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Occurrence of biopterin in the wings of *Morpho* butterflies

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Abstract. A blue fluorescent compound was isolated from *Morpho* butterfly wings. Based on thin layer chromatographic, UV and CD-spectrophotometric and HPLC analyses, the blue fluorescent compound was identified as L-*erythro* biopterin. Biopterin is a major component of blue fluorescent pteridines in both *M. sulkowskyi* and *M. adonis*. Pterin and isoxanthopterin can also be detected as minor components in these species. This paper is the first to report the presence of biopterin in butterfly wings.
Key words. Biopterin; pterin; isoxanthopterin; butterfly wings; *Morpho* butterfly.

The reduced form of pteridine, tetrahydrobiopterin (BH_4), plays an important role as a hydrogen donor cofactor for phenylalanine hydroxylase[1], as well as tyrosine and tryptophan hydroxylases[2,3], which are the rate-limiting enzymes in the synthesis of catecholamine and indoleamine, respectively.

Pteridines were originally described as pigments of insects and lower vertebrates[4]. Hopkins first isolated pteridine in 1889 as the yellow pigment from Pieridae butterfly wings[5]. Pteridines derive their name from the fact that they were first found in butterfly wings[6]. Although the occurrence of various pteridines has been reported in the wings of many butterflies, no comprehensive work has been documented on any but the Pieridae[7–10]. At present, pteridines in butterfly wings are considered to occur mainly in the family Pieridae and their presence is thought to be one of the systematic and taxonomic indicators of this group of organisms[11,12].

It is known that the brilliant blue color of tropical *Morpho* butterflies is due to interference caused by an elaboration of wing scales[13]. In the course of studies on the color mechanism and optical properties of *Morpho* butterflies' wings[14], we found that these butterflies contain blue fluorescent compounds in their wings. We isolated these compounds from *Morpho sulkowskyi* and *Morpho adonis*, and identified them as biopterin, pterin and isoxanthopterin. This is the first report of the presence of biopterin in butterfly wings.

Materials and methods

Male butterflies of the species *M. sulkowskyi* and *M. adonis* were purchased from the Okura Biological Institute (Tokyo). Four stereoisomers of biopterin were generous gifts from Prof. Matsuura of Fujita Health University College (Aichi). All other chemicals were obtained from commercial sources.

The high performance liquid chromatography (HPLC) system used consisted of an LC-6 A pump (Shimazu Co., Ltd.), FP-210 spectrofluorometer (Japan Spectroscopic Co., Ltd., JASCO) and a D-2500 chromato-integrator (Hitachi Ltd.). An Asahipak GS-320H column (7.6 × 250 mm, Asahi Chemical Industry Co., Ltd.) was used for the analysis and purification of pteridines. A μ-Bondasphere 5 μ C_{18}-100 Å column (19 × 150 mm, Nihon Waters Ltd.) was also used for purification of biopterin. A Shim-pack CLC-ODS column (6.0 × 150 mm, Shimazu Co., Ltd.) was utilized for the determination of the stereo form of biopterin. As the elution solvents, 5 mM ammonium acetate was used for the Asahipak column, 10% (v/v) aqueous methanol for the μ-Bondasphere column, and 10 mM potassium phosphate buffer pH 7.0 for the Shim-pack column, at a flow rate of 1.0 ml/min. Pteridines were detected by fluorimetry using an excitation wavelength of 360 nm and an emission wavelength of 445 nm.

The ultraviolet absorption spectra of the compounds were recorded on a U-2000 spectrophotometer (Hitachi, Ltd.). Circular dichroism (CD) studies were performed on a J-720 automatic recording spectropolarimeter (JASCO) at 0.1 M HCl, 25 °C.

After the wings of 20 *Morpho* butterflies had been cut into small pieces with scissors, they were homogenized with 10 volumes of 50% (v/v) aqueous ethanol using a Polytoron (Kinematica Co.) for 2 min. The homogenate was heated for 10 min at 70 °C in a water bath and then centrifuged at 10,000 × *g* for 10 min.

The supernatant was filtered through Miracloth (Calbiochem Co.). The pigments were extracted from the precipitate once more, using the same procedure as outlined above. After the combined extracts had been concentrated to a small volume by a rotary evaporator, the main fluorescent compound was purified by passing it through two different HPLC systems. After several

injections of other aliquots from the butterflies' wing extracts, fluorescent substances contained in each peak were isolated and concentrated to a small volume. The concentrated substances were separated by high performance thin layer chromatography (HPTLC) using an HPTLC cellulose plate (Merck Co., Ltd.) with five different solvent systems. Fluorescent spots were detected with a near-ultraviolet lamp at 365 nm.

Results

The profiles of the *M. sulkowskyi* and *M. adonis* extracts on an Asahipak column are shown in figure 1. Among several peaks in the profiles, compounds A, B and C coincided well in their retention times with biopterin, pterin and isoxanthopterin, respectively. Mixtures of the compounds with their corresponding authentic pteridines also resulted in elution of single peaks.

The Rf values of these three fluorescent substances from *M. sulkowskyi* coincided well with those of the authentic pteridines, as shown in the table. The results were the same for *M. adonis*.

As shown in figure 2, the ultraviolet spectrum of compound A from *M. sulkowskyi*, which was further purified by the use of a μ-Bondasphere column, was the same as that of authentic biopterin. The λ_{max} values of both ultraviolet spectra were 254 and 361 nm in 0.1 M NaOH, 246 and 317 nm in 0.1 M HCl, respectively. As for *M. adonis* similar spectra, with identical peaks, were obtained. Based on these data, we concluded that compound A from both *M. sulkowskyi* and *M. adonis* is indistinguishable from biopterin. Concerning the

Table. Rf values of purified pteridines from *Morpho sulkowskyi* wings on HPTLC.

Compound	Solvents				
	1	2	3	4	5
A	0.44	0.64	0.36	0.62	0.71
Biopterin	0.44	0.63	0.35	0.60	0.70
B	0.45	0.62	0.27	0.53	0.54
Pterin	0.46	0.60	0.28	0.52	0.54
C	0.29	0.45	0.15	0.35	0.35
Isoxanthopterin	0.30	0.45	0.15	0.35	0.34

Solvents: 1 n-Butanol:acetic acid:water (4:1:2, by vol.);
2 n-Propanol:1% ammonia (1:1, v/v);
3 n-Propanol:ethyl acetate:water (7:1:2, by vol.);
4 n-Propanol:2% ammonium acetate (1:1, v/v);
5 3% (w/v) ammonium chloride.

stereostructure of biopterin, the retention times of both authentic L- and D-*erythro* biopterin on a Shim-pack column were 23.9 min and those of both L- and D-*threo* biopterin were 36.5 min, respectively. In contrast, the retention times of biopterin from *M. sulkowskyi* and *M. adonis* were 23.9 and 23.8 min, respectively. These data correspond with the *erythro* biopterin standards. In circular dichroism (CD) studies, biopterin from both *M. sulkowskyi* and *M. adonis* was shown to give curves

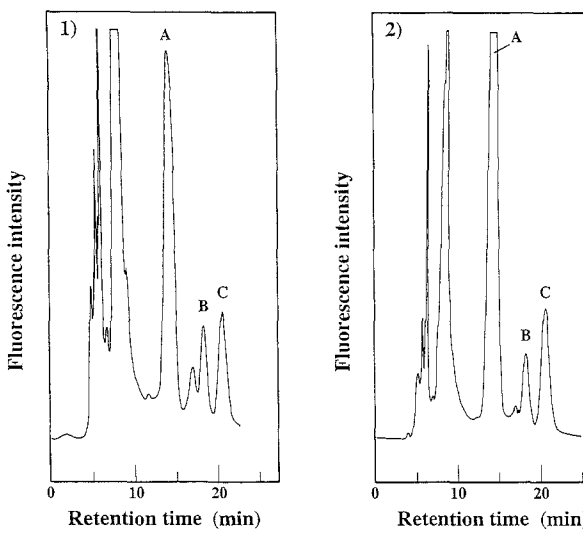

Figure 1. HPLC profiles of extracts from *Morpho* butterflies' wings. 1) *M. sulkowskyi*; 2) *M. adonis*. The analyses were performed on an Asahipak GS-320H column (7.6 × 250 mm) with 5 mM ammonium acetate as the mobile phase. Peak A, biopterin; peak B, pterin; peak C, isoxanthopterin.

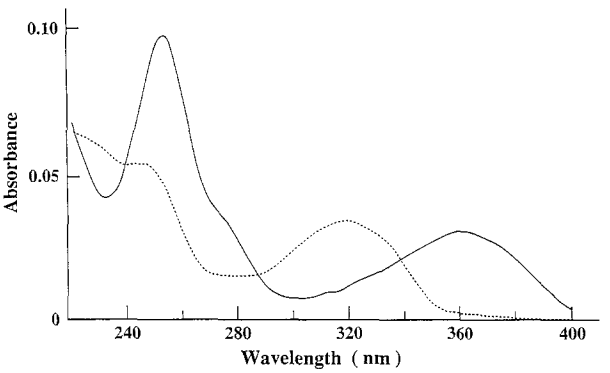

Figure 2. Ultraviolet absorption spectra of compound A purified from *M. sulkowskyi* wings. Solid line, in 0.1 M NaOH; dotted line, in 0.1 M HCl.

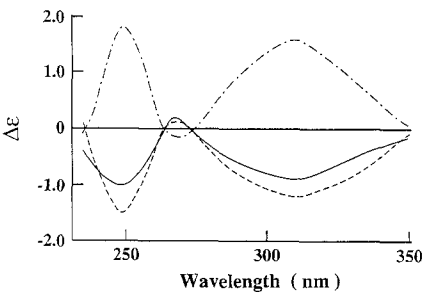

Figure 3. CD spectra of the stereoisomers of the authentic biopterin and of the natural compounds. Dotted line, L-*erythro* biopterin; dash-dotted line, D-*erythro* biopterin; solid line, biopterin from *M. sulkowskyi*. This profile is the same as biopterin from *M. adonis*.

coinciding with those of L-*erythro* biopterin (fig. 3). Based on these data we concluded that L-*erythro* biopterin is present in the wings of *M. sulkowskyi* and *M. adonis* butterflies. These butterflies were also found to contain pterin and isoxanthopterin in addition to biopterin. The content of these pteridines in the wings of the two species of *Morpho* butterflies was calculated on the basis of fluorescence intensities, using authentic pteridines as standards. The average contents of biopterin, pterin and isoxanthopterin in the wing of *M. sulkowskyi* were 1030 ± 430, 459 ± 183 and 150 ± 57 (pmol/individual) respectively, and in *M. adonis* 2890 ± 600, 363 ± 98 and 157 ± 99, respectively. These data reveal that biopterin is a major pteridine in the wings of at least two species of *Morpho* butterflies.

Discussion

The occurrence of pteridines in butterfly wings has been reported mainly in the Pieridae[7–10]. Shield detected stable pteridine derivatives by TLC analysis of the wings of several species of Libytheidae from northern neotropical areas. From phylogenic, taxonomic and morphological standpoints he discussed the possibility that Libytheidae may be descended from the Pieridae[11]. However, he took it for granted that the presence of pteridine derivatives in butterfly wings was characteristic of the Pieridae. After biopterin was first detected in human urine[15] as a growth factor for *Crithidia*[16], the wide distribution of L-*erythro* biopterin was reported[17]. L-*threo* biopterin was also found to occur in a ciliated protozoan, *Tetrahymena pyriformis*[18], and a recent paper reported the occurrence of D-*threo* biopterin in a slime mold, *Dictyostelium discoideum*[19].

The reduced form of L-*erythro* biopterin, BH_4, is a cofactor for aromatic amino acid hydroxylases[1–3]. This paper describes the presence of L-*erythro* biopterin in *Morpho* butterfly wings. Because the stereostructure of the *Morpho* butterfly pteridine is the same as that of the pteridine cofactor, biopterin in the wings of this butterfly may be derived from the pteridine cofactor by oxidation. In the past few years, the biosynthetic pathway of BH_4 from GTP *via* dihydroneopterin triphosphate and 6-pyruvoyl-tetrahydropterin (PPH_4) has been revealed[20–22]. It is reported that isoxanthopterin is produced from PPH_4 *via* dihydropterin and pterin in the metabolic pathway[23]. It has long been known that biosynthetic intermediates are carried *via* the haemolymph to the wings and body scales[24,25]. Harmsen documented that traces of them remain in the desiccated haemolymph after emergence, in those species which do not overproduce pteridines and ultimately deposit them in their scales[25].

Although the presence of biopterin has been reported in eyes and integument or in the whole body in several insect orders as Lepidoptera, Diptera, Hymenoptera, Hemiptera, Coleoptera and Phasmida[8,10], this paper is the first to report the presence of biopterin in the wings of a butterfly belonging to the order Lepidoptera.

Several reports[7,11,12] and our findings suggest that pteridines may be contained in butterflies' wings of Papilionoidea including four families i.e., Papilionidae, Pieridae, Lycaenidae and Nymphalidae[26].

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