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Improved Chemical Syntheses of 1- and 5-Deazariboflavin

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Abstract: The cofactor flavin adenine dinucleotide (FAD) is required for the catalytic activity of a large class of enzymes known as flavoenzymes. Because flavin cofactors participate in catalysis via a number of different mechanisms, isoalloxazine analogues are valuable for mechanistic studies. We report improved chemical syntheses for the preparation of the two key analogues, 5-deazariboflavin and 1-deazariboflavin.

The exceptional chemistry of the flavin cofactor allows flavoenzymes to play a wide variety of roles in vivo. These proteins participate in many biological processes including nitric oxide generation, photosynthesis, soil detoxification, and even apoptosis.[1] The diversity of functions performed by flavoproteins is due to the ability of the isoalloxazine ring system to participate in an assortment of catalytic mechanisms. It can perform redox and radical chemistry, act as a nucleophile or electrophile, or simply play a structural role.[1,2] With this range of potential functions, discerning the catalytic role of a flavin cofactor is often difficult.

Flavin analogues are invaluable tools for elucidating the role of the cofactor.[1,3] Most flavin adenine dinucleotide (FAD) analogues have been obtained through the chemical synthesis of the corresponding riboflavin derivatives, followed by coupling to the nucleotide diphosphate mediated by FAD synthetase to yield the full FAD analogue.[4] Previously, both 5-deazariboflavin **1**[5,6] and 1-deazariboflavin **2**[6,7] have been synthesized[3,8] and utilized in chemical and biological settings (Figure 1). Some analogues, however, such as 1-deazariboflavin, are seldom employed because of the difficulty of their synthesis. We found key

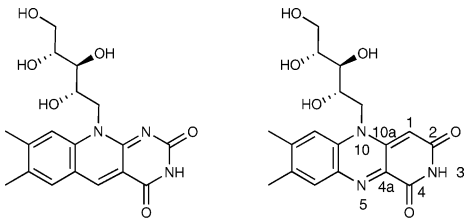

FIGURE 1. Flavin analogues are useful in the elucidation of the cofactor's role in catalysis.

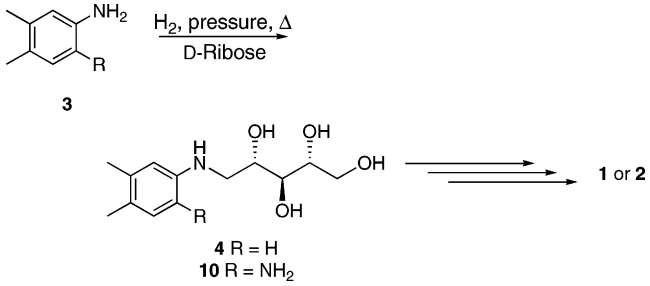

FIGURE 2. Coupling of ribose and **3** provides precursors to both analogues **1** and **2**. Previous syntheses required high pressure and temperature hydrogenation to perform this coupling.

steps within the routes to **1** and **2** to be irreproducible. To overcome these limitations, we developed new routes that afford efficient and reproducible syntheses of both analogues.

The syntheses of both **1** and **2** proceed via intermediates **4** and **10**, which possess common features (Figure 2). In previous routes, syntheses of these intermediates required high temperature and pressure hydrogenation steps, which are difficult to carry out on larger scales, and require specialized equipment (Figure 2).[3] To develop a more convenient route, we surveyed a series of reductive amination conditions. The use of $NaCNBH_3$ in refluxing methanol was found to be optimal, giving the ribitylated aniline **4** in a 90% yield and **10** in a 92% yield.

The published methods for conversion of **4** (or **10**) to compound **1** (or **2**) involved other problematic steps. Although several syntheses of compound **1** were published around 1970,[8] a number of alternative pathways have since appeared in the literature[9] because the key coupling step between intermediate **4** and 6-chlorouracil[10] was irreproducible (Figure 3). Typically, this reaction is run at high temperature and without any type of base or catalyst. We determined that a catalytic amount of

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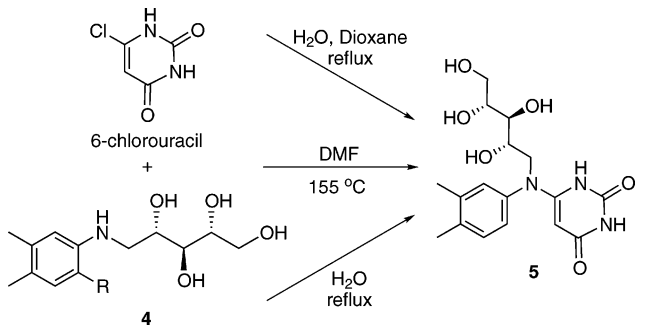
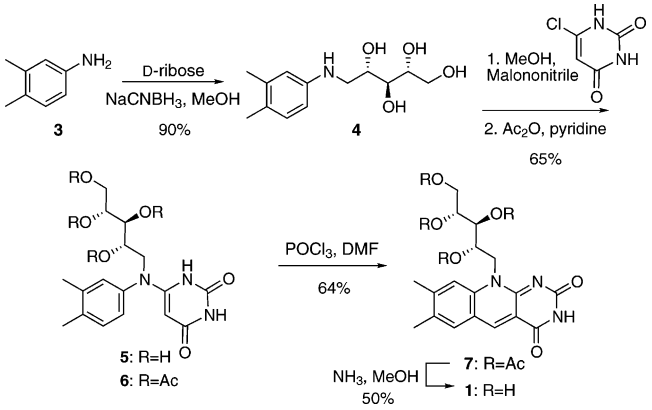


FIGURE 3. Development of reliable conditions for the key coupling reaction involved in the synthesis of **1** has been an elusive goal.

SCHEME 1



malononitrile, which has been reported to catalyze couplings with conjugated vinyl halides,[11] in refluxing methanol is required for efficient and reproducible coupling to form the functionalized uracil, **5** (Scheme 1).

Purification of **5** proved difficult as a result of its high polarity and acid sensitivity. To circumvent this problem, the crude material **5** was acetylated, and the product was isolated by extraction to give protected uracil **6** in a 65% yield over two steps. The last two steps of the synthesis of compound **1** were carried out as previously reported.[12] Treatment of compound **6** with the Vilsmeier reagent followed by removal of the acetate protecting groups provided crude 5-deazariboflavin, **1**. Purification by high performance liquid chromatography (HPLC) provided the yellow solid **1** as the trifluoroacetic acid (TFA) salt, in 50% yield (19% overall).

The synthesis of compound **2** also presented several challenges. As previously mentioned, the reductive amination conditions developed for the synthesis of **1** were effective en route to **2**. However, established strategies employed aniline **4**, which can be converted to the ribitylated intermediate **10** via further aromatic functionalization (Figure 4).[13] To avoid this late-stage functionalization, the commercially available diamine **8** was utilized. Initial attempts to monoribitylate the free

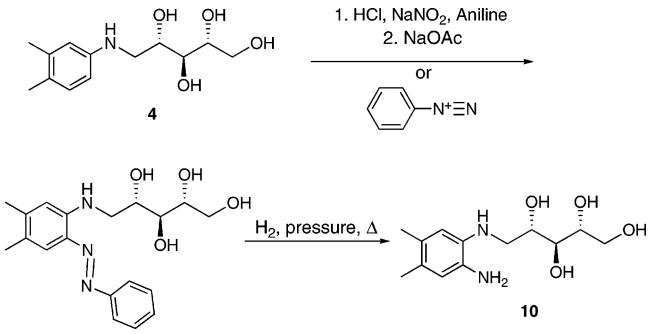
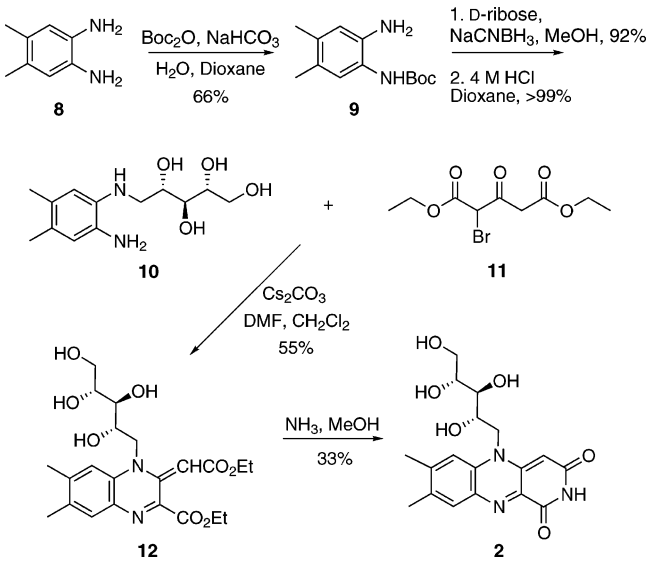


FIGURE 4. Previous methods used to introduce an *ortho*-amino group into **4**.

SCHEME 2



aniline proved problematic. When compound **8** was desymmetrized with *tert*-butyloxycarbonyl anhydride (Boc_2O), however, the protected aniline **9** was obtained in 66% yield (Scheme 2). Next, D-ribose was coupled with aniline **9** by reductive amination to afford the desired product in a 92% yield. The *t*-Boc protecting group was removed using HCl−dioxane to give intermediate **10** in near quantitative yield.

In previous syntheses, the formation of bicyclic intermediate **12** from compounds **10** and **11** was extremely low yielding (19%).[9] These routes utilized potassium carbonate as a base, which is rather insoluble in organic solvents. When cesium carbonate in a mixture of dimethylformamide and dichloromethane was employed, yields up to 55% of **12** were obtained. The isoalloxazine ring system was completed by stirring intermediate **12** in methanolic ammonia to give 1-deazariboflavin, **2**. The crude material was purified by HPLC to give the TFA salt of **2** as a purple solid in 33% yield (11% overall).

The routes described here address each of the problematic steps in previous syntheses of flavin analogues **1** and **2**. The resulting strategies are more efficient and alleviate the need for specialized equipment. Thus, they can readily be carried out on a multigram scale. Most importantly, the reactions are reproducible. We anticipate that the results presented here will facilitate the syntheses of flavin analogues for chemical and biological studies.

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Experimental Section

Synthesis of 4. Aniline **3** (3.0 g, 26 mmol), D-ribose (11.2 g, 74.3 mmol), and sodium cyanoborohydride (3.11 g, 49.5 mmol) were dissolved in methanol (150 mL), and the mixture was heated to 65 °C for 48 h. Solvent was removed under reduced pressure, and the residue was dissolved in 1 M HCl (50 mL) and swirled until gas evolution ceased. The solution was carefully neutralized using aqueous saturated sodium bicarbonate solution, and then the mixture was extracted with ethyl acetate (6 × 50 mL). The combined organic layers were washed with brine and dried with magnesium sulfate, and the solvent was removed under reduced pressure to yield **4** (5.67 g, 90%) as a white solid: ^{1}H NMR (300 MHz, d_3-MeOD) δ 6.88 (d, 1 H, J = 7.8 Hz), 6.55 (d, 1 H, J = 2.4 Hz), 6.47 (dd, 1 H, J = 7.8, 2.4 Hz), 3.82−3.91 (m, 1 H), 3.72−3.81 (m, 2 H), 3.60−3.67 (m, 2 H), 3.43 (dd, 1 H, J = 12.6, 3.6 Hz), 3.09 (dd, 1 H, J = 12.6, 8.1 Hz), 2.17 (s, 3 H), 2.12 (s, 3 H) ppm; ^{13}C NMR δ 148.1, 138.1, 131.2, 126.9, 116.9, 112.6, 74.9, 74.5, 72.3, 64.8, 20.9, 20.2, 18.9 ppm; ESI (m/z) [M + H] calcd for $C_{13}H_{21}NO_4$ 256.2, found 256.2.

Synthesis of 6. Ribitylated aniline **4** (300 mg, 1.18 mmol), 6-chlorouracil (207 mg, 1.41 mmol), and malononitrile (23 mg, 0.35 mmol) (a higher mol % of catalyst was necessary for gram-scale reactions) were suspended in dry methanol (5 mL) and heated at reflux for 48 h. Solvent was removed under reduced pressure, the crude material **5** was dissolved in pyridine (5 mL) and acetic anhydride (554 μL, 5.90 mmol), and the mixture was allowed to stir at room temperature for 1 h. The solvent was removed under reduced pressure, and the resulting residue was dissolved in dichloromethane (20 mL) and washed with water (10 mL) and brine (10 mL). The organic layer was dried with magnesium sulfate and filtered, and the solvent was removed under reduced pressure. The residue was recrystallized from hexanes and dichloromethane, resulting in the orange solid **6** (408 mg, 65% over two steps): ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.90 (br s, 1 H), 8.08 (br s, 1 H), 7.10−7.30 (m, 1 H), 6.86−7.04 (br s, 3 H), 5.09−5.42 (m, 3 H), 4.90 (s, 1 H), 4.29 (dd, 1 H, J = 12.3, 3.0 Hz), 4.00−4.12 (m, 2 H), 3.75 (dd, 1 H, J = 15.3, 2.7 Hz), 2.29 (s, 3 H), 2.28 (s, 3 H), 2.10 (s, 3 H), 2.02 (s, 3 H), 2.01 (s, 3 H), 1.91 (s, 3 H) ppm; ^{13}C NMR δ 170.4, 170.2, 169.9, 169.5, 145.4, 137.1, 130.2, 125.8, 114.8, 110.3, 70.9, 70.2, 69.6, 61.8, 43.9, 20.7, 20.7, 20.6, 20.5, 19.8, 18.5 ppm; ESI (m/z) [M + Na] calcd for $C_{25}H_{31}N_3O_{10}$ 556.2, found 556.2.

Synthesis of 7. Bicyclic compound **6** (360 mg, 0.680 mmol) was dissolved in DMF (1.2 mL) to which phosphorus oxychloride (119 μL, 1.28 mmol) was added dropwise. This solution was allowed to stir at room temperature for 30 min and then heated at 100 °C for 15 min. Ice was added, and the solution was adjusted to pH ∼6 with ammonium hydroxide. This solution was stirred at room temperature for 30 min, resulting in precipitation of product. Filtration resulted in the orange solid **7** (234 mg, 64%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.76 (s, 1 H), 8.46 (s, 1 H), 7.57 (s, 2 H), 5.58−5.64 (m, 1 H), 5.39−5.42 (m, 2 H), 4.40 (dd, 1 H, J = 12.0, 2.7 Hz), 4.22 (dd, 1 H, J = 12.0, 5.4 Hz), 2.51 (s, 3 H), 2.38 (s, 3 H), 2.26 (s, 3 H), 2.18 (s, 3 H), 2.04 (s, 3 H), 1.71 (s, 3 H) ppm; ^{13}C NMR δ 170.4 170.1, 169.8, 169.5, 161.6, 158.1, 155.0, 142.1, 139.3, 134.5, 131.4, 119.7, 116.4, 113.8, 70.4, 69.7, 69.1, 61.8, 44.7, 21.2, 20.8, 20.6, 20.5, 20.1, 19.0 ppm; ESI (m/z) [M + Na] calcd for $C_{26}H_{29}N_3O_{10}$ 566.2, found 566.2.

Synthesis of 5-Deazariboflavin (1). Compound **7** (32 mg, 0.059 mmol) was dissolved in methanolic ammonia (3 mL) and allowed to stir overnight. Solvent was removed under reduced pressure, and the residue was purified by using HPLC (100% → 50% A:B over 20 min, retention time about 16 min) to give 11 mg, a 50% yield of the yellow solid **1** as the trifluoroacetic acid (TFA) salt: mp > 300° (dec); ^{1}H NMR (300 MHz, d_6-DMSO) δ 11.06 (s, 1 H), 8.81 (s, 1 H), 7.93 (s, 1 H), 7.83 (s, 1 H), 4.90 (m, 1 H), 4.63 (d, 1 H, J = 13.5 Hz), 4.22 (m, 1 H), 3.72−3.59 (m, 3 H), 3.49−3.43 (m, 1 H), 2.45 (s, 3 H), 2.32 (s, 3 H) ppm; ^{13}C NMR δ 162.2, 157.5, 156.3, 146.0, 141.0, 140.0, 133.7, 130.6, 119.7, 117.9, 113.7, 73.7, 72.9, 69.6, 63.5, 47.3, 21.0, 18.7 ppm; ESI (m/z) [M + Na] calcd for $C_{18}H_{29}N_3O_{10}$ 398.1, found 398.1.

Synthesis of 9. Aniline **8** (1.0 g, 7.3 mmol), *tert*-butyl benzyloxy carbonyl anhydride (1.6 g, 7.3 mmol), and sodium bicarbonate (0.62 g, 7.3 mmol) were dissolved in dioxane (50 mL) and water (50 mL). The mixture was stirred for 5 h at room temperature. The reaction was diluted with water (150 mL) and then extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layers were washed with an aqueous solution of saturated bicarbonate and then brine, dried over magnesium sulfate, and concentrated under reduced pressure. The resulting red oil was purified by flash chromatography (silica, 4:1 hexanes/ethyl acetate) to yield **9** (1.1 g, 66%) as a pink solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.00 (s, 1 H), 6.54 (s, 1 H), 6.28 (br s, 1 H), 3.57 (br s, 2 H), 2.13 (s, 3 H), 2.12 (s, 3 H), 1.50 (s, 9 H) ppm; ^{13}C NMR δ 153.9, 137.5, 134.3, 127.5, 125.7, 122.3, 118.7, 80.1, 28.3, 19.2, 18.7 ppm; EMM (m/z) [M + H] calcd for $C_{13}H_{20}N_2O_2$ 237.1603, found 237.1613.

Synthesis of 10. Boc-protected aniline **9** (940 mg, 3.98 mmol), D-ribose (1.79 g, 11.9 mmol), and sodium cyanoborohydride (500 mg, 7.96 mmol) were dissolved in methanol (50 mL). The solution was heated to 65 °C degrees for 48 h. Solvent was removed under reduced pressure, and the residue was dissolved in 1 M HCl (10 mL) and swirled until gas evolution ceased. The solution was carefully neutralized using saturated sodium bicarbonate and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with brine (20 mL), dried with magnesium sulfate, and the solvent was removed under reduced pressure to give1.35 g, a 92% yield as a yellow/orange solid: ^{1}H NMR (300 MHz, d_3-MeOD) δ 6.87 (s, 1 H), 6.61 (s, 1 H), 3.97−3.62 (m, 5 H), 3.44 (dd, 1 H, J = 12.9, 3.3 Hz), 3.15 (dd, 1 H, J = 12.6, 7.8 Hz), 2.19 (s, 3 H), 2.13 (s, 3 H), 1.50 (s, 9 H) ppm; ^{13}C NMR δ 157.4, 142.8, 136.2, 128.9, 126.4, 123.1, 115.4, 81.1, 74.9, 74.5, 72.2, 64.8, 28.9, 20.0, 18.9 ppm; EMM (m/z) [M + Na] calcd for $C_{18}H_{30}N_2O_6$ 371.2182, found 371.2170. This compound (238 mg, 0.642 mmol) was dissolved in 4 M HCl in dioxane (11.6 mL), and the mixture was stirred at room temperature for 5 h. The dioxane was removed under reduced pressure, the residue was dissolved in water (100 mL), and the aqueous layer was washed with ether (3 × 20 mL). The water layer was removed by lyophilization to yield **10** as a brown foam in a near quantitative yield: ^{1}H NMR (300 MHz, d_3-MeOD) δ 6.77 (s, 1 H), 6.67 (s, 1 H), 3.37−3.43 (m, 1 H), 2.87−3.16 (m, 6 H), 1.58 (s, 3 H), 1.57 (s, 3 H) ppm; ^{13}C δ129.5, 120.2, 116.9, 75.0, 74.5, 72.1, 64.8, 19.5, 19.1 ppm; ESI (m/z) [M + Na] calcd for $C_{13}H_{22}N_2O_4$ 293.2, found 293.2.

Synthesis of 11. Diethyl 3-oxoglutarate (0.63 g, 3.1 mmol) was placed in a three necked round-bottom equipped with a condenser and two septa. Carbon dioxide gas was bubbled through the neat solution while it was heated at 65 °C. Bromine (320 μL, 6.30 mmol) was added slowly, and the solution was allowed to stir for 30 min. The reaction was cooled to room temperature and diluted with CH_2Cl_2 (20 mL). The organic solution was washed with 10% aqueous Na_2SO_3 (until color disappeared), then saturated sodium sulfate (10 mL), saturated sodium bicarbonate (10 mL), and brine (10 mL). The organic layer was dried with magnesium sulfate, and the solvent was removed under reduced pressure to yield a red oil. Crude **11** was used immediately, as purification or heat caused decomposition: ^{1}H NMR (mixture of mono and dibrominated products, 300 MHz, d_3-MeOD) δ 5.33−5.37 (m, 1 H), 4.28 (m, 4 H), 1.32 (m, 6 H) ppm; ESI (m/z) [M + Na] calcd for $C_9H_{13}BrO_5$ 303.0, found 303.0.

Synthesis of 12. Compounds **10** (179 mg, 0.583 mmol) and **11** (342 mg, 1.22 mmol) were dissolved in DMF (5 mL) and CH_2Cl_2 (6 mL). Cesium carbonate (570 mg, 1.75 mmol) was added to the reaction. After stirring at room temperature for 24 h, the solution was filtered and diluted with water (50 mL), and the aqueous layer was extracted (3 × 20 mL) with ethyl acetate. The organic layers were dried with magnesium sulfate, and the solvent was removed under reduced pressure. The resulting brown oil was purified with flash chromatography (silica, 3% MeOH in $CHCl_3$) to yield **12** (144 mg, 55%) as a brown oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.55 (s, 1 H), 8.24 (s, 1 H), 7.16 (br s, 1 H), 5.55 (br s, 1 H), 4.88−3.80 (m, 7 H), 4.54 (q, 2 H, J = 6.9 Hz), 4.20 (q, 2 H, J = 6.9 Hz), 2.64 (s, 3 H), 2.58 (s, 3 H), 1.46 (t, 3 H, J = 7.2 Hz), 1.23 (t, 3 H, J = 7.2 Hz) ppm; ^{13}C NMR δ 168.6, 162.9, 161.3, 153.0, 146.5, 145.6, 142.9, 131.3, 118.7, 117.7,

113.9, 73.0, 72.2, 71.6, 63.8, 62.8, 56.0, 37.7, 21.7, 20.1, 13.9, 13.7 ppm; ESI (*m*/*z*) [M + Na] calcd for $C_{22}H_{30}N_2O_8$ 473.2, found 473.2.

Synthesis of 1-Deazariboflavin (2). Compound **12** (25 mg, 0.056 mmol) was dissolved in ammonia-saturated methanol (3 mL), and the resulting solution was allowed to stir at room temperature for 48 h. Solvent was removed under reduced pressure. Crude material was purified by HPLC (100% → 60% A:B over 20 min, retention time about 18 min). This resulted in the purple solid **2** (7 mg, 33%) as the TFA salt: mp > 300° (dec); ^{1}H NMR (300 MHz, d_6-DMSO) δ 11.1 (s, 1 H, exchange D_2O), 7.61 (s, 1 H), 7.58 (s, 1 H), 7.22 (b s, 1H, D_2O exchange) 5.51 (s, 1 H), 5.20−3.95 (m, 7 H), 2.33 (s, 3 H), 2.23 (s, 3 H) ppm; ^{13}C NMR δ 164.4 (TFA), 159.9, 144.4, 142.1, 140.9, 133.1, 133.0, 132.5, 131.0, 115.9 (TFA), 86.7, 73.5, 72.9, 63.4, 48.2, 20.6, 18.6 ppm; ESI (*m*/*z*) [M + Na] calcd for $C_{18}H_{21}N_3O_6$ 398.1, found 398.0.

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Supporting Information Available: General experimental procedures and ^{1}H and ^{13}C NMR spectra for the complete syntheses of compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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