95	C, H, N
7e	*n*-pentyl	111	69	65–66	C, H, N
7f	CH_2CH_2OMe	98	83	97–99	C, H, N

[a] At 70 °C for 288 h, the yield was 77%.

Table II. Diaminopteridines **8** and *N*-Oxides **9**

no.	R	recrystn solvent[a]	yield, %	mp °C (dec)	anal.
8a	Me	A	70	248–251	C, H, N
8b	Et	A	56	247–248	C, H, N
8c	*n*-Pr	A	66	239–243	C, H, N
8d	2-Pr	A	81	285–286	C, H, N
8e	*n*-Bu	A	76	242	C, H, N
8f	*t*-Bu	A	66	286–287	C, H, N
8g	*n*-octyl	A	70	235	C, H, N
9a	Me	A	75	254–256	C, H, N
9b	*n*-Bu	A	44	>250	C, H, N
9c	*i*-Bu	B	72	>250	C, H, N
9d	*n*-pentyl	B	75	255	C, H, N
9e	CH_2CH_2OMe	A	65	241–242	C, H, N

[a] A = MeOH and B = $HOCH_2CH_2OMe$.

(20) This work was first reported by us in European Patent Application 0 108 890 (19 Mar 1983) from The Wellcome Foundation Ltd. Since the conclusion of our work, similar work was reported by Traub and Pfeiderer.[41]
(21) Pfleiderer, W. *J. Inherited Metab. Dis.* **1978**, *1*, 54.
(22) Bailey, S.; Ayling, J. *Biochemistry* **1983**, *22*, 1790.
(23) Nagatsu, T. *Neurochem. Int.* **1983**, *5*, 27.
(24) Armarego, W.; Waring, P.; Paal, B. *Aust. J. Chem.* **1982**, *35*, 785.
(25) Vicontini, M.; Bieri, J. *Helv. Chim. Acta* **1972**, *55*, 21.
(26) Taylor, E. C.; Jacobi, P. *J. Am. Chem. Soc.* **1974**, *96*, 6781.
(27) Taylor, E. C.; Perlman, K.; Sword, I.; Sequin-Frey, M.; Jacobi, P. *J. Am. Chem. Soc.* **1973**, *95*, 6407. Taylor, E. C.; Perlman, K.; Kim, Y.-H.; Sword, I.; Jacobi, P. *J. Am. Chem. Soc.* **1973**, *95*, 6413.
(28) Taylor, E. C.; Kobayashi, T. *J. Org. Chem.* **1973**, *38*, 2817.

Table III. 2-Amino-4(3*H*)-pteridinones **10** and 8-Oxides **11**

no.	R	reaction condtns[a]	temp °C	yield, %	anal.
10a	Me	A	70	78	C, H, N
10b	Et	A	75	92	C, H, N
10c	*n*-Pr	A	80	73	C, H, N
10d	2-Pr	A	90	68	C, H, N
10e	*n*-Bu	A	90	84	C, H, N
10f	*t*-Bu	B	reflux	83	C, H, N[b]
10g	*n*-octyl	A	90	73	C, H, N
11a	Me	A	70	89	C, H, N
11b	*n*-Bu	B	reflux	90	*c*
11c	*i*-Bu	B	reflux	85	*d*
11d	*n*-pentyl	B	reflux	49[e]	C, H, N[f]
11e	CH_2CH_2OMe	A	80	67	C, H, N

[a] A: 1 N NaOH. B: 1 N NaOH/EtOH. [b] Includes 0.2 H_2O. [c] Calcd for $C_{11}H_{15}N_5O_3$: C, 49.80; H, 5.70; N, 26.40. Found: C, 47.03; H, 5.37; N, 25.26. Contains 5.4% inorganics. [d] Calcd for $C_{11}H_{15}N_5O_3$: C, 49.80; H, 5.70; N, 26.4. Found: C, 47.6; H, 5.39; N, 25.21. Contains 4.4% inorganic impurity. [e] After recrystallization from H_2O. [f] Calcd for $C_{12}H_{17}N_5O_3 \cdot 0.5H_2O$: C, 49.99; H, 6.29; N, 24.29. Found: C, 49.95; H, 5.80; N, 24.26.

8 are δ 4.58 and 8.73. The ultraviolet spectra of **9** in 0.1 N HCl display maxima at 253, 282, 356, and 370 nm. See Table II.

The hydrolysis of **8** and **9** to pterins **10** and **11** was usually performed in 1 N NaOH at 75–90 °C. The progress of the reaction was followed by UV spectroscopy. Under these conditions, the hydrolysis of the more lipophilic analogues was slow, presumably because of pteridine insolubility, but the use of 0.5 N NaOH in 50% ethanol alleviated this problem. The pterins **10** and **11** were precipitated from basic solution by the addition of acetic acid and recrystallized from methoxyethanol when necessary. Yields for this step ranged from 67% to 92% (Table III). The spectra of pterin *N*-oxides **11** have common NMR peaks (Me_2SO-d_6) at δ 4.45 (Ar CH_2O) and δ 8.35 (H-7) and UV maxima at 262 and 388 nm in 0.1 N NaOH. The corresponding UV maxima of pterins **10** show a shift to 255 and 364 nm, and there is a shift in the NMR peaks to δ 4.56 and 8.68. The IR spectra of both sets of pterins have peaks near 1700, 1600, 1530, 1490, 1390, 1240, 1180, 1110, and 860 cm^{-1}.

The final step in the syntheses required the reduction of the pterins to the tetrahydro forms and isolation as the stable dihydrochloride salts. The reduction was most readily accomplished by catalytic hydrogenation over PtO_2 in trifluoroacetic acid (TFA) according to Bobst and Viscontini.[29] Following addition of 6 N HCl, the crude products were isolated and purified by recrystallization from 6 N HCl/MeCN. The dihydrochloride monohydrates were isolated in 48–84% yield. In large-scale preparations, 1:1 DMF/AcOH was substituted for TFA in the reduction step. The *tert*-butyl ether **3g**, which was unstable under the normal reaction conditions, was prepared by reduction in AcOH rather than TFA and was crystallized in 50% yield from cold, deoxygenated MeOH by the careful addition of excess cold 10 N HCl under N_2. The HCl salts **3** were generally stable once isolated and dried (see Table IV). Tetrahydropterins are autoxidized readily to the 7,8-dihydro form in neutral solutions with half-lives of 7 min in pH 7 phosphate and 21 min in pH 7 Tris buffer. The tetrahydropterins **3** are characterized by a strong UV band at 264 nm in 0.1 N HCl (ϵ = 14 600 ± 600, n = 13). Typical IR bands are observed at 1705, 1660 (s), 1609, 1460, 1370, 1220, 1120, and 750 cm^{-1}. The NMR spectra in Me_2SO-d_6 were not well-resolved at 80 MHz.

(29) Bobst, A.; Viscontini, M. *Helv. Chim. Acta* **1966**, *49*, 875.

Results and Discussion

The synthetic cofactor analogues described above were tested in isolated enzyme assays with tyrosine hydroxylase, phenylalanine hydroxylase, and tryptophan hydroxylase. The phenylalanine hydroxylase (PheH) was isolated from rat liver,[30] and the tryptophan hydroxylase (TrpH) was isolated from rat brain stem.[31] The tyrosine hydroxylase (TyrH) was isolated from bovine adrenal medulla.[32] The enzyme TyrH exists in two forms that have different cofactor affinities. In the cell, the low-affinity form is converted to the high-affinity form by phosphorylation. In the native state, the majority of the enzyme is in the low-affinity form. Partial trypsin digestion of TyrH produces a model of the high-affinity form.[33] Compounds **3** are also cofactors for purified, low-affinity enzyme from rat striatum.[34] The assay methods were described previously.[31,35-37]

In general, the synthetic analogues possessed excellent cofactor activity with all three hydroxylase enzymes (see Table V). With TyrH, the relative rate of hydroxylation driven by the best synthetic cofactors was equal to or greater than that when THB was the cofactor. In addition, the binding (K_m) of some analogues was tighter than that of THB or 6-MTHP. The K_m values were lowest with the propyl analogues **3c** and **3d**, whereas the *t*-Bu (**3g**) and methoxyethyl (**3i**) compounds were the least tightly bound. These results suggest lipophilicity and steric limitations on tight binding (see below).

Perhaps the most unique property of compounds **3** is their ability to bind and act as cofactors without dramatically decreasing substrate (tyr) binding. While the average K_m^{tyr} for **3** is 29 μM, the value for other cofactors (**2** and **13**) is >50 μM. The values for **13** and **3d** may reflect an unfavorable interaction caused by branching on the ring or side chain. The advantage of a low K_m^{tyr} is that the enzyme should not become subsaturated with tyr as easily as high-K_m^{tyr} enzyme–cofactor complexes. The enzyme should function at its maximum rate with the normal levels of endogenous tyrosine.

The study of cofactor analogues with PheH uncovered some interesting differences between that enzyme and TyrH. With PheH, the highest relative velocity was seen with 6-MTHP, **2**, but compounds **3a** and **3b** also had V_{rel} values greater than THB. However, as the chain length or size of the R group in **3** increased, the velocity decreased and in some cases the binding increased. All of the synthetic cofactors were less tightly bound to the enzyme than THB with the exception of **3j**, which may be an inhibitor because of its relatively tight binding and low V_{rel} value. The additional ether oxygen in **3i** was detrimental to binding to PheH and TyrH.

Results with TrpH resemble those with PheH. The best cofactor was **3b**, which was very close to THB in activity, but the rate decreased as the side chain became larger. The loss in activity was not so great as that observed with PheH. Compounds with larger side chains were bound less tightly, except highly lipophilic **3j**, which binds more

(30) Shiman, R.; Gray, D.; Pater, A. *J. Biol. Chem.* **1979**, *254*, 11 300.
(31) Friedman, P.; Kappelman, A.; Kaufman, S. *J. Biol. Chem.* **1972**, *247*, 4165.
(32) Petrack, B.; Sheppy, F.; Fetzer, V. *J. Biol. Chem.* **1968**, *243*, 743.
(33) Kuczenski, R. *J. Biol. Chem.* **1973**, *248*, 2261.
(34) Reinhard, J., to be published elsewhere.
(35) Nagatsu, T.; Levitt, M.; Udenfriend, S. *Anal. Biochem.* **1964**, *9*, 122.
(36) Bailey, S.; Ayling, J. *Anal. Biochem.* **1980**, *107*, 156.
(37) Cleland, W. *Methods Enzymol.* **1979**, *63*, 103.

Table IV. Tetrahydropterin Dihydrochloride Monohydrates **3**

no.	R	reduction conditions[a]	purification conditions[b]	yield, %	mp, °C (dec)	anal.
3a	Me	A	A	75	235–240	C, H, N
3b	Et	A	A	64	210–217	C, H, N
3c	*n*-Pr	A	A	68	194	C, H, N
3d	2-Pr	A	A	48	210	C, H, N
3e	*n*-Bu	A or B	A	77	208–209	C, H, N
3f	*i*-Bu	A	A	>46	220	C, H, N
3g	*t*-Bu	C	B	50	>202	C, H, N
3h	*n*-pentyl	A	A	67	220	C, H, N
3i	C_2H_4OMe	A	A	77	175–200	C, H, N
3j	*n*-octyl	A	A	84	215–230	C, H, N

[a] A: 1 atm of H_2/PtO_2/TFA. B: 50 psi of H_2/PtO_2/DMF + AcOH. C: 1 atm of H_2/PtO_2/AcOH. [b] A: Recrystallized from 6 N HCl/MeCN. B: Reduction product in cold MeOH plus excess 10 N HCl under N_2.

Table V. Biological Data for Cofactor Analogues

		TyrH[a]			PheH[b]		TrpH[c]		DHPR[d]	
compd	R	V_{rel}, %	K_m^{Pt}, μM	K_m^{tyr}, μM	V_{rel}, %	K_m^{Pt}, μM	V_{rel}, %	K_m^{Pt}, μM	V_{rel}, %	K_m^{Pt}, μM
THB		100	107	15	100	5.5	100	49	100	2
2	6-MTHP	154	133	51	238	21	154	355	216	13
13	6,6-DMTHP[e]	119	77	105	39	29	40	nd	239	260
3a	Me	126	84	22	138	17	65	33	421	9
3b	Et	154	52	23	155	21	85	44	419	6
3c	*n*-Pr	92	28	22	32	16	54	92	212	2
3d	2-Pr	137	45	55	15	9	44	129	277	4
3e	*n*-Bu	135	95	25	22	21	52	84	283	3
3f	*i*-Bu	110	80	nd	nd	nd	nd	nd	nd	nd
3g	*t*-Bu	130	120	nd	4	nd	nd	nd	222	5
3h	*n*-Pentyl	85	65	nd	nd	nd	nd	nd	nd	nd
3i	C_2H_4OMe	131	200	nd	36	65	nd	nd	nd	nd
3j	*n*-octyl	48	44	nd	3	1	32	11	nd	nd

[a] TyrH is tyrosine hydroxylase. [b] PheH is phenylalanine hydroxylase. [c] TrpH is tryptophan hydroxylase. [d] DHPR is dihydropteridine reductase. Compounds **3** were oxidized to the quinonoid dihydro form prior to analysis. [e] 6,6-Dimethyl-5,6,7,8-tetrahydropterin.[33] See Experimental Section for details.

tightly than THB. The weak binding of 6-MTHP, **2**, to TrpH was unexpected and suggests that a hydrogen-bonding atom in the side chain is necessary for good binding to this enzyme.

As shown in Scheme I, the oxidized form of the cofactor "quinonoid dihydrobiopterin" (q-DHB; bottom center structure) is reduced to the active tetrahydro form by the enzyme dihydropteridine reductase (DHPR). A desirable and perhaps necessary property of a useful cofactor analogue is the ability to be recycled by DHPR. As shown in Table V, the quinonoid forms of compounds **3** are good substrates for this enzyme. The relative velocities were 2–4 times greater and their K_m values were 1–4 times greater (weaker binding) than those of THB. The 6,6-dimethyl analogue **13** has a higher K_m (weaker binding) with DHPR than any other compound in the study. Should q-DHB rearrange to the more stable 7,8-dihydrobiopterin (Scheme I, bottom left), it would be lost to the catalytic cycle since it is *not* a substrate for dihydrofolate reductase (DHFR). In comparison, the 7,8-dihydro forms of compounds **3** are substrates for DHFR (to be published elsewhere).

Qualitatively, TyrH showed a cofactor specificity different from that of PheH or TrpH, which have a number of similarities. TyrH was much less subject to steric hindrance around the 6-position. We have shown previously that gem-substitution at the 7-position of the pteridine ring produces compounds that are selective cofactors for TyrH.[20] In the present study, chain elongation or branching in the 6-substituent produces a similar effect. Compare compounds **3b**, **3d**, and **3g**, which have high velocities with TyrH but stepwise diminished velocities with PheH. Cofactor selectivity differences can be due to differences in steric size or lipophilicity of the side chain or both. As the chain length of the R group became much longer, as in **3j**, velocities dropped significantly, and binding increased to the point that this compound could be a competitive inhibitor.

The oxygen atom in the side chains of **3** contributes in a significant way to cofactor activity. The mechanism of PheH has been studied by Lazarus et al.[38] The monooxygenase enzymes were found to contain an active-site iron atom, but the details of how the oxygen atom is transferred between O_2 and the substrate are not known. However, the role of the side-chain oxygens could be to hold the cofactor in the correct position for oxygen activation and transfer without interfering with the substrate. The unsubstituted cofactors **2** and **13** can activate oxygen, but the molecules may be in a slightly different position in the active site relative to THB or **3**. The greater lipophilicity of alkoxy over hydroxy is another advantage. THB is known to be poorly absorbed orally and to be excluded from the brain.[18] The physical properties of compounds **3** alleviate some of these problems.

Compounds of the general structure **3** are promising synthetic cofactor analogues that may be useful in the treatment of diseases caused by a deficiency of THB, the natural cofactor. By judicious side-chain choice, one can produce either a selective or nonselective cofactor that does not block the binding of the substrate and that can be recycled by DHPR. The efficacy of these compounds is now under study.

Experimental Section

Melting points were run on a Thomas-Hoover capillary melting point apparatus. Infrared spectra (KBr) were recorded on a

(38) Lazarus, R.; Wallick, D.; Dietrich, R.; Gottschall, S.; Benkovic, S.; Gaffney, B.; Shiman, R. *Biochemistry* **1981**, *20*, 6834. See also *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1982**, *41*, 2605.

Perkin-Elmer 267 spectrophotometer. NMR spectra were determied with Hitachi Perkin-Elmer R24 and Varian CFT-20, FT-80, and XL-100 spectrometers. Tetramethylsilane was used as internal standard in $CDCl_3$ and Me_2SO-d_6, and TSP was used in D_2O spectra. Ultraviolet data were obtained on a Varian SuperScan 3 spectrometer. Microanalyses were performed by Atlantic Microlabs, Inc., Atlanta, GA. All elemental analyses were within 0.4% of theoretical unless noted otherwise. The intermediate **4** was purchased from Aldrich Chemical Co.

(±)-5,6,7,8-Tetrahydro-6-methyl-4(3*H*)-pteridinone dihydrochloride (2) was made by catalytic reduction (see below) of 6-methylpterin, which was made by the method of Semb.[39]

5,6,7,8-Tetrahydro-6,6-dimethyl-4(3*H*)-pteridinone dihydrochloride (13) was made by the method of Armarego and Waring.[40]

The compounds shown in Tables I–IV were made by the general method exemplified below.

3-Amino-6-(*n*-butoxymethyl)-2-pyrazinecarbonitrile 4-Oxide (7c). The (chloromethyl)pyrazine **4** was heated at 70–80 °C in excess 1-butanol for 288 h. The solvent was evaporated, and the residue was dissolved in CH_2Cl_2. The cloudy liquid was filtered and evaporated. The residue was chromatographed on silica gel with 1:1 hexanes/EtOAc. The yield was 77%: mp 57–60 °C; NMR (Me_2SO-d_6) δ 0.84 (t, 3 H, Me), 1.0–1.8 (m, 4 H, CH_2), 3.41 (t, 2 H, OCH_2), 4.31 (s, 2 H, $ArCH_2O$), 7.86 (br s, 2 H, NH_2), 8.37 (s, 1 H, H-5). This reaction was repeated four times at reflux for 50–55 h. The excess butanol was evaporated. The residue was dissolved in CH_2Cl_2, filtered through silica gel (500 g/mol), and crystallized from a mixture of hexanes and CH_2Cl_2. The average yield was 58%: mp 77–79 °C.

2,4-Diamino-6-(*n*-butoxymethyl)pteridine 8-Oxide (9b). In analogy with the method of Taylor,[28] pyrazine **7c** was treated with 1.6 equiv of guanidine hydrochloride and 3.6 equiv of NaOMe in dry MeOH at reflux. The reaction was followed by TLC and UV and was stopped when complete (18 h). The mixture was cooled and filtered, and the dark green solid was washed well with MeOH. The yield at this stage was 65%. The crude sample was recrystallized from MeOH to give **9b** in 44% yield: mp >250 °C; UV (0.1 N HCl) λ_{max} 252.5 (ε 30900), 282 (10700), 343.5 (sh, 6200), 356.5 (8500), 369.5 (7800) nm; NMR (Me_2SO-d_6) δ 0.88 (t, 3 H, Me), 1.0–1.8 (m, 4 H, CH_2), 3.51 (t, 2 H, OCH_2), 4.49 (s, 2 H, Ar CH_2O), 7.01 (br s, 2 H, NH_2), 7.75 (br s, 2 H, NH_2), 8.37 (s, 1 H, H-7); IR (KBr) 3700–2800, 1632, 1546, 1461, 1356, 1331, 1163, 1053, 978 cm^{-1}. In later examples, the reaction mixture was neutralized with AcOH and evaporated to dryness, and the green or black solid was washed well with water to remove salts. The average yield was 75%, and the material could be used as is or purified further. Crude pteridines **8** were recrystallized from MeOH, and crude *N*-oxides **9** were recrystallized from 2-methoxyethanol.

2-Amino-6-(*n*-butoxymethyl)-4(3*H*)-pteridinone 8-Oxide (11b). Diaminopteridine **9b** was refluxed with a 1:1 mixture of EtOH and 1 N NaOH (100 mL/g of **9**) until UV showed no further change (1–6 h). The hot solution was filtered and acidified with AcOH. A fine precipitate formed. The mix was cooled overnight and filtered. The precipitate was washed with H_2O, EtOH, and finally Et_2O. The yield was 90%: UV (0.1 N NaOH) λ_{max} 261.5, 288 (sh), 390 nm; NMR (Me_2SO-d_6) δ 0.89 (t, 3 H, Me), 1.0–1.8 (m, 4 H, CH_2), 3.48 (t, 2 H, OCH_2), 4.45 (s, 2 H, Ar CH_2O), 6.25 (br s, 2 H, NH_2), 8.19 (s, 1 H, H-7); the N-3 H was not seen. The compound was used without further purification.

(±)-2-Amino-6-(*n*-butoxymethyl)-5,6,7,8-tetrahydro-4-(3*H*)-pteridinone (3e). The pterin 8-oxide **11b** (1 g) was dissolved in trifluoroacetic acid (25 mL) and hydrogenated at 1 atm of H_2 over 80 mg of PtO_2 until consumption was complete.[29] To the mixture was added 22 mL of 6 N HCl. The mixture was filtered, and the nearly colorless filtrate was evaporated to dryness. The crude product was dissolved in 10 mL of hot 6 N HCl, treated with charcoal, filtered, diluted with hot MeCN until cloudy, and cooled. The white, crystalline product **3e** was filtered, washed with MeCN, and dried under vacuum at 50 °C. The yield of dihydrochloride monohydrate was 77%: mp 208–209 °C; IR (KBr) 3100, 1705, 1668, 1609, 1460, 1372, 1220, 1120, 755, 740 cm^{-1}; NMR (Me_2SO-d_6) δ 0.87 (t, 3 H, Me), 1.0–1.7 (m, 4 H, CH_2), 3.0–3.8 (m, 7 H), 8.1 (br hump, NH); UV (0.1 N HCl) λ_{max} 264.5 nm (ε 14800).

Hydroxylase Assays. Tyrosine hydroxylase was purified from bovine adrenal medulla by partial trypsin digestion and ammonium sulfate fractionation as described by Petrack et al.[32] The phenylalanine hydroxylase (PheH) was isolated from rat liver and purified on phenylsepharose as described by Shiman et al.,[30] and the tryptophan hydroxylase source was a desalted, 40000*g* supernatant of rat brain stem.[31] TyrH was assayed by the release of ^3H_2O from L-(3,5-^{3}H)tyrosine (see also Scheme I).[30] PheH was assayed with use of liquid chromatography with fluorescence detection to measure the tyrosine produced. Assays of TyrH, PheH, and TrpH were described previously.[31,35,36] Kinetic data were analyzed by the computer-assisted paradigm of Cleland.[37]

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Registry No. (±)-**2**, 69113-63-9; (±)-**3a**, 104422-75-5; (±)-**3b**, 104422-76-6; (±)-**3c**, 104422-77-7; (±)-**3d**, 104422-78-8; (±)-**3e**, 104422-79-9; (±)-**3f**, 104422-80-2; (±)-**3g**, 104422-81-3; (±)-**3h**, 104422-82-4; (±)-**3i**, 104422-83-5; (±)-**3j**, 104422-84-6; **4**, 40127-89-7; **5**, 40127-91-1; **6a**, 40127-92-2; **6b**, 104422-48-2; **6c**, 104422-49-3; **6d**, 104422-50-6; **6e**, 92530-51-3; **6f**, 104422-51-7; **6g**, 104422-52-8; **7a**, 40127-90-0; **7b**, 104422-53-9; **7c**, 104422-54-0; **7d**, 104422-55-1; **7e**, 104422-56-2; **7f**, 104422-57-3; **8a**, 40110-13-2; **8b**, 104422-58-4; **8c**, 104438-50-8; **8d**, 104422-59-5; **8e**, 92530-52-4; **8f**, 104422-60-8; **8g**, 104422-61-9; **9a**, 40110-11-0; **9b**, 104422-62-0; **9c**, 104422-63-1; **9d**, 104438-51-9; **9e**, 104422-64-2; **10a**, 92530-43-3; **10b**, 104422-65-3; **10c**, 104422-66-4; **10d**, 104422-67-5; **10e**, 92530-53-5; **10f**, 104422-68-6; **10g**, 104422-69-7; **11a**, 104422-70-0; **11b**, 104422-71-1; **11c**, 104422-72-2; **11d**, 104422-73-3; **11e**, 104422-74-4; **13**, 84812-31-7; TyrH, 9036-22-0; PheH, 9029-73-6; TrpH, 9037-21-2; DHPR, 9074-11-7; guanidine hydrochloride, 50-01-1; 6-methylpterin, 708-75-8.

Supplementary Material Available: NMR data for compounds **6a–g**, **7a,b,d–f**, **8a–g**, **9a,c–e**, **10b–g**, **11a,c–e**, **3a–d,f–j**; UV data for **7c,d**, **9a,c–e**, **10a,c–g**, **11a**, **3a–d,f–j**; and IR data for **6b**, **7a,d–f**, **8b–g**, **9c–e**, **10b–g**, **11a,c,d**, **3a–d,f–j** (11 pages). Ordering information is given on any current masthead page.

(39) Semb, J. U. S. Patent 2 477 426, 1949; *Chem. Abstr.* **1950**, *44*, 1146c.
(40) Armarego, W.; Waring, P. *Aust. J. Chem.* **1981**, *34*, 1921.
(41) Traub, H.; Pfleiderer, W. *Biochemical and Clinical Aspects of Pteridines*; Walter de Gruyter: Berlin, 1985; Vol. 4 pp 45–56.