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Convenient synthesis of deazaflavin cofactor FO and its activity in F_{420}-dependent NADP reductase

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F_{420} and FO are phenolic 5-deazaflavin cofactors that complement nicotinamide and flavin redox coenzymes in biochemical oxidoreductases and photocatalytic systems. Specifically, these 5-deazaflavins lack the single electron reactivity with O_2 of riboflavin-derived coenzymes (FMN and FAD), and, in general, have a more negative redox potential than NAD(P)$^+$. For example, F_{420}-dependent NADP$^+$ oxidoreductase (Fno) is critical to the conversion of CO_2 to CH_4 by methanogenic archaea, while FO functions as a light-harvesting agent in DNA repair. The preparation of these cofactors is an obstacle to their use in biochemical studies and biotechnology. Here, a convenient synthesis of FO was achieved by improving the redox stability of synthetic intermediates containing a polar, electron-rich aminophenol fragment. Improved yields and simplified purification techniques for FO, from which F_{420} can be enzymatically generated, are described. Additionally, Fno activity was restored with FO in the absence of F_{420}. Investigating the FO-dependent NADP$^+$/NADPH redox process by stopped-flow spectrophotometry, steady state kinetics were defined as having a K_m of 4.00 ± 0.39 μM and a k_{cat} of 5.27 ± 0.14 s^{-1}. The preparation of FO should enable future biochemical studies and novel uses of F_{420} mimics.

Cofactor F_{420} (**2**, **Figure 1**), a 7,8-didemethyl-8-hydroxy-5-deazariboflavin derivative, was discovered in the 1970's[1,2] and is functionally similar to nicotinamide cofactors, NAD(P)$^+$, while structurally reminiscent of the isoalloxazine tricyclic system found in flavin cofactors, FAD and FMN. Found primarily in prokaryotes,[2-8] F_{420} and its precursor 5-deaza-7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO, **1a**), are unique organic redox-active coenzymes ($E_{1/2}$ = -340 to -350 mV) capable of reducing both NAD(P) (ca. -320 mV) and FAD/FMN (ca. -210 mV) in a thermodynamically favourable manner.[9] Due to the relatively acidic phenolic residue at C8, the activity of these species is pH dependent. In the neutral state, reduced FO (FOH_2) performs hydride transfer reactions with relatively enhanced reducing power as compared to NADH. Deprotonated FO was discovered to be a light-harvesting molecule for DNA photolyase in *Drosophila*.[10] We set out to prepare FO synthetically as part of our studies of F_{420} dependant NADP$^+$ oxidoreductase (Fno), an important enzyme for methanogens, which convert CO_2 to CH_4.[11]

The final intermediates of FO biosynthesis[12] were recently identified from the proposed shikimate pathway;[13] however, robust biological systems for the generation of FO and F_{420} are underdeveloped. One report isolated FO efficiently from the lysate of fruit flies.[10] Chemical approaches to deazariboflavins were defined prior to the discovery of F_{420}.[14] However, only one synthesis of each naturally occurring deazaflavin (FO, by Ashton et al.[15,16] and F_{420} by Yoneda and coworkers[17,18]) has been communicated (**Scheme 1**). These chemical preparations contain significantly unstable intermediates, making these synthetic achievements quite impressive, but leave the field without convenient preparations of FO or F_{420}.[14,19] This work

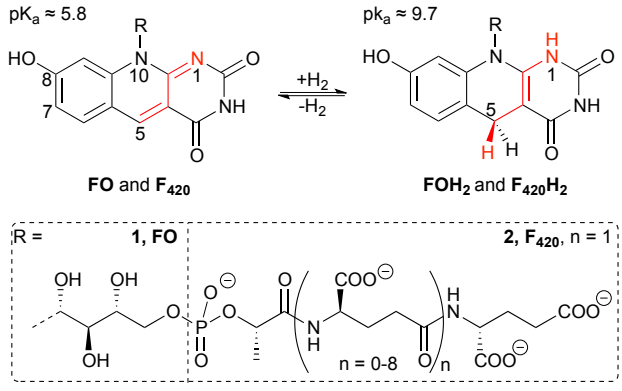

Figure 1. Structures and Properties of FO (**1**) and F_{420} (**2**).

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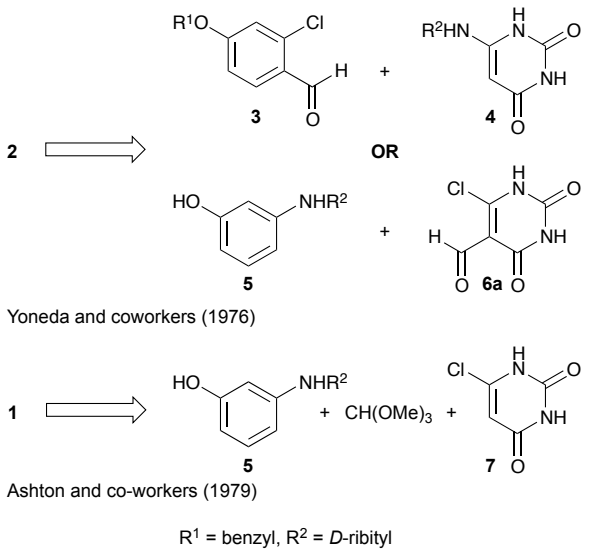
displays a preparation of FO in the context of prior methods, which were instructional to the overall synthesis. Stability of intermediates was gained by a protection strategy for 3-aminophenol that added a single step to the synthesis and allowed for normal phase chromatography. The reported procedure does not require anaerobic ion exchange chromatography. In addition to the synthesis of FO, a deuterated C5D-FO precursor was prepared and wild-type F_{420}-dependent $NADP^+$

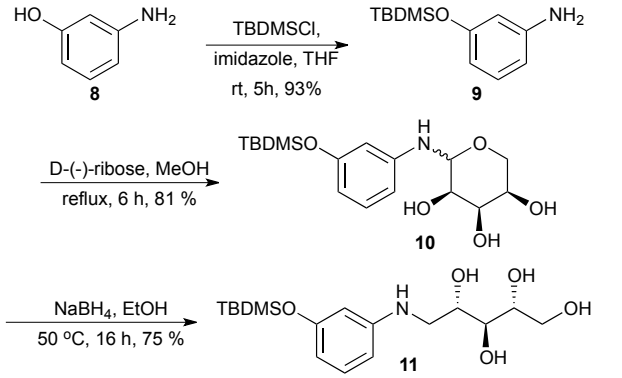


Scheme 1. Prior syntheses of compounds **(Fo, 1)** and **(F_{420}, 2)**

oxidoreductase (Fno) activity was studied directly with synthetic FO, displaying FO's function and kinetics in F_{420}-dependent enzymes.

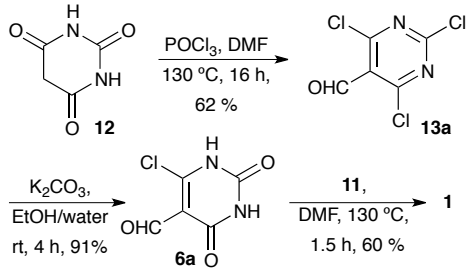
The photo-degradation of riboflavin and related cofactors to lumichrome or lumiflavin is indicative of the intermediate instability challenging syntheses of FO.[21] Purification challenges for natural and non-natural deazariboflavins and riboflavins pre-date FO and F_{420} syntheses, but the electron-rich and acidic 8-hydroxyl substituent of FO and F_{420} increase the challenges to these preparations.[22] Prior work (**Scheme 1**) required anaerobic and dark conditions for early-stage intermediates, specifically involving compound **5**, in addition to separations of polar, acidic intermediates by ion-exchange chromatography.[15,16,23]

We wondered if a combination of prior approaches and steric *O*-protection of 3-aminophenol could address the instability and related purification challenges of early-stage polar intermediates. Redox shuttle additives in batteries (e.g. di-*tert*-butyl-1,4-dimethoxybenzene) are kinetically stabilized by steric hinderance, rather than thermodynamically stabilized by alteration of their electron density.[24] Satisfyingly, the introduction of a *tert*-butyldimethylsilyl (TBDMS) protecting group to 3-aminophenol addressed numerous issues, including: chemoselective *O*- vs. *N*-protection, enhanced redox stability, and simplified purification. Furthermore, the protecting group imparted no apparent affect on later transformations and, as described below, concomitant TBMDS-*O*-deprotection was achieved during the final HCl-generating transformation. Related silyl-protecting group, TBDPS-, was investigated, but negatively impacted the final condensation cyclization and required an additional reaction for *O*-deprotection under more harsh conditions. Conversely, benzyl-protection, used in Yoneda's synthesis of F_{420}, yielded only 75% of the *O*-protected product, with the *N*-protected byproduct. Unfortunately, the *O*-benzyl protected analogue of **11** did not cyclize with **6a**, leading us to believe that the facile deprotection of TBDMS- reveals the more electron-rich phenolate, aiding in final cyclization.



Scheme 2. Preparation of stable, hydrophobic fragment **11**

Selective *O*-protection of commercial 3-aminophenol **8** was achieved in 93% isolated yield (Scheme 2).[25] Protected **9** and *D*-ribose were refluxed in dry methanol to yield *N*-ribosylaniline **10** as a white powder, which was purified by flash chromatography (silica). The resultant *N*-ribosyl compound was reduced to the corresponding *N*-ribitylaminophenol **11**,[23] as an amorphous white solid after purification, again by normal phase flash chromatography. In our experience with the prior literature, handling the unprotected ribityl species **5** was a major source of anguish, especially during purification. This was presumably due to (photo)oxidation products, i.e. careful aenaerobic and dark techniques did improve yields by limiting, but not eliminating, the formation of a brown multicomponent impurity, which could not be carried through subsequent reactions. Purified **11** was stable at room temperature for a few hours and could be stored at -20 ºC for over a month with no noticeable degradation.



Scheme 3. Synthesis of Uracil **6a** and Condensation to FO, **1**.

We found Yoneda's uracil derivative **6a** (**Scheme 3**) to be the best condensation partner for fragment **11** (in comparison to **7** or **13**). To prepare this species, barbituric acid **12** was converted to 2,4,6-trichloro-5-formyluracil **13a** by Vilsmaier-Haack conditions.[26] The resulting trichloroformyluracil **13a** was converted to 6-chloro-5-

formyluracil **6** in good yields by Yoneda's method.[27] This two-step procedure also allowed access to **deutoro-6** (**6b**) when d7-DMF was substituted for DMF (54% yield from **12**, see **6b** in SI). The convergent synthesis of **1** was completed by condensation of **6a** and **11** at 130 ºC in DMF for ca. 90 minutes. TBDMS-*O* was fully deprotected during the cyclization, which generates HCl.

With synthetic FO in hand, we investigated its reduction by NADPH in the place of F_{420} in wild-type Fno (wt-Fno, **Figure 2**) from *Archaeoglobus fulgidus*, which was expressed and purified in C41(DE3) *E. coli* cells. FO's activity in Fno (200 nM) was examined aerobically by steady-state kinetics with a saturating concentration of NADPH, 600 μM, and varying concentrations of FO from 2 μM to 30 μM. Standard Michaelis-Menten kinetics were observed at pH 6.5 and 23 ºC (Figure **2**). The k_{cat} for wt-Fno was 5.27 ± 0.14 s^{-1}. The

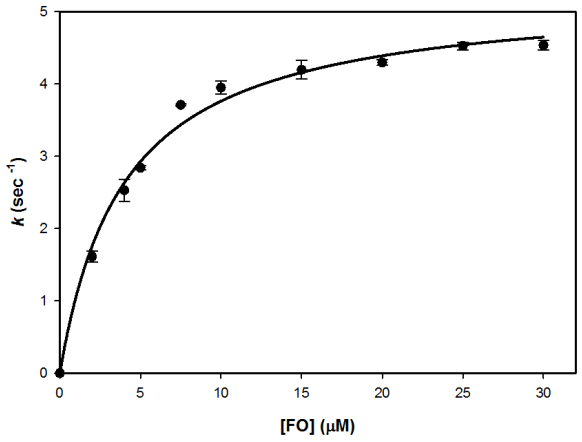


Figure 2. The steady state experiment of *wt*-Fno with constant [NADPH] and varying $\mathbf{F_O}$ concentration. The data points were fit with the Henri-Michaelis-Menten equation $\left(k = \frac{k_{cat}\,[S]}{K_m + [S]}\right)$.

K_m for oxidized FO under the same conditions was found to be 4.00 ± 0.39 μM. Previously reported K_m values of wt-Fno were: 20 μM at 65 ºC for the natural substrate, reduced $F_{420}H_2$; and 10 μM at 65 ºC for oxidized F_{420}.[28,29] FO lacks the charged poly-glutamate tail of F_{420} cofactor, eliminating putative points of contact between the coenzyme and Fno's binding site. However, oxidized FO still binds relatively tightly to Fno, in comparison to F_{420}. The detailed expression protocol and kinetic data for Fno using synthetic FO will be reported in due course.

Conclusions

F_{420} cofactor is believed to be a major electron transfer entity in methanogenic bacteria, where it exists in high concentration and transfers electrons from hydrogen or formate to the consecutive intermediates of methane biosynthesis. F_{420}'s relatively low redox potential enables specific energy producing reactions such as the conversion of carbon dioxide to methane.[5] A number of enzymes use F_{420} as a cofactor in the metabolism of key pathogens, making the synthesis of F_{420}, FO, and deazaflavin mimics valuable. The synthetic route employed in this work involves significantly stable and hydrophobic intermediates, which are suitable for purification by conventional methods with aerobic bench-top procedures. Furthermore, the four step synthesis from 3-aminophenol is achieved in 33% compared to the 13% by previous methods. Intermediate **6a** is generated in 56% yield. This preparation of FO should aid future biochemical researchers to investigate the different applications of F_{420} in nature, especially in the metabolism of various pathogens. The biochemical utility of FO is shown in place of F_{420} and the ability to use FO and related analogues may have great potential in biotechnology.

Acknowledgements

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