Tetrahedron Letters 55 (2014) 2487–2491

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

An extended scope synthesis of an artificial safranine cofactor

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ARTICLE INFO

Article history:
Received 16 February 2014
Revised 3 March 2014
Accepted 3 March 2014
Available online 11 March 2014

Keywords:
Safranine
Enzyme design
Reduction potential
Nitrogen chemical shift

ABSTRACT

Safranines hold great promise as artificial flavin-like electron transfer cofactors with tunable properties. We have previously reported the stepwise synthesis of a safranine analogue, *p*-methoxy safranine. We now report an improved synthetic pathway which enables the synthesis of safranine analogues containing electron donating phenyl substituents. Low potential safranine analogues were synthesized that extend the range of two electron midpoint reduction potentials to 249 mV, or 11.4 kcal/mol. NMR analysis of the safranine series demonstrates that the ^{15}N chemical shift at the N(5) position correlates with the two-electron reduction midpoint potential.

One of the anticipated advantages in designing artificial proteins is their capacity to utilize nonbiological artificial cofactors, an ability rarely found in natural proteins due to the high degree of shape and hydrogen bonding complementarity in their cofactor binding sites.[1,2] Artificial cofactors can ease the protein design process in that starting with a cofactor which is already optimized energetically and structurally for any particular catalytic function obviates the need for the protein itself to modulate or tune the cofactor reactivity. What is needed for these efforts is a palate of cofactors which encompass a large degree of variability with a small amount of structural alteration, as was done several years ago by the Kadish group in the case of the heme cofactors.[3]

Flavoproteins, which make up more than 10% of natural cofactor-containing proteins in the PDB,[4] are an attractive biodesign target due to the broad spectrum of catalytic functionality they exhibit in nature.[5] The structural features which determine the reactivity of any particular flavoprotein are poorly understood,[6] and in natural flavoproteins the active site mutations necessary to bind flavin analogues have often been found to relax catalytic and substrate specificity to an unacceptable extent. We have thus undertaken the synthesis of both modified flavins[7,8] and small molecule cofactors capable of performing a smaller, more directed subset of flavin catalytic function.[9]

One of the most common flavoprotein functions is that of electron transfer.[6] Many flavoprotein domains serve to take electrons from metabolic sources such as the nicotinamide cofactors and then either donate them directly to substrates or transfer them via other electron transfer cofactors such as hemes or iron–sulfur clusters to a distant active site where substrate reduction takes place.[10] Controlling the reduction potential of these flavin cofactors is critical for function in that this potential is a major determinant of both electron transfer rates[11,12] and reactivity.[13] Despite several decades of work on the topic, the structural features which determine flavoprotein reduction potentials remain poorly understood.[14] It is clear, however, that these potentials are the result of a complex combination of the electrostatic environment and conformational constraints imposed upon the cofactor by the protein.[15]

For these reasons we have set out to create a flavin-like cofactor in which the reduction potential can be significantly modulated by small covalent modifications at a single position.[9] We intend these molecules to be useful flavin substitutes in a manner similar to that of the high and low potential porphyrins of Kadish et al., which perform as tunable heme substitutes.[3] We chose the safranine O molecule because of the strong similarity between the phenazine moiety and the isoalloxazine headgroup in flavin cofactors (see Fig. 1), its demonstrated capacity to take part in electron transfer reactions without the oxygen activation side reactions that flavins are also structured to perform,[16] and the fact that the N(10) phenyl substituent, which is conjugated into the phenazine headgroup, offers the possibility of modifying the molecular reduction potential

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http://dx.doi.org/10.1016/j.tetlet.2014.03.010
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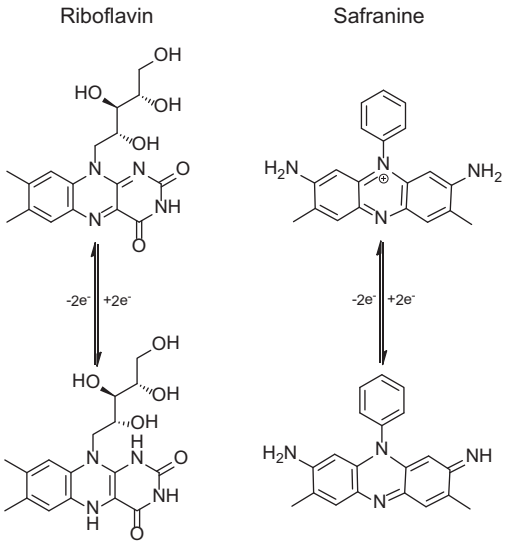

Figure 1. Oxidized and two electron-reduced forms of safranine O and riboflavin.

at a site remote from the phenazine headgroup. We have furthermore recently reported the design and characterization of an artificial safranine-binding protein.[17]

We previously reported the synthesis and characterization of the *p*-methoxy derivative of safranine O,[9] using the Ulmann condensation[18] to first create a triarylamine and then a nitrosative cyclization reaction[19] to form the phenazine moiety. This modification raised the reduction potential 125 mV, or 5.75 kcal/mol over the parent safranine compound. However, attempts to synthesize derivatives with neutral or electron withdrawing substituents failed in the nitrosative cyclization step.

We now report a modified synthesis with a greatly expanded scope, which has enabled the synthesis of lower potential safranine analogues. Because of our recent demonstration that the chemical shift tensor of the isoalloxazine N(5) nitrogen in flavin compounds is informative as to the chemical reactivity imparted upon flavins by their environments,[20,21] we retained the ability to incorporate isotopically labeled nitrogen at the equivalent N(5) position in the phenazine ring of these analogues. This has enabled the demonstration of a relationship between the N(5) solution chemical shift and the reduction potential of these analogues.

Synthesis

The amine substituents at positions 2 and 8 of the phenazine moiety are reactive and thus require protecting groups which shield them throughout both the Ulmann condensation and the nitrosative cyclization reaction steps required for safranine formation. Our original synthesis of *p*-methoxysafranine utilized di-tertbutyl dicarbonate (Boc) protecting groups for the condensation reaction, their removal, and then the introduction of phthalate protecting groups for the cyclization reaction which are removed during the final reductive deoxygenation step.[9] However, attempts to create safranine analogues containing *N*(10)-phenyl substituents bearing less electron donating substituents than the methoxy failed at the nitrosative cyclization step.

We thus set out to create a new synthetic route which utilizes protecting groups which are more electron-withdrawing. Boc protection[22] enabled the Ullmann condensation step with both the *p*-methoxy and the *p*-methyl derivative in 21% and 34% yields respectively, but with <5% yields when the N(10) benzene group encompasses less electron donating derivatives. Use of a more flexible bipyridine copper(I) ligand[23] and a stronger base (potassium *tert*-butoxide instead of potassium hydroxide) greatly broadened the scope of the triarylamine formation, enabling the creation of triarylamine intermediates bearing *p*-methoxy, *p*-methyl, unmodified benzene, and *p*-cyano substituents with yields of 78%, 52%, 38%, and 24% respectively. However, the acidic conditions required for the subsequent nitrosative condensation reaction deprotect each of these molecules, resulting in the accumulation of diazo side products with little or no discernible safranine formation.

This led us to find a protecting group more robust to the conditions required for the cyclization reaction. Allylic protecting groups have an electronegativity similar to that of the Boc moiety, but are relatively stable to both acid and base.[24] Our final synthetic

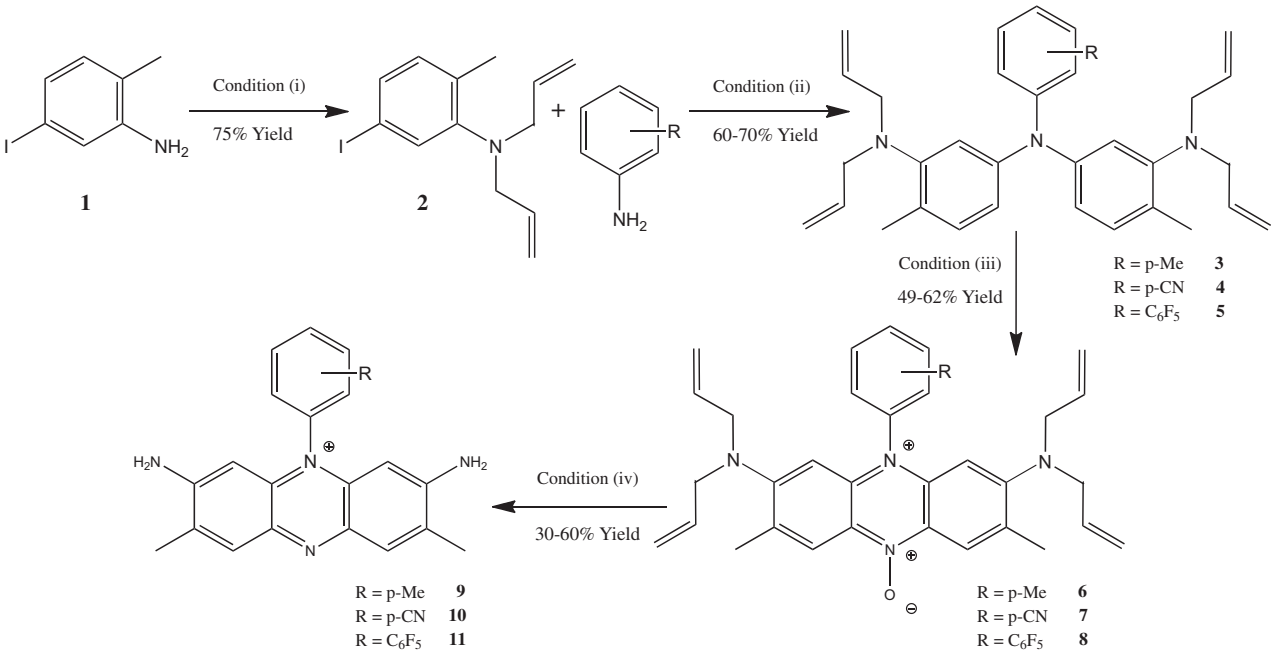

Figure 2. Synthetic route to safranine derivatives. (i) Allyl bromide, K_2CO_3, DMF, 80 °C, 2 h. (ii) NaH, CuI, 2,2′-bipyridine, Toluene, 120 °C, 4 h. (iii) $Na^{15}NO_2$, Acetic acid, 0 °C to RT. (iv) tetrakis(triphenylphosphine)palladium, *N*,*N*′-dimethyl barbituric acid, CH_2Cl_2, 35 °C, 2 h.