



Riboflavin derivatives for enhanced photodynamic activity against *Leishmania* parasites



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ABSTRACT

Riboflavin derivatives with various substituents (*O*-acyl, *N*-methyl, *N*-alkylcarboxyalkyl or *N*-alkyl(trialkyl) ammonium) have been prepared and spectroscopically characterized (absorption, emission and fluorescence quantum yields). Their quantum yields of photosensitized singlet molecular oxygen production (Φ_{Δ} 0.24–0.58) and octanol/water partition coefficients ($P_{ow} \leq 0.01$ –11) were measured. Preliminary studies indicate that all derivatives display higher phototoxicity against the human protozoan parasite *Leishmania* than the parent riboflavin, with negligible toxicity in the absence of light. Their photodynamic action shows a higher correlation with P_{ow} than with Φ_{Δ} , opening up their potential application to cutaneous diseases treatment.

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1. Introduction

Photodynamic therapy (PDT) is a technique with scarce or nil invasion for the treatment of different pathogenic diseases and cancers.¹ PDT involves incorporation of a photoactive compound (called photosensitizer, PS) into the target cells that upon irradiation at an appropriate wavelength within its absorption spectrum, forms an electronically excited triplet species.² The latter may generate radical species by electron transfer ('type I' photosensitization) or, in the presence of O₂, generate the highly reactive singlet molecular oxygen (¹O₂) molecule by energy transfer ('type II' photosensitization).³ Both PDT mechanisms cause cell damage leading to necrosis and/or apoptosis.⁴ The essential features of a PS for PDT have been established: (i) an efficient absorption of light, fast intersystem crossing and large singlet oxygen production quantum yield; (ii) a high affinity and selectivity for the target cells; (iii) low toxicity in the dark; (iv) enough chemical stability, and (v) a good solubility in water.

Riboflavin (RF, vitamin B₂, an essential nutrient in humans) and its natural derivatives, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), are present in aerobic organisms as

prosthetic groups of flavoenzymes.⁵ Being natural compounds, these molecules display low toxicity in the dark but produce a strong phototoxicity after absorbing light in the UV and blue regions.⁶ Flavins undergo efficient intersystem crossing in their lowest electronic excited state, forming transient triplet species,⁷ which in the presence of molecular oxygen and/or other biomolecules, generate ¹O₂ and/or radical species.⁸ Capitalizing on these features, natural and artificial flavin derivatives have been used as PSs in different applications such as water treatment,⁹ photodegradation of aminoacids,¹⁰ light-induced death of tumor cells,^{8b,11} photodynamic treatment of keratitis,¹² and blood disinfection.^{13,14} However, structure-activity relationships are missing for flavin photosensitizers.³

Leishmaniasis is a protozoal disease produced by infection with species of the genus *Leishmania*, reported in 98 countries mostly located in tropical and subtropical areas.¹⁵ Its impact on human health is only superseded by malaria among human protozoal maladies, with roughly two million new cases every year, ca. 75% of them associated to cutaneous forms of the disease and hence amenable to PDT treatment. Dyes such as phthalocyanines and methylene blue,¹⁶ different porphyrin scaffold surrogates absorbing at the blue region of the spectra,¹⁷ and the porphyrin precursor 5-aminolevulinic acid (ALA),¹⁸ have been tested as PSs against *Leishmania* parasites. Nevertheless, RF was only employed for the clearance of the microorganism in blood banks.¹⁴

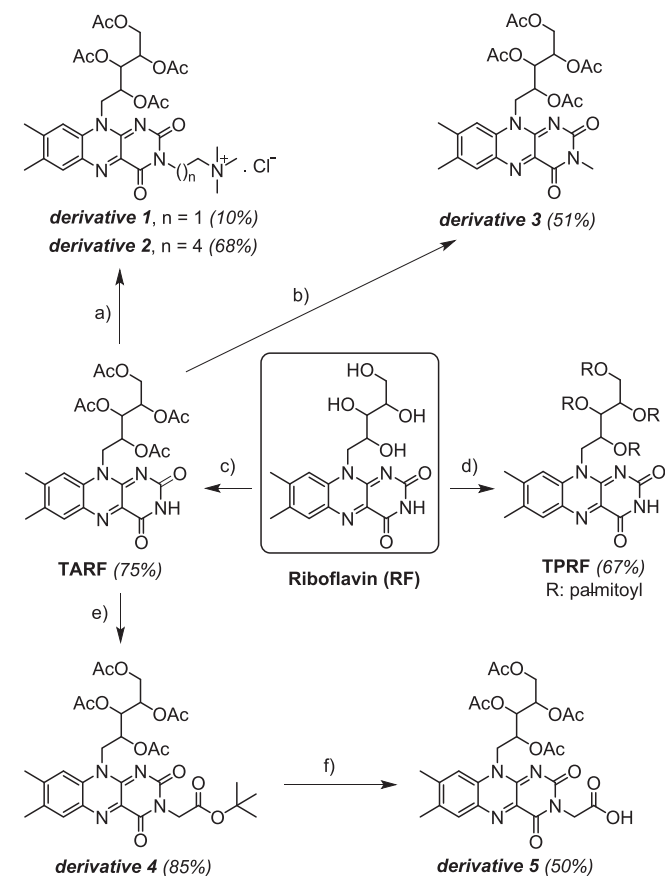
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In spite of the above mentioned uses of flavins as PS dyes, applying natural flavins in PDT displays some drawbacks, mainly related to their low solubility in physiological medium and poor photostability. Hence, we set out to synthesize riboflavin derivatives with diverse features and test them against *Leishmania* promastigotes.

2. Results and discussion

2.1. Synthesis

In order to improve the efficiency and the application of flavins in photodynamic processes, we modified RF derivatives with diverse motifs. The genesis of the different derivatives synthesized in this work from the parent RF is shown in Scheme 1.



Scheme 1. Synthesis of riboflavin derivatives. a) DMF/ $\text{BrC}_n\text{H}_{2n}\text{CH}_2\text{N}(\text{CH}_3)_3\text{Br}^-/\text{Cs}_2\text{CO}_3/\text{KI}/50^\circ\text{C}/4\text{ h}$; b) DMF/ $\text{CH}_3\text{I}/\text{Cs}_2\text{CO}_3/40^\circ\text{C}/24\text{ h}$; c) $(\text{CH}_3\text{CO})_2\text{O}/\text{DMAP}/40^\circ\text{C}/24\text{ h}$; d) DMF/ $\text{CH}_3(\text{CH}_2)_{14}\text{COCl}/\text{DMAP}/50^\circ\text{C}/24\text{ h}$; e) DMF/ $\text{BrCH}_2\text{CO}_2\text{C}(\text{CH}_3)_3/\text{K}_2\text{CO}_3/\text{KI}/40^\circ\text{C}/20\text{ h}$; f) $\text{CF}_3\text{CO}_2\text{H}/\text{CH}_2\text{Cl}_2/50^\circ\text{C}/5\text{ h}$; yields of isolated compounds are given (full details of the syntheses are provided in the Experimental section).

A first analog was obtained by acetylation of the four alkoxy groups of RF with good yield. Using a similar strategy, tetra-*O*-palmitoyl riboflavin (TPRF) was obtained with a significantly improved yield (67%) compared to those of reported methodologies (1–8% yield).¹⁹ Acyl derivatives of RF, more lipophilic and photostable than the natural compound, are known to keep its $^1\text{O}_2$ photosensitization and fluorescence properties.²⁰

To turn the acetylated RF into an amphiphilic photosensitizer, tetra-*O*-acetyl riboflavin was decorated with a tetraalkylammonium group, yielding the hitherto unknown derivatives **1** and **2**. Their synthesis was performed by nucleophilic substitution on the desired alkyl bromide with the flavin N3 atom using nucleophilic catalysis by I^- . After ion-exchange gel permeation chromatography,

the sought cationic flavins were obtained in 10% and 68% yields, respectively. Such disparate chemical yields are due to the strong difference of steric hindrance at the reactive brominated carbon atom. These novel RF derivatives display similar absorption and emission spectra to the parent compound, but they are significantly more soluble in water (see below). Moreover, the cationic RF derivatives **1** and **2** were also soluble in organic solvents (chloroform, acetone, ethanol, etc.). This amphiphilic character is expected to be useful for their implementation as pharmaceuticals.

The *N*-methyl RF derivative **3** has been described already and its $^1\text{O}_2$ quantum yield production was reported to be 20% higher than that of riboflavin.²¹ For the sake of comparison, we also synthesized it. Derivatives **4** and **5** have been prepared by Banekovich and Matuszczak, and the fluorescence spectrum of the latter reported.²² However, none of the flavins **3–5** has been tested as PS against human pathogens. The chemical structure of all synthesized flavins was confirmed by NMR and HRMS (see Supplementary data).

2.2. Photophysical properties and *n*-octanol/water partition

The electronic absorption and emission spectra of RF derivatives **1–5**, TARF and TPRF are all very similar to those of riboflavin (see Supplementary data for typical spectra exemplified for derivative **2**): two absorption maximums in the UV region at 267–273 nm and 327–358 nm, and an additional maximum in the vis region at 442–449 nm.²³ Their fluorescence peak appears at 515–529 nm in methanol. Due to the largely different solubility properties of the flavins, the spectra had to be recorded sometimes in a different solvent (Table 1). Similarly to RF, all the prepared derivatives are also significantly fluorescent (emission quantum yields from 0.4 to 0.5). Derivatives **3** and **5** are somewhat less fluorescent due to the higher intersystem crossing efficiency of the former (shows a higher $^1\text{O}_2$ production, see below) or specific hydrogen bonding to the solvent of the carboxylic group of the latter.

Table 1
Photophysical data of riboflavin and its derivatives^a

PS	λ_1/nm ($\epsilon/\text{M}^{-1}\text{cm}^{-1}$)	λ_2/nm ($\epsilon/\text{M}^{-1}\text{cm}^{-1}$)	λ_3/nm ($\epsilon/\text{M}^{-1}\text{cm}^{-1}$)	$\lambda_F^{\text{max}}/\text{nm}$	Φ_F
RF	267(33,000)	345(9050)	442(10,850)	529	0.39 ^b
TARF	267(32,030)	351(9575)	446(13,830)	525	0.46
TPRF	270(26,700) ^c	340(7850) ^c	446(10,780) ^c	512 ^c	0.49 ^d
1	270(27,400)	358(7230)	446(9485)	521	0.41
2	271(29,620) ^e	327(8240) ^e	449(9865) ^e	523	0.42
3	272(38,050)	353(9810)	447(12,800)	515	0.12
4	272(32,920)	357(9015)	449(12,060)	524	0.39
5	273(31,865)	351(8775)	445(10,940)	522	0.23

^a In methanol, except otherwise stated; λ_1 , λ_2 and λ_3 ($\pm 1\text{ nm}$) are the maximums in the absorption spectra, ϵ ($\pm 2\%$) is their molar absorption coefficient, λ_F^{max} ($\pm 1\text{ nm}$) is the fluorescence band maximum ($\lambda_{\text{exc}}=445\text{ nm}$) and Φ_F is the fluorescence quantum yield ($\pm 10\%$).

^b Standard from Ref. 24.

^c In chloroform.

^d In acetone.

^e In water.

The quantum yield of photosensitized $^1\text{O}_2$ production by riboflavin and its derivatives (Φ_{Δ} , Table 2) was measured in air-equilibrated solution and calculated from the area under the $^1\text{O}_2$ emission decay curve at 1270 nm (Fig. 1), using 1*H*-phenalen-1-one as a reference photosensitizer ($\Phi_{\Delta}=0.97$ in methanol).²⁵

The kinetic profiles of the investigated flavins are biphasic due to the small contribution of the tail of their luminescence. Consequently, only the area under the long-lived decay component, extrapolated to time zero, was taken into account for the $^1\text{O}_2$ production quantum yield measurements.

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