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Original paper

Lipophilic 5,6,7,8-tetrahydropterin substrates for phenylalanine hydroxylase (monkey brain), tryptophan hydroxylase (rat brain) and tyrosine hydroxylase (rat brain)

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Summary — A high yielding unambiguous synthesis of (±)-6-alkyl-5,6,7,8-tetrahydropterin **5a—f** hydrochlorides starting from ethyl α-isocyanoacetate **1** and the respective alkanoic anhydrides or alkanoyl chlorides in four steps is described. All the six pterins **5a—f** that have been synthesised are substrates for mammalian phenylalanine, tryptophan and tyrosine hydroxylases and their activities have been compared with those of natural 6*R*-tetrahydrobiopterin under similar conditions. The data allowed the choice of 6-*n*-propyl-5,6,7,8-tetrahydropterin **5c** for further studies as a candidate for tetrahydrobiopterin drug therapy.

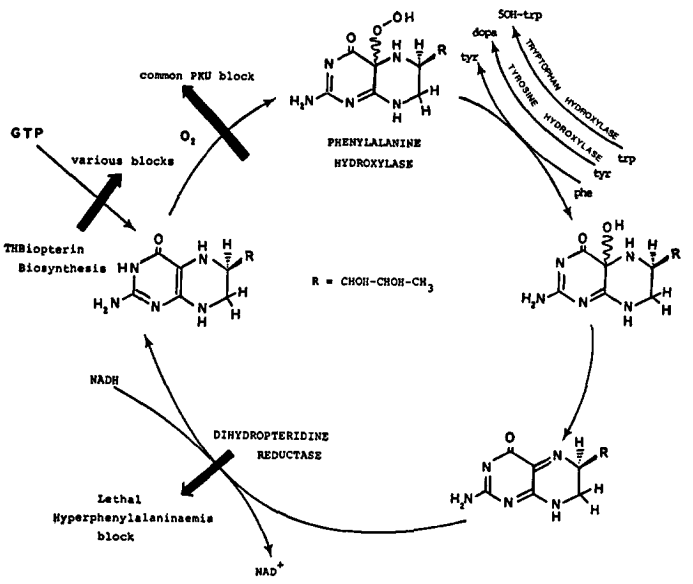
Résumé — **Tétrahydro-5,6,7,8 ptérines lipophiles, substrats de la phénylalanine hydroxylase (cerveau de singe), de la tryptophane hydroxylase (cerveau de rat) et de la tyrosine hydroxylase (cerveau de rat).** *On décrit une synthèse non équivoque des chlorhydrates des (±)alkyl-6-tétrahydro-5,6,7,8 ptérines* ***5a—f*** *avec de hauts rendements à partir de l'α-isocyanacétate d'éthyle* ***1*** *et les anhydrides ou les chlorures d'acides correspondants en quatre étapes. Les six ptérines* ***5a—f*** *que nous avons préparées sont des bons substrats des hydroxylases de la phénylalanine, du tryptophane et de la tyrosine provenant de mammifères, et leurs activités ont été comparées avec celles de la tétrahydrobioptérine naturelle dans des conditions semblables. Les résultats permettent de choisir la* n*-propyl-6 tétrahydro-5,6,7,8 ptérine* ***5c*** *pour des études futures.*

(±)-6-alkyltetrahydropterins / substrates / phenylalanine hydroxylase / tryptophan hydroxylase / tyrosine hydroxylase

Introduction

A variety of neurological disorders are characterised by decreased levels of the natural aromatic amino acid hydroxylase cofactor 6-*R*-5,6,7,8-tetrahydrobiopterin (6*R*-BH_4) in the nervous system, *e.g.*, brain, cerebrospinal fluid; and other tissue, *e.g.*, liver. The aromatic amino acid hydroxylases requiring the cofactor in question are phenylalanine hydroxylase (EC 1.14.16.1), tryptophan hydroxylase (EC 1.14.16.4) and tyrosine hydroxylase (EC 1.14.16.2), although a fourth enzyme, dihydropteridine reductase (EC 1.6.99.7), is essential for the proper functioning of these hydroxylases because it reduces the oxidised cofactor for recycling. The relationship between these enzymes is shown in Scheme 1. Decreased levels of 6*R*-BH_4 seriously affect the amounts of 5-hydroxytryptophan and 3,4-dihydroxyphenylalanine (dopa) by impairing the respective enzymes, tryptophan [1] and tyrosine [2] hydroxylases. As a consequence of this, the physiological levels of the essential neurotransmitters serotonin and catecholamines are decreased, resulting in serious neurological disturbances. Impairment of phenylalaanine hydroxylase function causes the accumulation of phenylalanine in tissues (including blood) and is responsible for the inherited metabolic disease phenylketonuria [3]. The classical variant of this disease is due to the absence or decreased levels of phenylalanine hydroxylase, but there are less common variants which are due to the absence of 6*R*-BH_4 or the cofactor recycling enzyme dihydropteridine

Abbreviations: **pterin:** 2-aminopteridin-4(3*H*)-one; **6*R*-BH_4:** 6*R*-(1′*R*, 2′*S*)-1′, 2′-dihydroxypropyl-5,6,7,8-tetrahydrobiopterin; **TPS:** sodium 3-(trimethylsilyl)propionate; **DMSO:** dimethyl sulfoxide-[$^2H_6$].



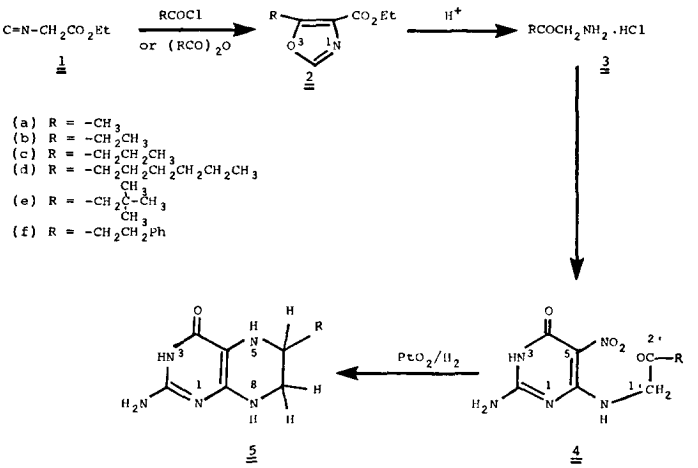
Scheme 1.

reductase. These are referred to as hyperphenylalaninaemia, malignant hyperphenylalaninaemia or hyperphenylalaninaemia due to BH_4 deficiency [4—8]. Other disorders that are characterised by decreased levels of 6*R*-BH_4 are Parkinson's disease [9, 10], Shy—Drager and Steel—Richardson syndromes, essential tremour, Huntington's chorea and Alzheimer's disease [9]. BH_4 therapy for hyperphenylalaninaemia [6] and for Parkinson's disease and endogenous depression [11] has had some success, although the advantages of this therapy have been questioned [12, 13]. More recently, some cases of dystonia have responded to BH_4 therapy [14]. The lack of uniform and outstanding success of BH_4 therapy has been attributed to the poor ability of BH_4 to penetrate the blood—brain barrier. Kapatos and Kaufman [15] have demonstrated the limited ability of BH_4 to traverse the blood—brain barrier compared with the apparently more liposoluble 6-methyl-5,6,7,8-tetrahydropterin. Recently, Ayling and Bailey [16] have shown that 6,6-dimethyl-5,6,7,8-tetrahydropterin also has a superior ability to penetrate the blood—brain barrier than BH_4.

In extending these studies, we synthesised a series of 5,6,7,8-tetrahydropterins substituted at position 6 with carbon side chains which increased the fat-soluble properties of the reduced pterin [17]. Before studying the ability of these pterins to penetrate the blood—brain barrier, and in order to find the best pterin for further study, it was necessary to evaluate these tetrahydropterins as substrates (or inhibitors) for the above mentioned aromatic amino acid hydroxylases and to determine if the hydroxylase oxidation products of these pterins, the quinonoid 7,8-dihydro(6*H*)pterins are substrates for the recycling enzyme dihydropteridine reductase. We have reported [17] that all the quinonoid dihydropterins **6a—f** with R = Me, Et, *n*-Pr, *n*-hexyl, neopentyl and phenethyl are good substrates for purified human brain dihydropteridine reductase and that the *n*-propyl and *n*-hexyl derivatives were the best substrates. In continuation of this work, we now present studies of the activities of the 5,6,7,8-tetrahydropterins **5a—f** (R = Me, Et, *n*-Pr, *n*-hexyl, neopentyl, phenethyl) and 6*R*-BH_4 (for comparison) as substrates for the above three aromatic amino acid hydroxylases and show that the pterins **5a—f** are also substrates of these enzymes, and, that on the whole, the *n*-propyl (and perhaps the *n*-hexyl) derivative is the best overall substrate of this group and warrants further studies as a potential drug for cofactor therapy in the above cited neurological disorders.

Chemistry

In the previous report [17], we described the preparation of 6-ethyl, *n*-propyl, *n*-hexyl, neopentyl and phenethyl pterins which were reduced to the respective 5,6,7,8-tetrahydropterins. The 6-alkylpterins were prepared by Isay reactions [18] as modified by Rosowsky and Chen [19], whereby 2,4,5-triaminopyrimidin-6(1*H*)-one was condensed with the respective 1-methylthioalkan-1-ol-2-one (or its precursor 1-methylsulfinylalkan-2-one). The 6- and 7-alkylpterins were formed as a mixture and were separated. This synthesis produced small amounts of pterins with structures that had to be assigned because the method was ambiguous. The small quantities of tetrahydropterins prepared in this way in our earlier work had been used for the reported enzymatic work and more material had to be prepared for the present study. Instead of using the previous synthesis, we chose to use an unambiguous one which produced only the required 6-substituted pteridines. This synthesis involves the preparation of the common intermediate ethyl α-isocyanoacetate **1**, obtained by dehydrating ethyl *N*-formylaminoacetate [20] (Scheme 2). When the isocyanide **1** was treated with an acid anhydride or acid chloride in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene in dimethylformamide, the oxazole carboxylic esters **2a—f** were formed in greater than 80% yields. The liquid esters were hydrolysed with 6 N hydrochloric acid to form the respective α-aminomethylketone hydrochlorides **3a—f** in close to quanti-



Scheme 2. Synthesis of 5,6,7,8-tetrahydropterins.

tative yields, using slight modifications of the method of Suzuki *et al.* [21]. The aminomethylketone hydrochlorides **3a**—**f** provided the 6-(alkan-2-on-1-yl)amino-2-amino-5-nitropyrimidin-6(1*H*)-ones **4a**—**f** when heated with 2-amino-6-chloro-5-nitropyrimidin-4(3*H*)-one in dimethylformamide in the presence of trimethylamine. The pyrimidinones **4** were reduced and cyclised in one step to the desired 6-alkyl-5,6,7,8-tetrahydropterins **5a**—**f** without isolation of the respective intermediate 7,8-dihydropterins **7**. The average overall yields of tetrahydropterin **5** from glycine ethyl ester hydrochloride were high and the substituent R in the pterin **5** is derived from the acid chloride or the anhydride used in forming the oxazole esters. By using a ^{14}C label in the original glycine ester, in the anhydride or acid chloride, or in the chloropyrimidine, it should be possible with this synthesis to prepare any of these 6-alkyltetrahydropterins **5** with a ^{14}C label at 7-C of the pyrazine ring, the alkyl side chain or in the pyrimidine ring, respectively.

Biological Results and Discussion

The 6-alkyltetrahydropterins **5a**—**f** were designed with 6-alkyl substituents so as to increase the fat solubility of the 5,6,7,8-tetrahydropterin hydrochloride. Thus, to show that this has been achieved, the solubilities of the **5a**—**f** hydrochlorides in *n*-octanol were determined by stirring the suspension in *n*-octanol overnight. The results in Table I show that the solubilities are indeed increased by increasing the size of 6-alkyl side chain. The natural cofactor 6*R*-BH_4 HCl exhibited rather high solubility. *n*-Octanol was used because it is the usual test solvent for fat solubility. However, from previous results [15], it could be extrapolated that the solubility of 6*R*-BH_4 HCl in body fat should be lower than that of 6-methyl-5,6,7,8-tetrahydropterin hydrochloride. Attempts to obtain partition coefficients between 4 mM aqueous hydrochloric acid and *n*-octanol were unsuccessful because of the high solubility of the pterin hydrochlorides in dilute acid. Partition studies between aqueous buffer at pH 7 and *n*-octanol were thwarted because of the facile aerobic oxidation of the tetrahydropterins in neutral aqueous solutions.

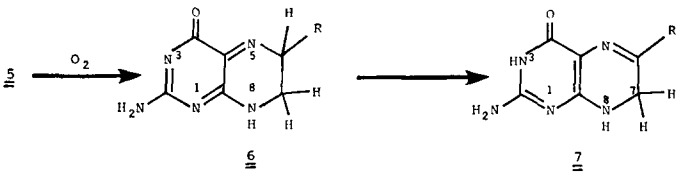
Table I. Solubilities of 5,6,7,8-tetrahydropterin hydrochlorides in *n*-octanol at 25°C.

5 Hydrochlorides	Wt. suspended (mg/ml)	Solubility (μmol/ml)	Solubility (mg/ml)
a $-CH_3$	3.26	0.162	0.045
b $-CH_2CH_3$	3.48	0.434	0.102
c $-CH_2CH_2CH_3$	3.16	0.515	0.215
d $-CH_2CH_2CH_2CH_2CH_2CH_3$	3.17	0.741	0.231
e $-CH_2C(CH_3)_2-CH_3$	3.26	1.863	0.574
f $-CH_2CH_2Ph$	3.65	0.458	0.148
$-CHOH-CHOH-CH_3$ (6*R*-BH_4)	3.55	1.012	0.314

Of the three aromatic amino acid hydroxylases, tryptophan and tyrosine hydroxylases are important for the biosynthesis of serotonin and catecholamine neurotransmitters. Phenylalanine hydroxylase, although useful for removing excess phenylalanine from tissues, is not directly associated with the production of neurotransmitters and is absent in the brain. However, we have studied the activities of phenylalanine hydroxylase with the 6-alkyltetrahydropterins because, if these compounds are to be used as drugs to enhance the production of neurotransmitters in the nervous system, then we should make certain that they are good substrates too. Otherwise, a phenylketonuria condition may be produced.

Phenylalanine hydroxylase activities

A monkey liver extract was used as the source of phenylalanine hydroxylase. A crude extract was preferred because we wanted to compare the hydroxylase activities using the alkyltetrahydropterins as substrates in the presence of as many cellular ingredients as possible. The extract was transparent at wavelengths longer than 325 nm and it was possible to use the spectrophotometric method of Ayling *et al.* [22] to assay hydroxylase activities. The assay involves the enzymatic oxidation of the tetrahydro(6*H*)pterin **5** in the presence of phenylalanine and oxygen (catalase is added to minimize non-enzymatic aerobic autooxidation of **5** to **6**), and the increase in absorbance due to formation of species **6** at a chosen wavelength is observed. Unfortunately, species **6** rearrange readily to the more stable 7,8-dihydro(3*H*)pterin tautomers **7** by general acid—base catalysis [23, 24] (Scheme 3). The UV spectral changes for the



Scheme 3.

rearrangement of **6** to **7** show several isosbestic points which can be conveniently used as analytical wavelengths. These wavelengths and the reaction extinction coefficients for the tetrahydropterins **5a**—**f** were determined by oxidising the tetrahydropterins in the required buffer with peroxidase and hydrogen peroxide which converts compounds **5** to **6** almost instantaneously and observing the spectral changes as the rearrangement proceeds to give **7**. An isosbestic point is chosen at a wavelength in which the absorbance of the original species **5** is as small as possible. The reaction (net) molar extinction coefficient (ϵ value) is the difference between the ϵ value at the isosbestic point (*i.e.*, of **6** and **7** which are the same) and the ϵ value of the original pterin **5** at the same pH. The net molar extinction coefficients and the respective analytical wavelengths (isosbestic points) are listed in Table II.

In order to obtain meaningful comparisons of activities it was necessary to determine the apparent K_m and V_{max}

Table II. Net molar extinction coefficients at the isosbestic points for the reaction **6** → **7**[a].

5	λ_{isosb} (nm)	Net ϵ_{isosb} ($M^{-1}\cdot cm^{-1}$)	λ_{max}[b]	ϵ_{max}[b]
a $-CH_3$	344	3030	268	13 433
b $-CH_2CH_3$	342	2627	266	12 500
c $-CH_2CH_2CH_3$	347	3687	268	14 474
d $-CH_2CH_2CH_2CH_2CH_2CH_3$	344	1063	265	10 600
e $-CH_2C(CH_3)_2-CH_3$	360	2146	266	12 160
f $-CH_2CH_2Ph$	380	3295	266	10 500
$-CHOH-CHOH-CH_3$[c] (6*R*-BH_4)	340	3000	265	16 800

[a]0.1 M Tris/HCl buffer, pH 7.2, at 25°C.
[b]Values for **5** in 4 mM HCl used to determine concentrations of solutions.
[c]From [25].

values for the pterins **5a**—**f** and 6*R*-BH_4 at an enzyme concentration at which the activity increased linearly with the protein concentration. The crude monkey liver extract exhibited phenylalanine hydroxylase activity with all the tetrahydropterins **5** (**a**—**f**) and 6*R*-BH_4 using the λ_{isosb} values and ϵ_{isosb} values in Table II, and, at concentrations ranging from 0.02 to 0.08 mM of the substrates, the initial rates of oxidation of the tetrahydropterins increased with the increased concentration of crude enzyme. These were linear with slopes of *ca.* 1, *i.e.*, the rates doubled with the doubling of the concentration of crude protein (at constant substrate concentration), up to a protein concentration of 1.92 mg/ml (60 μl), thereafter the plot was hyperbolic. The crude enzyme volume used for all the initial rate measurements for determining the kinetic parameters was 50 μl (1.3 mg/ml of crude protein) of extract per run. The apparent K_m and V_{max} values are given in Table III.

All the tetrahydropterins **5a**—**f** are effective cofactors for phenylalanine hydroxylase. Judging by the *V*/*K* values, the natural cofactor 6*R*-BH_4 is the most efficient substrate, and, in comparison, the second best is the 6-ethyl compound **5b**. The lower substrate activities compared with the natural cofactor reside in their higher K_m values (lower affinity for the enzyme). An extreme case is the 6-*n*-hexyl derivative **5d** where the *app* K_m is about 100-times higher, but the *app* V_{max} is only 4-times higher than the corresponding parameters for 6*R*-BH_4. The data for the neopentyl derivative **5e** imply that a bulky group at 6-C causes a decrease in hydroxylase activity. On the whole, these compounds are cofactors of the enzyme and are unlikely to upset the normal *in vivo* function of phenylalanine hydroxylase. In the absence of 6*R*-BH_4 they are all capable of turning over phenylalanine (to tyrosine) at moderate rates. It should be pointed out that the natural cofactor 6*R*-BH_4 is a pure diastereoisomer, whereas all the tetrahydropterins **5a**—**f** are equal mixtures of 6*R* and 6*S* enantiomers. In the final experiments, pure enantiomers will have to be used. It must be noted, however, that although Parniak and Kaufman ([26] and personal communication) showed that the non-natural diastereoisomer of tetrahydrobiopterin, 6*S*-BH_4, unlike 6*R*-BH_4, irreversibly inactivated phenylalanine hydroxylase, they also showed that the two enantiomers of 6-methyl-5,6,7,8-tetrahydropterin, *i.e.*, 6*R*- and 6*S*-**5a**, exhibited almost equal enzyme activities. *In vivo* experiments by Cotton [27], on the other hand, using a mouse model for hyperphenylalaninaemia due to BH_4 deficiency revealed that the elevated levels of phenylalanine produced in the model were considerably reduced after injection of 6*R*- or 6*S*-BH_4.

Tryptophan hydroxylase activities

The crude extracts from whole brain homogenates of Fischer (F344) rats were used after centrifugation at 39 000 × *g*. For this enzyme also it is advantageous to use a crude preparation because other cellular ingredients are present.

Table III. Kinetic parameters of monkey liver phenylalanine hydroxylase (0.1 M Tris/HCl, pH 7.2 and 25°C)[a].

5	*app* K_m (μM)	*app* V_{max}[b]	*V*/*K*	Relative activity
a CH_3	638 (± 80)	12.9 (±2.1)	0.041	0.15
b $-CH_2CH_3$	284 (± 28)	17.6 (±0.8)	0.062	0.23
c $-CH_2CH_2CH_3$	539 (±150)	10.5 (±1.5)	0.020	0.08
d $-CH_2CH_2CH_2CH_2CH_2CH_3$	2956 (± 50)	32.3 (±0.4)	0.011	0.04
e $-CH_2C(CH_3)_2-CH_3$	558 (± 37)	4.3 (±0.1)	0.008	0.03
f $-CH_2CH_2Ph$	225 (± 2)	4.4 (±0.02)	0.020	0.08
$-CHOH-CHOH-CH_3$[c] (6*R*-BH_4)	30 (± 3)	8.0 (±0.2)	0.265	1.00

[a]Crude extract, phenylalanine was 2.2 mM.
[b]μM pterin oxidised per min for 50 μl (1.6 mg) of protein.
[c]Values in [25] are *app* K_m 22 μM, and *app* V_{max} 300 μM/min/1.6 mg of partially purified rat liver protein.

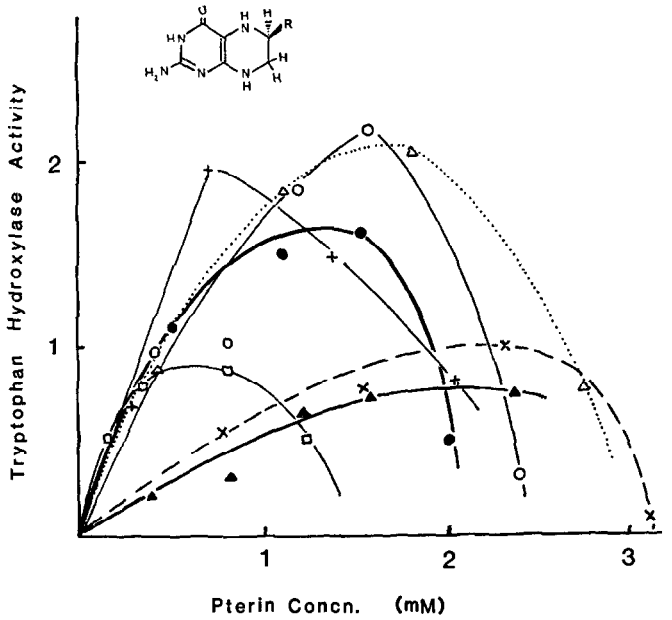


Fig. 1. Tryptophan hydroxylase activity is in nmol of 5-hydroxytryptophan produced in 20 min for 1.5 mg of crude protein in 1 ml of solution (0.12 M Tris/acetate buffer, pH 7.5, and 37°C).
R =
a: —○— $-CH_3$; **b**: ···△··· $-CH_2CH_3$; **c**: —+— $-CH_2CH_2CH_3$; **d**: —●— $-CH_2(CH_2)_4CH_3$; **e**: —□— $-CH_2C(CH_3)_2-CH_3$; **f**: --×-- $-CH_2CH_2Ph$; —▲— (6*R*)—CH(OH)CH(OH)CH_3.

The assays for tryptophan hydroxylase activity are essentially similar to the one used by Kato *et al.* [28] in which L-tryptophan was incubated with the crude extract in Tris/acetate buffer, pH 7.5, at 37°C for 20 min in the presence of a tetrahydropterin, catalase (to inhibit autooxidation of the pterin substrate), NSD 1015 (*m*-hydroxyphenylhydrazine, an amino acid decarboxylase inhibitor), and Ca^{2+} ions. The reaction was then stopped and the amount of 5-hydroxytryptophan formed was measured by its fluorescence at 527 nm (excitation 295 nm) in 2.3 M HCl. These wavelengths gave the highest fluorescence values. The blank contained D-tryptophan. The amount of 5-hydroxytryptophan formed was determined from a calibration curve using L-5-hydroxytryptophan. The assay was first performed using the pterin **5a** but in the absence of each ingredient in turn. It was found that all the above ingredients were necessary for maximum activity, and that it was unnecessary to add Fe^{2+} to the incubation mixture to keep the enzyme in the active form. The tryptophan hydroxylase activity, at a constant concentration of pterin cofactor **5a** (1 mM), increased linearly with the increase in the volume of crude extract (up to 300 μl $\equiv$ 3 mg of protein). It was not possible to obtain data which could give meaningful *app* K_m and *app* V_{max} by this method because initial rates could not be evaluated. However, a good comparison of the activity with the pterins **5a**—**f** can be made when the data are plotted as in Fig. 1. This shows that all the pterins are good substrates and generally better than the natural cofactor. They all exhibit strong substrate inhibition at higher concentrations. The pH of the mixture was checked carefully in all cases and was found to remain unaltered after the mixing of the test substances or after the incubation period. As with phenylalanine hydroxylase, the bulky C-6 branched chain in the 6-neopentyl derivative **5e** caused a decrease in activity.

Tyrosine hydroxylase activities

The same crude extracts from whole brain of Fischer (F344) rats as above were used for testing the tyrosine hydroxylase activities of the tetrahydropterins **5a**—**f**. Attempts to use the HPLC method for measuring the ratios of tyrosine to dopa formed after the enzymatic oxidation were unsuccessful because of the very low amounts of dopa formed. Similarly, attempts to use a spectrophotometric assay as discussed above for phenylalanine hydroxylase [21] gave negligible initial rates and when large volumes of brain extracts were used, the cuvettes were completely opaque to the light. We therefore resorted to the radioactive label method. The incubation was as described by Randles and Armarego [29] except that the amino acid decarboxylase inhibitor was *m*-hydroxyphenyl hydrazine. In the incubation, L-[side chain-2,3-^{3}H]tyrosine was used as the substrate and the work-up was based on that of Togari *et al.* [30] for the isolation of radioactive dopa for counting. The various amounts of radioactivity from dopa formed in 30 min using crude rat brain are shown in Table IV. The relative activities listed in Table IV are not a true indication of the relative efficiencies of these substrates because the K_m values for all but **5a** and 6*S*-BH_4 are not known. Therefore it is not known whether the concentrations of pterins used in Table IV were at saturating levels or not. Higher concentrations of pterins were not used for fear of inhibition by the substrate. All pterins are clearly substrates for tyrosine hydroxylase. However, if the K_m values for these pterins are qualitatively similar, then it could be said that the 6-*n*-propyl **5c** and 6-neopentyl **5a** are among the best synthetic substrates. Using bovine adrenal medulla, Ozawa and Suzuki [31] showed that the hydrochloride of 6-*n*-propylpterin **5c** caused the release of more tritium from L-[3,5-^{3}H]tyrosine than the hydrochlorides of 6-methyl-**5a** or 6,7-dimethyl 5,6,7,8-tetrahydropterins. The free bases, on the other hand, caused no release of tritium, *i.e.*, no formation of dopa. We cannot understand the latter results because the pH of the acetate buffer used (6.0) dictates the ionic state of the pterins. The inactivity may be attributed to a higher final pH (not stated in the reference) in order to keep the tetrahydropterin completely in the uncharged species. Tyrosine hydroxylases are known to be less active at higher pH values [2].

In conclusion all of the 6-alkyltetrahydropterins **5a**—**f** are substrates for the aromatic amino acid hydroxylases and are potential drugs for BH_4 replacement therapy. The compound that would warrant further studies for drug evaluation is perhaps the 6-*n*-propyl derivative **5c**. This choice is partly because the *n*-propyl derivative **5c** is at least as good a substrate as the other derivatives for the

Table IV. Tyrosine hydroxylase (crude rat brain) activity in NaOAc buffer, pH 6.0, at 37°C[a].

5	Concn. (mM)	[$^3H_2$]Dopa formed/30 min/ 5 mg of protein (dpm)	Relative activity
a —CH_3	0.92	13 363	0.14
b —CH_2CH_3	0.83	28 166	0.30
c —$CH_2CH_2CH_3$	1.47	41 903	0.44
d —$CH_2CH_2CH_2CH_2CH_2CH_3$	2.10	14 235	0.15
e —$CH_2C(CH_3)_2CH_3$	1.42	52 484	0.55
f —CH_2CH_2Ph	1.55	3 797	0.04
—CHOH—CHOH—CH_3 (6*R*-BH_4)	0.73	94 635	1.00
Boiled extract + **a** —CH_3	0.92	1 940	—
No tetrahydropterin	—	1 377	—

[a]500 μl of crude extract.

hydroxylases as well as dihydropteridine reductase, and partly because **5c** has the same side chain length (three carbon atoms) as the natural cofactor. The hepatotoxicity of the lipophilic tetrahydropterins will need to be evaluated in view of the suggestion that 6-methyltetrahydropterin therapy caused liver damage [32].

Experimental protocols

Chemistry

Instrumentation

NMR spectra at 90 MHz and 200 MHz were measured on Jeol FX90Q and Varian XL200E spectrometers. All spectra noted were at 90 MHz unless otherwise stated. UV spectra and kinetic measurements were recorded on Pye Unicam 1800 and Varian 219 spectrometers. IR spectra were obtained on a Pye Unicam 1050 spectrometer. Fluorescence intensities were measured using a Perkin—Elmer 3000 fluorescence spectrometer with a 0.5 cm quartz cell thermostated at 25°C. Radioactivity was measured using a Packard Tri-Carb 300D scintillation spectrometer and the counts were corrected for quenching, background and luminescence and recorded as dpm. Microanalyses were carried out by the Australian National University Analytical Unit. All evaporations were at < 40°C/18 mm in a Buchi evaporator.

Syntheses

Ethyl α-isocyanoacetate **1** [20] (50 mmol) in dry dimethylformamide (25 ml) was treated with a solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (75 mmol) followed by a solution of the acid—anhydride (65 mmol) or acid chloride (65 mmol) and the mixture was heated at 80°C for 6 h. It was poured into water (300 ml), extracted with ethyl acetate (3 × 250 ml), the extract was washed with water, dried (K_2CO_3) and evaporated. The residue was distilled *in vacuo* to yield pure *ethyl 4-alkyloxazole-5-carboxylate* **2**. The redistilled anhydrides were used for the preparation of **2a—c** and the acid chlorides (prepared in > 60% yields from the corresponding acids with 0.5 molar excess of oxalyl chloride in 3 vol of methylene chloride by stirring for 1.5 h at room temperature, evaporating and distilling the residue) were used for preparing **2e—f**.

When the ethyl 4-alkyloxazole-5-carboxylates **2a—f** were heated in 15—20 vol of 6 N HCl in an oil bath at 100°C for 6 h, a clear solution resulted. Evaporation gave an oily residue which solidified on trituration with dry ether and was collected. The hygroscopic *aminomethylketone hydrochlorides* **3** could be used directly for the next step, and all were recrystallised from ethanol—diethyl ether for microanalyses.

Triethylamine (1.5 ml) was added dropwise to a mixture of 2-amino-6-chloro-5-nitropyrimidin-4(3H)-one [33] (6 mmol) and aminomethylketone hydrochloride **3** (9 mmol) in dry dimethylformamide (4.5 ml) in a bath at 70°C with stirring. The temperature was gradually raised to 130°C, kept at 130°C for 5 min and the solution was evaporated to dryness. The residue was treated with water (20 ml), the solid was filtered off, washed with ethanol, and dried to yield the *pyrimidinones* **4a—f**. These were purified by dissolving in large volumes of boiling ethanol decolorizing with charcoal, filtering, evaporating to half the volume or less and cooled. The solid was collected by filtration or centrifugation, washed with ether and dried at 100°C for 4 h. The physical properties of the above compounds **2a—f**, **3a—f** and **4a—f** are given in Table V, and their NMR spectral data are in Table VI.

The preceding pyrimidinone ketones (100 mg) in trifluoroacetic acid (5 ml) were added to a pre-reduced suspension of platinum oxide (50 mg, Aldrich) in trifluoroacetic acid (5 ml) and shaken with hydrogen at room temperature and atmospheric pressure. At the end of the reduction, concentrated hydrochloric acid (0.5 ml) was added, and the solution was filtered through a plug of Celite under N_2 pressure. The filtrate was run into ice cold dry diethyl ether (100 ml). After standing in an ice bath for 30 min the white solid was collected and washed with dry ether (5 × 50 ml) by centrifugation. The residue was dried by blowing dry N_2 over it at *ca.* 37°C, then kept in a desicator (over P_2O_5 and KOH) *in vacuo* for 24 h and gave the 5,6,7,8-tetrahydropterin hydrochlorides **5a—f** in almost quantitative yields. These had spectral properties (IR, UV, NMR) identical with those which were analysed and reported by us [17].

Solubilities of tetrahydropterin hydrochlorides

The pterin hydrochlorides **5a—f** and 6*R*-BH_4, HCl (quantities in Table II) were suspended in *n*-octanol (1 ml) and stirred for 18 h at 25°C. The solutions were centrifuged at 3000 × *g* for 15 min and an aliquot (50 μl) of the clear supernatant was diluted with 4 mM HCl (950 μl) and the absorbances read at the respective UV maxima (Table II). The solubilities were calculated using the molar extinction coefficients (Table II) and are reported in Table I.

Phenylalanine hydroxylase activities

Monkey liver (50 g) was homogenized in a Waring blender with 3 vol (150 ml) of 0.1 M Tris/HCl and 10^{-4} M EDTA for 30 s at half speed then 30 s at full speed, centrifuged at 10 000 × *g* for 30 min, filtered through a muslin gauze and centrifuged again at 200,000 × *g* for 1 h all at 2—4°C [34]. The protein concentration, determined using the Bradford reagent (Bio-Rad, microassay) with bovine serum albumin as the standard, was 32 mg/ml. When assayed [22] using the substrates 6-methyltetrahydropterin (**5a**, 430 μM) and phenylalanine (2.2 mM), the crude extract (50 μl, 1.6 mg of protein) in 0.1 M Tris/HCl (pH 7.2) caused the oxidation of 3.6 μmol/min (λ_{anal}, 343 nm) of substrate

Table V. Physical data of intermediates.

R	2 (Oxazole ester)[a]	3 (Aminoketone HCl)[b]	4 (Pyrimidineketone)[c]
Me **a**	—[d]	—[d]	mp ⩾ 290°C (dec) 83% CHN
Et **b**	bp 80—81°C/1.8 mm 83% CHN	mp 126—127°C 99% CHNCl	mp > 253°C (dec) 79% CHN
n-Pr **c**	—[d]	—[d]	mp 246—247°C (dec) 89% CHN
n-Hexyl **d**	bp 120—121°C/1.5 mm 80% CHN	mp 162°C (dec) 96% CHNCl	mp 248—250°C (dec) 66% CHN
Neopentyl **e**	bp 106—108°C/2 mm 78% CHN	mp 173—174°C 85% CHNCl	mp > 250°C (dec) 66% CHN
Phenethyl **f**	bp 156—160°C/1.4 mm 78% CHN	mp 136—137°C 87% CHNCl	mp 224—225°C (dec) 97% CHN

[a]The IR spectra (film) typically had bands at *ca.* ν_{max} 1530 (C=C), 1614 (C=N), 1730 (C=O), 1880, 1940 and 3135 (C—H) cm^{-1}.
[b]The IR spectra (KBr) typically had bands at *ca.* ν_{max} 1730 (C=O) and 3000 ($-NH_3^+$) cm^{-1}.
[c]The IR spectra (KBr) typically had bands at *ca.* ν_{max} 1245—1525 (N=O), 1580 (C=N), 1660 (CON), 1700 (C=O) and 3300 (NH) cm^{-1}.
[d]Values as in [21].

at 25°C. The activity of the frozen aliquots was unchanged after storage at — 18°C for one year.

The ϵ values and reaction molar extinctions in Table II were determined for each tetrahydropterin and isosbestic wavelengths for the rearrangements of **4a**—**f** to **7a**—**f**, and $\epsilon_{isobestic}$ values were subtracted from the ϵ values of the corresponding tetrahydropterins **5a**—**f** at the same pH 7.2. The isosbestic points were obtained by oxidising **5a**—**f** as follows: 1M Tris/HCl (pH 7.2, 100 µl), peroxidase (100 µl, 10 µg, Boehringer), hydrogen peroxide (50 µl, 5 µmol), water (710 µl) and the tetrahydropterin hydrochloride (40 µl, *ca.* 250 µM in 4 mM HCl). The pterin was replaced by an equal volume of water in the blank. The UV spectra were scanned automatically from 650 nm up to 240 nm at intervals of *ca.* 2 min using an Apple II computer to drive the spectrometer until the final UV spectra were typically those of the 7,8-dihydro(3*H*)pterins **7**. The spectra of the corresponding pterins **5** at pH 7.2 were obtained by replacing the peroxidase and hydrogen peroxide solution by water in the above solutions. The isosbestic points were read off from the plot or deduced from the computer data. For the 6-*n*-propyl derivative **5c** see [17].

The solutions for determining the kinetic parameters contained 1M Tris/HCl (pH 7.2, 100 µl), phenylalanine (100 µl, 2.2 mM final concentration), catalase (50 µg, Sigma), water (700 µl), monkey liver extract (50 µl) [phenylalanine solution was replaced by water in the blank] and kept in the cuvettes for exactly 5 min (to activate the hydroxylase) and thermostated at 25°C. The reactions were initiated by simultaneous addition of the tetrahydropterin hydrochlorides (50 µl in 4 mM HCl at concentrations varying from 0.5 to 2 K_m) in platinum buckets which were shaken up and down 10 times in the cuvettes before activating the recorder. The initial rates for the various concentrations of substrates were determined at the isosbestic wavelengths (Table II) (duplicate or triplicate runs) and were linear over a 2—3 min period. The pH of the solution (7.2) was checked at the end of every run (glass electrode). The apparent K_m and V_{max} values (Table III) were calculated using a computer program kindly supplied by Dr. A. Cornish-Bowden [35].

Tryptophan hydroxylase activities

Fischer rats were killed in an atmosphere of CO_2, the heads were cut off and the brains were stored frozen at — 80°C. The thawed brains were homogenised in a Waring blender (2 min full speed) in 3 vo of 50 mM Tris/acetate buffer, pH 7.5, which contained 2 mmol of dithiothreitol. The homogenate was centrifuged at 39 000 × *g* for 1 h and the clear supernatant was stored in 2 ml aliquots in glass vials at — 80°C. All operations were carried out at 2—4°C. The protein concentration as determined using the Bradford reagent (see above) was 10 µg/ml.

The assay for tryptophan hydroxylase activity in the crude extract was as follows: 1.2 M Tris/acetate buffer (pH 7.5, 100 µl), 10 mM dithiothreitol (100 µl, Sigma), catalase (1 mg), 40 mM calcium chloride (50 µl), 10 mM L-tryptophan (100 µl), 10 mM *m*-hydroxyphenylhydrazine (NSD-1015, 100 µl, Aldrich), crude extract (150 µl) and tetrahydropterin hydrochloride (2—30 mM in 4 mM HCl) and water to complete to 1 ml. An equivalent amount of D-tryptophan replaced the L-isomer in the blank. These solutions and the respective blanks were shaken in a bath for 20 min at 37°C at such a rate as to allow the surface to break and aerate the solutions. The pH values of the solutions (glass electrode) were unaltered at the end of the incubations. The reaction was stopped by the addition of 70% $HClO_4$ (100 µl), allowed to stand at room temperature for 10 min and centrifuged. Concentrated HCl (200 µl) was added to 800 µl of clear supernatant and the fluorescence was measured at an excitation wavelength of 295 nm (slit 10 nm) and emission wavelength of 527 nm (slit 20 nm) in 0.5 cm quartz cells at 25°C. The cell blanks were determined with air in the cell and subtracted from fluorescence readings of the incubation solutions. Readings were taken after 2—3 min when they stabilized. The difference in fluorescence between the reaction solution with L-tryptophan and that with D-tryptophan was due to the 5-hydroxytryptophan formed. The amounts of the latter were determined from a calibration curve of fluorescence *versus* L-5-hydroxytryptophan in 2.5 M HCl. Plots are presented in Fig. 1.

Tyrosine hydroxylase activities

The crude Fischer rat brain preparation in the preceding studies was used. Larger volumes of extract (500 µl) were used in each assay than above because the tyrosine hydroxylase activity was low. The assay, in duplicate, consisted of incubating a mixture of 2 M sodium acetate buffer (pH 6.0, 100 µl), catalase (40 µl, 40 µg), β-mercaptoethanol (20 µl, 8 µg), *m*-hydroxyphenylhydrazine (80 µl, 40 µg), L-[side chain-

Table VI. NMR spectra at 90 MHz[a].

R	2 (Oxazole Ester)			3 (Amino-ketone HCl)			4 (Pyrimidine-ketone)		
	b A	δ (ppm)	c B	b A	δ (ppm)	c B	c A	δ (ppm)	c B
Me a	CCl_4 (TMS, ext, Stan-dard)	1.39(t,J 7.2) 2.62 (s) 4.29(q,J 7.2) 7.62 (s)	CH_3 (ester) $4CH_3$ CH_2 (ester) 2H	D_2O[d] (TPS)	2.31 (s) 4.12 (s)	$3CH_3$ $1CH_2$	DMSO[d] (TMS)	2.17 (s) 4.34(d,J 5.2) 7.00 (br.s) 7.63 (br.s) 9.78(t,J 5.2) 10.75 (s)	$3'CH_3$ $1'CH_2$ NH NH 6NH 3NH
Et b	$CDCl_3$ (TMS)	1.29(t,J 7.5) 1.40(t,J 7.1) 3.09(q,J 7.5) 4.30(q,J 7.1) 7.75 (s)	CH_3 (ester) $2'CH_3$ CH_2 (ester) $1'CH_2$ 2H	D_2O (TPS)	1.06(t,J 7.1) 2.61(q,J 7.1) 4.07 (s)	$4CH_3$ $3CH_2$ $1CH_2$	DMSO (TMS)	1.02(t,J 7.2) 2.53(q,J 7.2) 4.38(d,J 5.3) 7.28 (br.s) 9.83(t,J 5.3) 10.73 (s)	$3'CH_3$ $2'CH_2$ $1'CH_2$ NH_2 6NH 3NH
n-Pr c	$CDCl_3$ (TMS)	0.97(t,J 7.2) 1.40(t,J 7.2) 1.73(q,J 7.2) 3.04(t,J 7.2) 4.38(q,J 7.2) 7.77 (s)	$3'CH_3$ CH_3 (ester) $2'CH_2$ $1'CH_2$ CH_2 (ester) 2H	D_2O (TPS)	0.90(t,J 7.2) 1.61(m,J 7.2) 2.57(t,J 7.2) 4.11 (s)	$5CH_3$ $4CH_2$ $3CH_2$ $1CH_2$	DMSO (TMS)	0.87(t,J 7.1) 1.53(m,J 7.1) 2.48(t,J 7.1) 4.33(d,J 5.3) 7.35 (br.s) 9.78(t,J 5.3) 10.27 (s)	$5'CH_3$ $4'CH_2$ $3'CH_2$ $1'CH_2$ NH_2 6NH 3NH
n-Hexyl d	$CDCl_3$ (TMS)	0.88(t,J 7.2) 1.29 (br.s) 1.42(t,J 7.1) 1.69 (br.s) 3.06(q,J 7.2) 4.38(q,J 7.2) 7.75 (s)	$6'CH_3$ $5'4'3'-(CH_2)_3$ CH_3 (ester) $2'CH_2$ $1'CH_2$ CH_2 (ester) 2H	D_2O (TPS)	0.85(t,J 5.9) 1.29 (br.s) 1.58 (m) 2.60(t,J 7.0) 4.06 (s)	$8CH_3$ $7,6,5,-(CH_2)_3$ $4CH_2$ $3CH_2$ $1CH_2$	DMSO[d] (TMS)	0.86(t,J 6.2) 1.25 (br.s) 1.50(t,J 7.2) 4.33(d,J 5.1) 6.61 (br.s) 7.83(br.s) 9.80(t,J 5.1)	$8'CH_3$ $7'6'5'4'-(CH_2)_4$ $3'CH_2$ $1'CH_2$ NH NH 6NH
Neopentyl e	$CDCl_3$ (TMS)	1.00 (s) 1.40(t,J 7.2) 2.99 (s) 4.38(d,J 7.2) 7.79 (s)	$(CH_3)_3$ CH_3 (ester) $1'CH_2$ CH_2 (ester) 2H	D_2O (TPS)	1.01 (s) 2.50 (s) 4.02 (s)	$(CH_3)_3$ $3CH_2$ $1CH_2$	DMSO (TMS)	0.99 (s) 2.40 (s) 4.31(d,J 5.1) 7.20 (br.s) 9.77(t,J 5.1)	$(CH_3)_3$ $3'CH_2$ $1'CH_2$ NH 6NH
Phenethyl f	$CDCl_3$ (TMS)	1.38(t,J 7.2) 2.99 (m) 3.38 (m) 4.36(q,J 7.2) 7.24 (s) 7.75 (s)	CH_3 (ester) $2'CH_2$ $1'CH_2$ CH_2 (ester) C_6H_5 2H	DMSO (TMS)	2.86 (s) 3.93 (s) 7.25 (s) 8.27 (br.s)	$3,4-(CH_2)_2$ $1CH_2$ C_6H_5 + NH_3	DMSO (TMS)	2.84 (s) 4.35(d,J 5.7) 7.24 (s) 9.80(t,J 5.7)	$3'4'-(CH_2)_2$ $1'CH_2$ C_6H_5 6NH

[a] δ in ppm downfield of TMS or TPS (δ = 0 ppm); *J* values are in Hz.
[b] Solvent, DMSO is d_6-DMSO.
[c] Assignment of signals.
[d] Run at 200 MHz.

2,3-^{3}H]tyrosine (20 μl, 0.38 μCi, Amersham, U.K. TRK 282) and water (140 μl) totalling 400 μl taken from a stock solution, and a mixture containing the crude enzyme extract (500 μl) and the respective tetrahydropterin **5a**—**f** hydrochlorides and 6*R*-BH_4 HCl (100 μl, giving the final concentrations listed in Table IV) and shaken in a thermostated bath at 37°C for 30 min. Blanks were run with boiled enzyme or in the absence of pterin cofactor. The reactions were stopped by addition of 0.4 M $HClO_4$ (2 ml). The precipitated protein was centrifuged, the supernatant was decanted and the precipitate was washed by centrifugation with 0.2 M NaOAc buffer, pH 6.0 (2 × 5 ml). All the supernatants were combined and 2 M EDTA solution (200 μl) and L-dopa (150 μl, 30 μg, Sigma) as the carrier were added to them. The mixtures were stirred with alumina (300 mg, BDH Brockman II) in large centrifuge tubes for 5 min and allowed to settle. The pH of the solutions were adjusted to 8.6 with 3 M NH_4OH (*ca.* 0.34 ml, glass electrode) stirred for 2 min and centrifuged. The supernatant was carefully decanted and discarded, and the alumina was washed twice by stirring with water (20 ml) for 2 min followed by centrifugation. The supernatant washes were discarded. The alumina from the various reactions was transferred quantitatively into Pasteur pipettes plugged with cotton wool using large volumes of water. The radioactive L-dopa was then eluted from the alumina with 1M acetic acid (2 ml) directly into polypropylene counting vials. Scintillation fluid (10 ml, xylene and Triton X—114, 2:1, containing 0.5% of 2,5-diphenyloxazole) was added and counted 3 times for 10 min. The data are listed in Table IV.

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