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5-Thia-5-Deazaflavin, a 1e$^-$-Transferring Flavin Analog

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By sulfur substitution of the N-5 atom in flavins and flavocoenzymes a flavin analog is obtained, 5-thiaflavin, which is found to be isoelectronic and isosteric with natural flavin in the fully reduced and half-reduced states, but not in the oxidized state. Among the three 'redox shuttles' characterizing the flavin system, viz. upper 1e$^-$, lower 1e$^-$ and 2e$^-$ shuttle, only the second one is retained in thiaflavin, which limits the redox activity of this system to 1e$^-$ transfer.

The structure and properties of the molecular species participating in the thiaflavin redox system are discussed in comparison with the flavin system. The corresponding chemistry of a '2e$^-$ flavin', 5-deazaflavin, has been treated in the preceding paper.

5-Thiaflavin is found to exhibit a stable neutral radical, which is analogous to the 'blue' flavosemiquinone. Unlike normal flavin, where the radical is in a dismutation equilibrium, thiaflavin radical shows reversible formation of a covalent dimer, which is stable in aprotic solution and disproportionates only in water, with irreversible formation of a sulfoxide. The ultraviolet and infrared spectra of the dimer are in agreement with the structure of two 5-thiaflavin molecules linked covalently at their 4a carbons. This corroborates the earlier hypothesis that the essential intermediate in the dismutation of normal flavin is likewise a covalent dimer.

Thiaflavin is tightly bound by apoflavodoxin. The protein catalyses the autoxidation to the radical state. Thiaflavodoxin radical is even more stable (towards further oxidation) than is the free thiaflavin radical.

The redox potential of the couple reduced thiaflavin/thiaflavin radical ($sFl_{red}/sFl^{\cdot}$) is surprisingly high. From the reversible equilibrium established with ferricyanide, $sFl_{red} + Fe(CN)_6^{3-} \rightleftharpoons sFl^{\cdot} + Fe(CN)_6^{4-}$, the standard potential of the $sFl_{red}/sFl^{\cdot}$ couple, E_m at pH 7, has been estimated as + 0.38 V.

Recently, a modified type of flavocoenzyme, i.e. 5-deazaflavin [1–6], has been the subject of a steadily growing interest. In reviewing the available data on 5-deazaflavoproteins [7], we have reiterated the principles characterizing flavin-dependent redox catalysis, which were established earlier [8].

In contrast to the mandatory 2e$^-$ transfer agent nicotinamide, natural flavin appears to be ambiguous by its capability to catalyze 1e$^-$ as well as 2e$^-$ transfer.

In addition to 5-deazaflavin, which is a flavin analog lacking 1e$^-$-transfer activity [1], we have searched for a modification of the flavin nucleus which would remove the 2e$^-$-transfer activity in favor of 1e$^-$ transfer. Such a derivative has been found by replacing the N-5 atom of reduced flavocoenzymes by sulfur. We have reported structures and properties of the thus-formed '5-thiadihydroflavins' [9–12]. In the present paper we want to show that they retain only the 1e$^-$-transfer activities of the natural flavins. In accord with this concept, s^{5}FMN (the 5-thiaflavin analog of FMN) binds to apoflavodoxins forming redox-active stable holoprotein radicals.

Abbreviations. $sFl_{red}H$, thiaflavin (reduced state); $s\dot{F}l$, thiaflavin radical; sFl_{ox}^{+}, thiaflavin sulfonium cation; $HsFl_{ox}\rightarrow O$, thiaflavin sulfoxide; Fl_{ox}, oxidized flavin; $H\dot{F}l$, flavin radical; H_2Fl_{red}, reduced flavin; $(sFl)_2$, thiaflavin radical dimer; $(HFl)_2$, flavin radical dimer; Et, C_2H_5-; flavin = 10-alkylated-7,8-dimethylisoalloxazine; s^{5}FMN, ribo-5-thiaflavin 5'-monophosphate; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance.

RESULTS AND DISCUSSION

The structure and stability of molecular species making up the thiaflavin redox system is outlined in Scheme 1, along with the abbreviations used in this discussion and the absorption maxima, by which the species are characterized.

Scheme 1. *The thiaflavin redox system.* The values in brackets belong to the analogous flavin species whenever such analogy is found to exist (formula underlined). These values are taken from references [20,21,27,30]. The corresponding schemes for flavins and deazaflavins have been published in the preceding paper [1]. Bn = benzyl

The species underlined are those which are 'flavin-analogous', i.e. isoelectronic and isochromic with flavin species of the same state of protonation and oxidoreduction. In order to switch back from thia-flavin to natural flavin, the sulfur has to be replaced by NH. From this it is seen that the analogy is confined to the fully reduced and the half-reduced state, while the oxidized states behave differently. This must be compared with the 5-deazaflavin system, where the oxidized state is flavin-analogous, while the reduced and half-reduced states are not.

In the following paragraphs, we want to discuss the thiaflavin system in comparison with the natural flavin system, based upon the information given in Scheme 1. We begin with the reduced state, since the neutral reduced thiaflavin, $sFl_{red}H$, is the starting material obtained by total synthesis [11] from which all further preparations are derived.

The Reduced Species *sFl^{+}_{red}-1,4a-H_2, sFl_{red}-1H and sFl^{-}_{red}*

In contrast to the isochromic dihydroflavins, all sFl_{red} species are not autoxidizable, unless bound to apoflavodoxin (see below). From their absorption spectra, as shown in Fig. 1, the analogy with flavin is obvious for the neutral state and the anion. The end absorption in the visible region is somewhat less pronounced than in flavin which indicates that the free thiaflavin molecule is less planar than dihydroflavin, the anion being even more bent than the neutral $sFl_{red}H$ for reasons of π-electron overcrowding. This enhanced noncoplanarity is also the reason for the lack of O_2 reactivity. The sulfide group > S in position 5 is intermediate in reactivity between > NH and > $NCOCH_3$ [18]. The more planar a dihydroflavin-like species, the more easily autoxidizable [19] it is.

Under strongly acidic conditions, the reduced thiaflavin system deviates from flavin by the bathochromic shift shown in Fig. 1 as well as by a CH peak appearing at 5.2 ppm in the NMR spectrum. This clearly indicates C-4a protonation, which is due to the lack of proton affinity inherent to the sulfur as compared to nitrogen. The 4a-R-sFl^{+}_{red}-1-H [11] chromophors (λ_{max} = 380 nm) have their analogy in 4a-alkylated dihydroflavin cations 4a-RFl^{+}_{red}-1,5-H_2 (λ_{max} = 385 nm) [20,21]. This shows that in thiaflavin the reactivity of position 4a is even enhanced in compari-

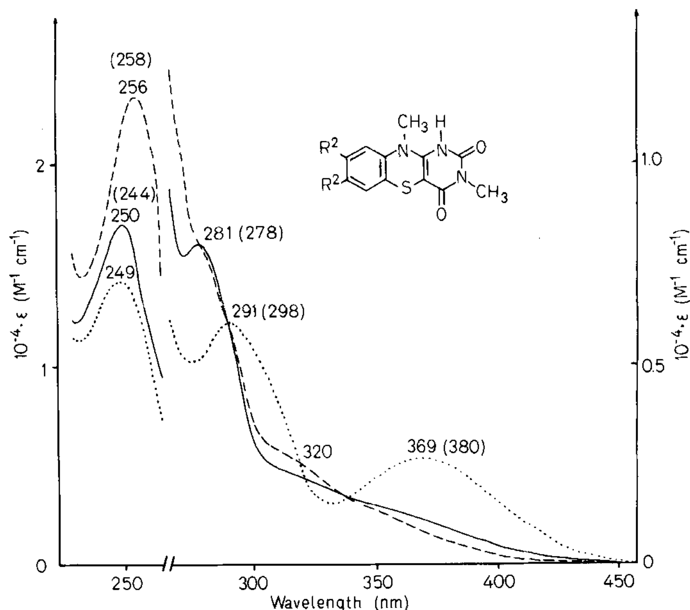


Fig. 1. *Absorption spectra of reduced thiaflavins.* R^2 = H. The values for the analogs $R^2 = CH_3$ are given in brackets. Solvent: (——) in methanol; (-----) in 0.1 M NaOH; (······) in 12 M HCl

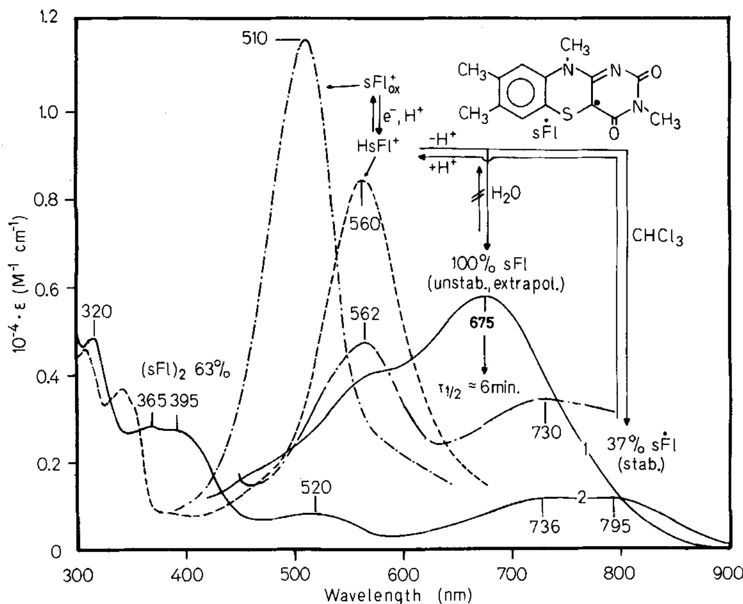


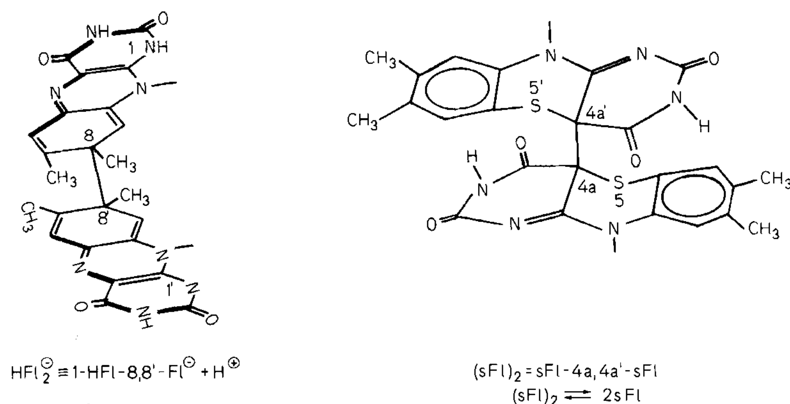
Fig. 2. *Absorption spectra of the half-reduced species.* (——) Spectrum 1, $s\dot{F}l$ in 0.1 M phosphate buffer pH 6.5; spectrum 2, 2 $s\dot{F}l \rightleftharpoons (sFl)_2$ equilibrium in chloroform; (·—·—·) thiaflavodoxin radical; (-----) $Hs\dot{F}l^+$ in $CHCl_3/CF_3COOH$ (1/1). For comparison: (·—··—·) sFl^+_{ox} in conc. H_2SO_4. Note that the neutral $s\dot{F}l$ disproportionates in water slowly and irreversibly with $\tau_{1/2} \approx 6$ min, while reacidification of the monomer-dimer equilibrium in $CHCl_3$ leads back to 100% radical cation $Hs\dot{F}l^+$

son with natural flavin, where previously only alkylation at C-4a [20] but not protonation has been observed.

The Half-Reduced Species *$s\dot{F}l$-1-H^+, $s\dot{F}l$ and sFl-4a,4a'-sFl*

Upon oxidation under acidic conditions, the very stable dark-red radical cation $s\dot{F}l$-1-H^+ is produced quantitatively, and can easily be crystallized, in analogy to the flavin radical cations described as early as 1937 by Kuhn and Ströbele [22]. Upon neutralization of the radical cation, the situation becomes more complex.

As indicated in Fig. 2, in neutral aqueous medium, a nearly quantitative amount of neutral, deep-green radical $s\dot{F}l$ is produced, which decays slowly, but irreversibly by disproportionation with a half time of



Scheme 2. *Dimers of flavin and thiaflavin*

about 6 min. Upon reacidification, after 1 h only small amounts of radical cation can be recovered unless concentrated H_2SO_4 is added.

Under aprotic conditions, such as in $CHCl_3$, however, no irreversible change is observed, although the amount of green neutral radical is far from quantitative, unless very low concentrations of thiaflavin are used. In concentrated solutions pale-yellow crystals can be isolated. When redissolved in $CHCl_3$, the same equilibrium is obtained as upon neutralisation of the radical cation. Upon reacidification, the radical cation state is restored quantitatively (Fig. 2). From this it is obvious that a reversible dimerisation occurs for which K_d can be estimated as 43.1 μM instead of irreversible dismutation that prevails in aqueous systems. Notwithstanding the striking similarity between the radical monomers $H\dot{F}l$ and $s\dot{F}l$, the first-mentioned natural flavosemiquinone decays by disproportionation, while the thiaflavin radical forms a σ-covalent colorless dimer. We take this as independent support of the concept, first proposed by Favaudon and Lhoste [23], that the disproportionation of the natural flavosemiquinone also occurs via a σ-covalent dimer as intermediate. $(HFl)_2$ is heterolyzed rapidly, while $(sFl)_2$ does so only slowly, requiring protic catalysis. We have proposed for $(HFl)_2$ the N-5-unprotonated structure 1-H-Fl-8,8'-Fl-H-1' [24] because of its long-wavelength absorption which we attributed to intramolecular charge transfer (Scheme 2, formula 1). For the pale yellow $(sFl)_2$, however, we must postulate the structure sFl-4a,4a'-sFl (Scheme 2, formula 2) since no proton can be dissociated from position 5.

This proposal is based on the agreement with other known 4a derivatives of thiaflavin [11], as documented by the carbonyl stretching range of the infrared spectra shown in Fig. 3. Since substitution at C-4a separates the 4-carbonyl from the chromophore, we must expect a hypsochromic shift along with a decrease in relative intensity in comparison with the 2-carbonyl. This behavior has been verified earlier for flavins substituted in position 4a [20, 25]. It is characteristic that

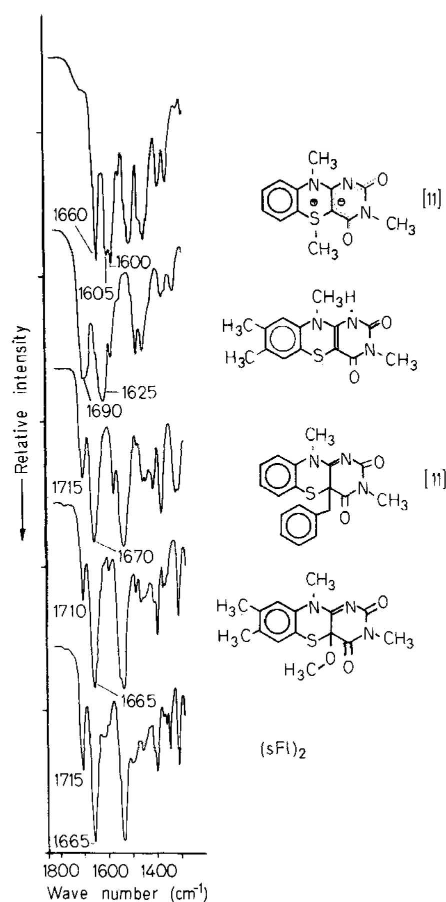


Fig. 3. *Comparison of the carbonyl stretching range of the infrared spectra of various thiaflavins.* Note that substitution at C-4a invariably leads to a characteristic weak band at 1710–1715 cm^{-1} for 4-CO and a strong band at 1665–1670 cm^{-1} for 2-CO. For the analogous flavins cf. [20, 25]

the absorption spectra of the 4a-substituted thiaflavin chromophore depends on the size and the bulkiness of the 4a substituents. Hence, sFl_{ox}-4a-OCH_3 shows in the first transition λ_{max} = 329 nm, $\varepsilon = 8300\ M^{-1}\ cm^{-1}$, while sFl_{red}-4a-benzyl produces the smaller

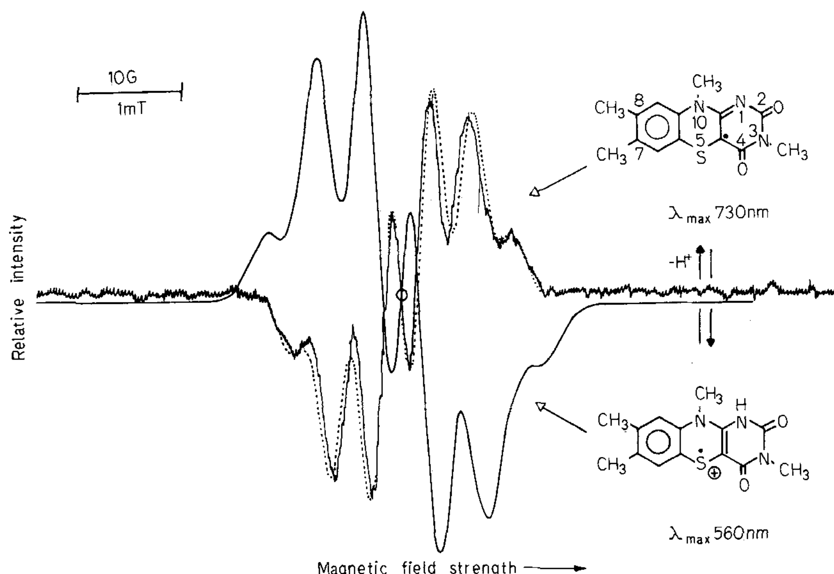


Fig. 4. *Low-resolution EPR spectra of thiaflavin radicals.* (——) sḞl in chloroform; (·····) sḞl calculated; (——) HsḞl$^+$ in CF_3COOH. Deuteration of the environment does not alter these spectra. The spectra are drawn at low resolution and one of them by reversed face for higher clarity

absorption of $\lambda_{max} = 334$ nm, $\varepsilon = 4600\ M^{-1}\ cm^{-1}$ [11]. The extremely bulky dimer $(sFl)_2$ exhibits $\lambda_{max} = 368$ nm, $\varepsilon = 3800\ M^{-1}\ cm^{-1}$, because of reduced planarity.

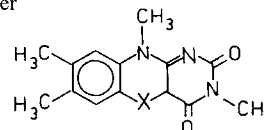
From the concentration dependence of the dimerization equilibrium, the spectrum of pure $(sFl)_2$ was found to exhibit maxima at 368 and 390(s) nm with absorption coefficients of 3800 and 3200 cm^2/mol mono-sḞl. This value is comparable with $\lambda_{max} = 335$ nm, $\varepsilon = 4600\ M^{-1}\ cm^{-1}$, for sFl_{red}-4a-benzyl [11]. From this it becomes apparent that there is a marked π-electron interaction between the two halves, which accounts for the ease of heterolytic cleavage under protic conditions. This dismutation reaction is even faster in alcohols than in water.

From the EPR spectra of the cationic and neutral radicals, as shown in Fig. 4, isotropic coupling constants are derived for N-10, N-10$-CH_3$, C-8$-CH_3$, and C-6$-$**H** which are similar to those of the natural flavin radical [26]. Coupling constants were calculated as described in Table 1. The calculation was not applied to the highest state of resolution which is shown in Fig. 5. Table 1 shows that the spin distribution of HḞl and sḞl is very similar. In particular, the pyrimidine subnucleus (positions 1–4) is nearly devoid of spin density in both cases, while the benzene subnucleus carries an appreciable spin density centered at C-8.

In Table 2 we have compared the absorption properties of HḞl and sḞl. The values of ε for sḞl are deduced from EPR integration with 3-carbamoyl-2,2,5,5-tetramethyl-pyrrolidin-1-yloxy as standard. The error limit over five measurements was 4.7%. The 2 sḞl $\rightleftharpoons$ $(sFl)_2$ equilibrium, as depending on solvent and temperature, is also listed in Table 2.

Table 1. *Comparison of the calculated coupling constants of sḞl with the analogous lumiflavin neutral radicals*

The corresponding four coupling constants of the natural flavin radical [26] applied with a line width of 0.23 mT and a lorentzian line shape were taken as start. Optimizing the coupling constants in steps of 0.01 mT led to the best values given in the table, which are shown to fit the experimental spectrum satisfactorily. The calculation was based on an experimental spectrum of low (0.1 mT) resolution in order to wipe out the minor contributions from couplings 10-fold weaker. The error limits for X = NH or NCH_3 are $\pm$ 0.02 mT. The computer program used was kindly placed at our disposal by Dr G. Kollmannsberger (FB Chemie, University of Konstanz) and the calculations were obtained from a Telefunken TR 440 computer



Atom	Coupling constant a for		
	X = S	X = NH [26]	X = NCH_3 [26]
	mT		
N-5	–	0.80	0.80
H-5α	–	0.76	–
CH_3-5β	–	–	0.76
N-10	0.36	0.36	0.36
CH_3-10β	0.36	0.39	0.39
H-6α	0.08	0.17	0.17
CH_3-7β	< 0.03	< 0.03	< 0.03
CH_3-8β	0.12	0.24	0.24
H-9α	< 0.03	< 0.03	< 0.03

The Oxidized Species
sFl^+_{ox}, sFl_{ox}-4a-X, $HsFl_{ox} \rightarrow O$ and $sFl_{ox} \rightarrow O^-$

The neutral radical sḞl and its dimer disproportionate under aqueous conditions, yielding one half

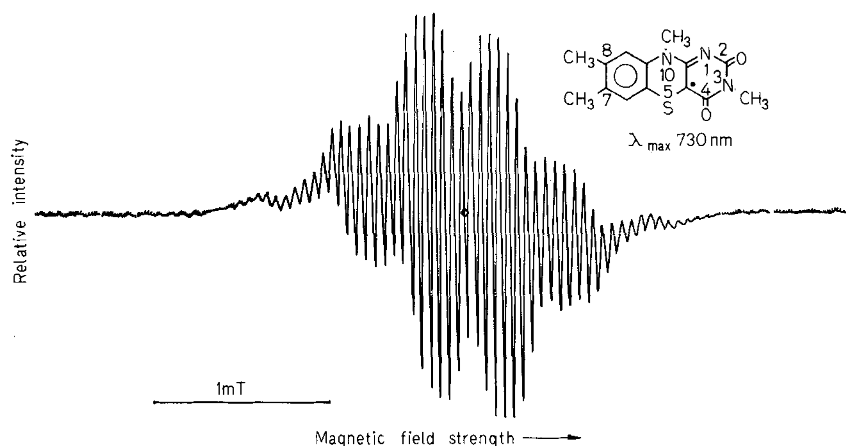


Fig. 5. *High-resolution EPR spectrum of sḞl in $CHCl_3$*

Table 2. *Dependence of the thiaflavin radical properties on the environment: temperature, solvent and protein binding*
An absorption coefficient of 5800 M^{-1} cm^{-1} in protic solvents was obtained by extrapolation to zero time. In the aprotic solvent chloroform it was calculated as 4500 ± 220 M^{-1} cm^{-1} (± 4.7%) by means of double integration of the low-resolution EPR spectrum of sḞl and comparison with 3-carbamoyl-2,2,5,5-tetramethyl-pyrrolidin-1-yloxy as a standard. We presume that the absorption coefficient is not changed considerably in the other aprotic solvents. The amount of the (thia)flavin radical relates to a total flavin concentration of 0.1 mM unless indicated otherwise. K_d was determined at ambient temperature, unless another temperature is stated; K_d = [(sḞl)]²/[(sFl)₂]. All values were obtained in this work unless indicated otherwise in the last column

Compound	Solvent	λ_{max}	ε	(s)Ḟl	$K_d \times 10^7$	T	Reference
		nm	M^{-1} cm^{-1}	%	M	K	
Thiaflavodoxin	0.1 M phosphate, pH 7.0	730	3200	≈ 100		288	
sḞl	pH 6.5	672	5800[b]	100[b]			
	$CHCl_3$	736	4500 (calc.)	37	431 ± 32[c]		
				10[a]	53	253	
				17[a]	159	278	
				26[a]	404	295	
				30[a]	585	313	
	CH_2Cl_2	740		24	137		
	$HCON(CH_3)_2$	740		11	27		
	CH_3CN	740		11	27		
	benzene	740		7.8	13		
	CCl_4	740		4.7	4.5		
	$(C_2H_5)_2O$	740		3.1	2.0		
Flavodoxin	pH 6.0[d]	580	4500	≈ 100		277	[32]
5-Et-Ḟl	pH 5.0, H_2O	580	3600	≈ 100			[30]
	EtOH	630	4500	≈ 100			[30]
	$CHCl_3$	642	4400	≈ 100			[30]
	benzene	655	4700	≈ 100			[30]

[a] The total thiaflavin concentration was 0.22 mM.
[b] These values were extrapolated to zero time.
[c] Mean value with standard deviation from 10 measurements.
[d] The buffered solution contained 0.15 M sodium acetate and 0.06 M EDTA.

molecule each of starting $sFl_{red}H$ and sulfoxide $HsFl_{ox} \rightarrow O$. The sulfoxide species are characterized by their very intense absorption below 320 nm (cf. Fig. 6). If methanol is used instead of water, the oxidized species sFl_{ox}-4a-OCH_3 is obtained (cf. Scheme 1). From this it might be concluded that, in the aqueous system, sFl_{ox}-4a-OH is an intermediate in the sulfoxide formation. This is, however, not true, since treatment of the 4a-methoxy compound with weak aqueous base does not yield sulfoxide anion, but total decay of the tricyclic system through ring opening at the C-4a – S bond. sFl_{ox}-4a-OCH_3 can, on the other hand, be converted into sulfoxide by acid via hydrolysis of the sulfonium cation sFl^+_{ox}. The latter can be obtained in pure form in concentrated H_2SO_4 solution. sFl^+_{ox} is more brownish red as compared to the purple radical HsḞl⁺ (Fig. 2), to which it is easily reduced by as weak a reductant as ethanol. It seems that sFl^+_{ox} in concentrated H_2SO_4 is even slowly reduced by its own methyl substituents. Thus, sFl^+_{ox} in H_2SO_4 is stable for days

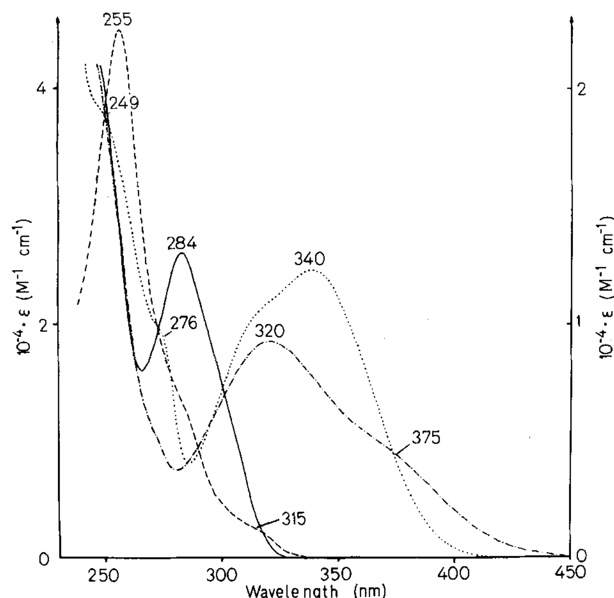


Fig. 6. *Absorption spectra of oxidized thiaflavin species.* (·····) sFl_{ox}-4a-OCH_3 in methanol; (–·–·–) 1H-sFl^{+}_{ox}-4a-OCH_3 in CF_3COOH; (——) $HsFl_{ox} \rightarrow O$ in methanol; (-----) $sFl_{ox} \rightarrow O^-$ in 0.1 M NaOH. For the latter spectrum the left-hand absorption scale is valid. sFl_{ox}-4a-OCH_3, 4a-Methoxy-3,7,8,10-tetramethyl-2,4-dioxo-2,3,4,5-tetrahydro-pyrimido[5,4-*b*][1,4]benzothiazine; $HsFl_{ox} \rightarrow O$, 3,7,8,10-Tetramethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrimido[5,4-*b*][1,4]-benzothiazine Sulfoxide

if unsubstituted in positions 7 and 8, but is reduced to $Hs\dot{F}l^+$ within 4 h when both positions are methylated. Among the oxidized species, only sFl^{+}_{ox} is, in principle, flavin-like, if compared with the analogous 5-Et-Fl^{+}_{ox} [20] species. It must be realized that protons add to Fl_{ox} not at N-5 but at N-1 [27], which accounts for the much smaller oxidation power of HFl^{+}_{ox} as compared to sFl^{+}_{ox}.

A further component of the complex equilibria in the oxidized thiaflavin system is the solvated sulfonium cation 1-$HsFl^{+}_{ox}$-4a-X, X = OH, halogen or *O*-alkyl which corresponds by its chromophore to the 4a-protonated cation, whose spectrum is shown in Fig. 1. This species is obtained pure in CF_3COOH solution (presumably X = $OOCCF_3$) (cf. Fig. 6). The bathochromic shift obtained upon N-1 protonation in the 4a-blocked system is characteristic, and it corresponds to the shift obtained upon C-4a-protonation of the N-1-blocked system (cf. Fig. 1).

Direct quantitative conversion of the reduced thiaflavin to the sulfoxide is, of course, achieved by peroxidation. Reduction of the sulfoxide under non-acidic conditions proved not to be feasible. This fact explains why thiaflavin functions as a reversible redox system at the radical and fully reduced levels only. The sulfoxide must be strongly bent according to this absorption spectrum and in agreement with the fact that in the planar state it would exhibit in the central subnucleus an antiaromatic 8 π configuration.

Thiaflavodoxin of Peptostreptococcus elsdenii

When apoflavodoxin is added anaerobically to a solution of s^5FMNH in 0.1 M phosphate pH 7, no long-wavelength absorption typical of the radical is formed, even over prolonged periods. However, on mixing with air, protein-bound thiaflavin radical is produced slowly, as detected by its characteristic absorption spectrum (Fig. 2). Thus it is clear that binding of the reduced s^5FMNH to the apoprotein results in a greater reactivity to O_2, since incubation of s^5FMNH without apoprotein results in no detectable radical formation or decay of the s^5FMNH. This increase must be due to a flattening of the $sFl_{red}H$ skeleton upon binding to the protein [19,28], which provides independent support for the flatness of the H_2Fl_{red} conformation in natural flavodoxin [29].

Upon autoxidation the yield of protein-bound radical is not complete, being approximately 40% of that obtained by titration with a reactive nitroxide radical (see below). The lower yield of radical with O_2 appears to be due to overoxidation of the protein-bound radical by the product of O_2 reduction, O_2^-, since the yield is considerably increased (up to 70%) in the presence of superoxide dismutase. Catalase has no effect on the yield.

The optimum conditions for formation of protein-bound radical were found to be by oxidation with the strong $1e^-$ acceptor, spirocyclohexyl porphyrexide, a nitroxide radical [16]. Addition of nitroxide radical results in the immediate production of $s\dot{F}l$, both free and protein-bound. However, while the protein-bound radical is stable, the uncomplexed radical is not, and at pH 7.0, 15 °C, has completely decayed after 40 min by irreversible dismutation. Thus, a mixture of apoflavodoxin and excess s^5FMNH, in 0.1 M phosphate, pH 7.0, was titrated with nitroxide radical until no further production of enzyme-bound thiaflavin radical was obtained. This procedure yielded the spectrum shown in Fig. 2, with $\varepsilon_{565} = 4625\ M^{-1}\ cm^{-1}$.

Oxidation-Reduction Potential

The oxidation reduction potential of the couple $sFl_{red}/s\dot{F}l$ is surprisingly high, as evidenced by the fact that equilibrium is established with the $Fe(CN)_6^{3-}$/$Fe(CN)_6^{4-}$ couple.

At pH values below 6.5, the radical decay is sufficiently slow so that direct spectrophotometric studies of this equilibrium may be made. For example, from a set of 12 individual experiments in 0.1 M phosphate pH 6.3, at 10 °C, ε_{675} is 6000 $M^{-1}\ cm^{-1}$ for $s\dot{F}l$ and the redox potential E'_m, pH 6.3, is + 0.45 V.

At pH above 7, radical disproportionation becomes sufficiently rapid so as to require determination of the radical by stopped-flow methods. In this way the oxidation rate of $sFl_{red}H$ by ferricyanide has been determined at pH 8.3 and 10 °C as $k_{ox} = 1.83 \times 10^6\ M^{-1}$

s^{-1} whereas for the radical and ferrocyanide we found $k_{red} = 2.2 \times 10^5\ M^{-1}\ s^{-1}$ which leads to $K = k_{red}/k_{ox} = 0.12$. Thus at pH 8.3, E'_m is + 0.37 V. From the pH dependence of this redox equilibrium, we calculate a p$K \approx 7.0$ for $sFl_{red}H$ and E'_m, pH 7, of + 0.38 V.

In the thiariboflavin series, where instead of methyl the substituent in position 10 is ribityl, this pK drops to 6.2, probably due to the intramolecular hydrogen chelation between the ribityl side chain and N-1.

It is remarkable that the neutral thiaflavin radical exhibits the same type of extremely strong negative solvatochromism (cf. Fig. 2, Table 2) as does the natural blue flavosemiquinone $H\dot{F}l$ (λ_{max} in $H_2O \approx 580$ nm, in benzene 655 nm) [30]. For the protein-bound flavosemiquinones λ_{max} is generally about 580 nm, which points to hydrophilic arrangement of the radical at the active site. This hydrophilicity is not due to an 'in-plane' hydration via N-5 – H, as is shown by the present data on $s\dot{F}l$, but must be a 'stacking interaction', in agreement with the data of Palmer and Mildvan [31] on proton relaxation rates of free and protein-bound flavin radicals.

The protein-bound thiaflavin radical, however, resembles the free radical in chloroform considerably more than in water.

Thus, both radicals, $H\dot{F}l$ and $s\dot{F}l$, reflect a stacking contact with water by a hypsochromic shift of > 50 nm. When protein-bound, this contact is eliminated for $s\dot{F}l$, but not for $H\dot{F}l$. This interesting fact still awaits explanation.

It is disappointing that the redox potential of thiaflavin is so high, since this prevents, of course, enzymatic activity of thiaflavoproteins, even if the modified flavin is strongly bound by the enzyme, as in the present case. We intend, therefore, to construct thiaflavin derivatives of lower potential though not altered in steric shape.

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Materials and Methods

Solvents and reagents were commercial products of the best available purity.
Thiariboflavin (I: R^1 = H, R^2 = CH_3, R^3 = ribityl) was synthesized as described by Janda & Hemmerich [10]. 5-Thia-FMN (I: R^1 = H, R^2 = CH_3) was prepared by an adaptation of the method of Scola & Hemmerich [13], for small scale phosphorylations. The crude product was purified by chromatography over an apoflavodoxin affinity column [14]. P. elsdenii apoflavodoxin was prepared as described by Mayhew [15]. The nitroxide radical spirocyclohexylporophyrexide, oxidizing the reduced thiaflavodoxin, was a gift from Dr. G. Blankenhorn, University of Konstanz, FB Biologie, and was prepared as described earlier [16].

All melting points are uncorrected and were run on a Linström or a Kofler heating block.
Elemental analyses were performed by the Analytische Abteilung des pharmazeutischen Institutes der Freien Universität Berlin. IR-spectra were run on a Perkin Elmer 621 spectrophotometer using KBr pills. Light absorption spectra were recorded with a Varian 635 M or a Varian Superscan 3 spectrophotometer.
^{1}H-NMR spectra were obtained with the following spectrometers: Varian A 60 A, Varian T-60 and Varian XL-100. Chemical shifts refer to tetramethylsilane as an internal standard and are given in ppm. All EPR measurements were recorded with a Varian E 3 X-Band spectrometer with 100 kHz field modulation using a Varian E-4531 multi purpose cavity. Oxygen was removed by flushing the solution in a quartz flat cell for 30 min with purified argon. Radical concentrations were determined by comparison of the low resolution EPR spectra with 3-carbamoyl-2,2,5,5-tetramethyl-pyrrolidin-1-yloxy as standard using the method of double integration described by Wyard [17]. The above mentioned nitroxide radical was purchased from EGA, Steinheim. Mass spectra were obtained using a Varian CH 7 MAT spectrometer at 70 eV.

3,7,8,10-Tetramethyl-2,4-dioxo-1,2,3,4-tetrahydro-10H-pyrimido [5,4-b][1,4] benzothiazine, $sFl_{red}H$ (I: R^1 = R^2 = R^3 = CH_3)

0.5 ml Sulfur dichloride was added to a suspension of 5 mmoles (1.2 g) 3-methyl-6-(N-methyl-3,4-xylidino)uracil in 5 ml chloroform. The solution was allowed to stand at room temperature, a precipitate forming after a short time. After a further 12 hr 50 ml chloroform was added and then the solvent was removed by filtration. The residue was dissolved in 10 ml dimethylformamide and poured into a 2 % sodium dithionite solution kept at pH 7 with 0.1 M buffer. The precipitate was collected and the filtrate shaken three times each with 70 ml chloroform. The chloroform was removed and the residue was combined with the former precipitate. The solid was dissolved in a small portion of boiling dimethylformamide; after cooling, methanol was added to the solution. This gave 670 mg of yellow crystals which also contained the undesired 6,7-dimethyl isomer as impurity, as analyzed by ^{1}H-NMR spectroscopy. Repeated recrystallization from dimethylformamide/methanol gave 300 mg of the pure 7,8-dimethyl isomer. Yield: 300 mg (20.7 %), m.p.: 317-321°C. Calculated for $C_{14}H_{15}N_3O_2S$ (289.4): C 58.11, H 5.23, N 14.52, S 11.08 %. Found: C 57.67, H 5.19, N 14.49, S 11.05 %. λ_{max} in methanol (ε): 345 sh (2300), 278 (10 000), 244 (22 000), 217 (20 000) nm ($M^{-1}cm^{-1}$), λ_{max} in 0.1 N NaOH (ε): 285 sh (8 000), 258 (26 000) nm ($M^{-1}cm^{-1}$), λ_{max} in CF_3COOH (ε): 380 (3 000), 298 (5 600) nm ($M^{-1}cm^{-1}$). ^{1}H-NMR in $(C^2H_3)_2SO$: 2.1 (3, s, 7-CH_3), 2.18 (3, s, 8-CH_3), 3.12 (3, s, 3-CH_3), 3.28 (3, s, 10-CH_3), 6.8 (1, s, 6-H), 6.85 (1, s, 9-H), 10.5-11.0 (1, s, 1-H). MS (70 eV): m/e 289 (100 %, M^+). IR in KBr: 3400 1-NH; 1690 4-CO; 1625 2-CO cm^{-1}.

3,10-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-10H-pyrimido [5,4-b][1,4] benzothiazinyl Radical Tribromide, $HsFl^+Br_3^-$ (II: R^1 = CH_3, R^2 = H, R^3 = CH_3)

2 mmoles (520 mg) of the corresponding reduced thialumiflavin was dissolved in 50 ml of refluxing glacial acetic acid. Fast addition of 10 mmoles (1.5 g) bromine immediately gave a violet crystalline precipitate, which was filtered off by suction. The crystals were washed with diethylether and dried over phosphorous pentoxide (80°C, 1.0 Pa, 8 h).
Yield: 580 mg (55 %), m.p.: 162°C (dec.). Calculated for $C_{12}H_{11}Br_3N_3O_2S$ (501.0): C 28.76, H 2.21, N 8.40 %. Found: C 28.29, H 2.16, N 8.23 %. IR in KBr: 3420 1-NH; 1710 4-CO; 1685 2-CO cm^{-1}. λ_{max} in CF_3COOH (ε): 522 (6 200), 345 (2 900), 334 (3 000), 299 (5 200) nm ($M^{-1}cm^{-1}$). EPR in CF_3COOH: a^N_{10} 0.45 mT, $a^H_{10\beta}$ 0.45 mT.

3,7,8,10-Tetramethyl-2,4-dioxo-1,2,3,4-tetrahydro-10H-pyrimido [5,4-b][1,4] benzothiazinyl Radical Perchlorate, $HsFl^+ClO_4^-$ (III: R^1 = CH_3, R^3 = CH_3, R^2 = CH_3)

30 mg (0.1 mmoles) of the corresponding reduced thialumiflavin was dissolved at 80°C in 20 ml glacial acetic acid, containing 5 vol. % acetic acid anhydride and 0.1 M sodium perchlorate. Addition of 1 ml of a solution containing 0.1 M bromine and 0.1 M sodium perchlorate in glacial acetic acid gave a violet precipitate which was filtered off by suction, washed with glacial acetic acid and diethylether and dried over phosphorous pentoxide (100°C, 13 Pa, 12 h). Yield: 28 mg (78 %), m.p.: 350°C.
Calculated for $C_{14}H_{15}ClN_3O_6S$ (388.8): C 43.26, H 3.89, N 10.81 %. Found: C 43.19, H 3.87, N 10.71 %. IR in KBr: 3450 1-NH; 1740 4-CO; 1650 2-CO; 1090 ClO_4 cm^{-1}. λ_{max} in CH_3CN (ε): 558 (7800), 343 (3400), 308 (4300), 290 sh (17 000), 260 (19 500), 252 (21 000) nm ($M^{-1}cm^{-1}$). EPR in CH_3CN: a^N_{10} 0.44 mT, $a^H_{10\beta}$ 0.44 mT MS: m/e: 289 (100 %, M^+-ClO_4).

4a-(3,7,8,10-Tetramethyl-2,4-dioxo-2,3,4,5-tetrahydro-10H-pyrimido [5,4-b][1,4] benzothiazinyl)-3,7,8,10-tetramethyl-2,4-dioxo-2,3,4,5-tetrahydro-10H-pyrimido [5,4-b][1,4] benzothiazine, $(sFl)_2$ (IV: R^1 = R^2 = R^3 = CH_3)

0.2 mmoles (75 mg) of the radical perchlorate was suspended in 50 ml dry dichloromethane at room temperature. On addition of 0.3 ml (2.6 mmoles) of freshly distilled 2,6-lutidine the colour of the suspension changed from violet to yellow over a period of 15 min.
The precipitate was allowed to stand overnight, then filtered off with suction, washed with diethylether, suspended in water to remove base and perchlorate and finally filtered and washed again with diethylether. The solid was dried over potassium hydroxide (80°C, 13 Pa, 8 h). Yield: 49 mg (85 %), m.p.: 260°C (dec.).
^{1}H-NMR-spectra could not be recorded, for the dimer was only sufficient soluble in chloroform, where it formed an equilibrium with its monomer radical. Calculated for $C_{28}H_{28}N_6O_4S_2$ (576.7): C 58.32, H 4.89, N 14.57, S 11.12 %. Found: C 57.86, H 4.90, N 14.29, S 10.92%. IR in KBr: 1715 4-CO; 1665 2-CO cm^{-1}. λ_{max} in benzene (ε): 390 sh (6400), 366 (7000), 309 (11 000) nm ($M^{-1}cm^{-1}$). EPR in chloroform: (a^N_{10} 0.365 mT, $a^H_{10\beta}$ 0.365 mT. MS: m/e 288 (100 %, $M^+/2$).

Sulfoxides $HsFl_{ox}$→O

General Syntheses:

A: 10 mmoles of the corresponding reduced thialumiflavin was suspended in 50 ml acetone. The suspension was heated and 20 mmoles of 30 % hydrogen peroxide solution added, which changed the colour from yellow to colourless. After cooling in an icebath, the solid was collected and washed with 0.1 N HCl, ethanol and diethylether before recrystallization from aqueous sodium hydroxyde solution. Yield: 90 %.
B: 10 mmoles of the corresponding reduced thialumiflavin was suspended in a concentrated methanolic ammonia solution. On cooling in an icebath a solution of 30 % hydrogen peroxide was added slowly and with vigorous stirring until the colour changed from yellow to colourless. Acidification with 50% acetic acid and subsequent evaporation of methanol yielded colourless crystals, which were recrystallized from methanol/water. Yield: 90 %.

10-Methyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrimido [5,4-b][1,4] benzothiazine Sulfoxide, $HsFl_{ox}$→O (V: R^1 = R^2 = H, R^3 = CH_3)

m.p.: 198°C. Calculated for $C_{11}H_9N_3O_3S$ (263.3): C 50.17, H 3.44, N 15.96%. Found: C 49.89, H 3.47, N 15.81 %. IR in KBr: 3450 1-NH; 1715, 1685 4-CO; 1635 2-CO cm^{-1}. λ_{max} in CF_3COOH (ε): 296 (5500), 282 (6500) nm($M^{-1}cm^{-1}$). ^{1}H-NMR in $(C^2H_3)_2SO$: 3.81 (3, s, 10-CH_3), 7.2-8.3 (4, m, ArH), 10.3-11.8 (2, 1-H, 3-H), MS: m/e 263 (1%, M^+), 247 (100 %, M^+-O).

3,10-Dimethyl-2,4-dioxo-1,2,3-4-tetrahydro-pyrimido [5,4-b][1,4] benzothiazine Sulfoxide, $HsFl_{ox}$→O (V: R^1 = CH_3, R^2 = H, R^3 = CH_3)

m.p.: 192°C. Calculated for $C_{12}H_{11}N_3O_3S$ (277.3): C 51.97, H 3.99, N 15.15 %. Found: C 51.73, H 3.99, N 15.24 %. IR in KBr: 3430 1-NH; 1715, 1690 4-CO; 1645 2-CO cm^{-1}. λ_{max} in $CHCl_3$ (ε): 298 sh (6800), 278 (11 000), 246 (24 500) nm ($M^{-1}cm^{-1}$); λ_{max} in CF_3COOH (ε): 294 (7 400), 278 (8700) nm ($M^{-1}cm^{-1}$); λ_{max} in 0.1 N NaOH (ε): 308 (2500) 256 (34 700) nm ($M^{-1}cm^{-1}$). ^{1}H-NMR in $(C^2H_3)_2SO$: 3.3 (3, s, 3-CH_3), 3.78 (3, s, 10-CH_3), 7.25-8.2 (4, m, ArH), 10.5-11.0 (1, 1-H). MS: m/e 277 (5 %, M^+), 261 (100 %, M^+-O).

3,7,8,10-Tetramethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrimido [5,4-b][1,4] benzothiazine Sulfoxide, $HsFl_{ox}$→O (V: R^1 = R^2 = R^3 = CH_3)

m.p.: 205-208°C. Calculated for $C_{14}H_{15}N_3O_3S$ (305.4): C 55.07, H 4.95, N 13.76 %. Found: C 54.78, H 4.98, N 13.43 %. IR in KBr: 3450 1-NH; 1700 4-CO; 1635 2-CO; 1050 5-SO cm^{-1}. λ_{max} in methanol (ε): 284 (12 600), 236 (33 900), 226 sh (30 900) nm ($M^{-1}cm^{-1}$); λ_{max} in 0.1 N NaOH (ε): 315 sh (2200), 255 (44 000) nm ($M^{-1}cm^{-1}$). ^{1}H-NMR in $(C^2H_3)_2SO$: 2.32 (3, s, 7-CH_3), 2.37 (3, s, 8-CH_3), 3.23 (3, s, 3-CH_3), 3.67 (3, s, 10-CH_3), 7.45 (1, s, 9-H), 7.65 (1, s, 6-H), 10.3-11.5 (1, 1-H). MS: m/e 305 (3%, M^+), 289 (100 %, M^+-O).

1,3,10-Trimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrimido [5,4-b][1,4] benzothiazine Sulfoxide, $HsFl_{ox}$→O (V: R^1 = R^3 = CH_3, R^2 = H)

m.p.: 210°C. Calculated for $C_{13}H_{13}N_3O_3S$ (291.3): C 53.59, H 4.49, N 14.42 %. Found: C 53.22, H 4.59, N 14.13 %. IR in KBr: 1695 4-CO; 1685 2-CO; 1020 5-SO cm^{-1}. ^{1}H-NMR in $(C^2H_3)_2SO$: 3.25 (3, s, 3-CH_3), 3.47 (3, s, 1-CH_3), 3.76 (3, s, 10-CH_3), 7.4-8.0 (4, m, ArH). MS: m/e 291 (10%, M^+), 275 (100 %, M^+-O).

4a-Methoxy-3,10-dimethyl-2,4-dioxo-2,3,4,5-tetrahydro-pyrimido [5,4-b][1,4] benzothiazine, sFl_{ox}-4a-OCH_3 (VI: R^1=CH_3, R^2 = H, R^3=CH_3)

10 mmoles (5.0 g) of the corresponding radical tribromide was stirred with 50 ml methanol for about 2 h. The resulting green solution changed to colourless and the precipitate was filtered off by suction. Further product was obtained by concentrating of the filtrate. Recrystallization from methanol yielded 2.1 g of white crystals.
Yield: 2.1 g (72 %). m.p.: 164°C. Calculated for $C_{13}H_{13}N_3O_3S$ (291.3): C 53.59, H 4.49, N 14.42 %. Found: C 53.35, H 4.47, N 14.45 %. IR in KBr: 1710 4-CO; 1660 2-CO cm^{-1}. λ_{max} in methanol (ε): 329 (8300), 245 sh (12900) nm ($M^{-1}cm^{-1}$); λ_{max} in 0.1 N NaOH (ε): 288 sh (7300), 252 (15 500) nm ($M^{-1}cm^{-1}$); λ_{max} in CF_3COOH (ε): 352 sh (4100), 305 (6200) nm ($M^{-1}cm^{-1}$). ^{1}H-NMR in C^2HCl_3: 3.28 (3, s, 3-CH_3), 3.34 (3, s, 10-CH_3), 3.85 (3, s, 4a-OCH_3), 7.1-7.6 (4, m, ArH). MS: m/e 291 (M^+).

4a-Methoxy-3,7,8,10-tetramethyl-2,4-dioxo-2,3,4,5-tetrahydro-pyrimido [5,4-b][1,4]benzothiazine, sFl_{ox}-4a-OCH_3 (VI: R^1 = R^2 = R^3 = CH_3)

This compound was synthesized by the same method described above. Yield: 68 %, m.p.: 250-256°C. Calculated for $C_{15}H_{17}N_3O_3S$ (319.4): C 56.40, H 5.37, N 13.16 %. Found: C 56.01, H 5.31, N 13.03 %. IR in KBr: 1710 4-CO; 1665 2-CO cm^{-1}. λ_{max} in methanol (ε): 340 (9800), 276 sh (8300), 255 sh (14 800) nm ($M^{-1}cm^{-1}$); λ_{max} in CF_3COOH (ε): 374 sh (4200), 318 (7800) nm ($M^{-1}cm^{-1}$). ^{1}H-NMR in C^2HCl_3: 2.28 (3, s, 7-CH_3), 2.32 (3, s, 8-CH_3), 3.28 (3, s, 3-CH_3), 3.35 (3, s, 10-CH_3), 3.77 (3, s, 4a-OCH_3), 7.13 (1, s, 6-H), 7.25 (1, s, 9-H); MS: m/e 319 M(+).