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Toward Catalytically Active Oligonucleotides: Synthesis of a Flavin Nucleotide and Its Incorporation into DNA

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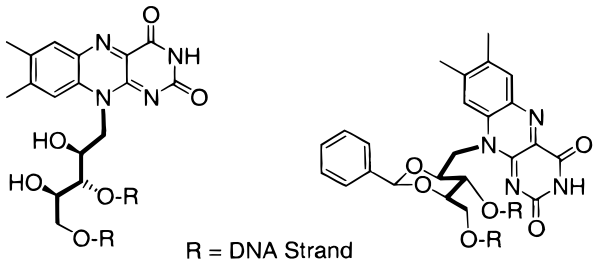
ABSTRACT



The synthesis of a vitamin B_2-derived flavin-nucleotide is described. A combined H-phosphonate/phosphoramidite protocol was developed for the first incorporation of flavin coenzymes into a DNA stack. The coenzyme–DNA is predicted to have novel biosensing and catalytic properties.

Coenzymes are small organic compounds which catalyze chemical reactions within the active site of coenzyme-dependent enzymes. The protein environment strictly controls the catalytic properties of the coenzyme by specific hydrogen bonding, π-stacking, and electrostatic forces.[1] These non-covalent interactions fine-tune the chemical reactivity of the coenzyme and enable the catalysis of specific biochemical reactions. Redox coenzymes such as FAD,[2] for example, require adjustment of the redox potential, which varies between different flavoproteins by several hundreds of millivolts. In this sense, the protein environment acts as a molecular device able to change the catalytic properties of the embedded coenzyme.

Because of the recent interest in developing novel biocatalysts based on oligonucleotide structures,[3] the incorporation of coenzymes into DNA or RNA deserves particular attention.[4] In such an oligonucleotide environment, specific hydrogen bonding and π-stacking contacts to nucleobases in proximity of the coenzyme could strongly modulate the catalytic properties.

If the coenzyme is able to function as an informational nucleobase in an oligonucleotide stack, its properties could become programmable by the choice of the counter base and the oligonucleotide sequence.[5] The potential to regulate the redox properties of coenzymes such as FAD within DNA or RNA could than be exploited for the design of bioanalytical devices.[6]

The FAD redox coenzyme contains as its central unit the riboflavin moiety **1** depicted in Figure 1. This riboflavin

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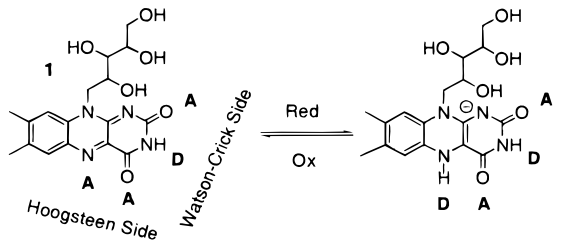


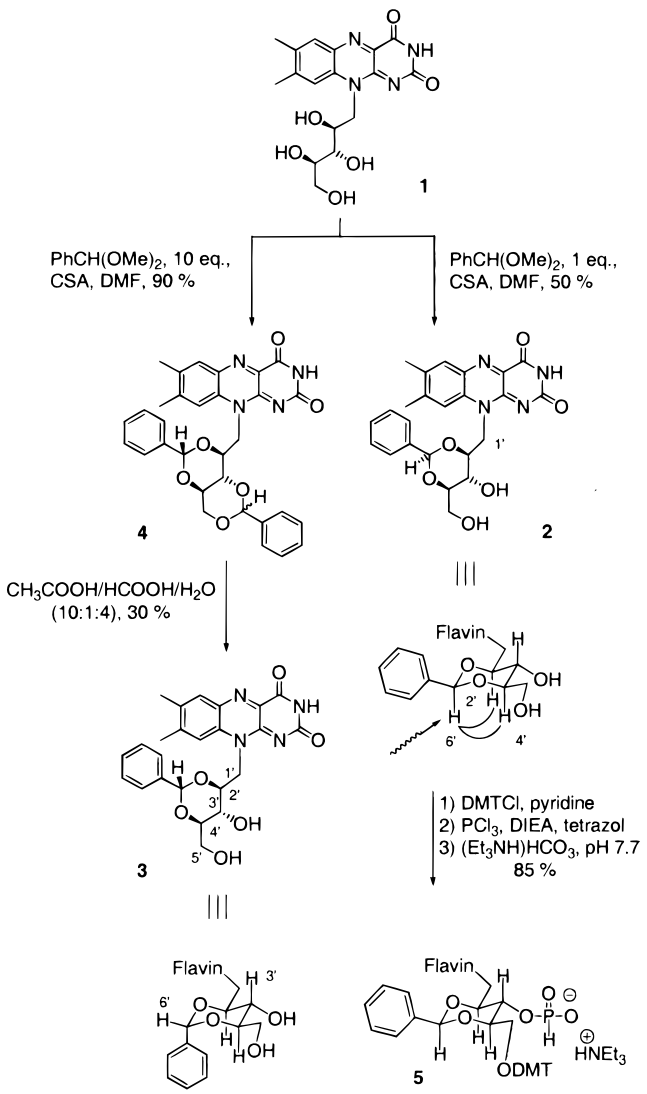
Figure 1. Hydrogen-bonding capabilities of riboflavin **1** in the oxidized and reduced state: **A**, hydrogen bond acceptor; **D**, hydrogen bond donor. Definition of the Hoogsteen and the Watson–Crick side of the flavin heterocycle.

possesses a hydrogen-bonding pattern (ADA) similar to that of the nucleobases thymidine or uridine at the "Watson–Crick" face of the flavin heterocycle. Changing the redox state of the coenzyme, however, influences the hydrogen-bonding pattern at the "Hoogsteen side", which switches from an AA pattern to a DA situation (Figure 1). Targeting this "Hoogsteen side" of the coenzyme with an opposite base is one potential possibility to modulate the redox and the fluorescence properties of flavin coenzymes in an oligonucleotide world.[7]

To achieve incorporation of a flavin unit into a base stack, we designed the benzylidene-protected riboflavin building block **2**, in which a 2′,4′-bridging benzylidene rigidifies the flexible ribityl chain of riboflavin. Computer modeling suggests that the diastereoisomer **2**, containing an all-equatorial substituted 1,3-dioxane ring, forces of the flavin nucleus in the DNA double helix with the "Hoogsteen side" toward the helix axis as the hydrogen-bonding face. The phenyl ring points in the all-equatorial diastereoisomer away from the double helix and avoids steric interference with the base-pairing process. Cleavage of the benzylidene group in the DNA strand allows for the synthesis of riboflavin (vitamin B_2) containing oligonucleotides. The synthesis of the monoprotected building blocks **2** and **3** is depicted in Scheme 1. Reaction of riboflavin **1** with an excess of benzaldehyde dimethyl acetal furnished bis-benzylidene-protected riboflavin **4** as a mixture of three diastereoisomers. Careful treatment of this mixture with a formic acid/acetic acid/water solution allowed deprotection of the 3′,5′-bridging benzylidene group and furnished monoprotected riboflavin compound **3** as a single diastereoisomer. Reaction of riboflavin **1** with just 1 equiv of benzaldehyde dimethyl acetal gave a mixture of the 3′,5′- and the 2′,4′-monoprotected riboflavin derivatives. The desired 2′,4′-benzylidene protected compound **2** was separated by chromatography and again obtained as a single diastereoisomer, which, however, was not identical with **3**. NOE experiments were performed in order to clarify the structures of compounds **2** and **3**.

In compound **2**, irradiation at the resonance frequency of the C(6′)H induces a strong NOE of the C(2′)H and of the

Scheme 1



C(4′)H as expected for an all-equatorial substituted 1,3-dioxane ring system. In contrast, diastereoisomer **3** showed a strong signal intensity enhancement of C(3′)H, which proves for this diatereoisomer an axial orientation of the phenyl ring at C(6′). For compound **2** we observe two C(1′)H broad and unresolved signals, which indicates hindered rotation of the flavin relative to the six-membered ring. This observation proves the desired rigidifying effect of the benzylidene group.

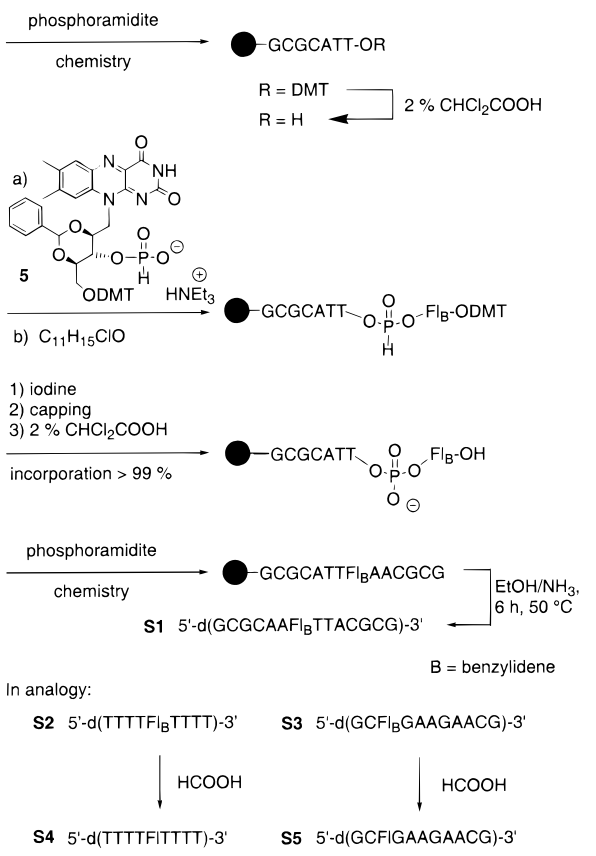
Conversion of the desired "all equatorial" 2′,4′-benzylidene-protected riboflavin **2** into the 5′-DMT 3′-H-phosphonate **5**, ready for incorporation into oligonucleotides by machine-assisted oligonucleotide synthesis, was possible using standard procedures. Because of the known rapid oxidation of a flavin phosphoramidite,[8] which excludes incorporation of such derivatives into oligonucleotides using standard phosphoramidite chemistry, a mixed phosphora-

(7) For the incorporation of related alloxazin nucleobases, see: (a) Pfleiderer, W.; Rösler, A. *Helv. Chim. Acta* **1997**, *80*, 1869–1881. (b) Wang, Z.; Rizzo, C. *Org. Lett.* **2000**, *2*, 227–230. (c) Chanteloup, L.; Thuong, N. T. *Tetrahedron Lett.* **1994**, *35*, 877–880.

(8) For the connection of a flavin to the 5′-end of an oligonucleotide, see: (a) Frier, C.; Décout, J.-L.; Fontacave, M. *J. Org. Chem.* **1997**, *62*, 3520–3528. (b) Frier, C.; Mouscadet, J.-F.; Décout, J.-L.; Auclair, C.; Fontecave, M. *Chem. Commun.* **1998**, 2457–2458.

midite/H-phosphonate/phosphoramidite protocol was developed with flavin H-phosphonate **5** (Scheme 2).

Scheme 2



To this end, all nucleotides at the 3′- and 5′-end of the incorporated flavin were coupled as PAC-protected nucleotides[9] using standard phosphoramidite chemistry. After DMT deprotection of the nucleotide to the 3′-side of the flavin, the synthesizer was programmed to pump simultaneously flavin H-phosphonate **5** and adamantyl acid chloride as the activating reagent.

After complete coupling, the H-phosphonate was oxidized with iodine to give an unprotected phosphodiester linkage. The phosphoramidite synthesis was continued after cleavage of the DMT protecting group of the flavin building block in the DNA strand.

The flavin-embedded DNA strands were subsequently cleaved from the support and fully deprotected with ammonia in a water/ethanol mixture at 50 °C for 6 h; purification was performed by reversed phase HPLC. Figure 2A shows the HPLC trace of a crude flavin−DNA sample, directly after cleavage from the solid support. Clearly evident is the high purity of the synthesized raw material. The inset shows the HPLC trace of the purified flavin-containing DNA strand. From the online trityl cation assay, indicating a coupling yield of the flavin H-phosphonate of 99%, and based upon these HPLC results, we calculated an incorporation yield of >98%.

(9) PAC amidites are commercially available from Pharmacia.

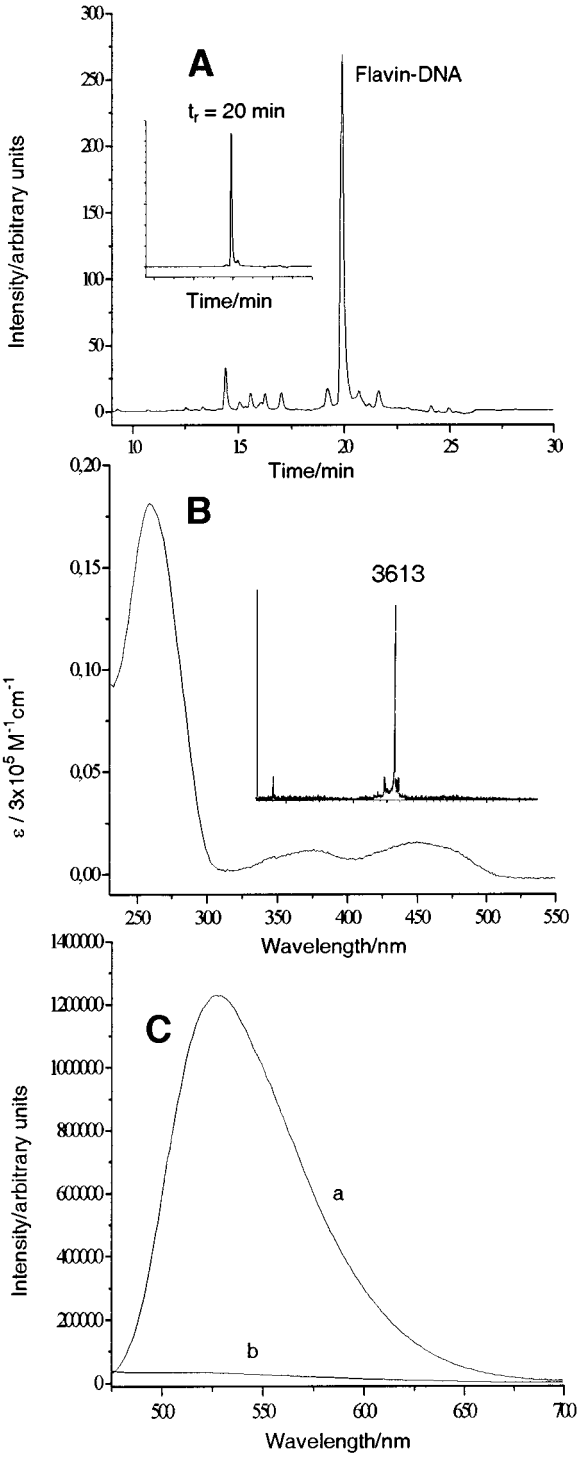


Figure 2. (A) HPL chromatogram of crude **S1** (260 nm). Inset: HPL chromatogram of purified **S1**. (B) UV spectrum of **S3**. Inset: MALDI-Tof MS of **S3**. (C) fluorescence spectra. (a) **S1** (MS: calcd 4434, found 4436). (b) **S3** (MS: calcd 3611, found 3613). MS for **S2**: calcd 2897, found 2898.

Figures 2B and 2C show the UV spectrum of oligonucleotide **S3** and the fluorescence spectra of **S1** and **S3** as examples. All spectra are identical with spectra recorded from riboflavin, proving the presence of the intact flavin chromo-

phor in the DNA environment. Additional support for the presence of the benzylidene-protected flavin building block was derived from MALDI-Tof mass spectra, which show as the exclusive signals the correct mass peaks of the benzylidene-protected flavin oligonucleotides. The inset in Figure 2B depicts as an example the MALDI-Tof spectrum of **S3**. These mass spectrometric data together with the HPLC results show that the benzylidene group is not cleaved during the DNA synthesis cycle, which includes treatment of the resin with dichloroacetic acid to achieve DMT deprotection.

Short treatment, however, of the benzylidene-protected flavin-containing oligonucleotide with formic acid caused rapid cleavage of the benzylidene group and furnished the unprotected riboflavin-containing DNA strands. HPLC monitoring of the deprotection reaction proved a clean educt to product conversion.

The fluorescence spectrum of the flavin inside the oligonucleotide environment was found to be strongly environment dependent (Figures 2Ca and 2Cb). We observe a strong flavin fluorescence at 520 nm only if we exclude guanosine (G) nucleobases in close proximity to the flavin. The fluorescence of the flavin is almost fully quenched in strands such as **S3**, possibly due to an electron transfer from the G residue to the photoexcited flavin and formation of a G radical cation ($G^{\bullet+}$).[10]

Although several reports describe light-induced DNA strand breaks in the presence of a flavin[11] (possibly due to initial $G^{\bullet+}$ formation), the flavin-embedded DNA strands are surprisingly stable. We observe no DNA cleavage if the flavin-containing oligonucleotides are handled in the laboratory under normal conditions. This surprising fact now allows detailed investigation of the pairing properties of a riboflavin and of the benzylidene-rigidified flavin nucleobase. It also enables studying of their catalytic and fluorescence properties in the oligonucleotide environment.[12] Initial melting point studies indicate that the rigidified flavin nucleoside indeed stabilizes DNA duplexe and participates in the hydrogen-bonding process.

In conclusion, we describe a straightforward synthesis of a riboflavin-derived H-phosphonate, containing a benzylidene-rigidified ribityl backbone. Using a combined phosphoramidite/H-phosphonate/phosphoramidite coupling protocol, the building block could be incorporated into oligonucleotides with a synthesis yield of >98%. High-yielding deprotection of the benzylidene group is possible in the DNA strand. Within an oligonucleotide environment, a strong modulation of the redox and of the fluorescence properties of the flavin coenzyme is predicted, due to π-stacking and hydrogen-bonding interactions in the base stack.

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