46. Pteridines

Part CVI[1])

Isolation and Characterization of Limipterin (1-*O*-(L-*erythro*-Biopterin-2′-yl)-*β*-*N*-acetylglucosamine) and Its 5,6,7,8-Tetrahydro Derivative from Green Sulfur Bacterium *Chlorobium limicola f. thiosulfatophilum* NCIB 8327

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A new pteridine compound was isolated from green sulfur photosynthetic bacteria, *Chlorobium limicola f. thiosulfatophilum* NCIB 8327. The structure of this pterin derivative was established to be 1-*O*-(L-*erythro*-5,6,7,8-tetrahydropterin-2′-yl)-*β*-*N*-acetylglucosamine (**1**) from ^{1}H-NMR and CD spectra as well as from various mass-spectrometric techniques and chemical-cleavage techniques. Upon acid hydrolysis of **1**, equimolar amounts of biopterin (**2**) and *N*-acetylglucosamine were produced. The structure of the hydrolysis product **2** was confirmed by comparing its NMR, UV, CD, and MS and its chromatographical behavior with those of an authentic specimen. *N*-Acetylglucosamine was identified by an enzymatic hydrolysis experiment as well as by NMR and thin layer chromatography. Electrospray (ES), fast-atom-bombardment (FAB), and thermospray (TS) mass spectrometry of **1** yielded an MH^+ at m/z 441. Periodate-oxidation experiments of the intact molecule **1** and of its hydrolysis product **2** are consistent with the proposed structure. Differential I_2 oxidation experiments with the native compound showed that the *in vivo* oxidation state of this pterin is its tetrahydro form. We propose the trivial name 'limipterin' for this new compound.

1. Introduction. – All naturally occurring pterin compounds including conjugated or unconjugated forms are biosynthesized from the common precursor GTP by GTP cyclohydrolase I [2–8]. The conjugated pterins refer to the linkage of *p*-aminobenzoylglutamate to pterin such as folate, whereas unconjugated pterins are biopterin, molybdopterin, and pterin pigments. Despite their structural similarity, these two types of cofactor have unrelated functions, namely C_1 transfer and redox reactions, respectively [2] [9] [10].

It was reported that microorganisms also contain various simple pterins as well as conjugated pterins [11]. Especially in a number of photosynthetic bacteria, the presence of relatively high contents of pterin compounds was demonstrated, and these contents were reported to be correlated with the method of cell culture [12].

In this publication, we report the purification and structural characterization of a new pterin glycoside, limipterin (**1**), isolated from one of anaerobic photoautotrophs, green sulfur bacteria, *Chlorobium limicola f. thiosulfatophilum* NCIB 8327.

2. Results and Discussions. – Isolation of the new natural product **1** was achieved by two different approaches – the *Dowex-DeltaPak* and the charcoal-*DeltaPak* method –

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which gave the same product. But, the yield of the second procedure was higher (19 mg/100 g wet weight cell) than that of the first one (7.5 mg/100 g wet weight cell). As shown in *Fig. 1*, limipterin (**1**) was, on monitoring at 276 nm, the major absorbing component present in the solution treated by *Dowex* or charcoal.

The purity of limipterin (**1**) was confirmed by anal. HPLC (*Fig. 2*) and capillary electrophoresis (*Fig. 3*). Since the fluorescence/absorbance ratio reveals very valuable information in identifying pteridine compounds [13], this feature and the retention time t_R of **1** on HPLC *NovaPak C18* was compared to the values of authentic pteridine compounds (*Table 1*). Limipterin (**1**) eluted at 4.82 min, which is longer than for biopterin

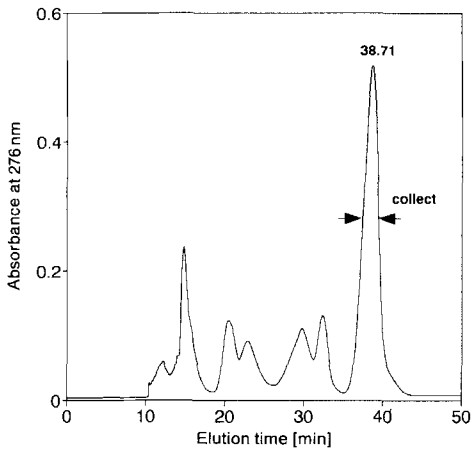


Fig. 1. *HPLC Profile on a column of* DeltaPak C18 (30 mm × 30 cm). Charcoal eluent was concentrated and injected isocratically with 2% aq. MeOH at a flow rate of 15 ml/min. Absorbance was monitored at 276 nm.

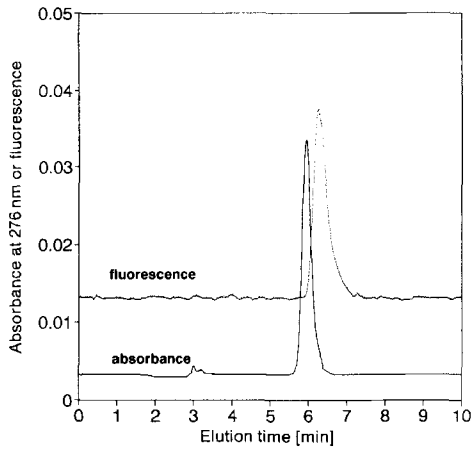


Fig. 2. *HPLC Profile of purified limipterin* (**1**) *on a column of* NovaPak C18 (2.5 mm × 15 cm). Purified **1** was injected and chromatographed isocratically with 2% aq. MeOH at a flow rate of 1.0 ml/min. Peak was detected by absorbance at 276 nm or by fluorescence (excitation at 360 nm, emission at 450 nm).

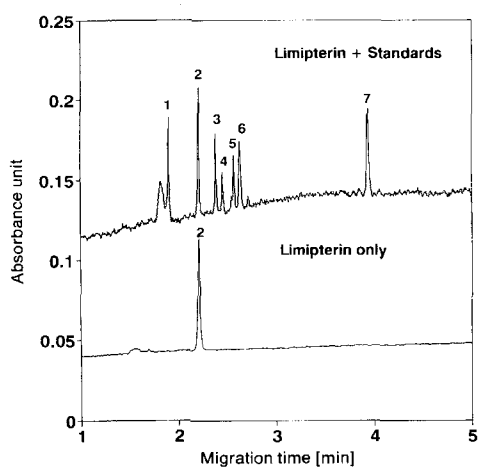


Fig. 3. *Capillary electrophoresis of limipterin* (**1**) *with and without standard pterins.* Purified **1** was injected by pressure for 5 s. Electrophoresis was carried out at 30 kV using running buffer 0.1M boric acid/sodium borate, pH 8.4. Legend: 1 = Sepiapterin, 2 = limipterin (**1**), 3 = biopterin, 4 = neopterin, 5 = pterin, 6 = monapterin, and 7 = 6-carboxypterin.

(4.05 min) or pterin (4.33 min), and **1** also had a slightly different fluorescence/absorbance ratio (1.061) than biopterin (1.00).

When purified limipterin (**1**) was subjected to acid hydrolysis, a new pteridine component **2** with a smaller t_R than **1** was produced. This hydrolysis product was purified from the hydrolysate using prep. *DeltaPak-C18* HPLC. The purity of **2** was verified by an anal. HPLC and capillary electrophoresis. The hydrolysis rate was pH-dependent, and **2** could be obtained when treated at pH 1.0–2.0. The released product **2** is very insoluble in H_2O and precipitated during the hydrolysis or during concentration of purified material. As shown in *Table 1*, the hydrolysis product **2** had the same t_R as authentic biopterin on the

Table 1. *Retention Times and Fluorescence/Absorbance Ratios of Limipterin* (**1**), *Hydrolysis Product* **2**, *and Authentic Pteridine Compounds*

	Retention times t_R [min][a])	Fluorescence absorbance ratio[b])
Biopterin (**2**)	4.05	1.000
Neopterin	2.07	0.986
Pterin	4.33	1.256
6-Carboxypterin	1.01	0.876
6-Formylpterin	9.96	0.630
6-(Hydroxymethyl)pterin	4.74	1.296
Sepiapterin	22.23	0.058
Monapterin	2.52	1.099
Isoxanthopterin	4.24	3.603
Leucopterin	2.88	0.015
Folic acid	1.03	0.357
Lumazine	4.07	0.146
6-Hydroxylumazine	2.11	9.171
Limipterin (**1**)	4.82	1.061
Hydrolysis product **2** of limipterin	4.04	1.003

[a]) Reversed-phase chromatography of pteridines was carried out with a *NovaPak C18* column (3.9 mm × 15 cm, *Waters*) using 5% aq. MeOH as mobile phase.

[b]) The fluorescence(450 nm)/absorbance(276 nm) ratio of biopterin was taken at 1.0.

anal. reversed-phase HPLC columns (*Lichrosorb C18* and *NovaPak C18*), and the fluorescence/absorbance ratio was also identical to biopterin (1.00). Thin-layer chromatography (TLC) on a cellulose plate (*Table 2*) and even capillary electrophoresis could not distinguish **2** from authentic biopterin (*Fig. 4*). The configuration of the side chain was shown to be L-*erythro* by a comparison of the CD spectra of hydrolysis product **2** and authentic biopterin (*Fig. 5*).

Limipterin (**1**) revealed also the typical fluorescence of pterin compounds, which emits at 450 nm when excited at 360 nm (*Fig. 6*). The UV/VIS spectrum of **1** shown in *Fig. 7a* was similar to that of biopterin (*Fig. 7c*) at wavelengths above 240 nm, but at shorter wavelengths, some minor differences were noticed. On the other hand, the hydrolysis product **2** (*Fig. 7b*) and authentic biopterin (*Fig. 7c*) had perfectly the same fluorescence and absorption spectra.

To obtain the molecular mass and more structural information about **1** and **2**, electrospray (ES), static fast-atom-bombardement (FAB), and thermospray (TS) mass spectrometry were carried out. For limipterin (**1**), the MH^+ peak at m/z 441 was obtained

Table 2. *Thin-Layer Chromatography of Limipterin* (**1**) *and of Its Hydrolysis Product* **2**[a])

	R_f						
	A	*B*	*C*	*D*	*E*	*F*	*G*
Biopterin (**2**)	0.52	0.50	0.64	0.47	0.48	0.39	0.28
Neopterin	0.35	0.47	0.59	0.30	0.35	0.22	0.11
Pterin	0.39	0.36	0.51	0.39	0.37	0.31	0.29
6-Carboxypterin	0.36	0.34	0.30	0.13	0.17	0.32	0.14
6-(Hydroxymethyl)pterin	0.37	0.34	0.51	0.33	0.35	0.26	0.21
Sepiapterin	0.22	0.20	0.31	0.27	0.37	0.31	0.27
Monapterin	0.37	0.45	0.59	0.26	0.32	0.17	0.10
Xanthopterin	0.30	0.28	0.37	0.17	0.21	0.18	0.27
Isoxanthopterin	0.24	0.22	0.31	0.18	0.22	0.20	0.15
Limipterin (**1**)	0.84	0.79	0.80	0.46	0.56	0.35	0.11
Hydrolysis product **2**	0.52	0.50	0.63	0.47	0.49	0.39	0.28

[a]) TLC was performed at room temperature by the ascending procedure. Precoated cellulose plates were developed in the following solvent systems: *A*, 3% NH_4Cl soln.; *B*, 4% sodium-citrate soln.; *C*, 5% AcOH soln.; *D*, i-PrOH/1% ammonia 2:1; *E*, i-PrOH/2% NH_4OAc soln. 1:1; *F*, i-PrOH/H_2O 7:3; *G*, BuOH/AcOH/H_2O 4:1:1.

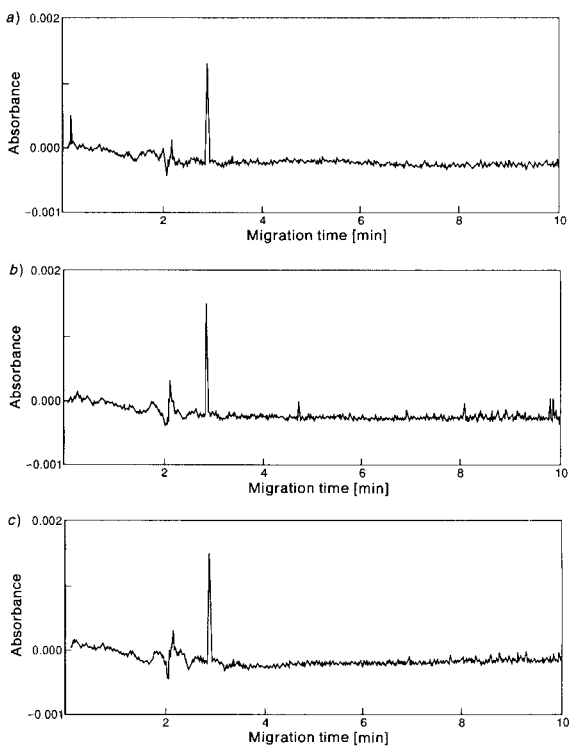


Fig. 4. *Capillary electrophoresis of the hydrolysis product* **2** *with and without biopterin*: a) *injection of biopterin only*, b) *injection of* **2** *only, and* c) *co-injection of biopterin and* **2**. For electrophoresis conditions, see *Fig. 3*.

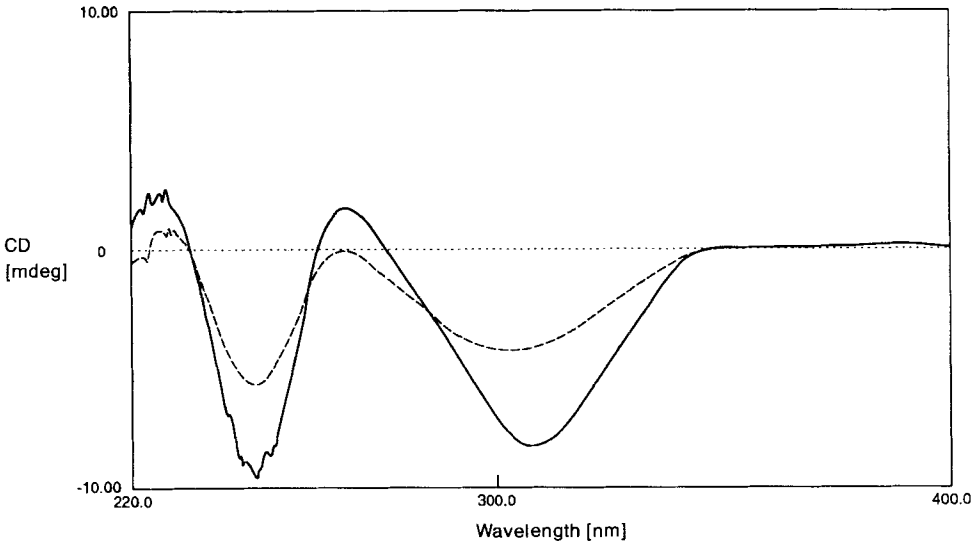


Fig. 5. *CD Spectra of hydrolysis product* **2** (———) *and of authentic biopterin* (------)

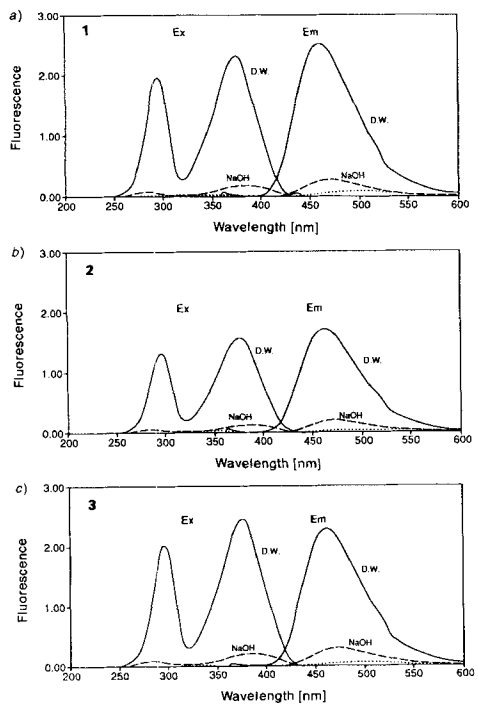


Fig. 6. *Fluorescence spectra* a) *of limipterin* (**1**), b) *of its hydrolysis product* **2**, *and* c) *of authentic biopterin.* Spectra were recorded in H_2O (———), in 0.1N HCl (· · · ·), and 0.1N NaOH (------).

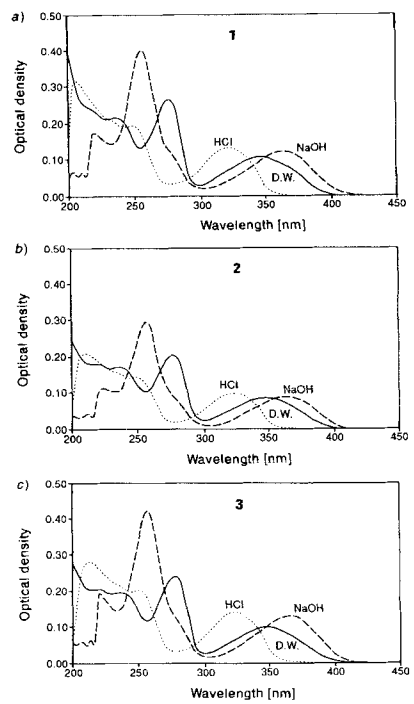


Fig. 7. *Absorption spectra* a) *of limipterin* (**1**), b) *of its hydrolysis product* **2**, *and* c) *of authentic biopterin.* Spectra were recorded in H_2O (———), in 0.1N HCl (· · · ·), and 0.1N NaOH (------).

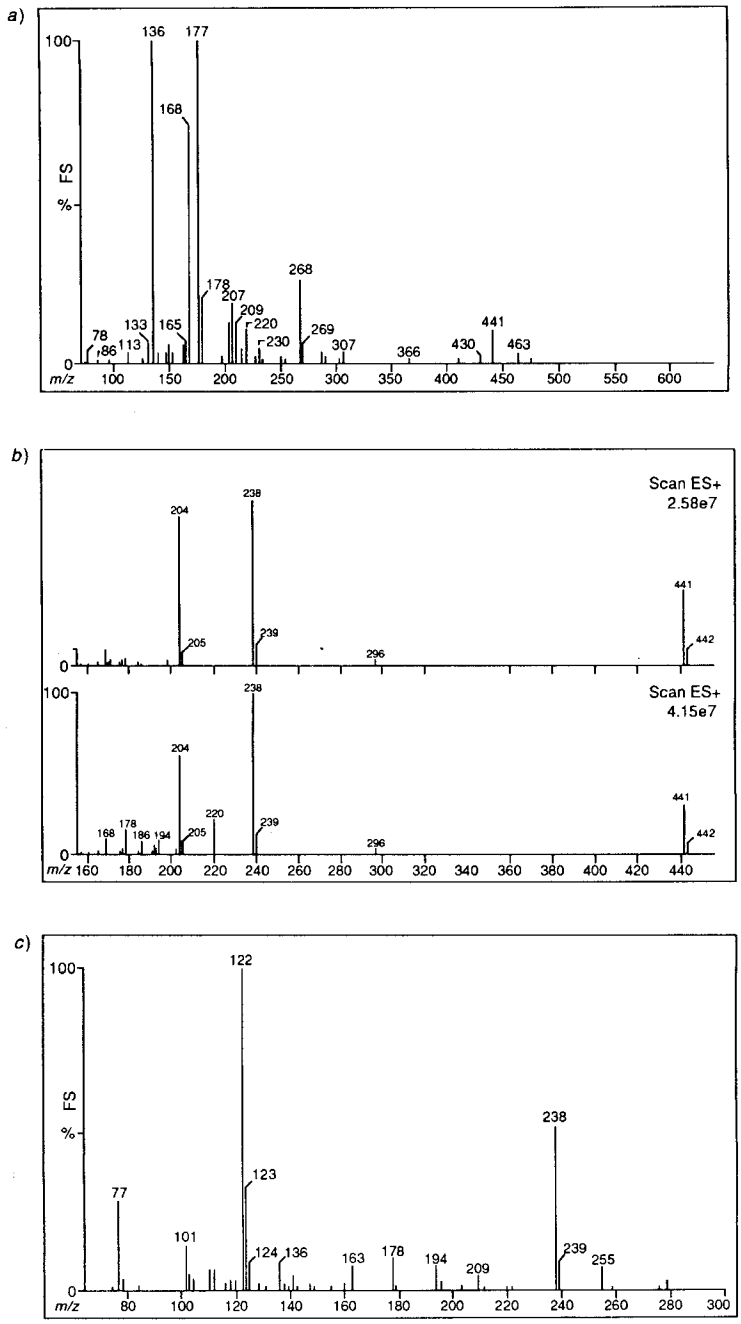
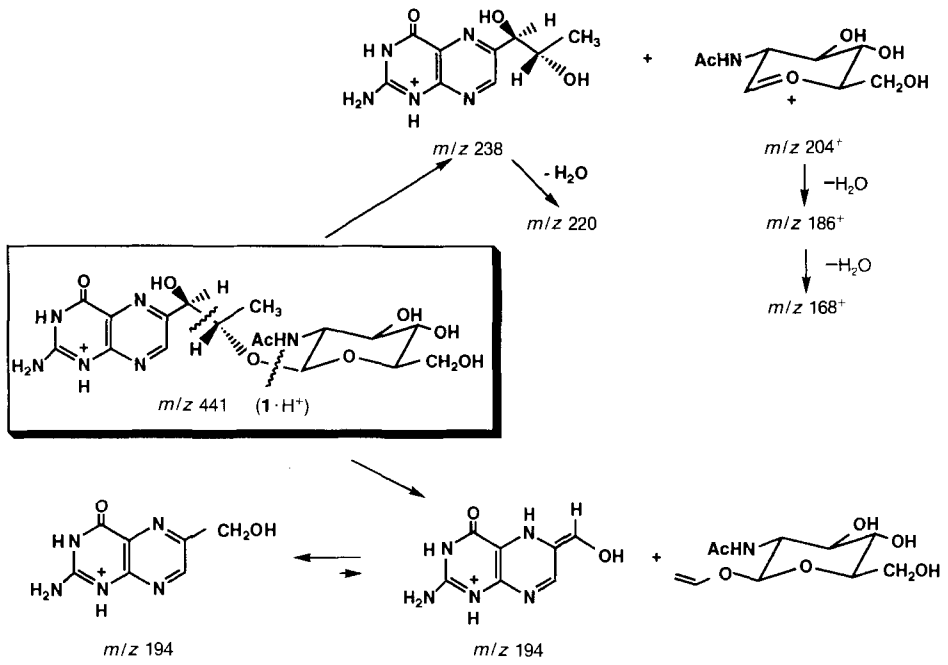


Fig. 8. a) *TS-MS of limipterin* (**1**), b) *ES-MS of* **1**, *and* c) *TS-MS of hydrolysis product* **2**

by all three methods (*Fig. 8, a* and *b*). These results suggest that the molecular mass of **1** is 440. The hydrolysis product **2** showed MH^+ at *m/z* 238 (*Fig. 8c*), which is in agreement with the heterobase biopterin. From a structural point of view, the ES-MS of **1** (*Fig. 8b*) is most informative, whereas the FAB- and TS-MS (*Fig. 8a*) are too complex for structural assignments. The peak at *m/z* 238 corresponds to protonated biopterin (see *Scheme*), and the second intense peak at *m/z* 204 is due to the other half of the molecule formed by cleavage of the glycosidic linkage. From the fragment at *m/z* 204, two molecules of H_2O eliminate successively (→*m/z* 186 and 168). Another cleavage of the side chain between C(1′) and C(2′) according to a *McLafferty* rearrangement leads to *m/z* 194 indicating that the D-glucosamine moiety is most likely attached to the 2′-OH group.

Scheme. *Mass Fragmentation of Limipterin* (**1**)



The structural assignment of **1** and **2** was finally based on ^{1}H-NMR-spectral investigations. The 500-MHz ^{1}H-NMR spectrum of the aglycon **2** of **1** in (D_6)DMSO (*Fig. 9*) perfectly superimposed on the spectrum of authentic biopterin. Chemical shifts, coupling constants, and splitting pattern fit into the structure of biopterin (*Table 3*). The ^{1}H-NMR spectrum of limipterin (**1**; *Fig. 9* and *Table 4*) showed the presence of additional signals not present in the aglycon, originating from a sugar part. They are in good agreement with a *N*-acetyl-D-glucosamine moiety. A straightforward assignment of all sugar and side-chain protons could be achieved by 2D-COSY-NMR spectra ((D_6)DMSO; *Fig. 10*).

In the ^{1}H-NMR spectrum of **2**, a *s* at 8.81 ppm (H–C(7)) is typical for 6-substituted pterins; moreover, two broad peaks for H–N(3) (11.58 ppm) and NH_2–C(2) (6.98 ppm) and a *d* for CH_3(3′) (1.17 ppm) appear. The other signals originate from H–C(1′) (*t* at 4.54 ppm), H–C(2′) (*m* at 4.02 ppm), OH–C(1′) (*d* at 5.68), and OH–C(2) (*d*

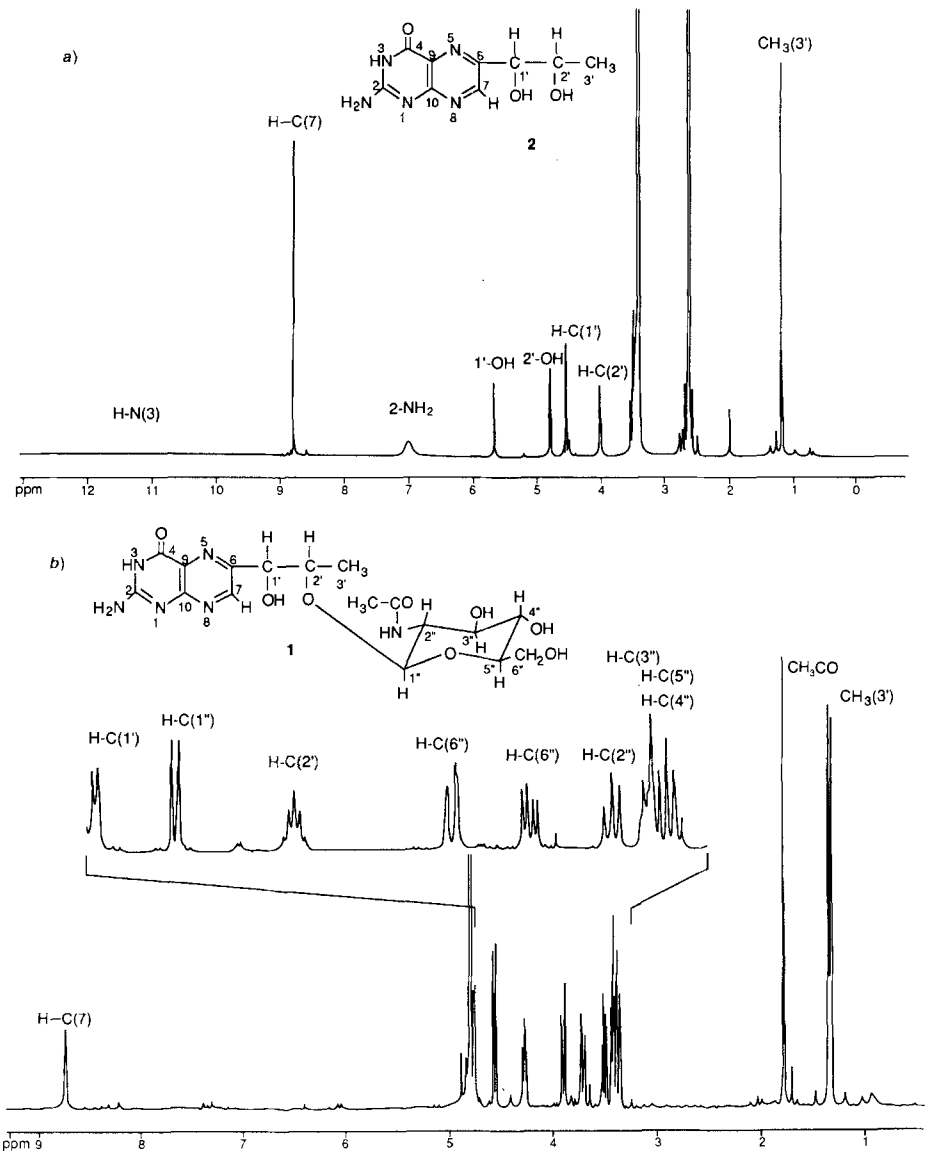


Fig. 9. *500-MHz ^{1}H-NMR Spectra* ((D_6)DMSO) a) *of aglycon* **2** *and* b) *of limipterin* (**1**)

at 4.80 ppm) of the side chain. The spectrum of **1** exhibits H–C(7) at 8.74 ppm, H–C(1′) at 4.76 ppm, H–C(2′) at 4.28 ppm and CH_3(3′) at 1.33 ppm, all arising from the biopterin moiety. H–C(1″) of the sugar moiety at 4.56 ppm reveals through its coupling constant ($J(1'',2'') = 6.8$ Hz) that we are dealing with a β-glycosidic linkage between the sugar and pterin moieties. The coupling constant $J(1,2)$ of β-glucosides are generally −7.4 Hz and that of the α-anomers −3 Hz [14–17].

The complex patterns in the 2D-COSY-NMR of **1** clearly illustrate the coupling between the various protons. It is worthwhile to mention that the signal at 3.53 ppm ((D_6)DMSO) is due to OH–C(6″), which exchanges relatively slowly, since it is still present in the D_2O spectrum at 3.71 ppm. The two H–C(6″) protons are

Table 3. *^{1}H-NMR Data* ((D$_6$)DMSO) *of Hydrolysis Product* **2** *of Limipterin and of Authentic Biopterin*

	Chemical shift [ppm]		Coupling pattern (J in Hz) and number of protons	
	2	authentic biopterin	**2**	authentic biopterin
CH_3	1.17	1.17	d (J = 6.28), 3 H	d (J = 6.30), 3 H
H–C(2′)	4.02	4.02	m, 1 H	m, 1 H
H–C(1′)	4.54	4.54	t (J = 5.34), 1 H	t (J = 5.22), 1 H
OH–C(2′)	4.80	4.81	d (J = 5.18), 1 H	d (J = 5.22), 1 H
OH–C(1′)	5.68	5.69	d (J = 4.75), 1 H	d (J = 4.80), 1 H
NH_2	7.00	6.98	s[a]), 2 H	s[a]), 2 H
H–C(7)	8.81	8.82	s, 1 H	s, 1 H
NH	11.4	11.6	s[a]), 1 H	s[a]), 1 H

[a]) Broad peak.

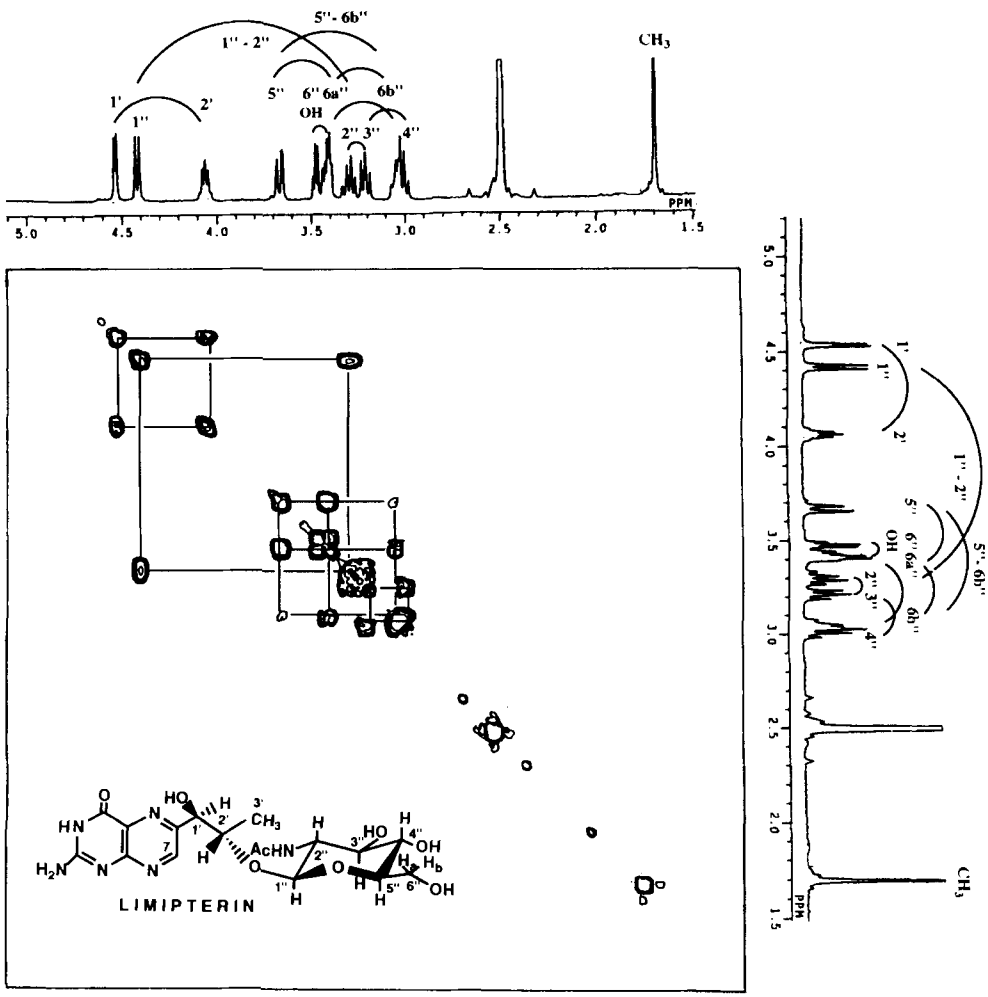


Fig. 10. *2D-COSY ^{1}H-NMR Spectrum* ((D$_6$)DMSO) *of limipterin* (**1**)

Table 4. *^{1}H-NMR Data of Limipterin* (**1**) *in D_2O and (D_6)DMSO*

		Chemical shift		Coupling pattern (J in Hz) and number of protons
		in D_2O	in (D_6)DMSO	
Glycosamine moiety	H–C(1″)	4.56	4.53	*d* (J = 8.4), 1H
	H_a–C(6″)	3.71	3.53	*dd* (J = 12.5, 5.8), 1H
	H–C(5″)	3.90	3.78	*dd* (J = 12.3, 1.0), 1H
	H–C(2″)	3.50	3.45	*t* (J = 9.25), 1H
	H–C(4″)	3.35	3.12	*m* (J = 9.25), 1H
	H–C(3″)	3.41	3.33	*m* (J = 8.85), 1H
	H_b–C(6″)	3.41	3.17	*m* (J = 12.5), 1H
	CH_3CO	1.77	1.81	*s*, 3H
	OH–C(6″)	3.71	3.53	
Biopterin moiety	H–C(7)	8.74	8.76	*s*, 1H
	H–C(1′)	4.76	4.65	*d* (J = 6.2), 1H
	H–C(2′)	4.28	4.18	*m* (J = 6.2), 1H
	CH_3	1.33	1.21	*d* (J = 6.2), 3H

diastereoisotopic and resonate at quite different chemical shifts (3.41 and 3.17 ppm in the 2D-COSY-NMR in (D_6)DMSO). The H–C(4′) signal is found at highest field of the sugar protons and shows, interestingly, a very small coupling to H–C(5′), which appears as a *dd* by interaction with only one of the H–C(6″) protons. The various NMR studies are in full agreement with the proposed structure of **1**.

IR Spectrometry was also useful for the structure elucidation of limipterin (**1**), confirming the functional groups in the proposed limipterin structure (*Fig. 11*; H-bonded OH (3290 cm^{-1}), C=O, conjugated C=O (1695 cm^{-1}), C–O, aliphatic CH (2800 cm^{-1}), NH (3450 cm^{-1}), aromatic C=C (1416–1538 cm^{-1}), CH_3, and C–N (1400–1300 cm^{-1})).

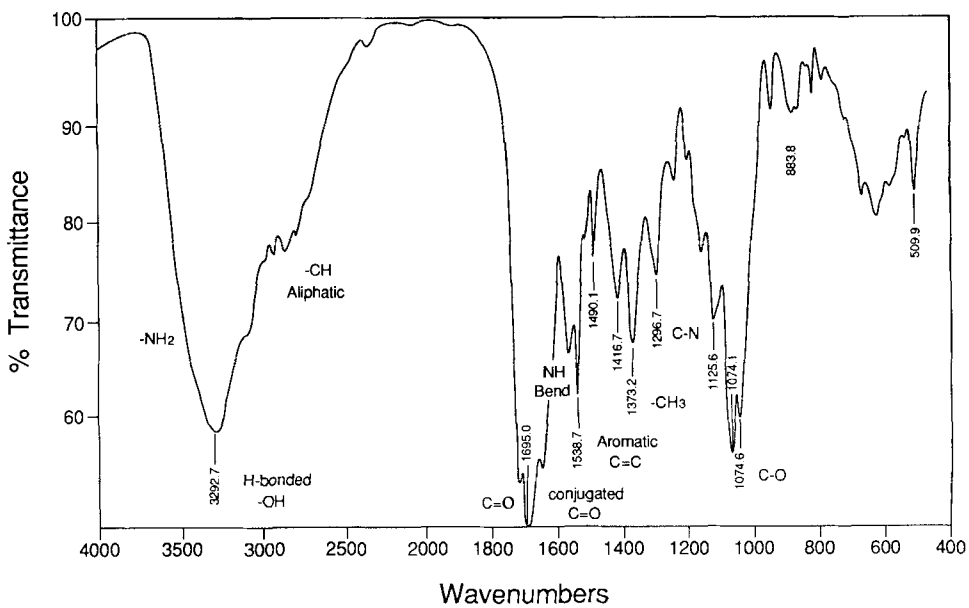


Fig. 11. *IR Spectrum of limipterin* (**1**)

Furthermore, the R_f values of limipterin (**1**) and of its hydrolysis products were compared with those of standard pteridine compounds and various sugar molecules. The R_f values for the aglycon **2** and authentic biopterin were the same, but **1** showed different mobility from any other standard pteridine (see above, *Table 2*): it tends to migrate faster in the aqueous solvent system such as 3% NH_4Cl or 4% sodium citrate solution than in the organic solvent system containing i-PrOH or BuOH. To identify the sugar moiety present in **1**, its acid-hydrolysis mixtures were subjected to TLC along with standard sugars. The results (*Table 5*) indicate clearly that **1** contains *N*-acetylglucosamine as the sugar moiety.

Table 5. *Thin-Layer Chromatography of the Limipterin-Hydrolysis Mixture and of Standard Sugars*[a])

	R_f	
	solvent *A*	solvent *B*
Glucose	0.49	0.56
Fructose	0.47	0.57
Ribose	0.50	0.64
Glucuronic acid	0.13	0.14
Galacturonic acid	0.08	0.09
Glucosamine	0.21	0.16
Galactosamine	0.18	0.13
N-Acetylglucosamine	0.49	0.68
CF_3COOH-Treated *N*-acetylglucosamine	0.48	0.69
Limipterin (**1**)	0.36	0.57
CF_3COOH-Treated limipterin (**1**)	0.48	0.67

[a]) TLC was performed as described in the *Exper. Part* using the following solvent systems: solvent *A*, PrOH/AcOEt/H_2O 7:1:2; solvent *B*, BuOH/pyridine/0.1N HCl 5:3:2.

Since the site of sugar attachment to the biopterin aglycon is still questionable, periodate oxidations with each limipterin (**1**) and biopterin (**2**) were performed. Whereas **2** was degraded easily to pterin-6-carbaldehyde, **1** was not. This result implies that either OH–C(1′) or OH–C(2′) of the side chain of **1** is blocked by *N*-acetylglucosamine through an acid-labile glycosidic linkage.

From the spectroscopic and the hydrolysis results, the complete structure of limipterin is proposed to be 1-*O*-(L-*erythro*-biopterin-2′-yl)-β-*N*-acetylglucosamine (= 6-{(1′*S*,2′*R*)-2′-[(2″-acetamido-2″-deoxy-β-D-glucopyranosyl)oxy] 1′-hydroxypropyl}-2-aminopteridin-4(3*H*)-one; **1**), with the molecular formula $C_{17}H_{24}O_8N_6$ and the molecular mass 440 Da. The attachment of *N*-acetylglucosamine *via* a glycosidic linkage was confirmed by acid hydrolysis as well as by enzymatic cleavage using β-*N*-acetylglucosaminidase (*Fig. 12*). Limipterin was susceptible to this enzyme, which is highly specific for β-linkage of *N*-acetylglucosamine [17].

The oxidation state of native limipterin was established by the differential I_2 experiments [18] described in *Fig. 13* and *Table 6*. It could be estimated that > 93% of limipterin is maintained as tetrahydro form *in vivo*. From *Table 6*, it was calculated that the *in vivo* concentration of limipterin is 1.85 μmol/g dry weight. This amount is *ca.* 10 fold higher than the biopterin content in pineal gland of rats [18], but comparable to the amount of methanopterin present in methanobacterium [19].

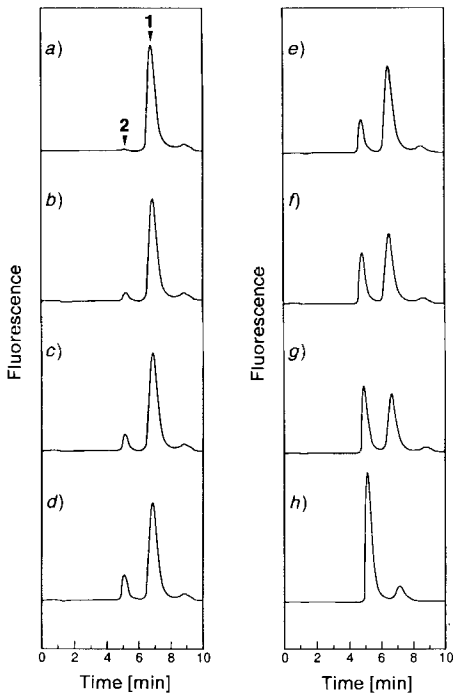


Fig. 12. *Time-dependent conversion of limipterin* (**1**) *to biopterin* (**2**) *by β-*N*-acetylglucosaminidase.* Each panel is a HPLC of the mixture obtained after digestion of **1** with *β-N*-acetylglucosaminidase for the following time. *a*) 0 min, *b*) 10 min, *c*) 25 min, *d*) 40 min, *e*) 60 min, *f*) 120 min, *g*) 180 min, and *h*) 15 h.

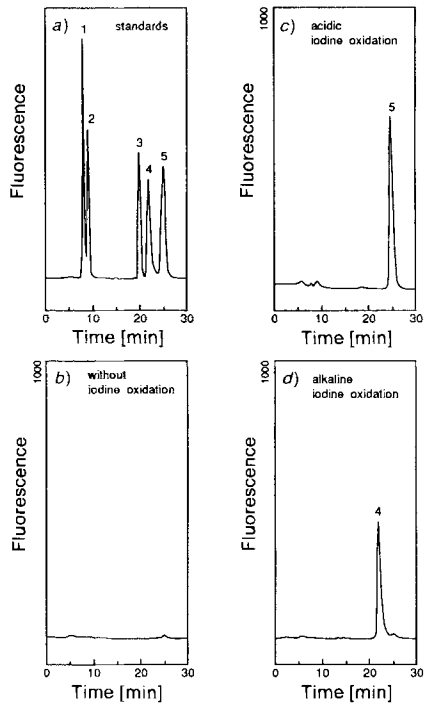


Fig. 13. *I_2-Oxidation pattern of native limipterin in acid or alkali.* After oxidation (*c*) and *d*)), the sample (5.0 µl) was analyzed by HPLC (*Partisil-5-ODS-3* column (4.6 mm × 25 cm), eluent 10 mM K_3PO_4, pH 5.5, flow rate 1.0 ml/min, detection by fluorescence). *a*) Pteridine standards: 1, pterin (7.95 min); 2, neopterin (8.91 min); 3, biopterin (**2**; 19.77 min); 4, 6-carboxypterin (21.82 min); 5, limipterin (**1**; 24.93 min). *b*) *Chlorobium* extract without oxidation. *c*) *Chlorobium* extract oxidized in 0.2N HCl. *d*) *Chlorobium* extract oxidized in 0.2N NaOH.

Table 6. *Oxidation State of Native Limipterin* in vivo

Total limipterin (mean ± s.d.)	Tetrahydrolimipterin	Dihydrolimipterin	Fully oxidized limipterin
1.846 ± 0.108[a]) (100)[b])	1.724 ± 0.122[a]) (93.4 ± 1.2)[b])	0.023 ± 0.019[a]) (1.6 ± 1.0)	0.093 ± 0.019[a]) (5.1 ± 1.1)[b])

[a]) In µmol/g dry cell. [b]) The values in parentheses represent rel. % ± standard deviation.

Relatively large amounts of pterin glycosides were found in various microorganisms including cyanobacteria [19] [20]. *Lin* and *White* [21] also reported that *Sulfolobus solfataricus*, one of the thermophilic archaebacteria, contained solfapterin (*erythro*-neopterin 3′-(2-deoxy-2-amino-D-glucopyranoside)) showing a close structural relationship to

limipterin (**1**). The biological functions of these pterin glycosides are still obscure, but suggestions were made that they may be involved in the redox reaction of photosynthetic dark reactions [22], photoreception and phototransduction [23], as well as carotenoid biosynthesis [24]. Further experimentations are needed to establish the biological role of **1** *in vivo*.

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Experimental Part

1. *Bacterial Strains and Cell Growth. Chlorobium limicola f. thiosulfatophilum* NCIB 8327, supplied by *S. Sirevag* (University of Oslo, Norway), was grown photoautotrophically under the light intensity of 50–80 par. for 5 to 6 days at 30°. The organism was cultured in narrow-neck polycarbonate carboys, each containing 19.8 l of modified *Pfennig* medium [25], which consists of 1.0 g of KH_2PO_4, 0.5 g of NH_4Cl, 1.0 g of $Na_2S_2O_3 \cdot 5H_2O$, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of NaCl, 0.05 g of $CaCl_2 \cdot 2H_2O$, 1.0 ml of vitamin B_{12} soln. (2.0 mg/100 ml), 1.0 ml of trace-element soln., 12.0 ml of 5% $Na_2S \cdot 9H_2O$, and 40 ml of 5% $NaHCO_3$ soln. (pH 6.8). Cells were harvested from the medium by continuous CEPA centrifugation. The yield of cells was 1.4 g/l of medium. The harvested cells were used to isolate limipterin at once or stored at −40° until further use.

2. *Purification of Limipterin* (**1**). Two methods were employed to isolate **1**. Both methods yielded pure samples of the same identity and quality.

Dowex-DeltaPak *Method.* The frozen cells (100 g) were suspended in 8 volumes of 0.1M H_3PO_4. The suspension was sonicated at 4° for 1 h (successively 5 s sonication followed by 10 s interval) using a sonicator (*Microson XL-2000, Heat-Systems-Ultrasonics Inc.*, USA). The material was centrifuged at 7000 rpm for 30 min. To the supernatant, 80 ml of 2.0N CCl_3COOH and 440 ml of acidic I_2 soln. (1% I_2/2% KI in 0.2N CCl_3COOH) were added. The material was stirred for 1 h and the resulting precipitate removed by centrifugation at 6000 rpm for 20 min. Solid ascorbic acid (2.0 g) was mixed to reduce excess I_2 until the color disappeared. The pale yellow soln. (1400 ml) was chromatographed on a column (V_t = 50 ml) of *Dowex 50W* × *8* (H^+ form, 100 mesh), which had been equilibrated with dist. H_2O. The column was washed with 500 ml of dist. H_2O and eluted with 500 ml of 1.0N NH_4OH at a flow rate of 120 ml/h. Fractions containing fluorescent materials were combined (200 ml) and acidified to pH 6.0 with AcOH. The soln. was concentrated to 21 ml and clarified by filtration through a nitrocellulose membrane (0.2 μm). The concentrated sample was injected in 1.2-ml aliquots onto a *DeltaPak C18* HPLC column (15 μm, 30 mm × 30 cm, *Waters Associates*) and chromatographed isocratically with 2%, aq. MeOH at a flow rate of 15 ml/min. The fluorescent peak with the retention time of 38.7 min in each run was collected and evaporated (7.8 mg) using a rotary evaporator and speed-vac. concentrator.

Charcoal-DeltaPak *Method.* The cells (200 g) were suspended in 8 volumes of buffer containing 50 mM K_3PO_4, pH 6.8, and 5.0 mM 2-mercaptoethanol. The suspension was sonicated and centrifuged as described previously. Solid $(NH_4)_2SO_4$ was added to the supernatant (1940 ml) to make 70% saturation. Upon CCl_3COOH and acidic I_2 soln. treatment and centrifugation, ascorbic acid was added to the soln. until the color disappeard. The soluble fluorescent compounds were adsorbed from the soln. by addition of 30 g of charcoal (*Fluka*). The charcoal was thoroughly washed with 400 ml of dist. H_2O and then eluted with 600 ml of EtOH/3% NH_4OH 1:1 (*v*/*v*). This eluate was acidified by the addition of AcOH (22.5 ml) until the pH reached 6.0, and concentrated to 12 ml using a rotary evaporator and speed-vac. concentrator. After *Sep-Pak-C18* and membrane clarification, the limipterin (**1**) was purified using *DeltaPak C18* HPLC by the method described above.

3. *Acidic Hydrolysis of Limipterin* (**1**). The purified **1** (18.4 mg) was dissolved in 1.0 ml of 0.1M Na_3PO_4 buffer pH 1.0, and incubated at 100° for 7.5 h. The extent of hydrolysis was followed by checking the samples at intervals using anal. HPLC (*NovaPak* column (3.9 mm × 15 cm), *Waters Associate*). The resulting precipitate was collected by microcentrifugation and redissolved in a minimum amount of 'milli-Q' H_2O. The sample was resolved by *DeltaPak C18* HPLC by the method used for the purification of **1**. Both anal. HPLC and capillary electrophoresis showed that the hydrolysis product **2** was pure.

4. *β-N-Acetylglucosaminidase Hydrolysis of Limipterin* (**1**). A mixture of 100 mM citrate buffer, pH 4.0, 5.0 μM **1**, and 0.4 U of *β-N*-acetylglucosaminidase (from *Jack* beans, *Sigma Chemical Co.*) was incubated at 25°. At timed invervals, aliquots of the hydrolyzed samples were analyzed by HPLC.

5. *Fast-atom-bombardment, Thermospray, and Electrospray Mass Spectrometry.* FAB-MS: *VG-Quattro* quadrupole mass spectrometer equipped with a static FAB probe; samples (0.5 mg/ml in thioglycerol) in stainless-steel probe were bombarded with 10 KeV energized CsI atoms; source temp. 200°, probe temp. 150°. TS-MS: system as for FAB, but equipped with a thermospray liquid chromatograph/mass spectrometer interface; the sample in 50% aq. MeCN containing 0.1M NH_4OAc was injected and analyzed by a flow rate of 0.6 ml/min; ion-source temp. 240°, probe temp. 220°. ES-MS: *VG-BIO-Q* mass spectrometer; $B1 = 100$ V and $B1 = 60$ V.

6. *^{1}H-NMR Spectrometry.* High-resolution ^{1}H-NMR spectra: *Bruker-AMX-500* spectrometer; 301 *K* in the *Fourier*-transform mode, resonance frequeny 500 MHz, accumulation number 800; purified **1** (1.2 mg) in 0.6 ml of D_2O or (D_6)DMSO in 5.0-mm tubes; chemical shifts δ in ppm rel. to $SiMe_4$ ($= 0$ ppm) as external reference, similarly for **2** and authentic biopterin.

7. *CD, UV/VIS, and Fluorescence Spectra.* CD-Spectra: 0.1N HCl soln.; spectropolarimeter *Jasco 600*. UV/VIS Spectra: *Hewlett-Packard-8452A* diode-array spectrophotometer. Fluorescence spectra: *Shimadzu-RF-540* spectrofluorophotometer.

8. *Capillary Electrophoresis.* Automated *P/ACE 2000* (*Beckman Instruments*) fitted with *P/ACE* software [26]; capillary cassette fitted with 75 μm i.d. fused-silica column (length 57 cm, 50 cm to the detector); injection of sample by pressure for 5 s; on-column detection by UV absorption at 214 nm; applied voltage of 25 kV with anode at the inlet and cathode at the outlet side. All buffer soln. was made of 'milli-Q' H_2O and filtered through the 0.2 μm membrane filter. The running buffer system was composed of 0.1M boric acid/sodium borate, pH 8.4. After filling the capillary with running buffer (1st rinse), 5 s pressure injection of sample was followed, and separation was carried out for 10 min using the running buffer. For good reproducibility, each run was followed by a 2.0 min rinse with 0.1N NaOH (2nd rinse), and 2.0 min rinse with H_2O (3rd rinse).

9. *Thin-Layer Chromatography.* At 100°, 100 μg of **1** were hydrolyzed in 2.0N CF_3COOH for 2 h. The mixture was evaporated and the residue dissolved in 40 μl of 50% aq. i-PrOH and applied to silica gel *60* plates (*Merck*) along with the sugar standards. Elution with PrOH/AcOEt/H_2O 7:1:2, (*v/v*) or BuOH/pyridine/0.1M HCl 5:3:2 (*v/v*) and detection by spraying with a reagent containing diphenylamine, aniline, and H_3PO_4 [27].

10. *Periodate Oxidation.* For 10 min, 50 μl each of 10 μM pterin compounds (**1**, biopterin, and hyrolysis product **2**) was oxidized by 0.2 μl of 0.2M $NaIO_4$. The product was analyzed by HPLC (*NovaPak C18*) and compared with standards.

11. *Determination of* in vivo *Oxidation State of Limipterin.* The *in vivo* oxidation state of limipterin was determined by the use of the methods described by *Fukushima* and *Nixon* [6] with some modifications. Cells, harvested at 8400*g* for 20 min in the presence of 0.02% ascorbic acid, were suspended in 10 volumes of 0.1M H_3PO_4 containing 0.2% ascorbic acid. After sonication, cell debris was removed by centrifugation at 40000*g* for 20 min. The crude supernatant (100 μl) was mixed with either 50 μl of 2.0N HCl or 2.0N NaOH and oxidized by the addition of 350 μl of 1% I_2/2% KI soln. After 2 h oxidation, the alkaline sample was acidified by the addition of 100 μl of 2.0N HCl. The resulting precipitate was removed by microcentrifugation at 12000 rpm. To the supernatant, 200 μl of 2% ascorbic acid was added, and the materials (5.0 μl aliquot) were analyzed by HPLC (*Whatman 5 ODS-3*, 4.6 mm × 25 cm; mobile phase containing 10 mM K_3PO_4, pH 5.5; flow of 1.0 ml/min).

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