Identification of (6*R*)-5,6,7,8-Tetrahydro-D-monapterin (=(6*R*)-2-Amino-5,6,7,8-tetrahydro-6-[(1*R*,2*R*)-1,2,3 -trihydroxypropyl]-pteridin-4(3*H*)-one) as the Native Pteridine in *Tetrahymena pyriformis*

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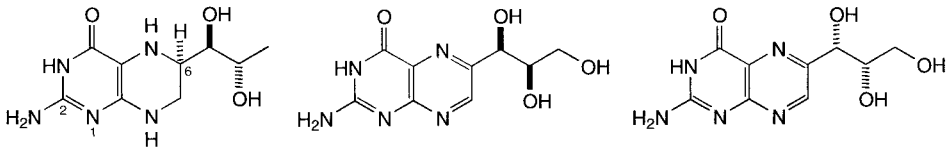
The structure of the native pteridine in *Tetrahymena pyriformis* was determined as (6*R*)-5,6,7,8-tetrahydro-D-monapterin (=(6*R*)-2-amino-5,6,7,8-tetrahydro-6-[(1*R*,2*R*)-1,2,3-trihydroxypropyl]pteridin-4(3*H*)-one; **4**). First, the configuration of the 1,2,3-trihydroxypropyl side chain was confirmed as D-*threo* by the fluorescence-detected circular dichroism (FDCD) spectrum of its aromatic pterin derivative **2** obtained by I_2 oxidation (*Fig. 1*). The configuration at the 6-position of **4** was determined as (*R*) by comparison of its hexaacetyl derivative **6** with authentic (6*R*)- and (6*S*)-hexaacetyl-5,6,7,8-tetrahydro-D-monapterins **6** and **7**, respectively, in the HPLC, LC/MS, and LC-MS/MS (*Figs. 3–6*). (6*R*)-5,6,7,8-Tetrahydro-D-monapterin (**4**) is a newly discovered natural tetrahydropterin.

1. Introduction. – The biosyntheses of catecholamines and indolamines from aromatic amino acids require (6*R*)-5,6,7,8-tetrahydrobiopteridin (=(6*R*)-2-amino-6-[(1*R*,2*S*)-1,2-dihydroxypropyl]-5,6,7,8-tetrahydropterin-4(3*H*)-one; **1**) as the natural cofactor of aromatic amino acid monooxygenases [1–3]. Compound **1** also acts as the natural cofactor of nitric oxide synthase [4]. These biogenic amines and nitric oxide play important roles as neurotransmitters, hormones, and chemical mediators in mammals. (6*R*)-5,6,7,8-Tetrahydrobiopterin (**1**) is synthesized from guanosine triphosphate (GTP) by the action of GTP cyclohydrolase I. The product, 7,8-dihydroneopterin triphosphate, is then converted to the final product *via* several enzymatic reactions. *Tetrahymena pyriformis* is a ciliated protozoa, and this animal cell is expected to synthesize **1** or analogous compounds rather than folic acid, since the protozoa has been reported to synthesize epinephrine and norepinephrine [5]. However, studies on the biosynthesis of pteridine compounds in *T. pyriformis* have been limited. The first report on the pteridine in *T. pyriformis* documented its structure as ciliapterin, a diastereoisomer of biopterin [6]; the structure was corrected later to D-monapterin (=2-amino-6-[(1*R*,2*R*)-1,2,3-trihydroxypropyl]pteridin-4(3*H*)-one; **2**) [7].

The presence of GTP cyclohydrolase I in *T. pyriformis* was confirmed for the first time in our recent study [8]. There, the major fluorescent pterin compound from *T. pyriformis* was neither biopterin nor neopterin. The pterin was identified as D- or L-monapterin (**2** or **3**, resp.) without confirming its absolute structure [8]. In addition, we obtained strong evidence that the pterin compound existed as a 5,6,7,8-tetrahydro



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derivative in the cell: oxidation of the crude extracts of the cell by I_2 [9] under acidic conditions afforded the fluorescent pterin in an amount increased by *ca.* 73% as compared to the oxidation under alkaline conditions.

In the present study, we establish the absolute structure of the native tetrahydropteridine in *T. pyriformis* as (6*R*)-5,6,7,8-tetrahydro-D-monapterin (**4**) by a highly sensitive fluorescence-detected circular dichroism study (FDCD) of its aromatic derivative **2** and by liquid chromatography/mass spectrometry (LC/MS) after transformation of the tetrahydropterin into the stable hexaacetyl derivative **6**.

2. Results and Discussion. – 2.1. *Configuration of the 1,2,3-Trihydroxypropyl Side Chain at C(6)*. The structure of the native tetrahydropterin **4** in *T. pyriformis* was determined first by confirming the absolute configuration of the side chain after transformation into its aromatic derivative as follows. The cells of *T. pyriformis* were disrupted by sonication in 2% (*w*/*v*) aqueous sodium dithionite solution and centrifuged. The lyophilized supernatant was subjected to I_2 oxidation under acidic conditions according to the method of *Fukushima* and *Nixon* [9]. Subsequent purification by column chromatography on *Florisil*®, a reversed-phase silica gel, and finally by prep. reversed-phase HPLC afforded 2.5 μg (from 28 g of wet cells) of 6-(*threo*-1′,2′,3′-trihydroxypropyl)pterin **2**. This compound and authentic D-monapterin (**2**) [10] exhibited the same HPLC retention volumes and FDCD spectra at wavelengths below 300 nm, with the first (−)-*Cotton* effect at 270 nm and the second (+)-*Cotton* effect at 230 nm (*Fig. 1*). L-Monapterin (**3**) [11] exhibited a mirror FDCD spectrum. Thus, the structure of the 6-(*threo*-1′,2′,3′-trihydroxypropyl)pterin from *T. pyriformis* was determined as D-monapterin (**2**). The FDCD spectra at the wavelength longer than 300 nm cannot be used for the structure determination of these pterin compounds, because FDCD signals are interfered by linear dichroism [12][13].

2.2. *Synthesis of (6*R*)- and (6*S*)-Hexaacetyl-5,6,7,8-tetrahydro-*D*-monapterins* **6** *and* **7**. Since tetrahydro-D-monapterin was present in *T. pyriformis* at very low concentration and was unstable toward air oxidation during purification steps, we aimed to determine the configuration at C(6) of a stable hexaacetyl derivative. As reference compounds for comparison, (6*R*)-hexaacetyl-5,6,7,8-tetrahydro-D-monapterin **6** and the (6*S*)-isomer **7** were synthesized, and their C(6) configurations were confirmed as follows. Catalytic hydrogenation of **2** on PtO_2 in HCl solution gave a mixture of (6*R*)-5,6,7,8-tetrahydro-D-monapterin (**4**) and the (6*S*)-isomer **5** (*Scheme*). Acetylation of this mixture by a large excess of Ac_2O at 110° yielded a mixture **6**/**7**, which was separated. The configuration at C(6) of **6** and **7** was determined by comparing their CD spectra with that of (6*R*)-pentaacetyl-5,6,7,8-tetrahydrobiopterin **8** [14], prepared similarly by acetylation of **1**. The CD spectrum of **8** showed the first (+)-*Cotton* effect at 320 nm,

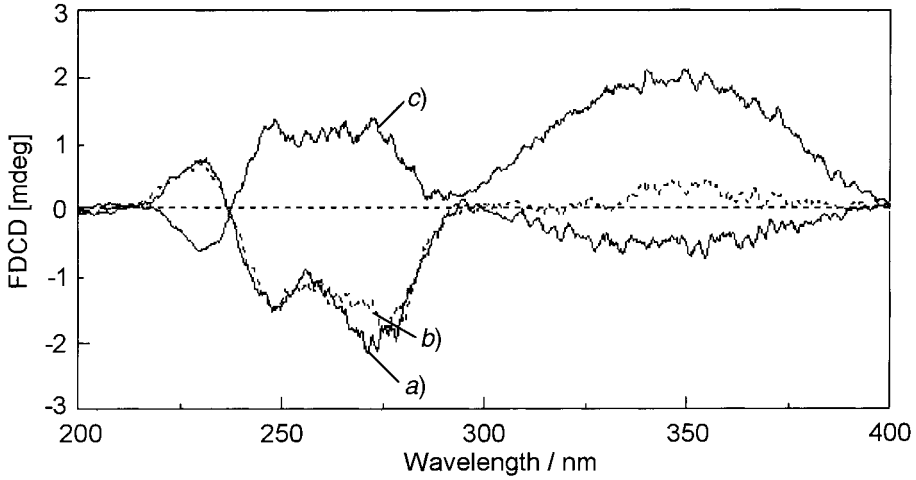
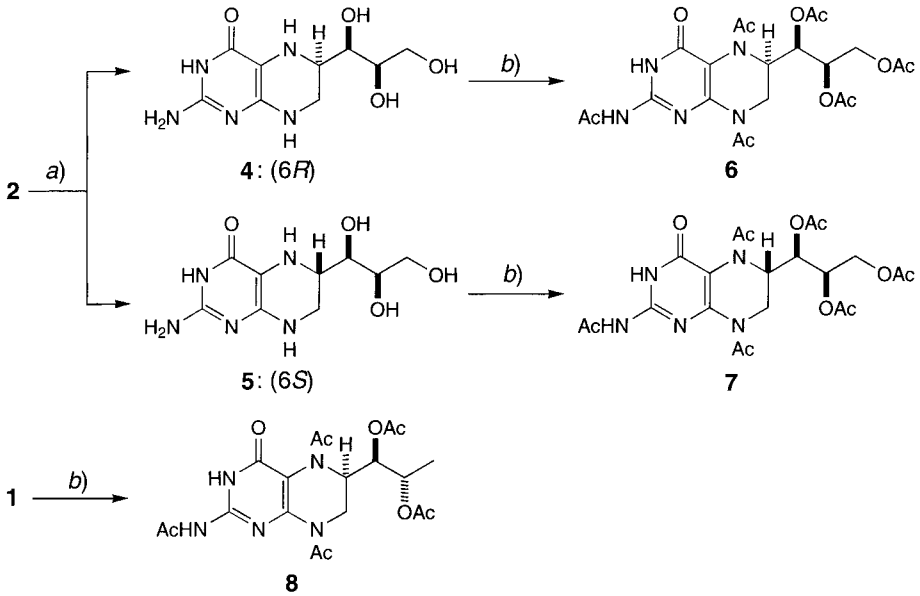


Fig. 1. *FDCD spectra of* a) D-*monapterin* (**2**) *produced by* I_2 *oxidation of cell extracts from* T. pyriformis, b) *authentic* D-*monapterin* (**2**), *and* c) L-*Monapterin* (**3**), *in 0.2*M *potassium phosphate buffer* (pH 5.4; $2 \cdot 10^{-5}$ M each)

the second (−)-*Cotton* effect at 248 nm, and the third (+)-*Cotton* effect at 227 nm (*Fig. 2*). One of the hexaacetyl-5,6,7,8-tetrahydro-D-monapterins, **6**, with an HPLC retention volume V_R of 19.4 ml, showed similarly *Cotton* effects at 319.5 (+), 249 (−), and 226.5 nm (+) (*Fig. 2*). On the other hand, the other hexaacetyl-5,6,7,8-tetrahydro-D-monapterin, **7**, with a retention volume of 17.8 ml, exhibited *Cotton* effects at 320.5 (−), 248 (+), and 227 nm (−) (*Fig. 2*). We have previously reported that CD spectra of tetrahydrobiopterin and related tetrahydropterins were most strongly affected by their C(6) configurations [15]. In addition, all of the three tetrahydropterin compounds **6**–**8** possess the same C(1′) configuration at the side chain. Therefore, the absolute configuration at C(6) of the hexaacetyl-5,6,7,8-tetrahydro-D-monapterin with retention volume 19.4 ml was determined as (*R*), and the other as (*S*) (**6** and **7**, resp.).

2.3. *Identification of (6*R*)-Hexaacetyl-5,6,7,8-tetrahydro-*D*-monapterin* **6** *from the Cell Extract of* T. pyriformis *after Acetylation.* The lyophilized cell extract of *T. pyriformis* was subjected to acetylation in a similar manner as above and then partially purified by supercritical-fluid carbon dioxide extraction, silica-gel column chromatography, and silica gel TLC. The HPLC of the crude sample from *T. pyriformis* showed a peak with the same HPLC retention volume (19.4 ml) and UV spectrum as those of (6*R*)-hexaacetyl-5,6,7,8-tetrahydro-D-monapterin **6** (*Fig. 3*,*a*), whereas no peak corresponding to the (6*S*)-isomer **7** was detected. The MS of (6*R*)-hexaacetyl-5,6,7,8-tetrahydro-D-monapterin **6** exhibited outstanding peaks at *m*/*z* 510, 532, and 548 corresponding to $[M\mathrm{H}]^+$, $[M+\mathrm{Na}]^+$, and $[M+\mathrm{K}]^+$ ions, respectively (*Fig. 4*,*a*). The peak at *m*/*z* 468 arose by loss of a ketene moiety (C_2H_2O), which is a typical fragment of an acetate compound. The (6*S*)-isomer **7** showed a similar MS as the (6*R*)-isomer (*Fig. 5*,*a*). Thence, we measured the LC/MS of the sample from *T. pyriformis* monitored by the signal at *m*/*z* 510, exhibiting V_R 19.4 ml like **6** (*Fig. 3*,*b*). No peak corresponding to the (6*S*)-isomer **7** was detected in this sample. The MS/MS of the

Scheme. *Synthesis of (6*R*)-Hexaacetyl-5,6,7,8-tetrahydro-*D*-monapterin* **6**, *the (6*S*)-Isomer* **7**, *and (6*R*)-Pentaacetyl-5,6,7,8-tetrahydrobiopterin* **8**



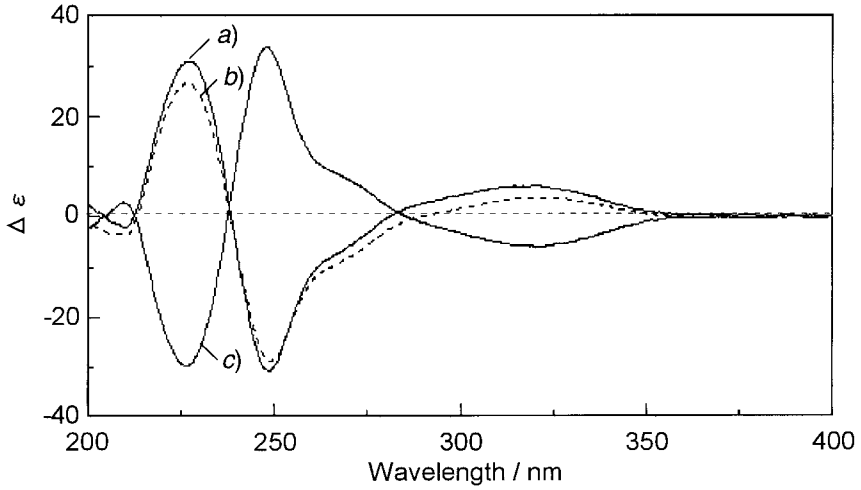


Fig. 2. *CD Spectra (MeCN) of* a) *(6*R*)-pentaacetyl-5,6,7,8-tetrahydrobiopterin* **8**, b) *(6*R*)-hexaacetyl-5,6,7,8-tetrahydro-*D*-monapterin* **6**, *and* c) *the (6*S*)-isomer* **7**

signal at *m*/*z* 510 from synthetic (6*R*)-isomer **6** and from hexaacetyl-5,6,7,8-tetrahydro-D-monapterin from *T. pyriformis* exhibited almost the same fragmentation pattern (*Figs. 4,b*, and *6,b*, resp.), with signals at *m*/*z* 468, 450 ($[468 - H_2O]^+$), 408 ($[450 - C_2H_2O]^+$), and 366 ($[408 - C_2H_2O]^+$). Although the ion at *m*/*z* 510 from the synthetic

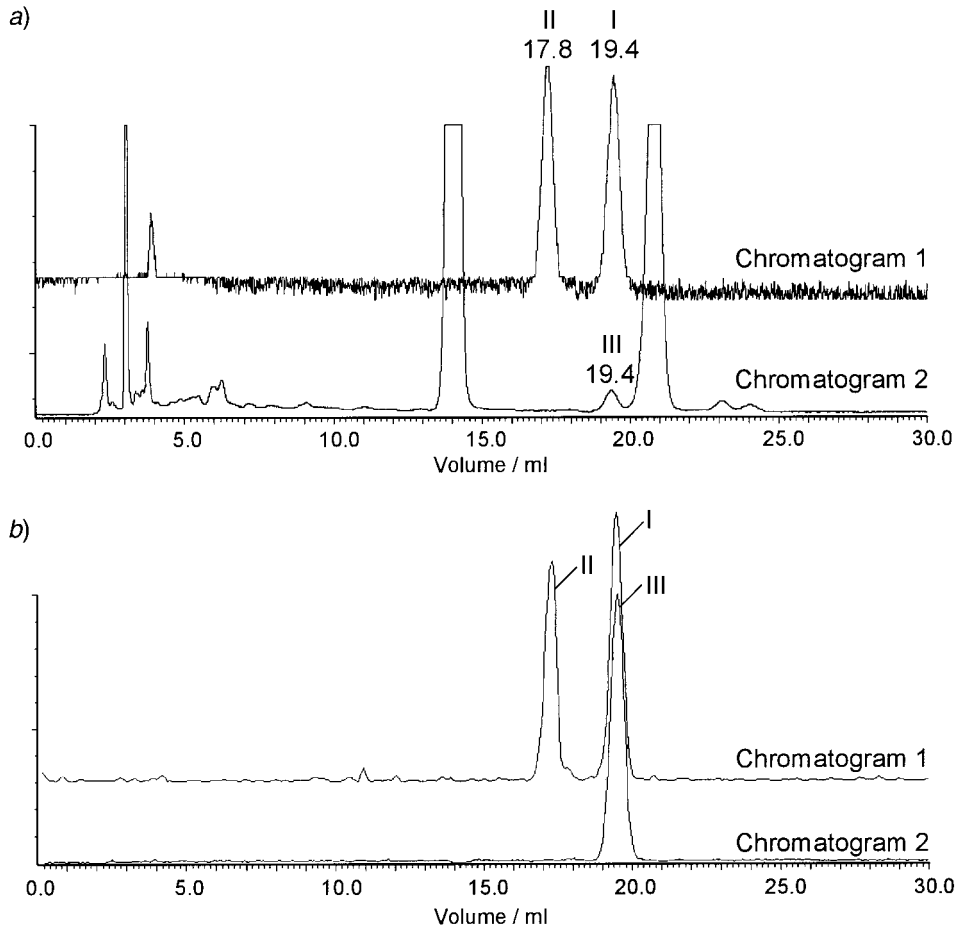


Fig. 3. a) *HPLC Trace monitored by UV absorption at 245 nm and* b) *HPLC/MS monitored by the signal at* m/z 510. Chromatogram 1: mixture of (6*R*)-hexaacetyl-5,6,7,8-tetrahydro-D-monapterin **6** (peak I) and the (6*S*)-isomer **7** (peak II). Chromatogram 2: the acetylated cell extracts of *T. pyriformis* with a peak for (6*R*)-hexaacetyl-5,6,7,8-tetrahydro-D-monapterin (peak III). HPLC Conditions: *Inertsil® ODS* (4.6 × 250 mm, with a 4.6 × 5 mm guard column), elution with 20% MeCN/H_2O

(6*S*)-isomer **7** produced the same fragment ions, their relative intensities were different compared to those of the (6*R*)-isomer **6** at the same collision energy (*Fig. 5,b*). A comparison of the UV spectra of the three samples is given in *Fig. 7*.

From these results, it is concluded that the hexaacetyl-5,6,7,8-tetrahydro-D-monapterin from *T. pyriformis* possesses the (*R*)-configuration at the C(6) position (structure formula, **6**), and that this microorganism contains (6*R*)-5,6,7,8-tetrahydro-D-monapterin (**4**) as the native pteridine. In addition, it should be emphasized that **4** is the natural compound in *T. pyriformis* and not an artifact obtained during the extraction steps of the cells with sodium dithionite since no (6*S*)-isomer **7** could be detected, even in the sample at a very crude purification stage. Attempts to detect (6*R*)-5,6,7,8-

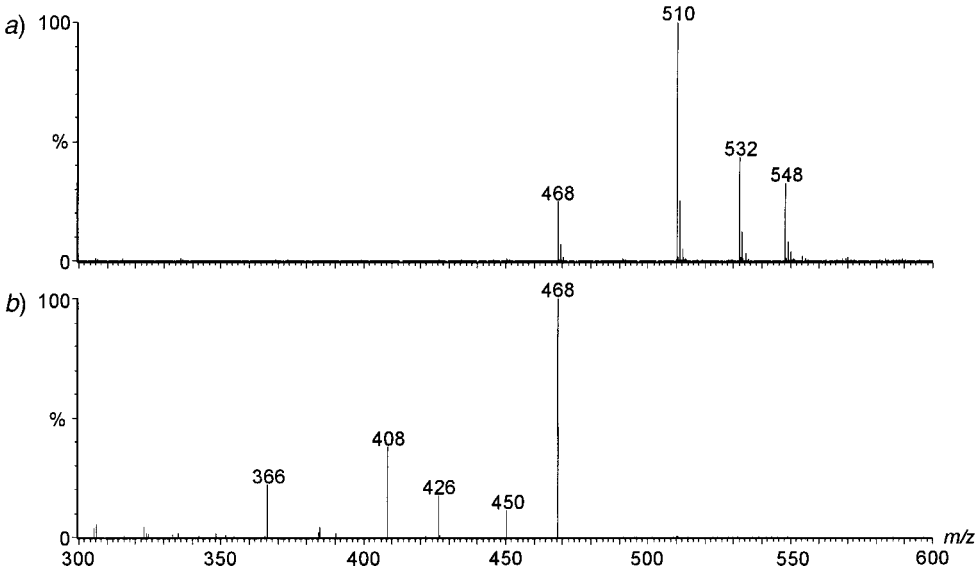


Fig. 4. a) *MS of (6*R*)-hexaacetyl-5,6,7,8-tetrahydro-*D*-monapterin* **6** (collision at 10 eV) *and* b) *MS/MS of the signal at* m/z *510* (collision at 20 eV)

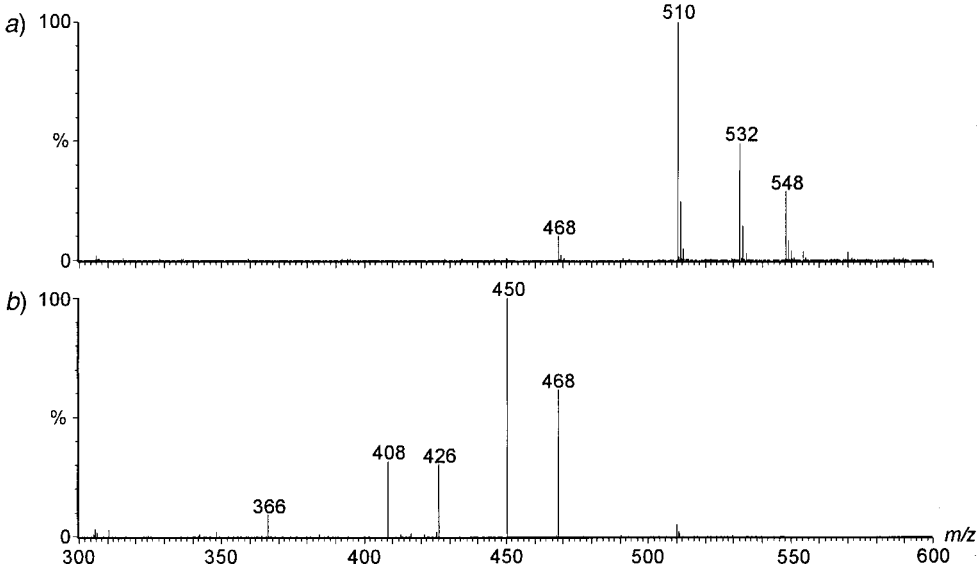


Fig. 5. a) *MS of (6*S*)-hexaacetyl-5,6,7,8-tetrahydro-*D*-monapterin* **7** (collision at 10 eV) *and* b) *MS/MS of the signal at* m/z *510* (collision at 20 eV)

tetrahydro-D-monapterin (**4**) directly in the cell extract were unsuccessful owing to its instability toward oxidation and its too-low concentration in the complex cell extract. The estimated concentration of **4** in *T. pyriformis* was *ca.* 0.1 μg/g of wet cells.

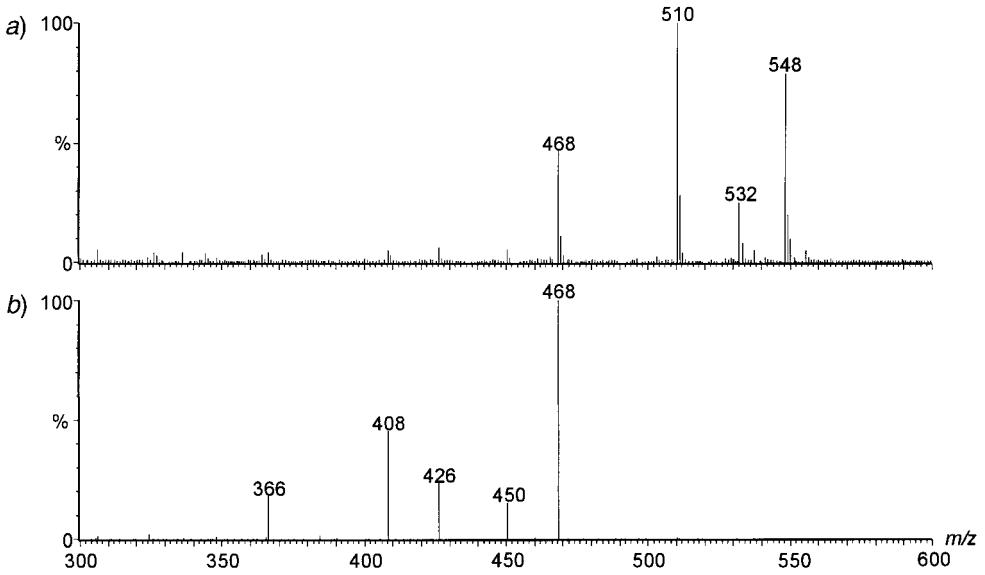


Fig. 6. a) *MS of hexaacetyl-5,6,7,8-tetrahydro-*D*-monapterin* **6** *from* T. pyriformis (collision at 10 eV) *and* b) *MS/MS of the signal at* m/z *510* (collision at 20 eV)

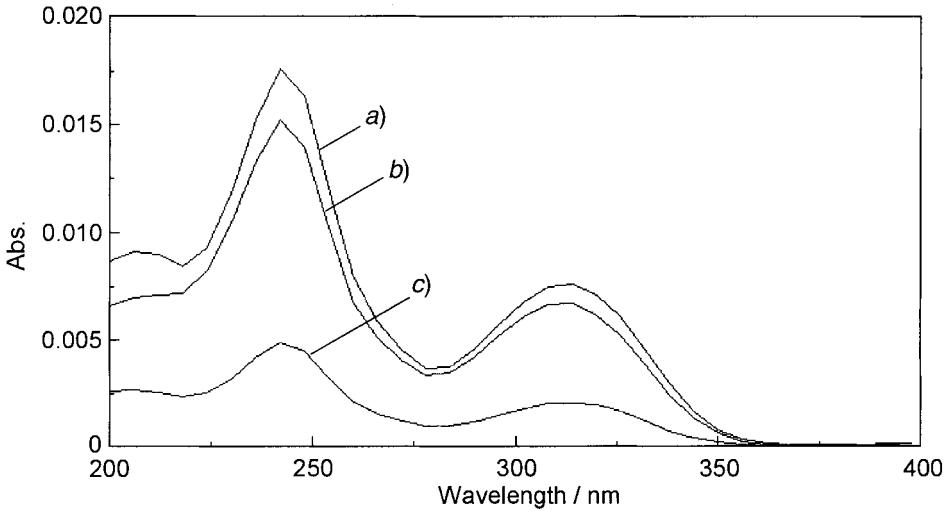


Fig. 7. *UV Spectra of* a) *(6*R*)-hexaacetyl-5,6,7,8-tetrahydro-*D*-monapterin* **6**, b) *(6*S*)-hexaacetyl-5,6,7,8-tetrahydro-*D*-monapterin* **7**, *and* c) *hexaacetyl-5,6,7,8-tetrahydro-*D*-monapterin* **6** *from* T. pyriformis, *in 20% MeCN/H_2O, measured with a multichannel HPLC detector*

Although D-monapterin (**2**) has been found in *T. pyriformis* [7] and human urine [16], its dihydro and tetrahydro derivatives have not been known in nature so far. Therefore, (6*R*)-5,6,7,8-tetrahydro-D-monapterin (**4**) is a newly discovered natural tetrahydropterin, after the recently confirmed natural (6*R*)-5,6,7,8-tetrahydrobiopterin

(**1**) [15], and determining the presence of **4** in higher animal species and its biological functions remain for further studies.

Experimental Part

1. *General.* Anal. and prep. TLC: *Merck*® silica gel *60 F_{254}* (thickness 0.25 mm). Column chromatography (CC): silica gel *Fuji Silia*® *BW-300*. Anal. HPLC, LC-MS, and LC-MS/MS: *Inertsil*® *ODS* (4.6 × 250 mm, with a 4.6 × 5 mm guard column), elution with 20% (*v*/*v*) MeCN/H_2O, unless otherwise indicated; detection with a *Jasco MD-1515* multichannel UV detector; V_R = retention volume. UV spectra: *Jasco MD-1515* multichannel UV detector or *Jasco V-550* λ_{max} (log ε) in nm. CD Spectra: *Jasco J-720* CD spectrometer; FDCD, *Jasco J-720* coupled to a *Jasco FDCD-357* $\lambda(\Delta\varepsilon)$ in nm. ^{1}H- and ^{13}C-NMR Spectra: *Jeol α400* spectrometer (400 MHz); δ in ppm rel. to internal $SiMe_4$, *J* in Hz. LC-MS and LC-MS/MS: *Micromass LCT* and *Quattro Ultima* mass spectrometers, resp.; in *m*/*z*.

2. *Cell Culture and Extraction of Intracellular Pteridines. T. pyriformis* strain W was grown in an enriched medium containing 2% (*w*/*v*) proteose peptone (*Difco*®), 0.2% (*w*/*v*) yeast extract (*Difco*®), and 0.5% (*w*/*v*) D-glucose by shaking at 26° for 48 h according to the previously described method [17]. Cells were collected by centrifugation (5000 rpm, 10 min), washed twice with 10 mM phosphate buffer saline (*ca.* 50 × the volume of the collected wet cells), and stored in a deep freezer at −80°.

The frozen cells of *T. pyriformis* (20 g) were disrupted in 2% (*w*/*v*) aq. sodium dithionite soln. (80 ml) by sonication on ice, with five bursts of 0.5 min at 60 W and 1 min interval, on a *Branson Sonifier 250*. The resulting mixture was centrifuged at 100000 × *g* and 4° for 20 min on a *Beckman XL-90* ultracentrifuge. The supernatant was immediately frozen and lyophilized to give a colorless powder (2.0 g), which was used as the intracellular extract of *T. pyriformis.*

3. *Isolation of* D-*Monapterin* (**2**) *after I_2 Oxidation of the Cell Extract from* T. pyriformis. The above colorless powder (2.8 g; equivalent to 28 g of wet cells) was dissolved in H_2O (30 ml). To the soln., a soln. of 8% (*w*/*v*) I_2 and 16% (*w*/*v*) KI in 1M HCl was added with shaking until the characteristic color of I_2 remained for 5 min. The mixture was kept in the dark at r.t. for 1 h. Then 20% (*w*/*v*) aq. ascorbic acid soln. was added until the mixture became colorless. Colorless precipitates were removed by centrifugation at 4000 rpm for 10 min. The supernatant was applied to CC (*Florisil*®, 1.5 × 13 cm), and H_2O (100 ml) was passed through the column until the eluted soln. became neutral. Then, 0.5M aq. ammonia was passed through the column. The blue fluorescent soln. that eluted between 20 and 70 ml was adjusted to pH 2 with 5M HCl and once again subjected to CC (*Florisil*®) in the same way as above. The soln. eluted between 20 and 70 ml was evaporated. The residue was mixed with 0.5M aq. ammonia (15 ml). A small amount of insoluble material was removed by centrifugation at 4000 rpm for 10 min. The supernatant was again evaporated. The residue was dissolved in H_2O (10 ml), and the soln. was subjected to reversed-phase CC (silica gel *Fuji Gel*® *ODSQ3*, 4 × 8 cm, 2% MeCN/H_2O (100 ml)). The soln. was concentrated to *ca.* 20 ml and then fractionated by prep. HPLC (*Inertsil*®*-ODS* column (20 × 250 mm, 20 × 50 mm, guard column), 2% MeCH/H_2O). On evaporation, the fraction with V_R 163–187 ml gave a residue containing 2.5 μg of **2** (estimated from UV absorption at 343 nm). HPLC (*Inertsil*®*-ODS-3* column, 4.6 × 250 mm, 2% MeCN/H_2O): V_R 8.5 ml.

4. *(6*R*)- and (6*S*)-2-Acetamido-5,8-diacetyl-5,6,7,8-tetrahydro-6-[(1*R*,2*R*)-1,2,3-tris(acetyloxy)propyl]pteridin-4(3*H*)-one* (= *(6*R*)- and (6*S*)-Hexaacetyl-5,6,7,8-tetrahydro*-D-*monapterin,* resp.; **6** and **7**, resp.). A mixture of **2** (200 mg) and PtO_2 (70 mg) in 1M HCl (10 ml) was shaken vigorously under H_2 for 4 h. The catalyst was removed by filtration and the filtrate evaporated. The residue was heated in the presence of AcOH (5 ml) and Ac_2O (15 ml) with stirring under Ar at 110° for 90 min. MeOH (5 ml) was added to the cooled soln., which was evaporated. The residue was dissolved in CH_2Cl_2 (5 ml), the soln. washed with H_2O (5 ml) and sat. aq. $NaHCO_3$ soln. (5 ml), dried ($MgSO_4$), and evaporated, and the oily residue fractionated by CC (silica gel, 11 × 120 mm, column, AcOEt/MeOH 95:5). The fraction with R_f 0.36 on TLC (silica gel, AcOEt/MeCN 8:2, the two diastereosiomers were not separated) was evaporated. The pale yellow residue was subjected to prep. HPLC (*Inertsil*® *Prep-ODS* column (20 × 250 mm, 20 × 50 mm, guard column) 20% MeCN/H_2O). The fractions with V_R 377–410 ml and 441–486 ml were separately evaporated. Each residue was subjected to prep. HPLC (3 times more under the same conditions): **6** (9 mg, 2.3%) from the latter fraction and **7** (8 mg, 2.0%) from the former fraction.

Data of **6**: Colorless prisms (from dioxane). M.p. 152–153.5° (dec.). HPLC: V_R 19.4 ml. UV (MeCN): 315.5 (3.93), 263 (sh, 3.85), 242.5 (4.28). CD (MeCN): 319.5 (2.5), 288.5 (0.0), 249 (−18.6), 238 (0.0), 226.5 (17.5), 213 (0.0), 210 (−3.8). ^{1}H-NMR ($CDCl_3$): 1.96 (*s*, 3 H); 2.07 (*s*, 3 H); 2.13 (*s*, 3 H); 2.14 (*s*, 3 H); 2.33 (*s*, 3

H); 2.54 (*s*, 3 H); 3.63 (*dd*, *J* = 6.4, 13.7, 1 H); 3.84 (*dd*, *J* = 6.8, 11.7, 1 H); 4.00–4.07 (*m*, 1 H); 4.15 (*dd*, *J* = 4.9, 11.7, 1 H); 4.94–5.00 (*m*, 1 H); 5.27–5.33 (*m*, 2 H); 9.49 (br., 1 H). ^{13}C-NMR ($CDCl_3$): 20.57; 20.64; 20.84; 22.07; 24.36; 27.29; 45.57; 47.52; 62.28; 68.34; 68.67; 105.00; 146.71; 150.66; 157.31; 169.94; 170.22; 170.56; 171.67; 172.13. MS: 548, 532, 510, 468.

Data of **7**: Viscous oil. HPLC: V_R 17.8 ml. UV (MeCN): 316 (3.96), 263 (sh, 3.88), 242.5 (4.30). CD (MeCN): 320.5 (–3.8), 285.5 (0.0), 248 (21.9), 237.5 (0.0), 227 (–19.2), 213 (0.0), 209.5 (1.9). ^{1}H-NMR ($CDCl_3$): 2.02 (*s*, 3 H); 2.03 (*s*, 3 H); 2.13 (*s*, 3 H); 2.16 (*s*, 3 H); 2.32 (*s*, 3 H); 2.53 (*s*, 3 H); 3.73 (*dd*, *J* = 6.8, 14.2, 1 H); 3.99 (*dd*, *J* = 6.8, 11.7, 1 H); 4.17–4.24 (*m*, 1 H); 4.32 (*dd*, *J* = 3.9, 11.7, 1 H); 5.01 (*dd*, *J* = 3.4, 9.3, 1 H); 5.24–5.37 (*m*, 2 H); 8.93 (br., 1 H). ^{13}C-NMR ($CDCl_3$): 20.64; 20.66; 20.74; 22.22; 24.41, 27.13; 47.60; 47.60; 62.51; 68.22; 69.27; 105.78; 146.68; 150.51; 157.11; 170.23; 170.31; 170.45; 170.62; 171.13, 172.74. MS: 548, 532, 510, 468.

5. *(6*R*)-2-Acetamido-5,8-diacetyl-6-[(1*R*,2*S*)-1,2-bis(acetyloxy)propyl]-5,6,7,8-tetrahydropteridin-4(3*H*)-one* (= *(6*R*)-Pentaacetyl-5,6,7,8-tetrahydrobiopterin*, **8**). A mixture of (6*R*)-5,6,7,8-tetrahydrobiopterin dihydrochloride (**1**·2 HCl; 30 mg) [15] and Ac_2O (12 ml) was heated under reflux and Ar for 2 h. The soln. was evaporated, the residue dissolved in CH_2Cl_2 (1 ml), and the soln. submitted to CC (silica gel, 6 × 70 mm column, AcOEt/MeCN 7:3). The fraction with R_f 0.37 on TLC (silica gel, AcOEt/MeCN 9:1) was evaporated. The residue was fractionated by prep. TLC (silica gel, 3 20 × 20 cm plates, AcOEt/MeCN 9:1). The scratched bands with R_f 0.17–0.45 were well mixed with MeCN (50 ml) and filtered. The filtrate was evaporated and the residue crystallized from AcOEt/hexane 4:6: **8** (12 mg, 27%) [14]. Colorless needles. M.p. 214–216.5° (dec.). HPLC: V_R 25.4 ml. UV (MeCN): 315.5 (4.01), 263 (sh, 3.91), 241 (4.36). CD (MeCN): 320 (6.2), 281 (0.0), 248 (–31), 238 (0.0), 227 (31.1), 212 (0.0), 210 (–2.1). ^{1}H-NMR ($CDCl_3$): 1.29 (*d*, *J* = 6.8, 3 H); 1.88 (*s*, 3 H); 2.07 (*s*, 3 H); 2.18 (*s*, 3 H); 2.30 (*s*, 3 H); 2.54 (*s*, 3 H); 3.50 (*dd*, *J* = 5.4, 13.7, 1 H); 4.12–4.19 (*m*, 1 H); 4.87 (*dt*, *J* = 2.0, 6.8, 1 H); 5.03–5.16 (*m*, 2 H); 8.43 (br., 1 H).

6. *Identification of (6*R*)-Hexaacetyl-5,6,7,8-tetrahydro-*D*-monapterin* (**6**) *from* T. pyriformis *Extracts.* A mixture of the lyophilized extract of *T. pyriformis* (0.5 g, equivalent to 5 g of the wet cells) and Ac_2O (12 ml) was refluxed under Ar with vigorous stirring for 90 min. The mixture was evaporated to remove volatile materials. The residue was transferred into the pressure vessel of the supercritical fluid extraction apparatus (*Jasco SUPER-200*). The residue was extracted in this apparatus with CO_2 (5 ml/min) for 2 h to remove unidentified less polar compounds (50 mg). Then, the extraction was continued with MeCN (3 ml/min) as an entrainer of CO_2 (5 ml/min) for 3.5 h. This extract was evaporated and the colored residue (35 mg) was applied to CC (silica gel, 5.5 × 70 mm column); washing with AcOEt (10 ml), then elution with AcOEt/MeCN 9:1 (50 ml). Evaporation of the eluate gave a brown residue (8 mg), which was dissolved in a small amount of MeCN and submitted to prep. TLC (silica gel, 2 20 × 20 cm plates, AcOEt/MeCN 7:3). The scratched bands with R_f 0.11–0.50 were well mixed with MeCN (20 ml) and filtered. Evaporation of the filtrate gave a light brown crude residue (1.4 mg). UV (20% MeCN/H_2O): 314, 242 (multichannel HPLC detector). LC-MS: 548, 532, 510, 468; HPLC V_R 19.4 ml.

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