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A flavin analogue with improved solubility in organic solvents

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Abstract—We report the synthesis and initial electrochemical characterization of a benzene-soluble flavin analogue: *N*(10)-2,2-dibenzylethyl-7,8-dimethylisoalloxazine (DBF, **1**). This analogue, which has an unmodified flavin headgroup, is intended for use in the spectroscopic examination of the electronic effects of flavin hydrogen bonding in simple model systems in aprotic, non-hydrogen bonding solvents. With future spectroscopic studies in mind, we have developed a synthetic route, which allows the incorporation of isotopic labels using inexpensive starting materials.

Flavins are versatile enzyme prosthetic groups or cofactors essential to enzymes found in all five kingdoms of life, where they catalyze a remarkably diverse chemistry that draws on its rich oxidation–reduction chemistry and strong coupling to proton release and uptake. This includes their role in light signaling and transcription activation, in light activated DNA repair, in respiratory electron chains, and in dehydrogenation, dehalogenation, hydroxylation and oxygenation reactions.[1] It is clear that for each enzyme the specific interactions between the cofactor and the protein control both the type and driving force behind specific types of flavin chemistries. Thus, flavoenzymes vary widely with respect to their one- and two-electron midpoint potentials and associated pK_a values in the multiplicity of states that describe the redox-coupled acid–base chemistry of the flavin cofactor.[1–4]

Despite many years of research, this area remains poorly understood. Powerful spectroscopic methods such as infrared spectroscopy, resonance Raman (RR) spectroscopy, Stark spectroscopy and solution nuclear magnetic resonance (NMR) spectroscopy have all been utilized in the examination of both free flavins (i.e., FMN and FAD in aqueous buffers) and a number of flavoproteins,[5–9] but global analysis of these data supports only qualitative conclusions due to the complexity of the protein environment and the attendant difficulty in deconstructing the simultaneous effects of the large number of flavin–protein interactions.

The Rotello group and others[10–14] have examined flavin oxidation–reduction in simplifying aprotic solvents. This work demonstrated that, in model hydrogen bonding complexes between a chloroform-soluble flavin analogue and mono- and diamidopyridines, the one-electron reduction potentials (from neutral oxidized to semiquinone radical anion) of the flavin are modulated by over 4 kcal/mol. These hydrogen bonded complexes offer the opportunity to examine, in the atomic detail we are currently lacking, the changes brought in the electronic structure of the flavin by individual flavin–cosolute interactions previously connoted only indirectly by the aforementioned changes in the flavin reduction potentials. Examination of these model systems promises significant new insights into the manner in which flavoproteins direct flavin reactivity.

The flavin analogue used in Rotello's pioneering experiments bears an isobutyl side chain at the N(10) position of the isoalloxazine moiety to increase the solubility in organic solvents (see Fig. 1). The oxidized form was examined by ^{1}H and ^{13}C solution NMR, and its reduction to the semiquinone was studied by cyclic voltammetry

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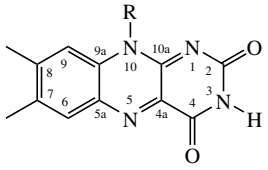


Riboflavin	R = $CH_2(CHOH)_3CH_2OH$
FMN	R = $CH_2(CHOH)_3CH_2OPO_3^-$
FAD	R = $CH_2(CHOH)_3CH_2(OPO_3)_2$-Adenine
TARF	R = $CH_2(CHOAc)_3CH_2OAc$
Rotello[11]	R = $CH_2CH(CH_2CH_3)_2$

Figure 1. The flavin moiety and some common natural and synthetic derivatives.

(CV), in conjunction with electron paramagnetic resonance (EPR).[4] The oxidized form of this molecule was found to be soluble to <1 mM in chloroform, less so in methylene chloride and almost insoluble in benzene (J. D. Walsh and A.-F. Miller, unpublished observations). Moreover, the reduced anionic forms of the flavin are considerably less soluble than the oxidized ones in any of these solvents. Sub-millimolar concentrations are insufficient for many forms of spectroscopic analysis. Additionally, chloroform, the solvent in which the magnetic resonance studies have by necessity been performed, is a solvent which possesses considerable hydrogen bond-donating ability.[15] Hydrogen bonding to solvents has been shown to significantly affect ^{15}N chemical shifts in other cases.[16] Pyridine, for example, has been demonstrated to exhibit a difference in chemical shift between vacuum and chloroform of over 30 ppm.[17] As an ideal model system should examine the effects of single or multiple hydrogen bonds in isolation, nonpolar solvents such as benzene, toluene, carbon tetrachloride or cyclohexane must be used. Therefore, the non-redox-active N(10) side chain must be highly soluble in these solvents.

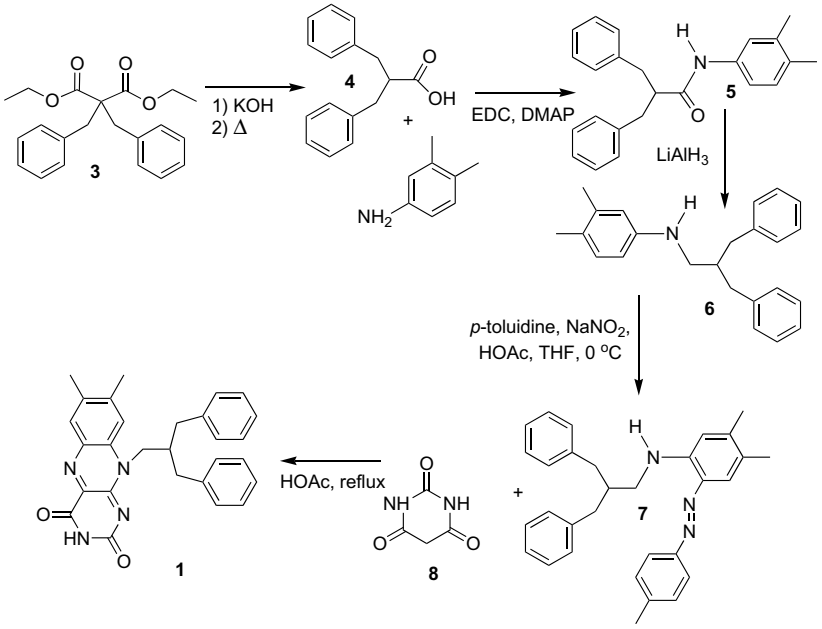
It will prove informative to expand the examination of these complexes to include ^{15}N NMR (both in solution and in the solid state[18]), IR, RR and Stark spectroscopies. In order to circumvent solubility problems, we have undertaken the synthesis of novel flavin analogues with improved solubility in aprotic, non-hydrogen bonding solvents. We have chosen benzene as a model solvent because its relatively high melting temperature is ideal for solid state NMR spectroscopy on frozen flavin solutions, although there remains a possibility that flavin energetics in this solvent will be perturbed by π stacking effects.[19] As each of NMR, infrared and raman spectroscopy necessitates the synthesis of multiple flavins, each isotopically labeled at different positions, our intent was to find a synthetic approach based on the most inexpensive isotopically labeled starting materials.

Some high-yield synthetic approaches to flavin analogues have been reported,[20,21] but each has the disadvantage of expensive and/or synthetically challenging isotopically labeled precursors. In 1947, Tishler et al. reported the condensation of 1-(D-ribitylamino)-2-arylazo-4,5-dimethylbenzene and barbituric acid to form riboflavin.[22] While this synthesis was performed in aqueous acid, which would not be amenable to the synthesis of more hydrophobic flavin analogues, the method does have the advantage that the N(5) nitrogen can be readily and inexpensively labeled using ^{15}N-sodium nitrite, and the uracil ring can be inexpensively labeled at many positions by synthesizing barbituric acid from urea and diethyl malonate.[23] Thus, every atom predicted to exhibit large changes in chemical shifts or vibrational frequencies[24] can be readily labeled using our strategy, with the exception of the N(10) nitrogen.

Our approach is outlined in Scheme 1 (synthetic details, including product characterization, are included in the Supplementary data). 2,2-Dibenzyl-diethylmalonate (**2**), synthesized using the method of Maslak,[25] was saponified with KOH and decarboxylated by heating to form 2,2-dibenzylacetic acid **3**.[27] Coupling of **3** with dimethylaniline using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride and dimethylaminopyridine followed by $LiAlH_4$ reduction[28] yielded secondary amine **5**, which was diazotinated with ^{15}N-sodium nitrite and *p*-toluidine using the method of Tishler,[22] substituting THF for water and performing the reaction at 0 °C to maximize the formation of the *meta*-substituted kinetic product. The resultant compound, **6**, was condensed with barbituric acid **7** in refluxing glacial acetic acid for 16 h forming *N*(10)-(2,2-dibenzylpropyl) isoalloxazine (DBF) **1** in 69% yield. The moderate yield of this final step is compensated for by the fact that this is the step in which the positions most likely to bear isotopic labels, those on the uracil ring of the dimethylisoalloxazine, are attached. This greatly reduces label loss in comparison to other possible approaches in which there are several synthetic steps subsequent to uracil ring formation, each of which would entail a geometrically growing loss of expensive labeled material.

DBF is soluble to 1.5 mM in benzene and 20 mM in methylene chloride as determined optically. This is a 15-fold improvement over the benzene solubility of tetraacetylriboflavin (TARF), the molecule most often used in spectroscopic investigations of free flavins.[6] Thus, DBF is sufficiently soluble for solution NMR, IR, RR and Stark spectroscopies. Importantly, DBF lacks the multiple ester functionalities of TARF, so it is less likely to form intermolecular hydrogen bonds to the alloxazine N(3) proton.[26] DBF's solubility in nonpolar solvents has already proven useful in the generation of dry, powdered, reduced flavin samples for solid state NMR spectroscopy.[18]

As expected, DBF is redox-active as demonstrated by CV, displaying a reversible single-electron oxidation/reduction transition between neutral oxidized and the anionic semiquinone radical at −1287 mV versus ferrocene (see Fig. 2). We observe a reduced peak area in the reduction half-wave, presumably due to some degree of protonation of reduced flavin anions by oxidized flavins in the bulk solution.[4] To our knowledge, this is the first report of flavin electrochemistry in benzene. Spectroscopic examination of DBF and its complexes is currently underway.



Scheme 1.

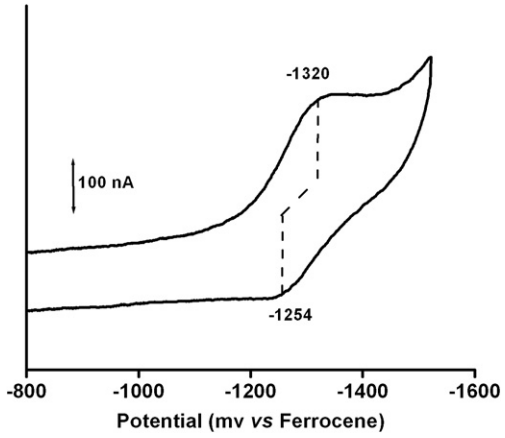


Figure 2. Cyclic voltammogram of 37 μM DBF in benzene with 0.2 M tetrahexylammonium perchlorate as a supporting electrolyte. A 1.6-mm diameter platinum electrode was used for the measurements. The voltammogram was recorded at a scan rate of 100 mV/s. The potential is internally referenced versus ferrocene and the positive peak denotes reduction of the flavin analogue.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2007.05.165.

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