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A CONVENIENT DETERMINATION OF CHIRAL PTERIDINES; APPLICATION OF FLUORESCENCE DETECTED CIRCULAR DICHROISM (FDCD) TO THE MAJOR PTERIN FROM *Escherichia coli*

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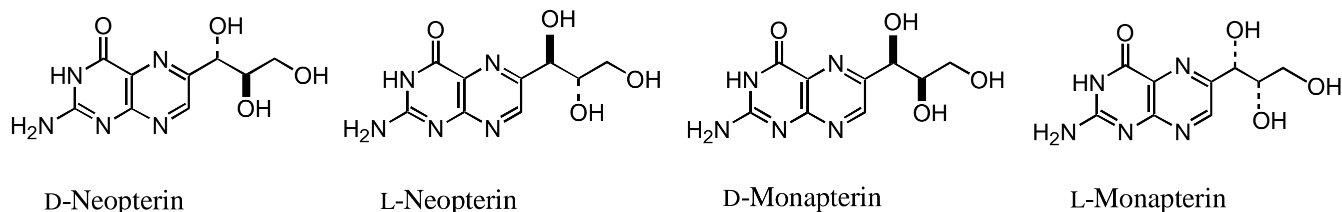
Abstract — The major pterin from *Escherichia coli* was determined as L-monapterin, by applying fluorescence detected circular dichroism (FDCD). FDCD was highly sensitive and specific to fluorescent chiral pterin, and allowed structure determination even in the presence of non-fluorescent contaminants.

The conversion of guanosine triphosphate (GTP) into 7,8-dihydroneopterin triphosphate by the action of GTP cyclohydrolase I is the first step in the biosynthetic pathway leading to biopterin cofactor in mammals.[1] Biopterin cofactor, (*6R*)-5,6,7,8-tetrahydrobiopterin, is a natural cofactor for pteridine dependent aromatic amino acid monooxygenases[2 – 4] and nitric oxide synthase.[5] In prokaryotes, 7,8-dihydroneopterin triphosphate is used as the substrate in the biosynthesis of folic acid.[6]



Neopterin [(+)-6-(D-*erythro*-1,2,3-trihydroxypropyl)pterin][7] is derived from 7,8-dihydroneopterin triphosphate and distributed in a wide range of biological materials.[8, 9] Elevated concentrations of neopterin were reported in human body fluids from patients of various kinds of cancer, viral infection, and allograft rejection.[10] These facts are explained by the action of macrophage, which produces neopterin on activation by interferon γ.[10] Thence, neopterin is considered as a biological marker to know an activation state of cell mediated immunity and to follow up the above diseases.

L-Monapterin [(+)-6-(L-*threo*-1,2,3-trihydroxypropyl)pterin][7] is one of the diastereomers of neopterin. This compound was isolated from *E. coli* and the structure was described based on the value of optical rotation.[11] It has been reported that L-monapterin exists in other microorganism,[12] plant,[13] and human body fluids including urine,[14] blood,[15] and saliva.[16] On the other side, D-monapterin[7] was found from a protozoa[17] and human urine;[18] the structure was characterized by chiral HPLC in the former case and by CD spectrum in the latter case. It is evident, however, that there is some confusion for the structural analysis of *threo*-diastereomers of neopterin, especially those from human resources. This confusion arose from the difficulty in the assignment of the stereochemical structure of pterin compounds that were contained in complex biological samples at very low concentrations. It is unable to recognize the absolute configuration of monapterin (D or L) by widely accepted HPLC analysis under achiral conditions. Therefore, enantiomeric structures of pterins have been generally discussed according to CD analysis on isolated samples. Since CD analyses were strongly affected by chiral contaminants, preparation of sufficient amount of pure samples was required.

In the present study we focused on the fluorescent nature of pterins. This paper describes an application of fluorescence detected circular dichroism (FDCD) [19] to the structure confirmation of the major blue fluorescent pterin from *E. coli*. FDCD is a CD spectroscopy with very high sensitivity and specificity to chiral fluorescent compound, and is practically free from non-fluorescent contaminants.

EXPERIMENTAL

Cell culture: E. coli BL21 was grown with shaking at 37 °C for 17 h in a solution of Bacto® LB Broth, Miller (25 g/L). Cells were harvested in the stationary phase by centrifugation at 6000 rpm and 4 °C for 20 min. The cells were washed twice by mixing well with phosphate buffered saline (pH 7.5, about 100 times volume) and centrifugation at 4000 rpm and 4 °C for 30 min. The cells were kept frozen at −80 °C.

Iodine oxidation of the extract from E. coli *under acidic and alkaline conditions:* Frozen cells of *E. coli* (2 g) were disrupted in 5 mM EDTA(2K) (3 mL) by sonication on ice using 25 bursts of 30 s at 55 W with 2 min interval (Branson Sonifier 250®), and the mixture was centrifuged at 100,000×g and 4 °C for 20 min. The supernatant was divided into two portions (1.2 mL each). One portion was subjected to oxidation under acidic condition by the procedures as follows: incubation with a solution of 1% (w/v) I_2 and 2% (w/v) KI in 1 M HCl (300 μL) in the dark at room temperature for 60 min; addition of 2% (w/v) ascorbic acid (70 μL) and water (130 μL); centrifugation at 3000 rpm for 10min; filtration through Chromatodisk® 13P. A portion of the filtrate (500 μL) was adjusted at pH 4−5 with 4 M NaOH (30 μL) and 1 M HCl (10 μL). This solution was used in analytical HPLC (Figure 1A). The other portion of the supernatant was subjected to oxidation under alkaline condition by the procedures as follows: incubation with a solution of 0.9% (w/v) I_2 and 1.8% (w/v) KI in 1 M NaOH (375 μL) in the dark at room temperature for 60 min; addition of 2% (w/v) ascorbic acid (70 μL) and 5 M HCl (55 μL). Subsequent procedures were essentially same as above, and the solution was subjected to HPLC analysis (Figure 1B).

Isolation of the major pterin after oxidation of E. coli *extract:* Frozen cells of *E. coli* (45 g) were disrupted by sonication in 0.2 M acetic acid (200 mL) using 10 bursts of 1 min at 55 W with 1 min interval, and centrifuged in the same way as above. The supernatant was immediately mixed with a solution of 8% (w/v) I_2 and 16% (w/v) KI in 1 M HCl (0.50 mL). The mixture was kept at room temperature in the dark for 60 min. A small amount of solid ascorbic acid was added till the brown color disappeared. The mixture was filtered to remove a small amount of precipitate. The filtrate was concentrated to about 30 mL and then subjected to a Florisil® column (ϕ 18×200 mm) chromatography. Water (100 mL) was passed through the column. Fluorescent materials were eluted with 0.5 M aqueous NH_3. The solution eluted between 50−130 mL, on evaporation to about 10 mL, formed solid which was removed by centrifugation at 3000 rpm for 10 min. The supernatant was evaporated to dryness and then dissolved in water (10 mL). The solution was applied to a Fuji Gel ODSQ3® column (60−100 mesh, ϕ 40×80 mm), after adjusting pH at 4. Water (100 mL) was passed through the column. The fraction

eluted with 1% (v/v) aq. CH_3CN (70 mL) was concentrated to about 0.8 mL. The solution was subjected to HPLC on an Inertsil® ODS-3 column (ϕ 20×250 mm) attached with a guard column (ϕ 20 ×50 mm), using 2% (v/v) aq. CH_3CN as the solvent. The solution of the fluorescent peak eluted at 152.5−164.0 mL was evaporated dryness and the residue (20 μg), estimated from UV absorption at 346 nm, was used as a crude sample of the major pterin from *E. coli* for the first CD and FDCD analyses. The sample was again subjected to preparative HPLC in the same way, obtaining 4.5 μg of residue which was used as the final sample.

Other procedures: D-Neopterin and D-monapterin were synthesized according to the methods described in literatures.[20, 21] Analytical HPLC was carried out on an Inertsil® ODS-3 column (ϕ 4.6×250 mm) eluted with 0.4% (v/v) aq. CH_3CN using JASCO 821-FP florescence detector (emission: 450 nm and excitation: 350 nm). The CD spectra were measured on a JASCO J-720 CD spectrometer in 0.2 M potassium phosphate buffer, pH 5.4. FDCD and LD were measured on J-720 attached with FDCD-357 and LD-324, respectively, in the same buffer.

RESULTS AND DISCUSSION

An HPLC chromatogram (Figure 1) of a mixture of authentic D-neopterin and L-monapterin clearly showed that *erythro* and *threo* diastereomers were separated completely (retention volume = 9.7 and 13.5 mL, respectively). Several fluorescent compounds obtained from iodine oxidation under acidic condition of *E. coli* extract are shown in Figure 1A. Among them, the major fluorescent compound (96% based on the peak area) showed the same retention volume as L-monapterin, the *threo* diastereomer. The major fluorescent pterin was subjected to FDCD and CD analyses. The FDCD spectrum of the pterin from *E. coli* was completely identical with that of L-monapterin at the wavelength shorter than 300 nm (Figure 2A). On the other hand, several differences between the CD spectra of the pterin and L-monapterin were recognized (Figure 2B), and it was impossible to say that these compounds were identical. The discrepancy of the CD spectra arose, most probably, from contamination of the sample from *E. coli* by non-fluorescent materials. Attempt to purify the sample by repeated HPLC gave another sample, whose FDCD was same. However, the CD spectrum of the sample still showed several differences, compared with authentic L-monapterin. A further purification was not carried out because of the loss during purification. In conclusion, the present FDCD method was highly specific for fluorescent compounds, and proved to be much better tool than CD or $[\alpha]_D$ for determining the stereochemical structure of pterin compounds from biological resources. By applying this method, the

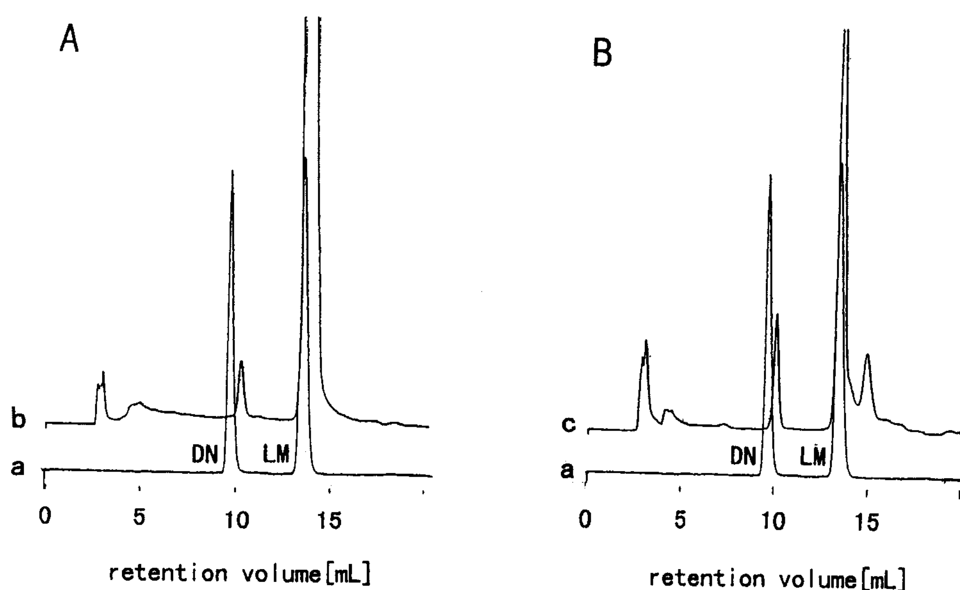


Figure 1. The reversed-phase analytical HPLC with fluorescence detection. **A**: authentic D-neopterin (**DN**) and L-monapterin (**LM**)(line **a**) and *E. coli* extract oxidized under acidic condition (line **b**); **B**: *E. coli* extract oxidized under alkaline condition (line **c**).

structure of the major pterin from *E. coli* was determined as L-monapterin.[22]

It is said that high linear dichroism (LD) gives fail signal to FDCD. [19, 23] Although the present examined three pterins exhibited rather low LD (Figure 2C), their CD amplitudes were too low at the wavelength longer than 300 nm (Figure 2B). This is the reason why the L- and D-monapterins did not exhibit symmetric figures in FDCD spectra. Since most of chiral pterin compounds exhibit low CD amplitude[18, 24, 25] over 300 nm, their FDCD should be used at a wavelength below 300 nm to discuss their chirality.

The existence of L-monapterin in human body fluids including urine, blood, and saliva has been reported by other groups.[14, 15, 16] It should be pointed that in most reports on L-monapterin in human body fluids, the structure assignments have been based on only HPLC under achiral conditions, by which enantiomers could not be separated. We have previously isolated pure D-monapterin (7 mg) from human urine (50 L) and determined the structure by using CD spectrum,[18] which required long purification procedures. In the present study, we could determine the structure of the chiral pterin from *E. coli* by using only 20 μg of the sample even contaminated with non-fluorescent impurities. We hope that our present report would

contribute to reduce the task required for determining the structure of chiral pterin compounds in nature.

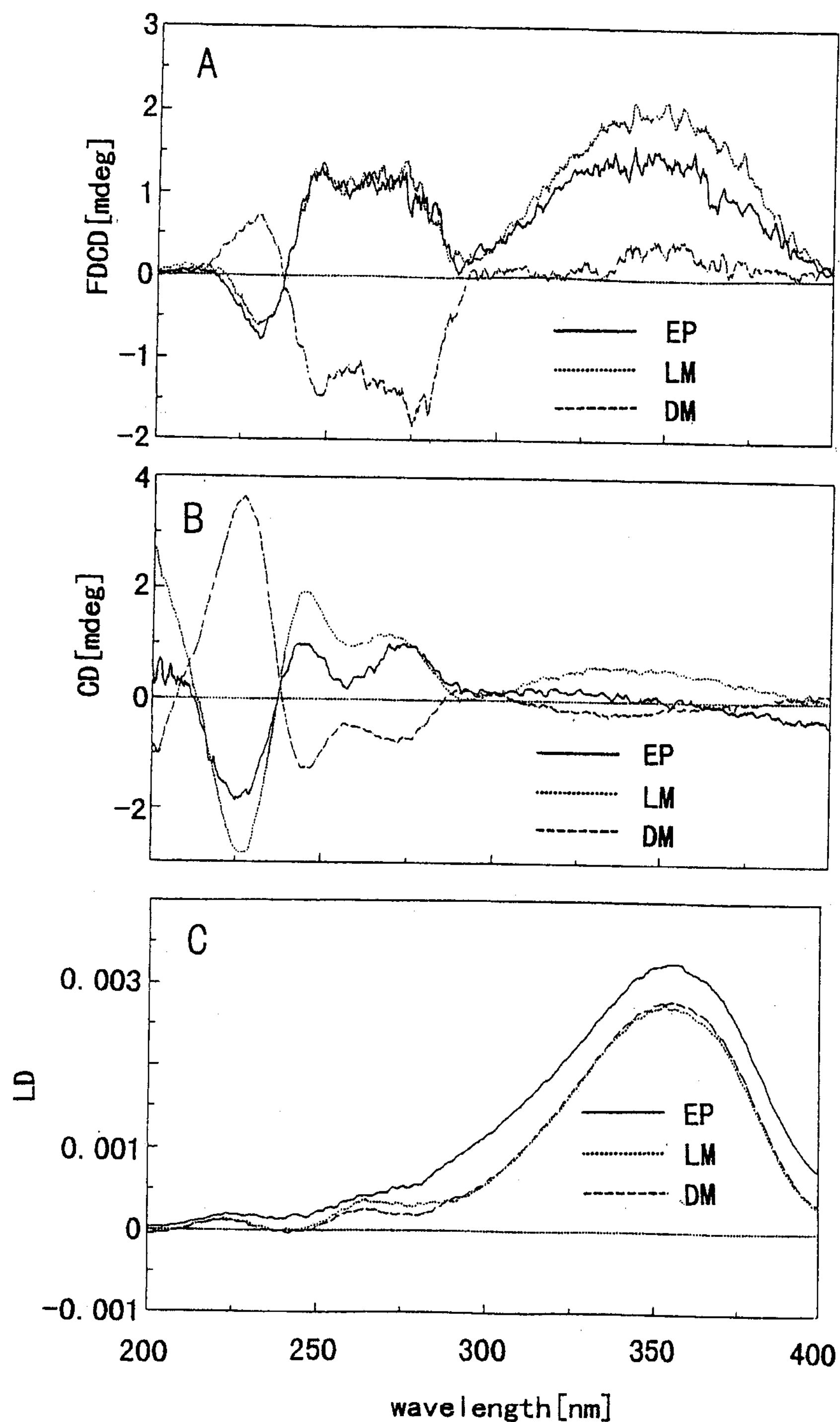


Figure 2. The FDCD (**A**), CD (**B**), and LD (**C**) spectra, respectively, of authentic L-monapterin (**LM**)(2.0×10^{-5} M), D-monapterin (**DM**) (2.0×10^{-5} M), and the pterin from *E. coli* (**EP**) (20 μg/2 mL).

In addition to the structure determination, it should also be noticed that the quantity of L-monapterin by oxidation of *E. coli* extract under acidic condition was three times larger than those under alkaline conditions (Figure 1A and 1B, respectively). This result strongly suggests the presence of a 5,6,7,8-tetrahydromonapterin in *E. coli*, according to the generally accepted method presented by Fukushima and Nixon.[26] The function of 5,6,7,8-tetrahydromonapterin in *E. coli* is ambiguous, in spite of a suggestion as a cofactor in the enzymatic conversion of phenylalanine to tyrosine in *Pseudomonas* sp.[27] However, such metabolism of aromatic amino acids is not known in *E. coli*. Then, further characterization of 5,6,7,8-tetrahydromonapterin in *E. coli* from chemical and biochemical points of view is required.

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22. The present structure determination by FDCD does not necessarily prove the optical purity of the major pterin. For such purpose, a combined use of FDCD and chiral HPLC, for example by using a chiral mobile phase (see Reference 17), would be useful.
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