# Studies on Biologically Active Pteridines. VI.[1] Biopterin Conjugated to β-D-Galactosidase and Bovine Serum Albumine

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(Received January 16, 1981)

**Synopsis.** 4-Hydroxy-6-(L-*erythro*-1,2-dihydroxypropyl)-2-(methylthio)pteridine was converted by ethylenediamine to 2-(2-aminoethylamino)analogue of biopterin, which condensed with *N*-hydroxysuccimidyloxy *m*-maleimidobenzoate to give the maleimidobenzamide. The latter compound and β-D-galactosidase afforded a biopterin-galactosidase conjugate which showed the enzyme activity about 40% as compared to the unmodified enzyme.

The biosynthesis of neurotransmitting dopamine and serotonine from phenylalanine and tryptophan requires tetrahydrobiopterin cofactor at the oxygenation step of these amino acids. Therefore, a decrease of biopterin concentration in those tissues generating the neurotransmitting amines may cause neurological disorders. Several examples to support such assumption have been found recently: atypical hyperphenylalaninemia due to deficiency of biopterin[2] and anormalous low biopterin concentrations in human brain from parkinonian patients.[3] Consequently, assay of biopterin in tissue or serum becomes essential for basic and clinical studies of such diseases.

We have recently developed a radioimmunoassay for biopterin, which makes a merit of high specificity, sensitivity, and reproducibility.[4] The method, however, suffers from a disadvantage of using a radioactive antigen. In order to avoid this inherent drawback of radioimmunoassay, we aimed to develop an enzymeimmunoassay for biopterin by using a conjugate of biopterin to β-D-galactosidase as a labelled antigen. This paper describes a synthesis of biopterin conjugated to the galactosidase and to bovine serum albumine (BSA), of which the latter is to be used as an immunogenic antigen.

Previously we synthesized a biopterin-BSA conjugate by a mixed anhydride method, in which 6-(biopterinylamino)caproic acid was condensed to the free amino group of the protein *via* an anhydride and the condensation product was treated with 2 M sodium hydroxide at the final step of preparation.[4] This anhydride method is inapplicable to the present required biopterin-galactosidase conjugate, because alkaline treatment may inactivate the enzyme. Accordingly we sought for a different conjugation method and applied *N*-hydroxysuccimidyloxy *m*-maleimidobenzoate (MBS)[5] to conjugating biopterin to the mercapto groups of galactosidase and BSA. The enzyme contains a number of mercapto groups unconcerned with the enzyme activity and hence the conjugation of biopterin at such sites will not reduce the enzyme activity.

4-Hydroxy-6-(L-erythro-1,2-dihydroxypropyl)-2-(methylthio)pteridine (**1**)[6] underwent aminolysis on heating with ethylenediamine to give the 2-(2-aminoethylamino) analogue (**2**), which was then converted to the *m*-maleimidobenzoyl derivative (**3**) by MBS. Conjugation of **3** with BSA was achieved by incubating BSA with a large excess **3** in a pH 7 phosphate buffer at 25 °C for 1.5 h, and the product was purified by chromatography on Sepharose 6B (Fig. 1). The number of moles of biopterin per mole of BSA was estimated 0.66 from the UV and fluorescence spectra. Conjugation of **3** to β-galactosidase was carried out

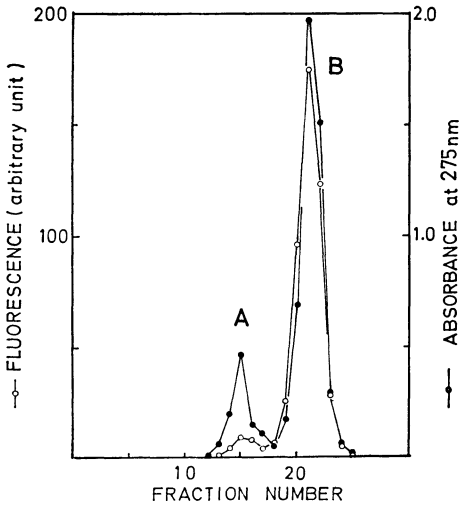



Fig. 1. Purification of biopterin-BSA conjugate on a Sepharose 6B column (1.8×30 cm) eluted by 0.05 M phosphate buffer, pH 7.0. The volume of each fraction was 5 ml. The intensity of fluorescence at 440 nm was measured by exciting at 360 nm. A: Conjugate, B: excess **3**.

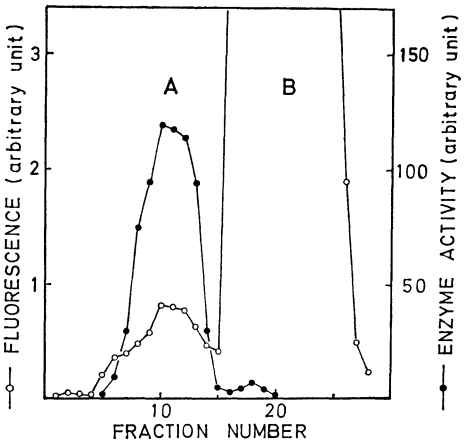
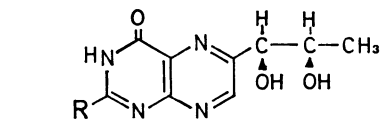



Fig. 2. Purification of biopterin-galactosidase conjugate on Sepharose 6B carried out in the same way as in Fig. 1. The enzyme activity was assayed as described in text.



R

1 -S CH3

2 -NH CH2 CH2NH2

3 -NH CH2CH2NH CO

4 -NH CH2CH2NH CO S-BSA

5 -NH CH2CH2NH CO S-βGAL

in the same way as above. The chromatography on Sepharose 6B was monitored by fluorescence spectra and by enzyme activity using 4-methylumbelliferyl β-D-galactoside as substrate.[7] As shown in Fig. 2, the enzymically active fractions show fluorescence due to the biopterin moiety, indicating a sucessful conjugation of **3** to galactosidase. The conjugate retained the enzyme activity about 40% as compared to the unmodified enzyme.

An enzymeimmunoassay for biopterin by employing the present synthesized conjugates is under progress and the detail will be reported elsewhere.

## Experimental

*2-(2-Aminoethylamino)-4-hydroxy-6-(*L-erythro-*1,2-dihydroxypropyl)pteridine (2).* A solution of **1**[6] (500 mg) in ethylenediamine (5 ml) was heated at 110 °C under nitrogen for 4 h. The solution was fractionated on a Florisil column (2.5×30 cm), eluted gradiently with 0—3% ammonia (1.0 l), and then on a Dowex 50 W×8 column (2.5×20 cm), eluted with 0—3% ammonia. The eluate was evaporated to give a jellied residue, which on standing in a refrigerator for several days crystallized into a pale yellow needles (220 mg) of **2**. The compound darkened at 245 °C; p$K_a$ at −1.0, 0.75, and 8.0 (measured by a spectroscopical method); $\lambda_{max}$ (log ε) at $H_0$ −3.0: 260 (4.08), 280 (sh., 3.99), 323 (3.65), and 392 (3.74); at $H_0$ −0.25: 255 (4.11), 280 (3.96), 321 (3.75), and 392 (3.68); at pH 5.0: 261 (4.40), 350 (3.82); at pH 10.0: 240 (4.09), 277 (4.27), and 365 (3.88).

N-[*2-[4-Hydroxy-6-(*L-erythro-*1,2-dihydroxypropyl)-2-pteridinyl]-aminoethyl*]-m-*maleimidobenzamide (3).* To a solution of **2** (100 mg) in *N,N*-dimethylformamide (10 ml), MBS[5] (120 mg) was added and the solution was stirred at 25 °C for 2.5 h. The solution was then participated with water (40 ml) and dichloromethane (3×40 ml). The aqueous solution was evaporated *in vacuo* to almost dryness. Addition of tetrahydrofuran (1 ml) and chilling gave a solid (80 mg) of **3** which was used for the next reaction without further purification.

*Preparation of Biopterin-BSA Conjugate (4).* A solution of **3** (1 mg) in 0.5 ml of 0.1 M phsphate buffer, pH 7.0, was added to a solution of BSA (10 mg) in the same buffer (1 ml). The mixture was stirred at 25 °C for 1.5 h and then fractionated on a Sepharose 6B column (1.8×30 cm), eluted by a 0.05 M phosphate buffer, pH 7.0. The eluate was monitored by the fluorescence spectra and UV absorbance at 275 nm.

*Preparation of Biopterin-galactosidase Conjugate (5).* A solution of **3** (1 mg) in a 0.1 M phosphate buffer (pH 7.0, 0.5 ml) was added to a solution of β-D-galactosidase from E. Coli [EC 3.2.1.23] (1 mg) in the same buffer (1 ml), and the mixture was stirred at 25 °C for 2 h. Fractionation of the reaction products on a Sepharose 6B column (1.8×30 cm) using a 0.05 M phosphate buffer (pH 7.0) as solvent gave a chromatogram shown in Fig. 2. The enzyme activity was assayed as below.

*Assay of β-D-Galactosidase Activity.* A mixture of 4-methylumbelliferyl β-D-galactoside (1 mg/30 ml of 0.01 M phosphate buffer, pH 7.0; 50 μl) and 100 μl of each fraction diluted 50-fold by a 0.01 M phosphate buffer (pH 7.0) containing 0.1% BSA was incubated at 37 °C for 10 m. A 0.1 M glycine buffer (pH 10.3; 3 ml) was added to the mixture and the intensity of the fluorescence at 440 nm (excited at 360 nm) was measured on a Hitachi MPF-2A fluorescence spectrometer.

We thank Mrs. N. Nishioka for measuring the fluorescence spectra.

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