JOC The Journal of Organic Chemistry

Article

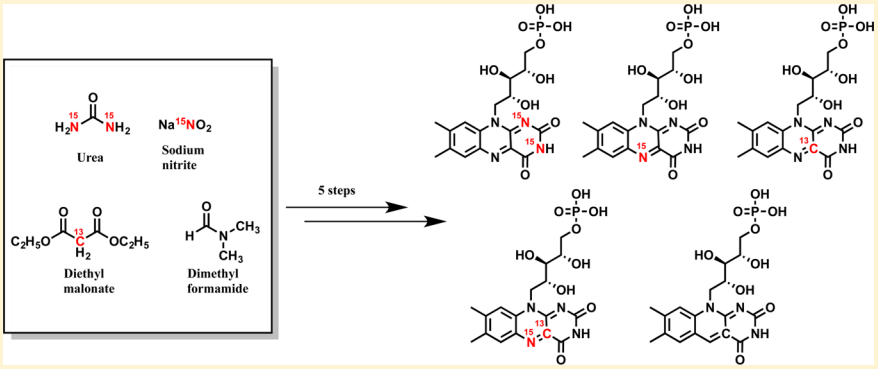
pubs.acs.org/joc

Site-Selective Synthesis of ^{15}N- and ^{13}C-Enriched Flavin Mononucleotide Coenzyme Isotopologues

Syam Sundar Neti and C. Dale Poulter*

Department of Chemistry, University of Utah, 315 South 1400 East, Salt Lake City, Utah 84112, United States

Supporting Information



ABSTRACT: Flavin mononucleotide (FMN) is a coenzyme for numerous proteins involved in key cellular and physiological processes. Isotopically labeled flavin is a powerful tool for studying the structure and mechanism of flavoenzyme-catalyzed reactions by a variety of techniques, including NMR, IR, Raman, and mass spectrometry. In this report, we describe the preparation of labeled FMN isotopologues enriched with ^{15}N and ^{13}C isotopes at various sites in the pyrazine and pyrimidine rings of the isoalloxazine core of the cofactor from readily available precursors by a five-step chemo-enzymatic synthesis.

INTRODUCTION

Flavins are ubiquitous in nature, where they serve as cofactors in a wide variety of biological processes. Three different flavins are found in nature (Figure 1). Riboflavin (vitamin B_2) is the biosynthetic precursor for flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are the biologically active cofactors. Typically, flavin cofactors mediate one- and two-electron redox reactions,[1,2] but they can carry out a variety of other functions which include electrophilic and nucleophilic substitution, blue light photoreceptors using flavin (BLUF), DNA repair,[3−5] signal transduction and light sensing.[6] In addition, many flavoenzymes are targets for drug development,[7,8] where a thorough understanding of the structures and reaction mechanisms could lead to the design of novel lead compounds as therapeutic agents. Flavoproteins constitute a large family with more than 100 000 entries in the NCBI database.[1,9]

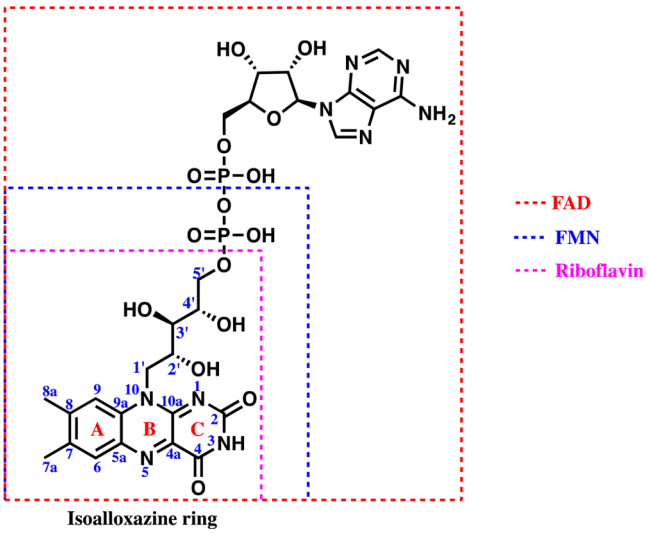


Figure 1. Structures of riboflavin, FMN, and FAD.

Isotopically labeled flavins and flavin analogs are useful probes for studying the structure and function of the cofactor in flavin-dependent processes. A few notable applications include investigation of flavin transport and metabolism,[10] elucidation of the mechanisms of flavoenzyme-catalyzed reactions where the cofactor is involved in the chemistry of enzyme catalysis,[11−14] and studies of the dynamics of light receptors.[15] Flavins uniformly enriched with ^{15}N and ^{13}C are employed to improve the NMR signal sensitivity, particularly when the cofactors are bound to proteins. Selective ^{15}N and ^{13}C enrichment is used to enhance signals for specific atoms of interest in an unlabeled background and for individual peak assignments.[16] Site-specifically ^{15}N and ^{13}C enriched flavins are

Received: March 24, 2016
Published: May 13, 2016

DOI: 10.1021/acs.joc.6b00640
J. Org. Chem. 2016, 81, 5087−5092

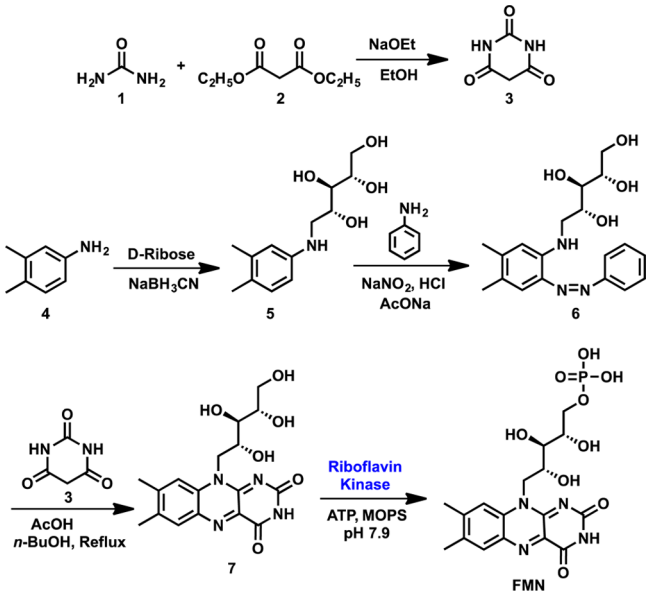
highly useful for unequivocal band assignments in IR and Raman spectroscopy.[17] ^{13}C-FAD isotopologues were used to study the BLUF-domain protein photoreceptor,[18] and both ^{13}C- and ^{15}N-FMN were used to investigate flavin−protein interactions in flavocytochrome b_2.[19]

Synthetic methods, biosynthetic methods, and a combination of the two have been used to insert ^{15}N- and ^{13}C-labels in riboflavin.[20] The Tishler condensation of *o*-aminoazo compounds with barbituric acid is a classic chemical approach for the synthesis of riboflavin.[21] GTP and ribulose 5-phosphate are precursors for the biosynthesis of riboflavin through the sequence of steps catalyzed by GTP cyclohydrolase II, lumazine synthase, and riboflavin synthase.[22] Recently, Fischer and co-workers reported an *in vivo* biotransformation method using an engineered bacterial strain to synthesize ^{15}N-, ^{13}C-, ^{17}O-, and ^{18}O-labeled flavins from isotopically labeled precursors.[23] In another report, Kohen and co-workers reported an enzymatic synthesis of ^{3}H-, ^{15}N-, and ^{13}C-labeled flavins from commercially available isotope-labeled riboflavin precursors.[24] We now report a combination of chemical and biotransformation steps for labeling nitrogen and carbon sites in the pyrazine and pyrimidine rings of the isoalloxazine core in FMN.

RESULTS AND DISCUSSION

Our synthetic approach consists of the five-step chemoenzymatic route shown in Scheme 1, where four chemical steps are followed by an enzyme-catalyzed phosphorylation of riboflavin **7**. The overall sequence is based on literature precedents for each chemical step through the Tishler condensation to give **7**, although the reaction conditions, workup procedures, and purification steps were substantially modified. We then used riboflavin kinase (RFK), which is regiospecific for the 5′-OH, to convert **7** to 5′-FMN.[25] In contrast, chemical phosphorylation of **7** is not regiospecific, giving a mixture of monophosphates, bisphosphates, and cyclic phosphates that are difficult to separate.[26] This approach is not practical when using expensive ^{15}N- and ^{13}C-enriched substrates.

Scheme 1. Strategy for Synthesis of ^{13}C and ^{15}N Labeled FMN



The substrates for the Tishler condensation, barbituric acid **3** and amino *o*-azoribitol derivative **6**, were synthesized from materials that permit us to introduce labels at N1/N3, N5, N10, C4a, and C2/C4 of **7** by the route outlined in Scheme 1. We reproduced the reported good yields for the synthesis of **3** from urea **1** and diethyl malonate **2**[27] and the reductive amination of 3,4-dimethyl aniline **4** with D-ribose and sodium cyanoborohydride ($NaCNBH_3$) to give *N*-ribityl 3,4-dimethyl aniline **5**.[28] The low yield (∼20%) for conversion of **5** to **6** with $NaNO_2$/HOAc/NaOH was improved considerably (95%) by adapting the procedure reported by Folkers and co-workers for the synthesis of L-lyxoflavin where HOAc/NaOH was replaced by NaOAc.[29]

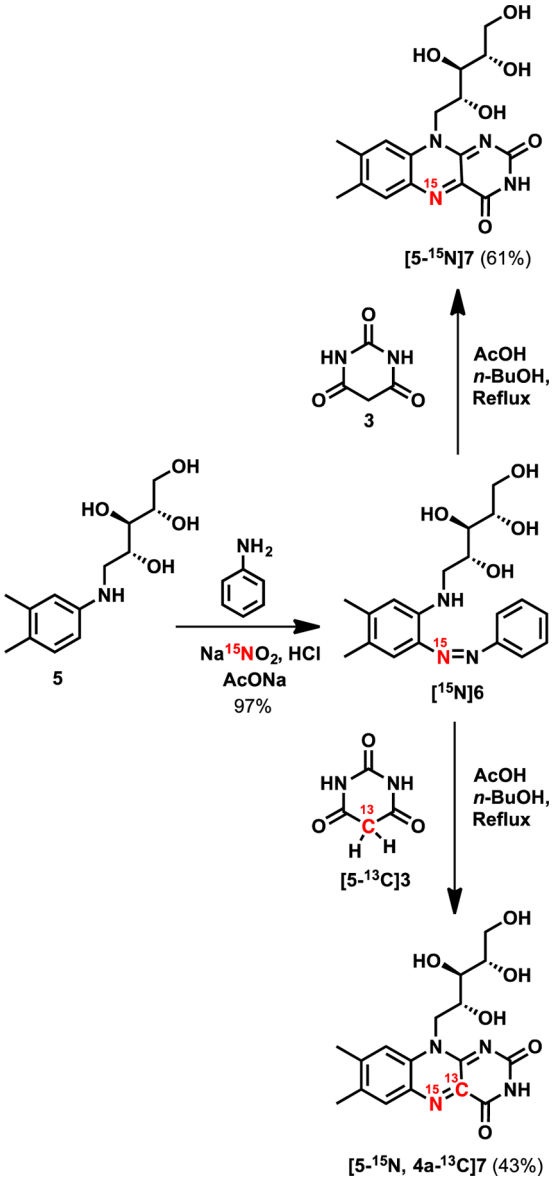
Purification of **7** following condensation of **6** with barbituric acid **3** proved to be difficult. We were not able to obtain good yields following the lengthy purification protocol, which includes several washes with water and MeOH, extractions with ether, and recrystallization with superoxol.[29] These procedures were necessary to provide pure **7** for a chemical phosphorylation. We verified that phosphorylation of **7** with $POCl_3$ gave a mixture of regioisomers that are hard to separate by HPLC.[26] Instead, the reaction mixture containing **7** from the condensation of **3** and **6** was washed with water and cold methanol and used directly for the RFK-catalyzed phosphorylation, thereby preventing a substantial loss of material during repeated washings, extractions, and recrystallization. Incubation of crude **7** with ATP and RFK gave 5′-FMN in >95% yield.

The reactions shown in Scheme 2 were used to synthesize **7** with ^{15}N at positions N1/N3 and N5, ^{13}C at C4a, and ^{13}C/^{15}N at C4a/N5. [N1,N3-$^{15}N_2$]- and [C4a-^{13}C]**7** were synthesized from labeled barbituric acid as outlined in Scheme 2. [1,3-$^{15}N_2$]- and [5-^{13}C]Barbituric acids were prepared from [1,3-$^{15}N_2$]urea and diethyl [3-^{13}C]malonate, respectively.[30,31] Condensation of the labeled barbiturates with azoribitol **6** gave the corresponding labeled riboflavins. [N5-^{15}N]- and [C4a-^{13}C, C5-^{15}N]Riboflavin were prepared as shown in Scheme 3. [N5-^{15}N]**7** was prepared by treatment of ribityl amine **5** with $Na^{15}NO_2$/HCl followed by coupling of the labeled diazonium intermediate with aniline to give ^{15}N-azoribitol **6**. Condensation of labeled **6** with barbituric acid gave [5-^{15}N]**7** and with [5-^{13}C]barbituric acid gave [C4a-^{13}C, C5-^{15}N]**7**.

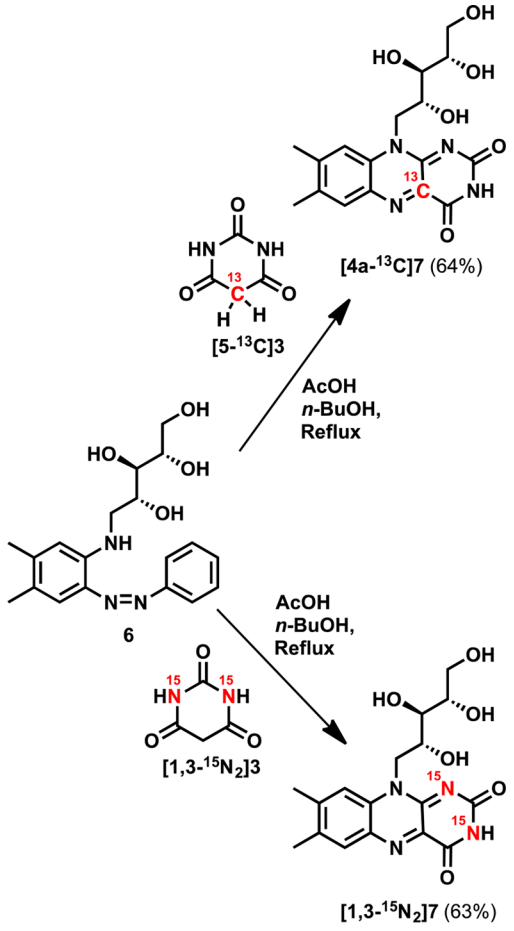
The overall yields of labeled riboflavins for the three steps from barbituric acid ranged from 29% to 43%. The poorest yields were for the final conversion of **6** to **7** (43−64%). We experienced additional losses at this step during attempts to purify **7** from the reaction mixture. Riboflavin is poorly soluble in water and common organic solvents, which severely limits its purification by chromatography, and we encountered substantial losses during recrystallization. As a result, *Homo sapiens* riboflavin kinase (*hs*RFK) was used to phosphorylate unpurified **7** with ATP. The labeled riboflavins were dissolved in a minimal volume of DMSO and added to *hs*RFK to give a reaction buffer that contained ∼1% DMSO. The enzyme-catalyzed phosphorylation was regioselective for synthesis of 5-FMN. Impurities in the labeled riboflavin samples did not interfere with the reaction, giving excellent conversions of **7** to FMN, which was purified by HPLC (Scheme 4, Table 1). The same procedure was used to phosphorylate 5-deazariboflavin **8** to give 5-deazaFMN **9**, an analogue frequently used in mechanistic studies of flavin-dependent enzymes.[32]

DOI: 10.1021/acs.joc.6b00640
J. Org. Chem. 2016, 81, 5087−5092

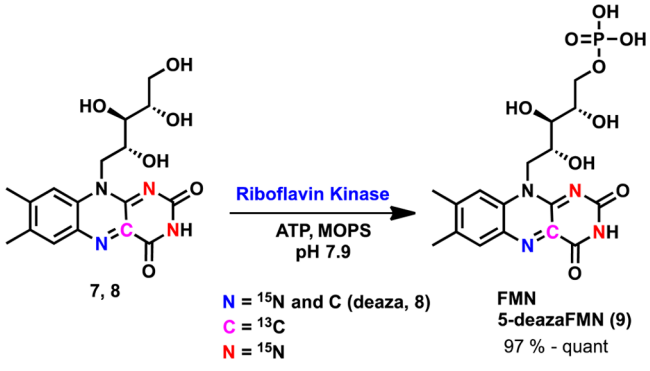
Scheme 2. Synthesis of [5-^{15}N]7 and [5-^{15}N, 4a-^{13}C]7



Scheme 3. Synthesis of [4a-^{13}C]7 and [1,3-^{15}N$_2$]7



Scheme 4. RFK Phosphorylation of Labeled Riboflavin Precursors and 5-Deazariboflavin (8)



CONCLUSIONS

In summary, we report an efficient five-step synthesis of ^{13}C and ^{15}N FMN isotopologues labeled at positions N1, N3, C4a, and N5 of pyrazine and pyrimidine rings in the isoalloxazine nucleus in yields ranging from 29% to 43% from commercially available labeled precursors. Our synthesis could be also adapted to label other carbon, nitrogen, hydrogen, oxygen, and phosphorus atoms in FMN by starting with suitable ^{15}N or ^{13}C enriched precursors. Although the final step involves an RFK-catalyzed phosphorylation requiring ATP to synthesize the labeled riboflavins to the corresponding FMN derivatives, an expression plasmid for RFK is available and ATP regeneration protocols can be used for larger scale syntheses.[33]

EXPERIMENTAL SECTION

Materials. Na^{15}NO$_2$ (98% ^{15}N) was purchased from Sigma-Aldrich, ^{15}N$_2$-urea (98%+ ^{15}N$_2$) and ^{13}C(2)-diethyl malonate (99% ^{13}C) were purchased from Cambridge Isotope Laboratories (CIL), and *Homo sapiens* (*hs*) riboflavin kinase plasmid (HS_RFK_EC_1_pQE-T7) was purchased from Qiagen.

Anhydrous reaction conditions were employed using anhydrous solvents under a nitrogen atmosphere in oven-dried glassware (100 °C). Anhydrous methanol (MeOH) and ethyl alcohol (ethanol) were obtained by passing through a column of activated alumina. Purification of the **5**, **6**, and [^{15}N]**6** was carried out using silica gel flash column chromatography (230−400 mesh, 60 Å). EMD Silica Gel 60 Å F254 TLC aluminum plates were for the thin-layer chromatographic analysis, and the spots were visualized under UV light. All the ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded at 25 °C, and chemical shifts were reported in δ ppm (parts per million) values. ^{1}H NMR spectra were recorded at 300 and 500 MHz and were referenced using CD$_3$OD, d_6-DMSO, and D$_2$O NMR solvents. ^{13}C NMR spectra were recorded at 75 and 125 MHz, and ^{31}P NMR spectra were recorded at 121 MHz. ^{13}C and ^{31}P NMR spectra recorded in D$_2$O were

DOI: 10.1021/acs.joc.6b00640
J. Org. Chem. 2016, 81, 5087−5092

Table 1. RFK-Catalyzed Phosphorylation of ^{15}N and ^{13}C-Enriched Riboflavins

substrate	purity[a] (area %)	product	yield (%)
$^{15}N(5)$-Riboflavin **7a** (10 mg)	75	$^{15}N(5)$-FMN (8.9 mg)	98
$^{13}C(4a)$-Riboflavin **7b** (10 mg)	80	$^{13}C(4a)$-FMN (9.4 mg)	97
$^{15}N(5)^{13}C(4a)$-Riboflavin **7c** (10 mg)	59	$^{15}N(5)^{13}C(4a)$-FMN (7.2 mg)	>98
$^{15}N(1)^{15}N(3)$-Riboflavin **7d** (10 mg)	86	$^{15}N(1)^{15}N(3)$-FMN (10.7 mg)	>98
5-deazaRiboflavin **8** (12 mg)	88	5-deaza-FMN (12.5 mg)	98

[a]Evaluated by RP C_{18}-HPLC.

unreferenced. HRMS-ESI data were recorded on LC-TOF and LTQ-FTMS mass spectrometers. HPLC purification of a crude isotopically labeled FMN mixture utilized a reverse phase column (XBridge C18, Waters, 19 × 100 mm) that had been equilibrated with 100% ammonium bicarbonate (NH_4HCO_3). The NH_4HCO_3–acetonitrile (ACN) gradient run was performed for a total run time of 45 min at a flow rate of 2 mL/min (rt). The retention time for FMN was 28.5 min. Fractions were combined, lyophilized, and stored at −80 °C.

*Barbituric Acid (**3**).* In a 50 mL round bottomed flask, sodium (250 mg, 10.8 mmol) was carefully added to 15 mL of ethanol. After the sodium metal had been consumed, diethyl malonate (**2**, 1.65 mL, 10.8 mmol) was added followed by urea (**1**, 0.65 g, 10.8 mmol). The stirred mixture was heated at reflux for 7 h in an oil bath, during which time a white solid precipitated. The reaction was acidified with 13 mL of H_2O and 1.2 mL of 12 N HCl to a pH ≈ 4. The clear solution was then filtered and cooled in an ice bath. The precipitate was removed by filtration and washed with 1 mL of cold H_2O to give 0.99 g (72%) of a white solid;[27] ^1H NMR (300 MHz, d_6-DMSO) δ ppm 11.13 (s, 2H), 3.46 (s, 2 H); ^{13}C NMR (300 MHz, d_6-DMSO) δ ppm 168.0, 151.8; GC-MS $[M]^+$ calculated for $C_4H_4N_2O_3$ 128.09, observed 128.1.

*[5-^{13}C]Barbituric Acid ([**5-^{13}C**]**3**).* Following the procedure described for **3**; sodium (200 mg, 8.699 mmol) in 5 mL ethanol, diethyl [2-^{13}C]malonate ([**2-^{13}C**]**2**) (0.5 mL, 6.204 mmol), and **1** (0.372 g, 6.204 mmol) were combined to give 0.54 g (68%) of a white solid; ^1H NMR (500 MHz, d_6-DMSO) δ ppm 11.09 (s, 2H), 3.47 (d, 2H, $^1J_{C-H}$ = 130 Hz) ppm; ^{13}C NMR (500 MHz, d_6-DMSO) δ ppm 167.3 (d, $^1J_{C-C}$ = 39.18 Hz), 151.31. HRMS (ESI^-) calculated for $^{13}C_1C_3H_3N_2O_3$ $[M - H]^-$ (m/z) = 128.0183, found 128.0182.

*[1,3-$^{15}N_2$]Barbituric Acid ([**1,3-$^{15}N_2$**]**3**).* Following the procedure described for **3**; sodium (90 mg, 3.91 mmol) in 5 mL of ethanol, **2** (0.5 mL, 3.223 mmol), and [**1,3-$^{15}N_2$**]**1** (0.20 g, 3.22 mmol) were combined to give 0.275 g (66%) of a white solid; ^1H NMR (400 MHz, d_6-DMSO) δ ppm 11.12 (d, 2H, $^1J_{N-H}$ = 92 Hz), 3.46 (s, 2H); ^{13}C NMR (400 MHz, d_6-DMSO) δ ppm 167.4 (d, $^1J_{C-N}$ = 11 Hz), 151.3 (t, $^1J_{C-N}$ = 18.1 Hz). HRMS (ESI^-) calculated for $C_4H_3{}^{15}N_2O_3$ $[M - H]^-$ (m/z) = 129.0090, observed 129.0090.

*N-Ribityl 3,4-Dimethyl Aniline (**5**).* In a 250 mL round bottomed flask, 3,4-dimethyl aniline (**4**) (2.06 g, 17 mmol), D-ribose (7.9 g, 53.0 mmol), and sodium cyanoborohydride (2.07 g, 33.0 mmol) were dissolved in 100 mL of methanol. The mixture was heated at reflux for 48 h with stirring. Solvent was removed under reduced pressure, and the residue was dissolved in 1 M HCl (40 mL) and swirled. A saturated solution of $NaHCO_3$ was added, and the mixture was extracted with ethyl acetate (6 × 35 mL). The combined organic layers were washed with brine and dried over $MgSO_4$. Solvent was removed under reduced pressure to give 3.81 g (88%) of a white solid;[28] ^1H NMR (300 MHz, d_3-MeOD) δ ppm 6.88 (d, 1H, J = 9 Hz), 6.55 (d, 1H, J = 2.3 Hz), 6.47 (dd, 1H, J = 2.4, 8 Hz), 3.91 (ddd, 1H, J = 8, 6.1, 3.5 Hz), 3.81–3.6 (m, 4H), 3.43 (dd, 1H, J = 12.8, 3.5 Hz), 3.08 (dd, 1H, J = 12.8, 8 Hz), 2.17 (s, 3H), 2.12 (s, 3H); ^{13}C NMR (300 MHz, d_3-MeOD) δ ppm 148.2, 138.1, 131.2, 126.9, 116.9, 112.7, 75, 74.5, 72.2, 64.8, 48.2, 20.2, 19.

*N-Ribityl-2-phenylazo-4,5-dimethyl Aniline (**6**).* Aniline (0.22 g, 2.36 mmol), 12 N HCl (0.66 mL), and H_2O (1.55 mL) were mixed in a 50 mL round bottomed flask and allowed to stir at 0 °C for 10 min, followed by slow addition of solid $NaNO_2$ (0.163 g, 2.36 mmol) in small portions. The solution was maintained at 0 °C for 30 min. In a second 50 mL round bottomed flask, *N*-ribityl 3,4-dimethyl aniline **5** (0.5 g, 1.95 mmol) was dissolved in 4 mL of H_2O followed by the addition of 0.7 mL of 12 N HCl and 0.66 g (8.04 mmol) of sodium acetate. The mixture was cooled to −5 °C, the solution of diazotized aniline was added, and the resulting solution was stirred at −9 to −5 °C for 1 h and at 0 °C for 2 h. After warming to 20 °C, a solution of 0.33 g (4.02 mmol) of sodium acetate in 2.5 mL of H_2O was slowly added to maintain pH ≈ 3 and the temperature at 17–20 °C. The resulting mixture was allowed to stir at 22–25 °C for 17 h to give a dark orange suspension. The solid was removed by filtration and purified by flash chromatography (1:10 v/v MeOH/DCM) to give 0.67 g (95%) of an orange solid;[29] R_f 0.35 (1:10 v/v MeOH/DCM); ^1H NMR (300 MHz, d_3-MeOD) δ ppm 7.82–7.79 (m, 2H), 7.52 (s, 1H), 7.47–7.42 (m, 2H), 7.36–7.32 (m, 1H), 6.72 (s, 1H), 4.05–3.97 (m, 1H), 3.82–3.61 (m, 5H), 3.39 (dd, 1H, J = 13.1, 7.7 Hz), 2.28 (s, 3 H), 2.22 (s, 3 H); LC-MS $[M + Na]^+$ calculated for $C_{19}H_{25}N_3O_4Na$ 382.92, observed 382.2.

*[^{15}N]-N-Ribityl-2-phenylazo-4,5-dimethyl Aniline ([^{15}N]**6**).* Following the procedure described for **6**; aniline (0.109 g, 1.18 mmol), 0.33 mL of 12 N HCl, 0.77 mL of H_2O, and $Na^{15}NO_2$ (0.0825 g, 1.18 mmol) were combined to prepare the corresponding diazonium salt. The diazonium salt was added to **5** (0.25 g, 0.979 mmol) to give 0.34 g (97%) of an orange solid; R_f 0.35 (1:10 v/v MeOH/DCM); ^1H NMR (300 MHz, d_3-MeOD) δ ppm 7.85–7.81 (m, 2H), 7.53 (s, 1H), 7.49–7.44 (m, 2H), 7.39–7.34 (m, 1H), 6.75 (s, 1H), 4.05–3.99 (m, 1H), 3.84–3.61 (m, 5H), 3.39 (dd, 1H, J = 13.2 Hz, 7.7 Hz), 2.29 (s, 3H), 2.23 (s, 3H); ^{13}C NMR (300 MHz, d_3-MeOD) δ ppm 154.5 (d, J_{C-N} = 5.1 Hz), 144.1, 143.8, 136.7, 131.2 (d, J_{C-N} = 6.75 Hz), 130.4, 130.2, 125.2 (d, J_{C-N} = 2.92 Hz), 123 (d, J_{C-N} = 3.75 Hz), 114.3, 74.7, 72.5, 64.8, 46, 20.8, 18.9; HRMS (ESI^+) calculated for $C_{19}H_{25}N_2{}^{15}N_1O_4Na$ $[M + Na]^+$ (m/z) = 383.1713, found 383.1726.

*Riboflavin (**7**).* To *N*-ribityl-2-phenylazo-4,5-dimethyl aniline **6** (100 mg, 0.278 mmol) in a 10 mL round bottomed flask, 2 mL of *n*-butanol, barbituric acid **3** (56.5 mg, 0.44 mmol), and 0.5 mL of AcOH was added. The mixture was stirred and heated to reflux for 5 h, stirred for am additional 1 h in an ice bath, and then filtered. The solid on the filter was washed with hot H_2O and methanol to give 62.5 mg of a crude yellow solid; HRMS (ESI^+) calculated for $C_{17}H_{21}N_4O_6$ $[M + H]^+$ (m/z) 377.1461, observed 377.1463.

*[5-^{15}N]Riboflavin ([**5-^{15}N**]**7**).* Following the procedure described for **7**; [**5-^{15}N**]**6** (100 mg, 0.278 mmol), 2 mL of *n*-butanol, **3** (56 mg, 0.44 mmol), and 0.5 mL of AcOH were combined to give 85.5 mg of a crude yellow solid (75% pure as determined by HPLC); HRMS (ESI^+) calculated for $C_{17}H_{20}N_3{}^{15}NO_6Na$ $[M + Na]^+$ (m/z) 400.1251, observed 400.1255.

*[5-^{15}N, C4a-^{13}C]Riboflavin ([5-^{15}N, 4a-^{13}C]**7**).* Following the procedure described for **7**; [**^{15}N**]**6** (54.6 mg, 0.1515 mmol), 1.5 mL of *n*-butanol, [**5-^{13}C**]**3** (40 mg, 0.3121 mmol), and 0.3 mL of AcOH were combined to give 42 mg of a crude yellow solid (59% pure as determined by HPLC); HRMS (ESI^+) $[M + H]^+$ calculated for $C_{16}{}^{13}CH_{21}N_3{}^{15}NO_6$ 379.1460, observed 379.1459.

*[4a-^{13}C]Riboflavin ([**4a-^{13}C**]**7**).* Following the procedure described for **7**; **6** (60 mg, 0.166 mmol), 1.5 mL of *n*-butanol, [**5-^{13}C**]**3** (40 mg, 0.3121 mmol), and 0.3 mL of AcOH were combined to give 50 mg of a crude yellow solid (80% pure as determined by HPLC); HRMS (ESI^+) $[M + Na]^+$ calculated for $C_{16}{}^{13}CH_{20}N_4O_6Na$ 400.1309, observed 400.1286.

*[1,3-$^{15}N_2$]Riboflavin ([**1,3-$^{15}N_2$**]**7**).* Following the procedure described for **7**; **6** (100 mg, 0.2766 mmol), 1.5 mL of *n*-butanol,

DOI: 10.1021/acs.joc.6b00640
J. Org. Chem. 2016, 81, 5087–5092

[**1,3-$^{15}N_2$**]**3** (56 mg, 0.43 mmol), and 0.3 mL of AcOH were combined to give 76.8 mg of a crude yellow solid (86% pure as determined by HPLC); HRMS (ESI$^+$) $[M + H]^+$ calculated for $C_{17}H_{21}N_2{}^{15}N_2O_6$ 379.1396, observed 379.1401.

*5-Deazariboflavin (**8**).* 5-Deazariboflavin (88% pure as determined by HPLC) was synthesized as previously described[28] and was a generous gift from Dr. Seoung Ryoung-Choi. HRMS (ESI$^+$) $[M + H]^+$ calculated for $C_{18}H_{22}N_3O_6$ 376.1503, observed 376.1505.

Expression and Purification of Homo sapiens (hs) Riboflavin Kinase (RFK). *E. coli* BL21 (DE3) cells were transformed with *Homo sapiens* (*hs*) riboflavin kinase plasmid (HS_RFK_EC_1_pQE-T7) containing the gene for RFK with a N-terminal His_6 affinity tag (Qiagen). Starting from a single colony, the cells were grown in 1 L of MDG [Luria Broth[34] + 1% glucose] containing 34 μg/mL kanamycin at 37 °C, 250 rpm to $OD_{600} \approx 0.6$. Expression of the protein was induced by addition of isopropyl-β-D-thiogalactoside (IPTG, final concentration of 1 mM). Incubation was continued for 4 h at 37 °C. The cells were harvested by centrifugation (6000*g*, 25 min, 4 °C) and stored at −80 °C until used. A frozen cell pellet was suspended in lysis buffer (50 mM sodium phosphate, pH 8.0, containing 300 mM NaCl and 10 mM imidazole), and the cells were lysed by incubation with lysozyme, one protease inhibitor tablet (Roche), and DNase I (2 mg) on ice for 30 min followed by sonication (6 cycles of 30 s, 1 min cooling on ice). The cell lysate was centrifuged (12 000 rpm, 25 min, 4 °C), and the resulting supernatant was mixed with 10 mL of Ni-NTA Agarose resin (Qiagen) and loaded onto a column. The resin was washed with 80 mL of 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and 20 mM imidazole and then eluted with 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and 500 mM imidazole. Fractions were analyzed by SDS gel electrophoresis, and those containing RFK were pooled, concentrated by centrifugation with a 10 kDa MWCO filter (Centriprep, Millipore), and dialyzed against 10 mM Tris-HCl buffer, pH 8, containing 10% glycerol. Protein concentration was determined by the BCA assay (Pierce).

FMN. In a 100 mL round bottomed flask, crude riboflavin **7** (25 mg, 0.066 mmol, 0.66 mM) and ATP (275.57 mg, 0.5 mmol, 5 mM) were incubated with riboflavin kinase (18.84 mg, 1 μmol, 10 μM) in 100 mL of buffer (100 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.9) containing 5 mM $MgCl_2$ for 12 h at 30 °C. After incubation, the enzyme was removed by filtration (Millipore Centricon, 10 000 molecular weight cutoff (MWCO)). The solvent was then removed under lyophilization, and the solid was purified using high performance liquid chromatography equipped with an RP-C18 column to give 19.2 mg of a yellow solid; ^{1}H NMR (300 MHz, D_2O) δ ppm 7.69 (s, 1H), 7.48 (s, 1H), 4.97 (t, *J* = 11.7 Hz, 1H), 4.6 (d, *J* = 13.8 Hz, 1H), 4.34−4.31 (m, 1H), 4.11−3.97 (m, 4H), 2.45 (s, 3H), 2.31 (s, 3H); ^{13}C NMR (75 MHz, D_2O) δ ppm 160.8, 157.8, 150.8, 149.8, 139.6, 134.2, 133.7, 131.6, 130.3, 117.1, 72.6, 71.4 (d, J_{C-P} = 7.95 Hz), 69.3, 66, 47.6, 20.9, 18.7; ^{31}P NMR (121 MHz, D_2O) δ ppm 2.72; HRMS (ESI$^-$) calculated for $C_{17}H_{20}N_4O_9P$ $(M - H)^-$ *m*/*z* = 455.0973, found 455.0971.

[5-^{15}N]FMN. Following the procedure described for FMN; crude [**5-^{15}N**]**7** (10 mg, 0.0265 mmol, 0.53 mM) and ATP (137.785 mg, 0.25 mmol, 5 mM) were incubated with riboflavin kinase (9.423 mg, 0.5 μmol, 10 μM) in 50 mL of buffer containing 5 mM $MgCl_2$ and purified by HPLC to give 8.94 mg (98%) of a yellow solid; ^{1}H NMR (500 MHz, D_2O) δ ppm 7.77 (s, 1H), 7.61 (s, 1H), 5.04 (t, *J* = 10 Hz, 1H), 4.69 (d, *J* = 15 Hz, 1H), 4.39−4.36 (m, 1H), 4.16−3.99 (m, 4H), 2.51 (s, 3H), 2.38 (s, 3H); ^{13}C NMR (125 MHz, D_2O) δ ppm 163.5 (d, $^2J_{C-N}$ = 6.3 Hz), 160.3, 153.3, 152.4, 142, 136.8, 136.4 (d, J_{C-N} = 4.28 Hz), 134.2, 132.9 (d, $^2J_{C-N}$ = 7.56 Hz), 119.5, 75, 73.8 (d, J_{C-P} = 7.56 Hz), 71.8, 50.1, 23.4, 21.2; ^{31}P NMR (121 MHz, D_2O) δ ppm 1.92; HRMS (ESI$^-$) calculated for $C_{17}H_{20}{}^{14}N_3{}^{15}NO_9P$ $(M - H)^-$ *m*/*z* = 456.0938, found 456.0950.

[5-^{15}N, 4a-^{13}C]FMN. Following the procedure described for FMN; crude [**5-^{15}N, 4a-^{13}C**]**7** (10 mg, 0.0264 mmol, 0.53 mM) and ATP (137.785 mg, 0.25 mmol, 5 mM) were incubated with riboflavin kinase (9.423 mg, 0.5 μmol, 10 μM) in 50 mL of MOPS buffer containing 5 mM $MgCl_2$ and purified by HPLC to give 7.19 mg (98% yield) of a yellow solid; ^{1}H NMR (500 MHz, D_2O) δ ppm 7.76 (s, 1H), 7.60 (s, 1H), 5.03 (t, *J* = 10 Hz, 1H), 4.68 (d, *J* = 15 Hz, 1H), 4.38−4.35 (m, 1H), 4.15−3.97 (m, 4H), 2.49 (s, 3H), 2.37 (s, 3H); ^{13}C NMR (125 MHz, D_2O) δ ppm 163.6 (dd, $^1J_{C-C}$ = 76.1 Hz, $^2J_{C-N}$ = 7.56 Hz), 160.3, 153.2, 152.4 (d, $^1J_{C-C}$ = 55.44 Hz), 142, 136.8, 136.4 (d, J_{C-N} = 3.78 Hz), 134.2 (d, $^3J_{C-C}$ = 6.17 Hz), 132.9 (t, $^3J_{C-C}$ = 6.3 Hz), 129, 119.5, 75, 73.8 (d, J_{C-P} = 7.56 Hz), 71.8, 68.8, 50, 23.3, 21.2; ^{31}P NMR (121 MHz, D_2O) δ ppm 1.95; HRMS (ESI$^-$) calculated for $C_{16}{}^{13}CH_{20}{}^{14}N_3{}^{15}NO_9P$ $(M - H)^-$ *m*/*z* = 457.0972, found 457.0971.

[4a-^{13}C]FMN. Following the procedure described for FMN; crude [**4a-^{13}C**]**7** (10 mg, 0.0265 mmol, 0.53 mM) and ATP (137.785 mg, 0.25 mmol, 5 mM) were incubated with riboflavin kinase (9.423 mg, 0.5 μmol, 10 μM) in 50 mL of buffer containing 5 mM $MgCl_2$ and purified by HPLC to give 9.38 mg (97% yield) of a yellow solid; ^{1}H NMR (500 MHz, D_2O) δ ppm 7.74 (s, 1H), 7.56 (s, 1H), 5.01 (t, *J* = 10 Hz, 1H), 4.65 (d, *J* = 15 Hz, 1H), 4.38−4.34 (m, 1H), 4.13−3.98 (m, 4H), 2.48 (s, 3H), 2.35 (s, 3H); ^{13}C NMR (125 MHz, D_2O) δ ppm 163.4 (d, $^1J_{C-C}$ = 76.86 Hz), 160.3, 153.2, 152.3 (d, $^1J_{C-C}$ = 55.44 Hz), 142, 136.7, 136.3, 134.1 (d, $^3J_{C-C}$ = 6.04 Hz), 132.8 (d, $^3J_{C-C}$ = 6.1 Hz), 128.8, 119.5, 75, 73.8 (d, J_{C-P} = 7.2 Hz), 71.8, 68.6, 50, 23.3, 21.2; ^{31}P NMR (121 MHz, D_2O) δ ppm 1.85; HRMS (ESI$^-$) calculated for $C_{16}{}^{13}CH_{20}N_4O_9P$ $(M - H)^-$ *m*/*z* = 456.1001, found 456.0995.

[1,3-$^{15}N_2$]FMN. Following the procedure described for FMN; crude [**1,3-$^{15}N_2$**]**7** (10 mg, 0.0265 mmol, 0.53 mM) and ATP (137.785 mg, 0.25 mmol, 5 mM) were incubated with riboflavin kinase (9.423 mg, 0.5 μmol, 10 μM) in 50 mL of buffer containing 5 mM $MgCl_2$ and purified by HPLC to give 10.66 mg (>98% yield) of a yellow solid; ^{1}H NMR (500 MHz, D_2O) δ ppm 7.75 (s, 1H), 7.57 (s, 1H), 5.02 (t, *J* = 10 Hz, 1H), 4.67 (d, *J* = 15 Hz, 1H), 4.39−4.36 (m, 1H), 4.17−3.99 (m, 4H), 2.50 (s, 3H), 2.36 (s, 3H); ^{13}C NMR (125 MHz, D_2O) δ ppm 163.4 (d, J_{C-N} = 13.86 Hz), 160.3 (t, J_{C-N} = 11.0 Hz), 153.3, 152.3 (d, J_{C-N} = 7.93 Hz), 142, 136.8, 136.3, 134.1, 132.9, 119.5, 75, 73.8 (d, J_{C-P} = 7.56 Hz), 71.8, 68.8, 50.1, 23.5, 21.2; ^{31}P NMR (121 MHz, D_2O) δ ppm 1.9; HRMS (ESI$^-$) calculated for $C_{17}H_{20}{}^{14}N_2{}^{15}N_2O_9P$ $(M - H)^-$ *m*/*z* = 457.0909, found 455.0913.

*5-deazaFMN (**9**).* Following the procedure described for FMN; crude **8** (12 mg, 0.032 mmol, 0.53 mM) and ATP (137.785 mg, 0.25 mmol, 5 mM) were incubated with riboflavin kinase (9.423 mg, 0.5 μmol, 10 μM) in 50 mL of buffer containing 5 mM $MgCl_2$ and purified by HPLC to give 13 mg (>98% yield) of a yellow solid;[35] ^{1}H NMR (500 MHz, D_2O) δ ppm 7.78 (s, 1H), 7.45 (s, 1H), 7.03 (s, 1H), 4.27−3.97 (m, 7H), 2.3 (s, 3H), 2.12 (s, 3H); ^{13}C NMR (125 MHz, D_2O) δ ppm 164.7, 161, 158.7, 152.1, 143.6, 141.2, 138.9, 132.6, 121.6, 119.5, 113.3, 74.9, 74 (d, J_{C-P} = 7.3 Hz), 72.3, 68.8, 49.3, 23.2, 20.8; ^{31}P NMR (121 MHz, D_2O) δ ppm 2.5; HRMS (ESI$^-$) calculated for $C_{18}H_{21}N_3O_9P$ $(M - H)^-$ *m*/*z* = 454.1015, found 454.1016.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b00640.

^{1}H, ^{13}C, ^{31}P NMR spectral data of the unlabeled, labeled intermediates, unlabeled FMN, and labeled FMNs [**5-^{15}N**]**FMN**, [**4a-^{13}C**]**FMN**, [**5-^{15}N,4a-^{13}C**]**FMN**, [**1,3-$^{15}N_2$**]**FMN**, and **5-deazaFMN** (Figures S3−S31); analytical and prep HPLC chromatograms (Figures S32−S35) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: poulter@chem.utah.edu.

Notes

The authors declare no competing financial interest.

DOI: 10.1021/acs.joc.6b00640
J. Org. Chem. 2016, 81, 5087−5092

■ ACKNOWLEDGMENTS

We thank Dr. Jack Skalicky for the assistance with NMR spectroscopy and Dr. James Muller and Dr. Krishna Parsawar for the mass spectrometry measurements. We also thank Dr. Seoung Ryoung-Choi for providing 5-deazariboflavin. This work was supported by NIH Grant GM 25521 from the Institute for General Medical Sciences (CDP).

■ REFERENCES

(1) Walsh, C. *Acc. Chem. Res.* **1980**, *13*, 148−155.
(2) Edwards, A. M. In *Flavins and Flavoproteins: Methods and Protocols, Methods in Molecular Biology*; Weber, S., Schleicher, E., Eds.; Springer Science + Business Media: New York, 2014; Vol. *1146*, pp 3−14.
(3) Massey, V. *Biochem. Soc. Trans.* **2000**, *28*, 283.
(4) Briggs, W. R. In *Flavins: Photochemistry and Photobiology*; Silva, E., Edwards, A. M., Eds.; RSC: Cambridge, 2006; pp 183−216.
(5) Teufel, R.; Miyanaga, A.; Michaudel, Q.; Stull, F.; Louie, G.; Noel, J. P.; Baran, P. S.; Palfey, B.; Moore, B. S. *Nature* **2013**, *503*, 552.
(6) Moglich, A.; Yang, X.; Ayers, R. A.; Moffat, K. *Annu. Rev. Plant Biol.* **2010**, *61*, 21−47.
(7) De Ruyck, J.; Janczak, M. W.; Neti, S. S.; Rothman, S. C.; Schubert, H. L.; Cornish, R. M.; Matagne, A.; Wouters, J.; Poulter, C. D. *ChemBioChem* **2014**, *15*, 1452−1458.
(8) Makarov, V.; et al. *Science* **2009**, *324*, 801−804.
(9) Chaiyen, P.; Fraaije, M. W.; Mattevi, A. *Trends Biochem. Sci.* **2012**, *37*, 373−380.
(10) Rhead, W.; Roettger, V.; Marshali, T.; Amendt, B. *Pediatr. Res.* **1993**, *33*, 129.
(11) Ghisla, S.; Massey, V. *Biochem. J.* **1986**, *239*, 1.
(12) Chaiyen, P.; Sucharitakul, J.; Svasti, J.; Entsch, B.; Massey, V.; Ballou, D. P. *Biochemistry* **2004**, *43*, 3933.
(13) Ballou, D. P.; Massey, V. *Biochemistry* **1997**, *36*, 15713.
(14) Haines, D. C.; Sevrioukova, I. F.; Peterson, J. A. *Biochemistry* **2000**, *39*, 9419−9429.
(15) Mehlhorn, J.; Steinocher, H.; Beck, S.; Kennis, J. T. M.; Hegemann, P.; Mathes, T. *PLoS One* **2013**, *8*, e79006.
(16) Muller, F. In *Flavins and Flavoproteins: Methods and Protocols, Methods in Molecular Biology*, Weber, S., Schleicher, E., Eds.; Springer Science + Business Media: New York, 2014; Vol. *1146*, pp 229−306.
(17) Haigney, A.; Lukacs, A.; Zhao, R. K.; Stelling, A. L.; Brust, R.; Kim, R. R.; Kondo, M.; Clark, I.; Towrie, M.; Greetham, G. M.; Illarionov, B.; Bacher, A.; Römisch-margl, W.; Fischer, M.; Meech, S. R.; Tonge, P. J. *Biochemistry* **2011**, *50*, 1321−1328.
(18) Lukacs, A.; Haigney, A.; Brust, R.; Zhao, R. K.; Stelling, A. L.; Clark, I. P.; Towrie, M.; Greetham, G. M.; Meech, S. R.; Tonge, P. J. *J. Am. Chem. Soc.* **2011**, *133*, 16893.
(19) Fleischmann, G.; Lederer, F.; Muller, F.; Bacher, A.; Rüterjans, H. *Eur. J. Biochem.* **2000**, *267*, 5156−5167.
(20) Bacher, A.; Illarionov, B.; Eisenreich, W.; Fischer, M. In *Flavins and Flavoproteins: Methods and Protocols, Methods in Molecular Biology*; Weber, S., Schleicher, E., Eds.; Springer Science + Business Media: New York, 2014; Vol. *1146*, pp 65−78.
(21) Tishler, M.; Pfister, K., III; Babson, R. D.; Ladenburg, K.; Fleming, A. *J. Am. Chem. Soc.* **1947**, *69*, 1487−1492.
(22) Haase, I.; Grawert, T.; Illarionov, B.; Bacher, A.; Fischer, M. In *Flavins and Flavoproteins: Methods and Protocols, Methods in Molecular Biology*; Weber, S., Schleicher, E., Eds.; Springer Science + Business Media: New York, 2014; Vol. *1146*, pp 15−40.
(23) Illarionov, B.; Zhu, F.; Eisenreich, W.; Bacher, A.; Weber, S.; Fischer, M. *J. Org. Chem.* **2015**, *80*, 2539−2544.
(24) Mishanina, T. V.; Kohen, A. *J. Labelled Compd. Radiopharm.* **2015**, *58*, 370−375.
(25) Kasai, S.; Nakano, H.; Maeda, K.; Matsui, K. *J. Biochem.* **1990**, *107*, 298−303.
(26) Nielsen, P.; Rauschenbach, P.; Bacher, A. *Methods Enzymol.* **1986**, *122*, 209.
(27) Dickey, J. B.; Gray, A. R. *Organic Syntheses* **1943**, *2*, 60.
(28) Carlson, E. E.; Kiessling, L. L. *J. Org. Chem.* **2004**, *69*, 2614−2617.
(29) Heyl, D.; Chase, E. C.; Koniuszy, F.; Folkers, K. *J. Am. Chem. Soc.* **1951**, *73*, 3826−3827.
(30) Muller, F.; Vervoort, J. J.; Lee, J.; Horowitz, M.; Carreira, L. A. *J. Raman Spectrosc.* **1983**, *14*, 106−117.
(31) Grande, H. J.; Gast, R.; van Schagen, C. G.; van Berkel, W. J. H; Muller, F. *Helv. Chim. Acta* **1977**, *60*, 367−379.
(32) Fisher, J.; Spencer, R.; Walsh, C. *Biochemistry* **1976**, *15*, 1054−1064.
(33) Calhoun, K. A.; Swartz, J. R. *Methods Mol. Biol.* **2007**, *375*, 3−17.
(34) Bertani, G. J. *Bacteriology* **1951**, *62*, 293−300.
(35) Hersh, L. B.; Walsh, C. *Methods Enzymol.* **1980**, *66*, 277−287.

DOI: 10.1021/acs.joc.6b00640
J. Org. Chem. 2016, 81, 5087−5092