Studies on Biologically Active Pteridines. IV.[1] Synthesis of Several Biopterin Derivatives as an Antigen in Radioimmunoassay for Biopterin

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The synthesis of biopterin, D(and L)-*erythro*-neopterins, and their derivatives having a substituent at the 2-amino group *via* the 2-methylthio analogues is described. The 2-(methylthio)pteridines were synthesized by condensation of 4,5-diamino-6-hydroxy-2-(methylthio)pyrimidine with 5-deoxy-L-arabinose and D(and L)-arabinose phenylhydrazones, followed by oxidation with $K_3[Fe(CN)_6]$ and O_2 in the presence of KI in an acidic solution, respectively. Aminolysis of these thioethers with ammonia and other amines in the presence of acetic acid gave the title compounds.

The aromatic amino acid hydroxylases, phenylalanine hydroxylase,[2] tyrosine hydroxylase,[3] and tryptophan hydroxylase,[4] play key roles in the biosynthesis of catecholamine neurotransmitters or indoleamine neurotransmitter. Since these hydroxylases require tetrahydrobiopterin as cofactor, defficiency of the biopterin cofactor causes serious neuropathological disorders. Indeed, hyperphenylalaninemia due to defficiency of biopterin has been reported as a variant form of phenylketonuria.[5,6] In order to diagnose such disease, assay of biopterin in serum or urine is required.

Recently we studied a radioimmunoassay for biopterin (**3a**) by employing 6-[[4-hydroxy-6-(L-*erythro*-1,2-dihydroxypropyl)-2-pteridinyl]amino]hexanoic acid–bovine serum albumin conjugate (**1a**) as the immunogen and *N*-[2-(4-hydroxy-3-[^{125}I]phenyl)ethyl]-6-[[4-hydroxy-6-(L-*erythro*-1,2-dihydroxypropyl)-2-pteridinyl]-amino]hexanamide (**1b**) as the radioactive antigen.[7] The antiserum produced by rabbits against **1a** showed high specificity toward biopterin and did not cross-react with other pteridines including D- and L-*erythro*-neopterins (**4a** and **5a**) and 6,7-dimethylpterin. During a preliminary experiment, we found that the sensitivity of the radioimmunoassay increased about ten-fold on preincubating the antiserum with 6-[(4-hydroxy-6,7-dimethyl-2-pteridinyl)amino]hexanoic acid (**2**); most probably, antibodies having specificity against the hexanoic acid moiety were removed by the preincubation.

This finding suggests that the sensitivity of the assay could be more increased even when the time-consuming preincubation procedure is omitted, if a radioactive antigen having no hexanoic acid moiety were used. In order to examine this possibility, we synthesized several biopterin and neopterin derivatives to be used as antigen carrying a hydroxyphenyl or methoxyphenyl group linked to the amino nitrogen of biopterin and neopterin directly or indirectly through a carbon chain smaller than a pentyl group.

In the present paper, we studied a synthetic route for such biopterin derivatives by a nucleophilic replacement of 4-hydroxy-6-(L-*erythro*-1,2-dihydroxypropyl)-2-(methylthio)pteridine (**3b**) with an appropriate amine to save reaction steps and time. The required common intermediate (**3b**) was synthesized by condensation of 4,5-diamino-6-hydroxy-2-(methylthio)pyrimidine and 5-deoxy-L-arabinose phenylhydrazone, followed by oxidation of the unstable condensate with potassium hexacyanoferrate(III) and oxygen in the presence of a small amount of potassium iodide.[8,9] Two blue fluorescent compounds were obtained in this reaction. The minor

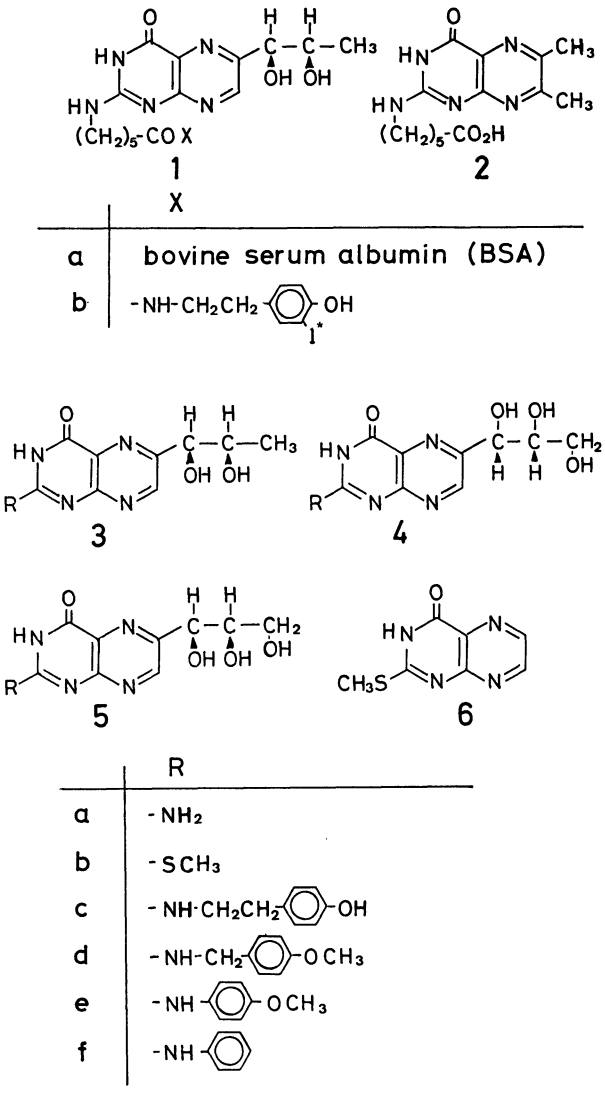

Table 1. The pK_a and UV spectra of 6-(dihydroxypropyl)- and 6-(trihydroxypropyl)pteridines

Compound	pK_a	pH of buffer[a]	λ_{max} (log ε)[b]	Ionic species[c]
3b	−1.01±0.05	−3.05	289(4.15), *300(4.11)*, 386(3.81)	+
	6.55±0.01	3.0	231(3.99), 282(4.21), 336(3.85)	○
		9.0	247(4.03), 269(4.32), 353(3.86)	−
4b	−1.10±0.06	−3.05	289(4.17), *300(4.13)*, 386(3.84)	+
	6.52±0.01	3.0	230(3.96), 282(4.24), 336(3.90)	○
		9.0	244(4.04), 270(4.35), 353(3.90)	−
5b	−1.04±0.05	−3.05	289(4.19), *300(4.15)*, 386(3.87)	+
	6.51±0.01	3.0	230(3.95), 282(4.22), 336(3.89)	○
		9.0	244(4.04), 270(4.36), 353(3.90)	−
3c	1.86±0.04	−0.5	240(4.18), *252(4.15)*, 325(3.86)	+
	8.02±0.02	5.0	224(4.20), 281(4.28), 354(3.75)	○
	10.2±0.5	9.0	222(4.21), 267(4.32), 370(3.80)	−
		12.0	241(4.24), 265(4.43), 372(3.86)	2−
4c	1.86±0.05	−0.5	241(4.22), *252(4.19)*, 325(3.88)	+
	7.94±0.03	5.0	224(4.27), 282(4.32), 353(3.78)	○
	9.5±0.5	9.0	222(4.25), 267(4.37), 370(3.85)	−
		12.0	240(4.27), 265(4.46), 373(3.90)	2−
5c	1.94±0.05	−0.5	241(4.21), *252(4.18)*, 325(3.88)	+
	7.84±0.03	5.0	224(4.25), 282(4.31), 354(3.78)	○
	9.6±0.5	9.0	221(4.25), 267(4.36), 370(3.84)	−
		12.0	240(4.27), 265(4.46), 373(3.89)	2−
3d	1.66±0.04	−0.5	226(4.33), *252(4.24)*, 324(3.70)	+
	7.95±0.05	5.0	226(4.29), 281(4.35), 352(3.80)	○
		10.0	*225(4.26)*, 265(4.44), 370(3.88)	−
4d	1.60±0.05	−0.5	226(4.35), *250(4.26)*, 324(3.90)	+
	7.92±0.07	5.0	226(4.30), 281(4.35), 352(3.80)	○
		10.0	222(4.25), 265(4.44), 370(3.88)	−
5d	1.63±0.05	−0.5	227(4.34), *250(4.26)*, 324(3.90)	+
	7.91±0.03	5.0	227(4.25), 281(4.35), 352(3.80)	○
		10.0	222(4.24), 265(4.43), 370(3.88)	−
3e	1.70±0.03	−1.0	228(4.26), 256(4.20), 325(3.94)	+
	7.40±0.04	4.5	236(4.16), 287(4.37), 354(3.81)	○
		9.5	*265(4.28)*, 287(4.38), 377(3.93)	−
4e	1.66±0.02	−1.0	228(4.27), 256(4.20), 325(3.94)	+
	7.35±0.05	4.5	233(4.17), 287(4.35), 353(3.79)	○
		9.5	*265(4.25)*, 287(4.35), 377(3.90)	−
5e	1.63±0.04	−1.0	228(4.26), 256(4.19), 325(3.92)	+
	7.31±0.05	4.5	235(4.16), 287(4.34), 354(3.78)	○
		9.5	*264(4.26)*, 287(4.35), 377(3.90)	−
3f	1.43±0.05	−1.0	255(4.20), 324(3.90)	+
	7.14±0.01	4.5	236(4.03), 289(4.32), 352(3.78)	○
		9.5	*243(4.05)*, 284(4.38), 374(3.91)	−
4f	1.43±0.03	−1.0	255(4.21), 324(3.91)	+
	7.21±0.04	4.5	237(4.05), 289(4.33), 353(3.80)	○
		9.5	*240(4.06)*, 284(4.39), 374(3.92)	−
5f	1.44±0.03	−1.0	255(4.21), 324(3.91)	+
	7.20±0.04	4.5	237(4.05), 289(4.34), 353(3.80)	○
		9.5	*240(4.06)*, 285(4.38), 375(3.92)	−
6	−1.01±0.05	−3.05	287(3.81), *296(3.77)*, 379(3.81)	+
	6.51±0.01	3.0	230(3.92), 278(4.15), 331(3.83)	○
		9.0	240(3.92), 268(4.27), 349(3.85)	−

a) Negative figures are H_0 values. b) Wave length in nm measured in aqueous buffer of given pH; shoulders or influexions in italics. c) Ionic species are shown by + (cation), ○ (neutral molecule), − (monoanion), and 2 − (dianion).

product was found to be 4-hydroxy-2-(methylthio)-pteridine (**6**) which was unambiguously synthesized by condensation of 4,5-diamino-6-hydroxy-2-(methylthio)-pyrimidine and glyoxal. The structure of the main product was determined to be **3b** from the elemental analyses and UV spectra similar to those of **6** (Table 1). This conclusion was further confirmed by aminolysis of the main product into biopterin (**3a**). The 6-(D-*erythro*-1,2,3-trihydroxypropyl) and 6-(L-*erythro*-1,2,3-trihydroxypropyl) analogues (**4b** and **5b**) of **3b** were similarly synthesized by condensation of 4,5-diamino-6-hydroxy-2-(methylthio)pyrimidine with D- or L-arabinose phenylhydrazone and oxidation of the condensates with the above oxidizing agents; their structures were confirmed by aminolysis to the known D- or L-*erythro*-neopterin (**4a**, **5a**),[10] respectively. A considerable amount of the condensate again lost the trihydroxypropyl substituent during the oxidation. It is to be noted that an analogous condensate from 2,4,5-triamino-6-hydroxypyrimidine and D-arabinose phenylhydrazone did not loose the trihydroxypropyl group even when the condensate was oxidized by air in an alkaline solution;[10] under such conditions, the present condensate entirely lost the trihydroxypropyl group to give **6**.

The (methylthio)pteridine (**3b**) proved to be a good precursor for the synthesis of the present required biopterin derivatives carrying a substituent at the 2-amino group. When **3b** was heated with tyramine in 2-methoxyethanol or *N,N*-dimethylformamide in the presence of acetic acid, **3b** was easily converted into 4-hydroxy-2-[2-(*p*-hydroxyphenyl)ethylamino]-6-(L-*erythro*-1,2-dihydroxypropyl)pteridine (**3c**); in the absence of acetic acid, decomposition of **3b** into several unclarified substances took place to a fair extent. Analogous treatment of **3b** with *p*-methoxybenzylamine, *p*-anisidine, or aniline gave the biopterin derivatives (**3d—f**) possessing respectively a *p*-methoxybenzyl, *p*-methoxyphenyl, or phenyl group at the 2-amino nitrogen. The thioethers (**4b** and **5b**), on aminolysis with those amines, gave the corresponding derivatives of D-*erythro*-neopterin (**4c—f**) and L-*erythro*-neopterin (**5c—f**).

The (methylthio)pteridines (**3b**, **4b**, and **5b**) showed very weak basicity (pK_a −1.0) and a characteristic bathochromic shift on protonation (see Table 1). The aminolysis products exhibited two pK_a values at about 1.6 and 7.5: the former is attributed to the protonation of the pteridine ring to form cations and the latter to the ionization of the 4-hydroxyl group to form anions. Both values are slightly lower than those of biopterin (pK_a 2.23 and 7.89[9]). An additional pK_a value at 10 was observed with **3c**, **4c**, and **5c** owing to the ionization of the phenolic hydroxyl group. However, the ionization changed the UV spectra only slightly, because the hydroxyl group is not conjugated to the main pteridine chromophore.

A study of radioimmunoassay for biopterin and neopterin using the present synthesized compounds will be reported eleswhere.

Experimental

The elemental analyses were conducted at The Analytical Section, Meijo University, Nagoya. The pK_a values were determined by a spectroscopical method,[11] and the UV spectra in an appropriate buffer solution on a Shimadzu UV-300 spectrophotometer.

*4-Hydroxy-6-(*L-erythro-*1,2-dihydroxypropyl)-2-(methylthio)-pteridine (**3b**) and the Trihydroxypropyl Analogues (**4b** and **5b**).* 4-Amino-6-hydroxy-2-methylthio-5-nitrosopyrimidine[12] (18 g) was hydrogenated over 5% palladium on carbon (10 g) in 0.5 M KOH (500 ml) at room temperature and atmospheric pressure till the calculated amount of hydrogen was absorbed. The catalyst was removed by filtration and the filtrate was adjusted to pH 3—4 with formic acid. A considerable amount of colorless needles of the 5-aminopyrimidine deposited on neutralization. Without isolating the needles, the mixture was added to 5-deoxy-L-arabinose phenylhydrazone[9] (32 g) in methanol (400 ml). The resulting solution was stirred under nitrogen at 25 °C for 90 min, and then under reflux for 30 min. After chilling, it was added to a solution of potassium hexacyanoferrate(III) (100 g), potassium iodide (5 g), and formic acid (10 ml) in water (500 ml). The solution was stirred under bubbling oxygen at pH 3—4 and 25 °C for 20 h. The solution was concentrated to about 500 ml under reduced pressure and made alkaline (pH 9—10) with ammonia. The nonfluorescent precipitate was removed by filtration and the filtrate was fractionated on a Florisil column (5×60 cm) using water as the elution solvent to give two blue fluorescent fractions. The major fraction, after concentration to about 150 ml, was chromatographed again on Florisil in the same way as above. The eluate was evaporated to dryness under reduced pressure and the residue was extracted with hot methanol (500 ml). Concentration of the extract to about 50 ml and chilling gave colorless needles (5.5 g) of 4-hydroxy-6-(L-*erythro*-1,2-dihydroxypropyl)-2-(methylthio)pteridine (**3b**), which decomposed above 210 °C without melting (from water) (Found: C, 40.27; H, 4.45; N, 18.65%. Calcd for $C_{10}H_{12}N_4O_3S\cdot 2H_2O$: C, 39.46; H, 5.31; N, 18.41%). The minor fraction, after purification as above, yielded 1.9 g of 4-hydroxy-2-(methylthio)pteridine (**6**), mp 268—269 °C (dec) (from water) (Found: C, 43.59, H, 3.08; N, 28.72%. Calcd for $C_7H_6N_4OS$: C, 43.29; H, 3.11; N, 28.85%). This compound was synthesized alternatively by heating 4,5-diamino-6-hydroxy-2-(methylthio)pyrimidine and equimolar glyoxal in water under reflux for 30 min (68% yield).

By employing D- or L-arabinose phenylhydrazone in palce of 5-deoxy-L-arabinose phenylhydrazone in the above reaction, 4-hydroxy-6-(D-*erythro*-1,2,3-trihydroxypropyl)-2-(methylthio)-pteridine (**4b**) [70% yield, mp 156—158 °C (dec) (from water) (Found: C, 39.63; H, 4.68; N, 18.03%. Calcd for $C_{10}H_{12}N_4O_4S\cdot H_2O$: C, 39.72; H, 4.68; N, 18.54%)] and the L-isomer (**5b**) [62% yield, mp 156—158 °C (dec) (from water) (Found: C, 39.75; H, 4.63; N, 18.17%. Calcd for $C_{10}H_{12}N_4O_4S\cdot H_2O$: C, 39.72; H, 4.68; N, 18.54%)] were respectively obtained.

*Biopterin (**3a**) and* D *(and* L*)-*erythro*-Neopterins (**4a** and **5a**).* A solution of **3b** (200 mg), ammonium acetate (0.6 g), and 28% aqueous ammonia (5 ml) was heated under reflux for 4 h. The solution was adjusted to pH 2 with hydrochloric acid and fractionated on a Florisil column (3.5×20 cm) using water as the elution solvent. The eluate was evaporated to dryness under reduced pressure and the residue was extracted with 1% ammonia (50 ml). The extract was concentrated to about 10 ml, adjusted to pH 3, and chilled to give ivory needles (90 mg) of biopterin, which was identified with

the authentic sample.[9])

In a similar way, **4b** and **5b** were converted to D-*erythro*-neopterin (**4a**) (62% yield) and L-*erythro*-neopterin (**5a**) (72% yield), respectively.

*4-Hydroxy-2-[2-(*p*-hydroxyphenyl)ethylamino]-6-(*L-erythro-*1,2-dihydroxypropyl)pteridine (3c) and the Trihydroxypropyl Analogues (4c and 5c).* A solution of **3b** (1.0 g), tyramine (3 g), and acetic acid (0.8 g) in 50% aqueous 2-methoxyethanol (8 ml) was heated at 100—105 °C for 6 h. The solution was adjusted to pH 1—2 with hydrochloric acid and placed on a Florisil column (3.5×40 cm). The column was washed with 2 M formic acid (0.5 l) and water (0.5 l) to remove the excess tyramine and a small amount of **3b**. The column was then eluted gradiently with 0—2% ammonia (1 l). The eluate, after concentration to about 150 ml, was again chromatographed in the same way as above and the final eluate was evaporated to dryness. The residue was extracted with 2% ammonia (200 ml), which was concentrated to about 100 ml. The solution was adjusted to pH 3 with formic acid and chilled to give yellow needles (34% yield) of **3c**, which decomposed at 165 °C (from water) (Found: C, 54.56; H, 5.62; N, 18.38%. Calcd for $C_{17}H_{19}N_5O_4 \cdot H_2O$: C, 54.39; H, 5.64; N, 18.66%).

Similar treatnent of **4b** and **5b** with tyramine gave **4c** [30% yield, mp 180—182 °C (dec) (from water) (Found: C, 49.88; H, 5.05; N, 16.72%. Calcd for $C_{17}H_{19}N_5O_5 \cdot 2H_2O$: C, 49.87; H, 5.66; N, 17.11%)] and **5c** [32% yield, mp 180—182 °C (dec) (from water) (Found: C, 49.88; H, 5.23; N, 16.90%. Calcd for $C_{17}H_{19}N_5O_5 \cdot 2H_2O$: C, 49.87; H, 5.66; N, 17.11%)], respectively.

*4-Hydroxy-6-(*L-erythro-*1,2-dihydroxypropyl)-2-(*p*-methoxybenzylamino)pteridine (3d) and the Trihydroxypropyl Analogues (4d and 5d).* A mixture of **3b** (1.0 g), *p*-methoxybenzylamine (2 g), and acetic acid (0.6 g) was heated at 100—105 °C for 4 h. The solution was adjusted to pH 3 with 50% formic acid and placed on a Florisil column (3.5×30 cm). The column was washed with 2M formic acid (500 ml) and then eluted gradiently with 0—2% ammonia (2 l). The eluate was concentrated to about 100 ml, made acid with formic acid, and then evaporated to dryness. The residue was extracted with hot methanol (100 ml). Concentration of the extract to about 5 ml and dilution with water (5 ml) gave pale yellow needles (50% yield) of **3d**, mp 226—227 °C (dec) (from 50% aqueous methanol) (Found: C, 54.70; H, 5.35; N, 18.33%. Calcd for $C_{17}H_{19}N_5O_4 \cdot H_2O$: C, 54.39; H, 5.64; N, 18.66%).

A similar reaction of **4b** and **5b** with *p*-methoxybenzylamine gave respectively **4d** as pale yellow needles [26% yield, darkened above 230 °C without melting (from methanol) (Found: C, 54.60; H, 5.02; N, 18.56%. Calcd for $C_{17}H_{19}N_5O_5$: C, 54.68; H, 5.13; N, 18.76%)] and **5d**[50% yield, darkened above 230 °C without melting (Found: C, 54.57; H, 5.06; N, 18.44%. Calcd for $C_{17}H_{19}N_5O_5$: C, 54.68; H, 5.13; N, 18.76%)].

*4-Hydroxy-6-(*L-erythro-*1,2-dihydroxypropyl)-2-(*p*-methoxyphenylamino)pteridine (3e) and the Trihydroxypropyl Analogues (4e and 5e).* A solution of **3b** (1.0 g), *p*-anisidine (8 g), and acetic acid (3 g) in *N,N*-dimethylformamide (15 ml) was heated at 80 °C for 8 h. The solution was fractionated on Florisil as above to give pale yellow needles (78% yield) of **3e**, which decomposed at 273 °C (from water) (Found: C, 56.08; H, 4.86; N, 20.23%. Calcd for $C_{16}H_{17}N_5O_4$: C, 55.97; H, 4.99; N, 20.40%).

In a similar way, the D-trihydroxypropyl analogue (**4e**) [43% yield, decomposed above 250 °C without melting (Found: C, 53.43; H, 4.63; N, 19.28%. Calcd for $C_{16}H_{17}N_5O_5$: C, 53.48; H, 4.77; N, 19.49%)] and the L-trihydroxypropyl analogue (**5e**) [30% yield, decomposed above 250 °C without melting (Found: C, 53.30; H, 4.64; N, 19.24%. Calcd for $C_{16}H_{17}N_5O_5$: C, 53.48; H, 4.77; N, 19.49%)] were obtained from the respective 2-(methylthio)pteridines (**4b** and **5b**).

*4-Hydroxy-6-(*L-erthro-*1,2-dihydroxypropyl)-2-phenylaminopteridine (3f) and the Trihydroxypropyl Analogues (4f and 5f).* These compounds were synthesized from the corresponding 2-methylthio precursors (**3b**, **4b**, and **5b**) and aniline in a way similar to that for the *p*-methoxybenzylamino analogues. The (1,2-dihydroxypropyl)pteridine (**3f**) was obtained as pale yellow needles (22% yield), mp 270—271 °C (dec) (from methanol) (Found: C, 57.38; H, 4.77; N, 21.83%. Calcd for $C_{15}H_{15}N_5O_3$: C, 57.50; H, 4.83; N, 22.34%). The (D-*erythro*-trihydroxypropyl)pteridine (**4f**) was obtained in 57% yield as pale yellow needles which decomposed above 260 °C without melting (Found: C, 54.80; H, 4.37; N, 21.04%. Calcd for $C_{15}H_{15}N_5O_4$: C, 54.71; H, 4.59; N, 21.27%). The (L-*erythro*-trihydroxypropyl)pteridine (**5f**), obtained in 33% yield as pale yellow needles, decomposed above 260 °C without melting (Found: C, 54.82; H, 4.46; N, 21.12%. Calcd for $C_{15}H_{15}N_5O_4$: C, 54.71; H, 4.59; N, 21.27%).

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