ChemPubSoc Europe

DOI: 10.1002/cbic.201200670

Spectroscopic and Theoretical Study on Electronically Modified Chromophores in LOV Domains: 8-Bromo- and 8-Trifluoromethyl-Substituted Flavins

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Two chemically synthesized flavin derivatives, 8-trifluoromethyl- and 8-bromoriboflavin (8-CF_3RF and 8-BrRF), were photochemically characterized in H_2O and studied spectroscopically after incorporation into the LOV domain of the blue light photoreceptor YtvA from *Bacillus subtilis*. The spectroscopic studies were paralleled by high-level quantum chemical calculations. In solution, 8-BrRF showed a remarkably high triplet quantum yield (0.97, parent compound riboflavin, RF: 0.6) and a small fluorescence quantum yield (0.07, RF: 0.27). For 8-CF_3RF, the triplet yield was 0.12, and the fluorescence quantum yield was 0.7. The high triplet yield of 8-BrRF is due to the bromine heavy atom effect causing a stronger spin–orbit coupling. Theoretical calculations reveal that the decreased triplet yield of 8-CF_3RF is due to a smaller charge transfer and a less favorable energetic position of T_2, required for intersystem crossing from S_1 to T_1, as an effect of the electron-withdrawing CF_3 group. The reconstitution of the LOV domain with the new flavins resulted in the typical LOV photochemistry, consisting of triplet state formation and covalent binding of the chromophore, followed by a thermal recovery of the parent state, albeit with different kinetics and photophysical properties.

Introduction

LOV (light, oxygen, voltage) domain proteins, such as YtvA of *Bacillus subtilis*, are members of a large blue-light-sensitive photoreceptor family. Their plant counterparts are called phototropins.[1,2] The response towards light in a LOV domain depends strongly on the photophysical properties of the incorporated chromophore, flavin mononucleotide (FMN). Light excitation generates the triplet state, which, during its decay over a few μs, forms a covalent bond to the protein through the side chain of a closely positioned cysteine residue (Cys62 in *YtvA*-LOV), concomitantly with a rearrangement of the hydrogen bonding network in the chromophore pocket.[3–5] This photoadduct (LOV_{390}) is considered the signaling state of YtvA. In the dark, the covalent bond in LOV_{390} is slowly reopened over several hours, yielding the initial (parent) state of the photoreceptor (LOV_{447}).[6]

LOV domains have recently attracted attention because of their very versatile properties, which make them interesting candidates for potential biotechnological applications.[7–9] Accordingly, effects of structural modifications of the photophysical/photochemical properties of these biological photoreceptors are now being intensely investigated.[10] The main strategies for controlling the signaling mechanism are through changes in the protein by site-directed mutations or through replacement of the native chromophore with chemically synthesized or modified flavins. The objective of the latter approach is based on the assumption that a new functional group at a modified chromophore might change its electronic properties and also might interfere with the hydrogen bonding network between the chromophore and the protein. As well as the natural chromophore (FMN), riboflavin (RF) and (in small amounts) flavin adenine nucleotide (FAD) have also been identified in LOV domains.[11] In the dark state, the isoalloxazine ring of flavin is involved in many interactions between the chromophore and protein that are important for regulation of the kinetics of the LOV domain photocycle, whereas the phosphate group at the ribityl moiety establishes electrostatic interactions with the side chains of two arginine residues on the surface of the protein.[12]

Here we report the synthesis and photophysical properties of two electronically modified flavins (Scheme 1)—8-(trifluoromethyl)riboflavin (8-CF_3RF) and 8-bromoriboflavin (8-BrRF)—in H_2O and after incorporation into the LOV domain of YtvA from *B. subtilis*, in combination with high-level quantum chemical calculations. There are still very few reports on the use of flavin derivatives as chromophores in LOV domains. One interesting case was the in vivo incorporation of roseoflavin (8-dimethylamino FMN), which showed a strong bathochromic shift of its absorbance band ($\lambda_{max} = 520$ nm).[13] However, those authors demonstrated that roseoflavin did not form a covalent bond

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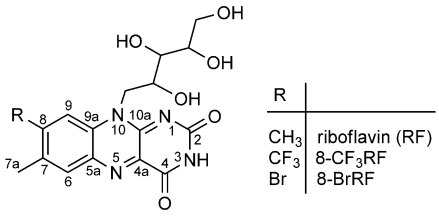


Scheme 1. Structural formulas of riboflavin and its halogenated derivatives. The corresponding lumiflavin compounds each carry a methyl group at N_{10}.

(i.e., a photoadduct) with the protein. We were recently able to show that the naturally occurring flavin chromophores RF, FMN, and FAD, bearing differently substituted ribityl chains, differ only slightly[14] in their photophysical characteristics, with the largest variations in the thermally driven dark recovery kinetics; accordingly, the RF forms of the modified flavins were used in the experiments reported here. Both substituents should disturb the steric interactions with the protein cavity only marginally, because their van der Waals radii are only slightly larger than that of the substituted methyl group. However, the introduction of a bromine atom should increase electron density in the flavin and modify the triplet formation yield strongly, whereas the trifluoromethyl group should have a strongly electron-withdrawing effect. Selection of these substituents and their position was motivated by previous findings that indicated that the transition dipole moment extends from position 8 of the isoalloxazine ring system to position 3.[15,16] Both in vivo[13,17] and in vitro[12,14,18] chromophore exchange procedures are available; here we followed our recently reported protocol for chromophore exchange, based on unfolding of the wild-type (WT) protein, removal of the native chromophore, and introduction of a modified flavin upon refolding of the protein into its native conformation.[14] The quantum chemical calculations performed here explain the observed excited state behavior (these calculations were performed for the lumiflavin forms, i.e., the sugar-free forms of the modified flavins).

Results and Discussion

The two flavins reported here were designed especially because of the strong electronic changes induced by the halogenated substituents at position 8, because calculations reveal the direction of both transition dipole moments from the phenyl ring to the uracil component. In particular, the S_2 transition dipole moment is oriented between positions 8 and 3. In addition, the bromine should exert a strong heavy-atom effect on the fluorescence/triplet reaction channels such that the triplet yield should increase at the expense of the fluorescence.

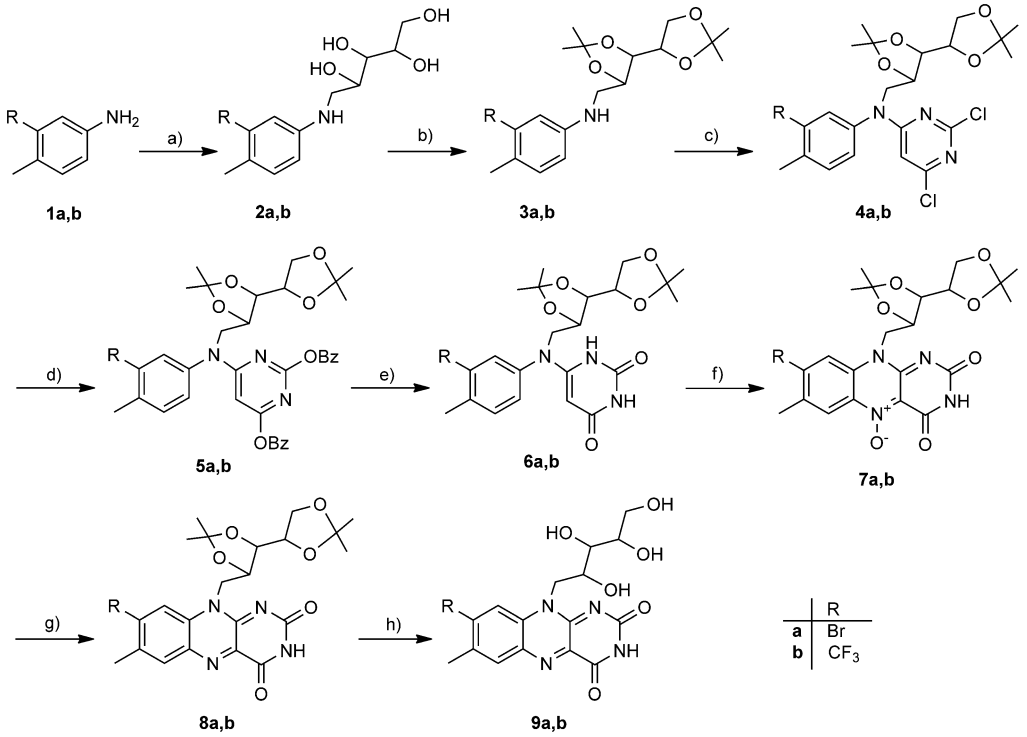
Chemical synthesis

Several methods for the synthesis of flavin derivatives have been reported;[19–21] they are based either on displacement reactions on flavins containing good leaving groups at the required positions[22,23] or on multistep synthesis from appropriately substituted aromatic building blocks as starting materials.[24] However, the synthesis of 8-halogenated flavins is not straightforward, because halogen substituents reduce the nucleophilicities of the amine functions of the *meta*-halogenated aryl systems from which the synthesis starts. On the other hand, selective introduction of halogens into the complete isoalloxazine ring is rather troublesome due to the presence of many competing functional groups in this complex molecule.

Here, the synthesis of the both 8-substituted chromophores was performed in similar manner to published synthetic routes (Scheme 2, for details see the Experimental Section).[24,25] The previous authors reported the protection group chemistry applied here in great detail. In brief, the free OH groups in the ribitylated anilines **2**[26] were protected by treatment with 2,2-dimethoxypropane, and the resulting compounds **3** were allowed to react with 2,4,6-trichloropyrimidine, yielding mixtures of isomers with compounds **4** as major components. Substitution on the pyrimidine ring with benzyloxy functions, followed by hydrogenation (Pd/C), resulted in intermediates **6** in good yield. The completion of the isoalloxazine ring (ring closure reaction) was performed by treatment with sodium nitrite[27] in the presence of acetic acid, yielding the N-oxides **7**. The desired compounds **9** were then obtained by reduction with sodium thiosulfite, followed by deprotection of the ribityl OH groups. The overall yields were 3% and 4.3% for 8-CF_3RF and 8-BrRF, respectively. These apparently moderate yields should be seen in the context of the reaction sequence of eight reaction steps. In addition, **3**→**4** involves the formation of mixtures of isomers of which only one (with the correct steric configuration) could be used in each case for further synthesis; furthermore, the substituent change (chloride to benzyl, **4**→**5**) and the deprotection chemistry had to be processed for both positions. For the good yields of the individual steps see the Experimental Section.

Spectroscopic properties of 8-CF_3RF and 8-BrRF in buffered solution (pH 7)

Both flavins had typical flavin double-peaked absorption spectra (Figure 1). The spectrum of 8-BrRF was essentially identical to that of riboflavin, also with respect to its extinction coefficient ($\lambda_{max}=450$ nm, $\varepsilon_{max}=12460\ M^{-1}cm^{-1}$). This finding is in excellent agreement with the theoretical calculations, which yield vertical absorption energies of 8-BrLF that closely match those of LF. Interestingly, the S_2 transition of 8-CF_3RF showed a hypsochromic shift (330 nm, compared to 360 nm for the parent compound, RF, or for 8-BrRF, vide infra), and this compound also exhibited a lower extinction coefficient ($\lambda_{max}=$ 450 nm, $\varepsilon_{max}=8450\ M^{-1}cm^{-1}$, Table 1). The theoretically calculated vertical absorption energies of 8-CF_3LF show convincing agreement with the measured absorbance band maxima and reveal the origin of the hypsochromic shift mentioned above. For the parent compound, the energetic position of the second absorption band is known to be solvent-dependent, ranging between 332 nm (3.73 eV) in dioxane and 367 nm (3.38 eV) in water.[28] The underlying electronic transition is ac-



Scheme 2. Synthesis of 8-substituted flavins. a) D-Ribose, $NaCNBH_3$; b) 2,2-dimethoxypropane/acetone, TsOH; c) 2,4,6-trichloropyrimidine, K_2CO_3, Bu_4NBr; d) $PhCH_2ONa$; e) H_2/Pd; f) $NaNO_2$, AcOH; g) $Na_2S_2O_3$; h) TFA.

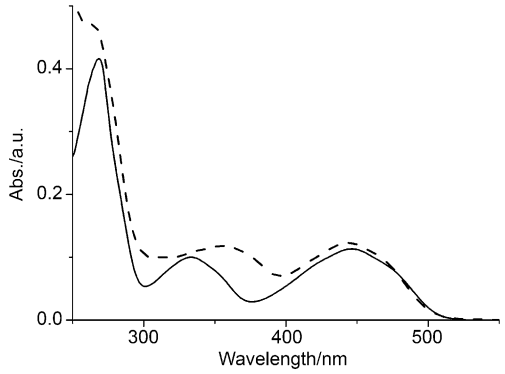


Figure 1. UV/VIS spectra of 8-BrRF (dotted line) and 8-CF_3RF (solid line) in H_2O, pH 7. The absorption spectrum of 8-BrRF fully coincides with that of riboflavin.

companied by a transfer of charge density from the benzene to the pteridine moiety of the isoalloxazine ring, thus increasing the molecular dipole moment with respect to the ground state.[29] It is thus not surprising that the electronically excited state is preferentially stabilized by polar solvents. The more polar and protic the surrounding medium, the more this band shifts to lower energy. Our calculations reveal that for the trifluoromethyl derivative even the electronic transition in the isolated molecule requires higher excitation energy. In the parent compound all heteroatoms are concentrated in one half of the molecular scaffold, whereas in the trifluoromethyl compound, polar heteroatoms with increased electronegativity are added on the aliphatic half of the molecule. A transfer of charge density from the benzene to the pteridine moiety, as takes place in the excited state, is thus less favorable. In addition, the impact of the solvent on the energetic position of the absorbance band in question is less than it is in the parent compound. The origin of this variation can be explained by the significantly decreased dipole moment of the 8-CF_3LF.

Table 1. Spectroscopic properties of 8-substituted riboflavins and of the parent compound (RF) in water, pH 7. In addition, calculated vertical excitation wavelengths are listed.

	λ_{abs} (max) [nm]	λ_{abs} (calcd) [nm]	ε_{max} [$M^{-1}cm^{-1}$]	λ_{em} (max) [nm]	τ_F [ns]	Φ_F
RF	360, 450	357, 432	12460	520	4.8	0.27
8-CF_3RF	330, 450	333, 435	8450	520	4.9	0.70
8-BrRF	360, 450	359, 433	12460	520	3.5 (85%), 7.0 (15%)	0.07

Fluorescence and triplet state properties of halogenated flavins

The fluorescence maxima for both synthetic flavins were similar to that for RF (520 nm, H_2O). However, both 8-CF_3RF and 8-BrRF significantly differed from RF in their fluorescence quantum yields (0.7 and 0.07, respectively, in comparison with 0.27 for RF[30]). Time-resolved fluorescence measurements yielded a biexponential decay for 8-BrRF [τ_{F1} = 3.5 ns (85%), τ_{F2} = 7.0 ns (15%)], whereas the decay of 8-

CF_3RF was monoexponential ($\tau_F = 4.9$ ns) and in the same range as that of the parent compound (Table 1).

Study of the triplet parameters revealed significant variations with respect to those of the parent compound: both modified riboflavins showed broad transient absorptions with maxima at 520 and at approximately 700 nm, similarly to RF (Figure 2, Table 2),[31] but the quantum yield determined for 8-CF_3RF ($\Phi_T = 0.12$, with a decay time τ_T of 10 μs) was much smaller than that for RF ($\Phi_T = 0.6$, $\tau_T = 29$ μs).[31;32] In earlier work on RF, we found that the second $^3\pi\pi^*$ state (T_2) acts in aqueous solution as a doorway state for intersystem crossing from the S_1 to the T_1 state.[29] Like its singlet counterpart that gives rise to the second band in the absorption spectrum, the T_2 state of 8-CF_3RF experiences a smaller solvent shift than that of the parent compound. It may therefore be assumed that the crossing point between the S_1 and T_2 potential energy surfaces of 8-CF_3RF occurs at higher energies with respect to the S_1 minimum than in RF, thus explaining the smaller triplet quantum yield. In contrast, the quantum yield of 8-BrRF was remarkably high: $\Phi_T = 0.97$ (decay time = 5 μs), which is clearly attributable to the heavy-atom effect of the introduced bromine.

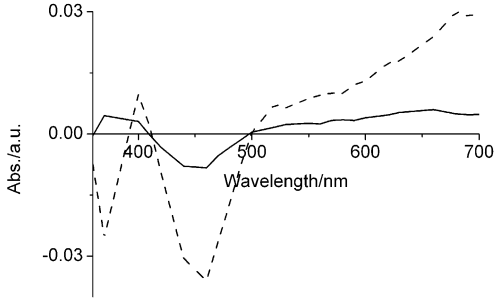


Figure 2. Transient spectra of 8-CF_3RF (solid line) and 8-BrRF (dotted line) in H_2O, pH 7. Compound 8-CF_3RF: interval 1.5–3.5 μs; 8-BrRF: 0.5–1.5 μs.

Table 2. Transient absorption properties of modified flavins (triplet decay).

	λ_T (max) [nm]	τ_T [μs]	Φ_T
RF	520, 700	29	0.60
8-CF_3RF	520,[a] 650	10	0.12
8-BrRF	520, 700	5	0.97

[a] Very weak signal.

Photocycle and photophysical properties of 8-CF_3RF and 8-BrRF in LOV

Both 8-substituted chromophores were successfully incorporated into the blue light photoreceptor YtvA by the chromophore exchange protocol reported previously.[14] The newly reconstituted proteins were relatively unstable relative to LOV domains reconstituted by the chromophore exchange protocol with naturally occurring flavins (RF, FMN, FAD), and also with the alkyl-modified flavins in the former work;[14] the halogenated flavin chromophores were lost in relatively short times, causing the apoprotein to aggregate and precipitate. Because the riboflavin forms were used here, one might speculate that interaction was less stable, due to the lack of the phosphate group. Previous experiments,[14] however, revealed no destabilizing effect from the removal of the phosphate group: a riboflavin reconstituted LOV domain showed no change in its spectral properties, even when kept for several weeks at 4 °C.

Nevertheless, both 8-CF_3LOV and 8-BrLOV showed the functional properties of the LOV photocycle: an absorption maximum around 450 nm, the photoproduct formation after illumination with blue light, and the thermally driven dark recovery (Figure 3). The hypsochromically shifted S_2 absorption band of 8-CF_3 seen in aqueous solution was identically present in the protein-incorporated form, allowing in this case the identification of three isosbestic points during the generation of the

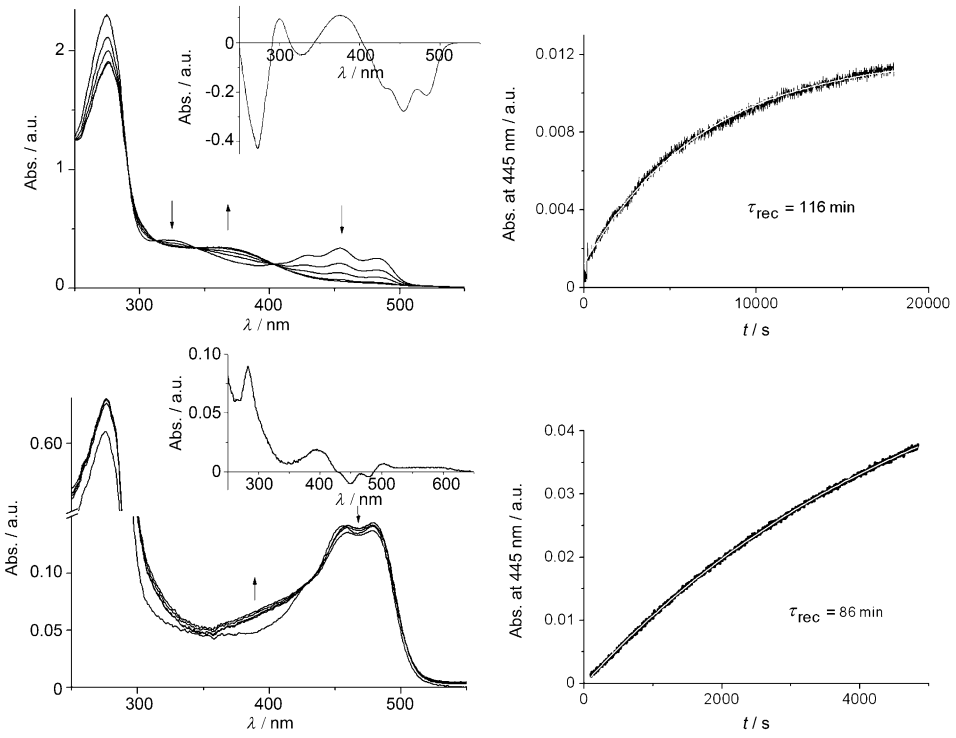


Figure 3. Photoproduct formation in 8-CF_3LOV (top left) and in 8-BrLOV (bottom left) upon blue light exposure. The insets show the difference spectra between the lit and the dark states. Dark recovery kinetics are shown on the right for 8-CF_3LOV (top) and for 8-BrLOV (bottom) with the exponential fit curves given in white.

photoproduct (λ_{max} = 370 nm, Figure 3). Quantitatively, the formation of the photoproduct was calculated for the first 30 s of irradiation and then after saturating blue light irradiation, by using YtvA WT as reference. Dark recoveries were monitored at λ_{max} = 445 nm for both reconstituted proteins. The instability of reconstituted 8-CF_3LOV mentioned above, however, impeded complete dark recovery. The summarized data for the protein photochemistry of 8-CF_3LOV and 8-BrLOV are presented in Table 3.

Table 3. Photocycle parameters of LOV-reconstituted flavins.

	t_{ph}[a] [min]	τ_{rec}[b] [min]	Photoproduct formation after 30 s [%]	sat. irradiation [%]
YtvA WT	7	129	27	100
8-CF_3RF LOV	6.5	116[c]	45	100
8-BrRF LOV	6.5[d]	86	8	15

[a] Time of photoproduct formation. [b] Dark-state recovery kinetics. [c] The recovery was not complete, probably due to partial denaturation of the sample during the experiment. [d] There were no further changes after the indicated time and the complete lit state was not achieved.

A very interesting behavior was observed for 8-BrLOV. Whereas the S_2 absorption band for this compound was very pronounced when measured in H_2O (λ_{max} = 360 nm, Figure 1), in the protein-bound state only the S_1 absorption band was clearly detected (Figure 3). In addition, this S_1 absorption band showed a peculiarity in that the high-energy shoulder of the 450 nm band is of very low intensity, in contrast to the "normal" flavin chromophores that exhibit a very characteristic "three-peaked" 450 nm absorbance band. Interestingly, blue light irradiation of 8-BrLOV yielded a very limited bleaching of the absorption at 450 nm, yet the increase at 390 nm is clearly present (Figure 3). Estimation of the vertical excitation energies of 8-BrLOV shed light on this behavior. As elucidated in earlier work,[33] two distinct conformations (confA and confB) at the sulfur atom of Cys62 have been detected in the X-ray structure of *YtvA*-LOV, and these give rise to slightly different vertical absorption spectra. The calculated difference in the vertical excitation energies is most pronounced for the excitation that yields the S_2 band and results from the conformation-dependent interaction of the sulfur lone-pair orbital of Cys62 (Figure 4). In both conformers, remarkable red shifts of this band relative to the water-solvated cofactor are observed. In confA, in which the sulfur atom of Cys62 is located above the benzene moiety of the isoalloxazine ring, the stabilization of the upper electronic state is particularly strong. It can therefore be assumed that the unusual shape of the measured absorption spectrum is due to merging of the S_1 and S_2 bands.

The reasons for the reduced yield of photoconversion are not clear. Careful observation of the fluorescence lifetime identified a biexponential decay, with one species (≈19%) showing τ_F = 3.4 ns (Table 4), identical to that of the major species in the fluorescence lifetime decay of the unbound 8-BrRF (in H_2O, pH 7). However, the absence of the S_2 band is clear evidence for the bound chromophore, and no signal at 360 nm

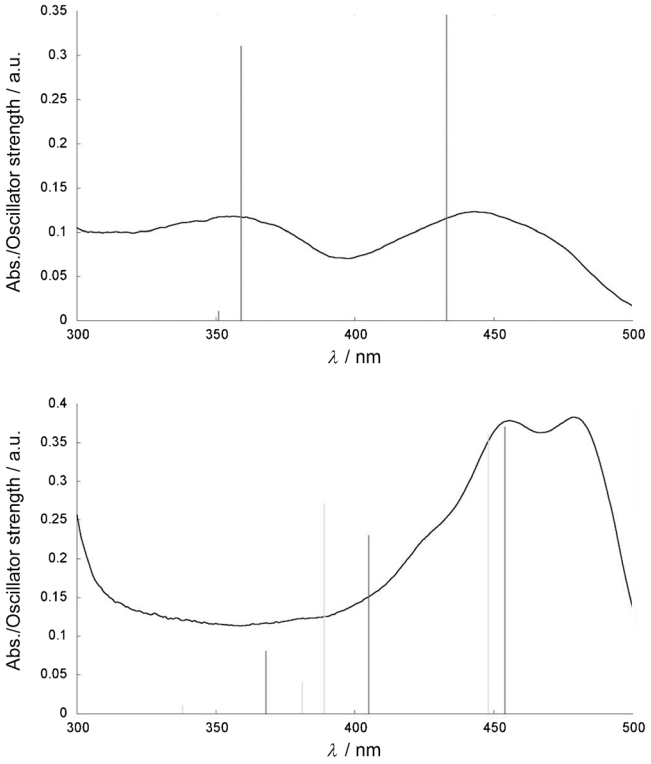


Figure 4. Comparison between experimentally measured absorption spectra of 8-BrRF/8-BrLOV and DFT/MRCI vertical excitation energies and intensities of the lumiflavin derivatives. The top panel shows the results for aqueous solution and the bottom panel for the protein environment. The calculated vertical excitation energies (bottom) correspond to the two different conformers of the 8-BrLOV complex (confA: gray lines; confB: light gray lines). For further explanation see text.

Table 4. Spectroscopic properties and triplet decays of 8-CF_3RF and 8-BrRF in the LOV domain.

	τ_F [ns]	Φ_F	τ_T [μs]
YtvA WT	2.2 (85%), 4.6 (15%)	0.22	1.7
8-CF_3LOV	3.3 (70%), 0.7 (30%)	0.10[a]	1.7
8-BrLOV	0.5 (81%), 3.4 (19%)	0.07	3.6

[a] The protein was not stable over longer times (i.e., a decrease in protein amount by 40% was found upon repetition of the measurement after 24 h).

(which would be indicative of chromophore release) was detected upon blue light irradiation. Moreover, the observed distribution (3.3 ns (70%) and 0.6 ns (30%); Table 4) was the opposite of the fluorescence decay of 8-CF_3LOV; this could lead to the conclusion that the 19% component in 8-BrLOV (3.4 ns) might be the active 8-BrLOV species. If it is assumed that this fraction of the chromophore is the active one, the typically observed acceleration of the fluorescence decay in the protein-bound form is not detected. Still, both lifetimes of 0.5 and 3.4 ns are within the reported range of LOV proteins.[34–37]

Another possible explanation for the low level of conversion to the photoproduct could be steric hindrance introduced by

the bulkier bromine atom or, alternatively, its electronic influence on the surrounding amino acid network in the chromophore pocket, possibly resulting in an increased distance between the C-4a position of the isoalloxazine ring and the reactive Cys62 residue. These steric or electronic restrictions in the chromophore pocket might also contribute to the low stability of the protein–chromophore complex.

The data in Tables 3 and 4 do not allow for a discussion in quantitative terms with comparison to time-resolved triplet state determination. However, the extent of photoadduct formation after 30 s is higher for the trifluoromethyl derivative than for the WT protein. This is even more surprising if its lower extinction coefficient and, in addition, the very low triplet yield in water are taken into account. This interpretation would point to an additional reaction channel involving the singlet state, as already proposed for the LOV2 domain of the alga *Chlamydomonas reinhardtii*.[38]

The bromo derivative has different properties, if the finding of a large unreactive fraction is taken into account. Only 15% of the sample could converted even under saturating irradiation conditions (Table 3). If this 15% fraction is assumed to be the functionally active species, we would already have converted more than 50% (8% of 15%) after 30 s of irradiation. This calculation would indeed—especially for the bromo derivative—demonstrate the correlation of an increased triplet yield and, in parallel, increased photoadduct formation.

Conclusions

Both, 8-CF_3RF and 8-BrRF were synthesized in good overall yields and could be incorporated into the LOV domain of *B. subtilis* photoreceptor YtvA. Interestingly, the positions and shapes of the absorption bands, the fluorescence properties, and the triplet lifetimes and yields of the flavin derivatives in water are significantly different from those of RF: the introduced bromine substituent produces a remarkably high triplet quantum yield, whereas the properties of the trifluoromethyl compound are similar to those of the parent compound. Also in the protein-incorporated form, both flavin derivatives showed significant changes in photochemical behavior. Especially strong effects were observed for 8-BrRF, for which the S_2 state absorption was practically absent and the high-energy shoulder of the S_0 absorption band was significantly less pronounced. No such strong effects were found in the case of the CF_3 substituent; this chromophore showed properties very similar to those of the RF-assembled protein, except that the S_2 absorption appears at higher energy. The large changes in triplet yields (much higher for the bromo derivative and lower for the trifluoromethyl derivative) observed in water could not be identified similarly clearly in the protein-bound forms, due to the relatively unstable derivative–LOV domains. However, the apparently higher yield of photoproduct formation of the trifluoromethyl flavin might be indicative of (with all reluctance) an alternative pathway for photoadduct formation to that of the WT-LOV under identical conditions. Site-directed mutagenesis might help in clarifying this preliminary observation. All these findings are evidence for the strong modifying effects of the introduced halogen substituents on the electronic properties of the flavin chromophore and reveal different types of interactions between the chromophore and its surrounding amino acid network.

Experimental Section

Synthesis of 8-substituted flavins

General: All chemicals were obtained from Sigma–Aldrich, except for 3-isopropyl-4-methylaniline, which was purchased from Daxian Chemical Institute, Ltd. All moisture- and oxygen-sensitive reactions were carried out under inert atmosphere (Ar). All reported yields are for isolated pure products. Flash chromatography (FC) purifications were performed on Merck silica gel 60 (230–400 mesh). HPLC purifications were carried out on Kromasil C18 ODS-5-100 (RP-C_{18}, 5 μm, 250 mm × 21 mm, flow rate 0.8 mL min^{-1}), with elution with systems A (H_2O + 2% CH_3CN + 0.05% TFA) and B (30:70 H_2O + CH_3CN + 0.05% TFA), if not indicated otherwise. The NMR spectra were recorded with a Bruker AVANCE DRX 400 spectrometer. Chemical shifts are given as δ values (ppm) downfield relative to tetramethylsilane as an internal standard. MS characterization was carried out with a Finnigan MAT 8200 (EI) or Bruker Esquire 3000 (ESI) instrument; exact mass determinations (HRMS) were performed with a Finnigan MAT 95 instrument.

General procedure for riboaniline formation: Compound **1** (1 mmol) was dissolved in MeOH (10 mL), and $NaCNBH_3$ (2 equiv) was added with stirring, followed by D-ribose (2 equiv). The resulting mixture was stirred under reflux for 43–48 h, the solvent was then removed under reduced pressure, and the remaining material was added to HCl (1 M, 2 mL) and swirled until gas generation finished. The solution was then carefully neutralized with saturated $NaHCO_3$, extracted into EtOAc (3 × 25 mL), dried over Na_2SO_4, and concentrated. FC on silica gel, with elution with CH_2Cl_2/MeOH (90:10), provided the product **2**.

3-Bromo-4-methylriboaniline (***2a***): Yield: 78%; ^{1}H NMR (400 MHz, CD_3OD): δ = 7.20 (d, 1H; Ar), 6.62 (s,1H; Ar), 6.43 (d, 1H; Ar), 4.81 (br s; 4OH), 3.91 (m, 1H; 5′-C*H*H), 3.76 (m, 2H; 5′-CH*H*, 2′-CH), 3.65 (m, 2H; 3′-CH, 4′-CH), 3.30 (dd, 1H; 1′-C*H*H), 3.13 (m, 1H; 1′-CH*H*), 2.25 ppm (s, 3H; CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ = 149.8, 138.9, 133.4 (Ar), 116.5, 113.6, 112.0 (Ar), 74.7 (C-3′), 74.6 (C-2′), 72.2 (C-4′), 64.6 (C-5′), 47.6 (C-1′), 23.1 ppm (CH_3); MS (EI): *m/z*: 319 [*M*], 198 [*M*−$CH_2OH(CHOH)_3$].

3-Trifluoromethyl-4-methylriboaniline (***2b***): Yield: 85%; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.05 (d,1H; Ar), 6.93 (d, 1H; Ar), 6.77 (dd, 1H; Ar), 4.80 (br s; 4OH), 3.91 (m, 1H; 5′-C*H*H), 3.80–3.72 (m, 2H; 5′-CH*H*, 4′-CH) 3.66–3.61(m, 2H; 2′-CH, 3′-CH), 3.44 (dd, 1H; 1′-C*H*H), 3.15 (dd, 1H; 1′-CH*H*), 2.29 ppm (s, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 148.5, 133.7 (Ar), 130.2, 129.9 (CF_3), 127.7, 124.6, 117.0, 111.3 (Ar), 74.6 (C-3′), 74.4 (C-2′), 72.2 (C-4′), 64.4 (C-5′), 47.3 (C-1′), 18.3 ppm (CH_3); MS (EI): *m/z*: 309 [*M*], 188 [*M*−$CH_2OH(CHOH)_3$].

General procedure for OH protection: A riboaniline **2** (1 mmol) was dissolved in a mixture of dimethoxypropane/dry acetone (1:2, 12 mL), and TsOH·H_2O (1.0 equiv) was added. The reaction mixture was stirred at RT for 1.5 h, and the solvents were then removed in vacuo. CH_2Cl_2 (20 mL) was added, and the resulting mixture was washed with saturated $NaHCO_3$ (5 mL), followed by H_2O (5 mL). FC on silica gel with elution with $CH_2Cl_2 \rightarrow CH_2Cl_2/Et_2O$ (99:1) resulted in the desired 2,3:4,5-bis(methylethylidene)riboaniline and in 2,5:3,4-bis(methylethylidene)riboaniline as a side product.

*3-Bromo-4-methyl [2,3:4,5-bis(methylethyliden)ribo]aniline (**3a**)*: Yield: 45%; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.00$ (d, $J = 8.5$ Hz, 1H; H-5), 6.89 (m, 1H; H-2), 6.57 (m, 1H; H-6), 4.37 (m, 1H; 2′-CH), 4.11 (m, 2H; 3′-CH, 4′-CH), 4.02 (m, 1H; 5′-C*H*H), 3.92 (m, 1H; 5′-CH*H*), 3.40 (m, 1H; 1′-C*H*H), 3.32 (m, 1H; 1′-CH*H*), 2.26 (s, 3H; 4-CH_3), 1.43, 1.40, 1.35, 1.32 ppm (4s, 12H; 4CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 146.3$ (C-3), 131.1 (C-5), 131.0 (C-1), 125.4 (C-4), 117.2 (C-6), 113.2 (C-2), 110.0, 108.9 (2C; 2*C*(CH_3)$_2$), 78.2 (C-3′), 75.4 (C-2′), 73.2 (C-4′), 68.1 (C-5′), 44.1 (C-1′), 28.0, 26.9, 25.45, 25.39 (4C; 4CH_3), 21.7 ppm (CH_3); MS (EI): *m/z* (%): 401, 399 (32) [*M*], 386, 384 (20) [*M*−CH_3], 200 (95), 198 (100) [*M*−CH_2(OH)(CHOH)$_3$]. Yield of isolated 3-bromo-4-methyl [1,5:3,4-bis(methylethyliden)ribo]aniline: 6%.

*3-Trifluoromethyl-4-methyl [2,3:4,5-bis(methylethyliden)ribo]aniline (**3b**)*: Yield: 41%; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.04$ (m, 1H; H-5), 6.88 (m, 1H; H-2), 6.68 (m, 1H; H-6), 4.38 (m, 1H; 2′-CH), 4.14 (m, 2H; 3′-CH, 4′-CH), 4.02 (m, 1H; 5′-C*H*H), 3.93 (m, 1H; 5′-CH*H*), 3.46 (m, 1H; 1′-C*H*H), 3.34 (m, 1H; 1′-CH*H*), 2.33 (s, 3H; 4-CH_3), 1.43, 1.41, 1.35, 1.33 ppm (4s, 12H; 4CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 145.7$ (C-3), 132.7 (C-5), 132.6 (C-1), 129.5 (d, CF_3), 124.8 (C-4), 115.9 (C-6), 110.7 (C-2), 110.0, 108.9 (2C; 2*C*(CH_3)$_2$), 78.2 (C-3′), 75.7 (C-2′), 73.2 (C-4′), 68.1 (C-5′), 43.6 (C-1′), 28.0, 26.8, 25.4, 25.3 (4C; 4CH_3), 18.2 ppm (CH_3); MS (EI): *m/z* (%): 389 (23) [*M*], 188 (100) [*M*−CH_2(OH)(CHOH)$_3$].

3-Trifluoromethyl-4-methyl [2,5:3,4-bis(methylethyliden)ribo]aniline: Yield: 38%; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.03$ (d, $J = 8.0$ Hz, 1H; H-5), 6.92 (s, 1H; H-2), 6.71 (m, 1H; H-6), 4.13 (m, 1H; 2′-CH), 3.94 (m, 2H; 3′-CH, 4′-CH), 3.89 (m, 1H; 5′-C*H*H), 3.81 (m, 1H; 5′-CH*H*), 3.52 (m, 1H; 1′-C*H*H), 3.06 (m, 1H; 1′-CH*H*), 2.32 (s, 3H; 4-CH_3), 1.51, 1.35 (2C), 1.24 ppm (3s, 12H; 4CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 145.3$ (C-3), 132.7 (C-5, C-1), 129.5 (d, CF_3), 123.3 (C-4), 116.5 (C-6), 110.8 (C-2), 108.6 (dioxolane-*C*(CH_3)$_2$), 102.0 (dioxepane-*C*(CH_3)$_2$), 76.7 (C-3′, C-2′, hidden under $CDCl_3$ signal), 68.6 (C-4′), 58.4 (C-5′), 45.9 (C-1′), 28.4, 25.6, 25.2, 23.7 (4C; 4CH_3), 18.2 ppm (CH_3); MS (EI): *m/z* (%): 389 (28) [*M*], 188 (100) [*M*−CH_2(OH)(CHOH)$_3$].

General procedure for condensation with 2,4,6-trichloropyrimidine: A protected riboaniline **3** (1 mmol), $KHCO_3$ (4 equiv), and tetrabutylammonium bromide (cat. amount) were suspended in toluene (4 mL). A solution of 2,4,6-trichloropyrimidine (2 equiv) in toluene (1 mL) was added, and the resulting mixture was stirred under argon at 100 °C for 22–26 h. After filtration from solid material, the mixture was directly loaded on top of a FC column and eluted with CH_2Cl_2/Et_2O (95:5). Three compounds were isolated in the following order: undesired side-product (20–27%), starting compound, and desired product (35–42%).

*8-Bromo intermediate **4a***: Yield: 42%; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.33$ (m, 1H; H-6), 7.22 (s, 1H; H-9), 7.14 (m, 1H; H-5a), 6.00 (m, 1H; H-4a), 4.47 (m, 1H; 2′-CH), 4.35 (m, 1H; 3′-CH), 4.09 (m, 2H; 4′-CH, 5′-C*H*H), 3.95 (m, 3H; 5′-CH*H*, 1′-CH_2), 2.34 (s, 3H; CH_3-7a), 1.40, 1.34, 1.29, 1.28 ppm (4s, 12H; 4CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 164.2$ (C-10a), 159.7 (C-4), 140.3 (C-2), 138.7 (C-9a), 132.1 (C-6), 129.0 (C-9), 128.2 (C-5a), 127.1 (C-7), 125.3 (C-8), 109.9, 109.3 (2C; 2*C*(CH_3)$_2$), 102.0 (C-4a), 77.8 (C-3′), 74.4 (C-2′), 73.2 (C-4′), 68.1 (C-5′), 50.6 (C-1′), 27.8, 26.9, 25.6, 25.3 (4C; 4CH_3), 22.6 ppm (CH_3-7a); MS (EI): *m/z* (%): 545 (3) [*M*], 530 (12) [*M*−CH_3], 344 (15) [*M*−CH_2(OH)(CHOH)$_3$], 331 (60) [*M*−ribose]; HRMS: *m/z* calcd: 568.037608 [*M*+Na]$^+$; found: 568.037204.

*8-Trifluoromethyl intermediate **4b***: Yield of desired **4b**: 35%; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.60$ (m, 1H; H-6), 7.40-7.24 (m, 2H; H-9, H-5a), 6.00 (m, 1H; H-4a), 4.49 (m, 1H; 2′-CH), 4.12 (m, 2H; 3′-CH, 4′-CH), 4.02 (m, 1H; 5′-C*H*H), 3.85 (m, 3H; 5′-CH*H*, 1′-CH_2), 2.53 (s, 3H; CH_3-7a), 1.41, 1.31, 1.29, 1.27 ppm (4s, 12H; 4CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, signals that could be interpreted): $\delta = 164.4$ (C-10a), 163.0 (C-4), 139.7 (C-6), 133.9 (C-2), 131.4 (C-5a), 131.3 (C-9), 109.8, 109.1 (2C; 2*C*(CH_3)$_2$), 101.9 (C-4a), 77.9 (C-3′), 74.5 (C-2′), 73.2 (C-4′), 68.1 (C-5′), 50.9 (C-1′), 27.7, 26.8, 25.5, 25.1 (4C; 4CH_3), 19.1 ppm (CH_3-7a); MS (EI): *m/z* (%): 535 (3) [*M*], 520 (20) [*M*−CH_3], 376 (15) [*M*−CF_3(C_6H_3)CH_3], 334 (35) [*M*−CH_2(OH)(CHOH)$_3$], 321 (90) [*M*−ribose].

Yield of the side product: 20%; ^{1}H NMR (400 MHz, $CDCl_3$, only signals that differ from **4b** are listed): $\delta = 7.30$ ppm (1H; pyrimidine-H); ^{13}C NMR (100 MHz, $CDCl_3$, signal that differs from **4b**): $\delta = 109.9$ ppm (pyrimidine-CH); MS (EI): *m/z* (%): 535 (6) [*M*], 520 (25) [*M*−CH_3], 376 (15) [*M*−CF_3(C_6H_3)CH_3], 334 (40) [*M*−CH_2(OH)-(CHOH)$_3$], 321 (100) [*M*−ribose].

General procedure for substitution of 4 with sodium benzoate: An intermediate **4** (1 mmol) was added to a solution of sodium benzoate (5 M in benzyl alcohol, 5 equiv) in toluene (10 mL), and resulting mixture was heated at reflux with stirring for 20–24 h. Afterwards, the reaction mixture was allowed to cool down, Celite (1 g) was added, and the crude product was filtered out, washed with toluene (2×10 mL), and concentrated in vacuum. FC on silica gel (CH_2Cl_2/toluene 98:2) afforded the desired product as a colorless solid.

*8-Bromo intermediate **5a***: Yield: 71%; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.55$–7.28 (m, 13H; aromatic), 5.41, 5.28 (2s, 4H; 2CH_2–Bz), 5.22 (s, 1H; H-4a), 4.54 (m, 2H; 2′-CH, 3′-CH), 4.07 (m, 2H; 4′-CH, 5′-C*H*H), 3.93 (m, 1H; 5′-CH*H*), 3.85 (m, 1H; 1′-C*H*H), 3.67 (m, 1H; 1′-CH*H*), 2.40 (s, 3H; CH_3-7a), 1.32, 1.27, 1.25 ppm (3s, 12H; 4CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, DEPT): $\delta = 171.3$ (C-4), 165.1 (C-2), 164.0 (C-10a), 142.6 (C-9a), 140.9, 137.2, 137.0, 136.7 (Bz), 132.7 (C-6), 131.4 (C-9), 128.5, 128.4, 128.3, 128.1, 128.0, 127.9 (Bz), 127.8 (C-5a), 127.7, 127.6 (Bz), 127.1 (C-7), 125.0 (C-8), 109.8, 108.5 (2C; 2*C*(CH_3)$_2$), 82.1 (C-4a), 78.1 (C-3′), 75.4 (C-2′), 73.0 (C-4′), 69.5, 68.1 (2C; 2CH_2–Bz), 67.9 (C-5′), 50.4 (C-1′), 27.9, 26.7, 25.5, 25.3 (4C; 4CH_3), 22.5 ppm (CH_3-7a); MS (EI): *m/z* (%): 689 (3) [*M*], 674 (12) [*M*−CH_3], 488 (15) [*M*−CH_2(OH)(CHOH)$_3$], 475 (45) [*M*−ribose].

*8-Trifluoromethyl intermediate **5b***: Yield: 49%; ^{1}H NMR (400 MHz, $CDCl_3$, HH-COSY): $\delta = 7.66$–7.16 (m, 13H; aromatic), 5.40, 5.28 (2s, 4H; 2CH_2–Bz), 5.22 (s, 1H; H-4a), 4.69 (m, 1H; 1′-C*H*H), 4.55 (m, 1H; 2′-CH), 4.06 (m, 2H; 4′-CH, 5′-C*H*H), 3.94 (m, 1H; 3′-CH), 3.85 (m, 1H; 5′-CH*H*), 3.58 (m, 1H; 1′-CH*H*), 2.48 (s, 3H; CH_3-7a), 1.32, 1.25, 1.24 ppm (3s, 12H; 4CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, HMBC, HMQC): $\delta = 171.3$ (C-4), 165.0 (C-2), 163.7 (C-10a), 141.8 (C-9a), 137.2, 136.7, 133.1, 132.1, 129.0, 128.5 (2C), 128.4 (2C), 128.2, 128.1 (2C), 128.02 (2C), 128.98, 127.9, 125.3 (17C; Bz, C-5a, C-6, C-7, C-8, C-9), 115.0 (d, $J = 81$ Hz, CF_3), 109.8, 108.9 (2C; 2*C*(CH_3)$_2$), 82.1 (C-4a), 78.2 (C-3′), 75.5 (C-2′), 73.2 (C-4′), 68.6, 68.0 (2C; 2CH_2–Bz), 68.2 (C-5′), 50.6 (C-1′), 27.8, 26.7, 25.4, 25.3 (4C; 4CH_3), 17.8 ppm (CH_3-7a); MS (EI): *m/z* (%): 679 (5) [*M*], 664 (10) [*M*−CH_3], 520 (8) [*M*−(CH_3)(CF_3)C_6H_3], 478 (18) [*M*−CH_2(OH)(CHOH)$_3$], 465 (85) [*M*−ribose].

General procedure for the reduction of the pyrimidine ring: A flask containing a dibenzyl compound **5** (0.5 mmol) and Pd/C (25 mg, 10%) in methanol (2.5 mL) was flushed with argon, and the reaction mixture was stirred under hydrogen for 2 h and then flushed again with argon to remove the hydrogen. After removal of solid material by filtration and the concentration of the solvent, the corresponding product **6** was purified by FC on silica gel with elution with CH_2Cl_2/methanol/toluene 98:1:1.

*8-Bromo intermediate **6a***: Yield: 79%; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 9.32$ (br s, 1H; NH), 8.58 (br s, 1H; NH), 7.28 (d, 1H; H-6), 7.20

(m, 2H; H-9, H-5a), 4.70 (s, 1H; H-4a), 4.39 (m, 1H; 2′-CH), 4.04 (m, 3H; 3′-CH, 4′-CH, 5′-C*H*H), 3.94 (m, 1H; 5′-CH*H*), 3.89 (m, 1H; 1′-C*H*H), 3.75 (m, 1H; 1′-CH*H*), 2.40 (s, 3H; CH_3-7a), 1.42, 1.36, 1.31 ppm (3s, 12H; $4CH_3$); ^{13}C NMR (100 MHz, $CDCl_3$, HMBC, HMQC): $\delta = 164.6$ (2C; C-4, C-2), 154.0 (C-10a), 139.9 (C-9a), 138.7 (C-6), 132.0 (C-9), 126.9 (C-5a), 125.7 (C-7), 125.2 (C-8), 110.2, 109.7 (2C; $2C(CH_3)_2$), 78.9 (C-4a), 77.8 (C-3′), 74.8 (C-2′), 73.0 (C-4′), 67.9 (C-5′), 52.5 (C-1′), 27.6, 26.7, 25.3, 25.1 (4C; $4CH_3$), 22.5 ppm (CH_3-7a); MS (EI): *m/z* (%): 509 (6) [*M*], 494 (5) [$M-CH_3$], 308 (10) [$M-CH_2(OH)(CHOH)_3$], 295 (45) [*M*−ribose].

8-Trifluoromethyl intermediate ***6b***: Yield: 77%; ^1H NMR (400 MHz, $CDCl_3$, HH COSY): $\delta = 8.55$ (brs, 1H; NH), 8.47 (brs, 1H; NH), 7.57 (m, 1H; H-6), 7.36 (m, 1H; H-5a), 7.24 (s, 1H; H-9), 4.64 (s, 1H; H-4a), 4.39 (m, 1H; 2′-CH), 4.10 (m, 2H; 1′-C*H*H, 5′-C*H*H), 4.00 (m, 2H; 3′-CH, 4′-CH), 3.90 (m, 1H; 5′-CH*H*), 3.74 (m, 1H; 1′-CH*H*), 2.50 (s, 3H; CH_3-7a), 1.43, 1.35, 1.32, 1.31 ppm (4s, 12H; $4CH_3$); ^{13}C NMR (100 MHz, $CDCl_3$, HMBC, HMQC): $\delta = 164.2$ (C-4), 154.1 (C-2), 150.4 (C-10a), 139.3 (C-9a), 137.4 (C-7), 134.0 (C-6), 131.2 (C-9), 128.5 (d, $J = 81$ Hz, CF_3), 125.8 (C-5a), 122.3 (C-8), 110.3, 110.0 (2C; $2C(CH_3)_2$), 79.0 (C-4a), 77.8 (C-3′), 75.0 (C-2′), 73.1 (C-4′), 68.0 (C-5′), 52.8 (C-1′), 27.6, 26.7, 25.3, 25.1 (4C; $4CH_3$), 19.0 ppm (CH_3-7a); MS (EI): *m/z* (%): 499 (15) [*M*], 484 (10) [$M-CH_3$], 298 (15) [$M-CH_2(OH)(CHOH)_3$], 285 (100) [*M*−ribose].

General procedure for ring closure: Sodium nitrite (5 equiv) was added to a solution of a compound **6** (0.5 mmol) in glacial acetic acid (2.5 mL) in a dark environment. The resulting mixture was stirred at room temperature for 3 h, and then water (2 mL) was added. The resulting suspension was stirred for an additional 3 h, after which the solvents were evaporated in vacuum. The crude product was purified on silica gel with elution with CH_2Cl_2/methanol/toluene (96:3:1) to afford the corresponding compound **7** as an orange solid.

8-Bromo-5-N-oxide-isoalloxazine (***7a***): Yield: 69%; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 8.27$ (brs, 1H; NH), 7.24 (m, 1H; H-6, signal partially covered by signal of $CDCl_3$), 7.18 (m, 1H; H-9), 5.12 (m, 1H; 2′-CH), 4.74 (m, 2H; 3′-CH, 4′-CH), 4.23 (m, 3H; 5′-CH_2, 1′-C*H*H), 4.00 (m, 1H; 1′-CH*H*), 2.56 (s, 3H; CH_3-7a), 1.55, 1.46, 1.37, 1.23 ppm (3s, 12H; $4CH_3$); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 155.8$, 153.7 (2C, C-4, C-2), 137.3 (C-10a), 134.1 (C-4a), 133.7 (C-9a), 133.3 (C-6), 129.0 (C-9), 128.2 (C-5a), 122.1 (C-7), 121.7 (C-8), 110.4, 110.3 (2C; $2C(CH_3)_2$), 77.9 (C-3′), 74.7 (C-2′), 73.6 (C-4′), 68.2 (C-5′), 45.9 (C-1′), 27.6, 26.9, 25.6, 25.3 (4C; $4CH_3$), 22.8 ppm (CH_3-7a); MS (EI): *m/z* (%): 507 (10) [$M-NO$], 521 (3) [$M-O$].

8-Trifluoromethyl-5-N-oxide-isoalloxazine (***7b***): Yield: 73%; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 8.37$ (brs, 1H; NH), 8.34 (m, 1H; H-6), 8.25 (m, 1H; H-9), 5.24 (m, 1H; 2′-CH), 4.75 (m, 2H; 1′-C*H*H, 5′-C*H*H), 4.23 (m, 3H; 3′-CH, 4′-CH, 5′-CH*H*), 4.00 (m, 1H; 1′-CH*H*), 2.63 (s, 3H; 7a-CH_3), 1.47, 1.43, 1.38, 1.21 ppm (4s, 12H; $4CH_3$); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 155.6$, 153.7 (2C; C-4, C-2), 153.3 (C-10a), 137.9, 135.5, 135.1, 132.6, 126.2, 125.3, 124.3 (C-4a, C-5a, C-6, C-7, C-8, C-9, C-9a), 110.5, 110.1 (2C; $2C(CH_3)_2$), 78.0 (C-3′), 74.6 (C-2′), 73.6 (C-4′), 68.2 (C-5′), 46.1 (C-1′), 27.5, 26.8, 25.5, 25.2 (4C; $4CH_3$), 19.1 ppm (CH_3-7a); MS (EI): *m/z* (%): 497 (10) [$M-NO$], 511 (15) [$M-O$].

General procedure for reduction of N-oxides 7: $Na_2S_2O_4$ (1.5 equiv) in water (3 mL) was added to a solution of a compound **7** (0.085 mmol) in EtOH (3 mL) under argon. After the system had been stirred at RT for 1.5 h, the solvents were evaporated, and the crude yellow solid was washed with water (2×2 mL) and dried under vacuum. FC on silica gel (elution with CH_2Cl_2/MeOH 97:3) yielded the corresponding compound **8** (89–95%).

8-Bromo-[2,3:4,5-bis(methylethyliden)ribo]flavin (***8a***): Yield: 89%; ^1H NMR (400 MHz, $CDCl_3$, HH COSY): $\delta = 8.61$ (brs, 1H; NH-3), 8.10 (2s, 2H; H-6, H-9), 5.09 (m, 1H; 1′-C*H*H), 4.91 (m, 1H; 1′-CH*H*), 4.76 (m, 1H; 2′-CH), 4.22 (m, 3H; 3′-CH, 4′-CH, 5′-C*H*H), 4.02 (m, 1H; 5′-CH*H*), 2.55 (s, 3H; CH_3-7a), 1.50, 1.39, 1.21 ppm (3s, 12H; $4CH_3$); ^{13}C NMR (100 MHz, $CDCl_3$, HMQC, HMBC): $\delta = 159.0$, 154.5 (2C; C-4, C-2), 150.6 (C-10a), 137.5, 135.0, 134.2, 133.2 (C-6 or C-9), 132.1 (C-9), 129.0 (C-5a), 128.2 (C-7), 120.5 (C-6 or C-9), 110.4 ($2C(CH_3)_2$), 78.0 (C-3′), 74.2 (C-2′), 73.6 (C-4′), 68.2 (C-5′), 46.1 (C-1′), 27.6, 26.9, 25.5, 25.3 (4C; $4CH_3$), 22.6 ppm (CH_3-7a); MS (EI): *m/z* (%): 520 (3) [*M*], 504 (10) [$M-O$], 462 (8) [$M-C(CH_3)_2O$], 320 (5) [$M-CH_2(OH)(CHOH)_3$], 304 (10) [*M*−ribose]; HRMS: *m/z* calcd: 520.095762 $[M+Na]^+$; found: 520.095525.

8-Trifluoromethyl-[2,3:4,5-bis(methylethyliden)ribo]flavin (***8b***): Yield: 95%; ^1H NMR (400 MHz, $CDCl_3$, HH COSY): $\delta = 9.10$ (brs, 1H; NH), 8.18, 8.17 (m, 2H; H-6, H-9), 5.22 (m, 1H; 1′-C*H*H), 4.86 (m, 1H; 1′-CH*H*), 4.76 (m, 1H; 2′-CH), 4.24 (m, 3H; 3′-CH, 4′-CH, 5′-C*H*H), 4.00 (m, 1H; 5′-CH*H*), 2.62 (s, 3H; CH_3-7a), 1.46, 1.44, 1.37, 1.18 ppm (4s, 12H; $4CH_3$); ^{13}C NMR (100 MHz, $CDCl_3$, HMBC, HMQC): $\delta = 158.7$ (C-4), 154.7 (C-2), 150.8 (C-10a), 139.7 (C-4a), 136.6 (C-6 or C-9), 135.2 (C-7), 134.6, 131.2, 124.6, 121.9, 115.5 (C-9 or C-6), 110.5 (2C; $2C(CH_3)_2$), 78.0 (C-3′), 74.3 (C-2′), 73.6 (C-4′), 68.2 (C-5′), 46.2 (C-1′), 27.4, 26.7, 25.5, 25.2 (4C; $4CH_3$), 18.9 ppm (CH_3-7a); MS (EI): *m/z* (%): 452 (23) [$M-OC(CH_3)_2$], 495 (30) [$M-CH_3$], 510 (5) [*M*].

General procedure for deprotection of OH groups: A compound **8** (0.04 mmol) was stirred in TFA/H_2O solution (10:1, 0.5 mL) at RT for 4 h. The solvents were then evaporated, and ether (2 mL) was added to the crude product. The formed precipitate was centrifuged (10 °C, 3000 rpm, 10 min), and washed with ether (1 mL). The combined ethereal extracts were washed with water, and the aqueous solution was lyophilized. The solid was dried in vacuo to provide the corresponding pure compound **9** (85–95%).

8-Bromoriboflavin (***9a***): Yield: 85%; ^1H NMR (400 MHz, [D_6]DMSO, HH COSY): $\delta = 11.42$ (brs, 1H; NH-3), 8.35 (s, 1H; H-6), 8.08 (s, 1H; H-9), 5.10 (m, 1H; 5′-C*H*H), 4.85 (m, 1H; 5′-CH*H*), 4.66 (m, 3H; 2′-CH, 3′-CH, 4′-CH), 4.44 (m, 1H; 1′-C*H*H), 4.22 (m, 1H; 1′-CH*H*), 3.62 (m, 4H; 4OH), 2.48 ppm (s, 3H; CH_3-7a); ^{13}C NMR (100 MHz, [D_6]DMSO, HMQC): signals that could be identified: $\delta = 159.5$, 155.1 (2C; C-4, C-2),135.4, 134.2, 131.2, 130.0, 117.0, 131.8 (C-9, from HMQC), 121.0 (C-6, from HMQC), 73.3 (C-3′), 72.4 (C-2′), 68.9 (C-4′), 63.3 (C-5′), 49.2 (C-1′), 21.8 ppm (CH_3-7a); MS (ESI): *m/z* (%): 385 (50) [$M-Br+H+Na]^+$, 463 (100) $[M+Na]^+$, 903 (5) $[2M+Na]^+$; HRMS: *m/z* calcd: 463.022930 $[M+Na]^+$; found: 463.023083.

8-(Trifluoromethyl)riboflavin (***9b***): Yield: 95%; ^1H NMR (400 MHz, [D_6]DMSO, HH COSY): $\delta = 11.52$ (brs, 1H; NH), 8.38 (m, 1H; H-6), 8.17 (m, 1H; H-9), 4.80 (m, 2H; 1′-CH_2, signal partially covered by signal of H_2O), 4.50 (m, 1H; 2′-H), 4.22 (m, 1H; 4′-CH), 4.00 (m, 1H; 3′-CH, 5′-C*H*H), 3.62 (m, 1H; 5′-CH*H*), 2.57 ppm (s, 3H; 7a-CH_3); ^{13}C NMR (100 MHz, [D_6]DMSO, HMQC): $\delta = 159.4$, 153.4 (2C; C-2, C-4), 148.0 (C-10a), 140.9 (C-4a), 136.0 (C-9), 134.0, 132.4, 131.8, 133.3 (C-6), 129.0 (C-9), 73.4 (C-3′), 72.6 (C-2′), 68.7 (C-4′), 63.3 (C-5′), 47.4 (C-1′, from HMQC), 18.0 ppm (CH_3-7a); MS (ESI): *m/z* (%): 453 (100) $[M+Na]^+$; HRMS: *m/z* calcd: 453.099242 $[M+Na]^+$; found: 453.099723.

Isolation of YtvA protein and chromophore exchange: The isolation of recombinant YtvA has been described elsewhere.[4,39,40] Incorporation of both 8-CF_3RF and 8-BrRF as chromophores in the YtvA protein was achieved by a recently developed protocol based on the unfolding/refolding of the protein on a His-Trap column by treatment with a high concentration of urea and the exchange of

the native FMN chromophore with the artificial flavins during refolding.[14] The yield of chromophore incorporation was assessed by determining the A_{280}/A_{450} absorption ratios; this gave values of 7:1 for 8-CF_3RF (WT YtvA: 4:1) and 5:1 for 8-BrRF (note that 8-CF_3RF has a lower extinction coefficient than FMN: $\varepsilon_{max}=8450\ cm^{-1}\,M^{-1}$, in comparison with $\varepsilon_{max}=12500\ cm^{-1}\,M^{-1}$). Both chromophores were used in their riboflavin forms, due to low yields of the phosphorylation reactions. Our previous study showed that presence of the 5′-phosphate group does not influence the photochemistry within the chromophore pocket in the protein and causes only a minor change in the dark recovery kinetics.[14]

Analytical methods: Absorption spectra and kinetics traces were recorded with a UV2401-PC spectrophotometer (Shimadzu). Steady-state fluorescence was measured on a Varian Eclipse fluorimeter by using a 5 nm emission slit width and a scanning speed of 300 nm min^{-1}. Fluorescence quantum yields were calculated with RF ($\Phi_F=0.27$ in H_2O)[30] and wild-type YtvA ($\Phi_F=0.22$ in phosphate buffer)[34] as references. Time-resolved fluorescence was measured with a single-photon-counting apparatus model FL900 (Edinburgh Analytical Instruments, Livingston, UK). Transient absorbance changes after nanosecond-laser flash excitation were recorded with a detection system (LFP111) from Luzchem, Ontario, Canada. For excitation, a Nd:YAG-driven tunable OPO laser was used (Nd:YAG, Innolas, Garching, Germany; OPO, GWU Lasertechnik, Erftstadt, Germany). The experiments were performed in the linear laser-energy dependence region of the transient absorbance changes with $\lambda_{exc}=450$ nm. Triplet quantum yields (Φ_T) of the flavin derivatives were determined by comparison with the known triplet quantum yield of RF. For this the literature value of $\Phi_T=0.6$ was used as reference.[32] Data analysis was performed with Origin software. All measurements were performed at 20 °C by using 1 cm light-path quartz cuvettes. Photoequilibrium conditions, with accumulation of the photoactivated state, were achieved by illuminating the sample with a blue-light-emitting Led-Lenser V8 lamp (Zweibrüder Optoelectronics, Solingen, Germany).

Computational methods: Calculations on 8-BrLF and 8-CF_3LF (without the sugar side chain) were carried out by using DFT for the geometry optimizations and a combination of DFT and multireference configuration interaction (MRCI) for the determination of the excitation energies and transition moments. Throughout, a basis set of valence triple zeta quality with polarization functions (TZVP) was used in the quantum chemical treatments. Water solution was mimicked by adding four explicit water molecules forming hydrogen bonds with the nitrogen and oxygen atoms of the chromophore combined with a continuum solvation model (COSMO) for describing the solvent polarity. Further details on the methods and procedures can be found in earlier work[29] (solvation protocol IV), from which the theoretical results for the parent compound were also taken. Spectroscopic properties of 8-BrLF in the LOV domain of YtvA were studied by combined quantum-mechanical/molecular-mechanical (QM/MM) methods. In addition to the modified FMN cofactor, several important amino acid side chains, including Gln66, Asn94, Asn104, and Gln123, as well as the reactive residue Cys62, were treated quantum mechanically, whereas the protein environment was represented by the CHARMM force field (refer to Salzmann et al.[33] for a detailed description of the protocol). Because of the lack of force-field parameters suited for describing the nonbonded interactions between bromine and the protein environment, the active regions of the two conformers (confA and confB) of the modified protein–cofactor complex were not fully geometry optimized at the QM/MM level. Instead, we made use of the fact that the steric requirements of a bromine substituent resemble those of a methyl group. Starting from the optimized structures of the wild-type complexes,[33] we replaced the methyl group in the 8-position by a bromine atom and relaxed the C_8–Br interatomic distance at the QM level while freezing the geometric positions of all other centers and of the point charges representing the MM environment.

Keywords: chromophore–protein interactions · flavins · photoreceptors · photophysics · QM/MM calculations

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Received: October 25, 2012
Published online on March 1, 2013