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Rapid Photolytic Release of Cytidine 5′-Diphosphate from a Coumarin Derivative: a New Tool for the Investigation of Ribonucleotide Reductases

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Abstract—In order to study the long-range radical transfer in the *Escherichia coli* ribonucleotide reductase (RNR), caged cytidine 5′-diphosphate (CDP) **1** was synthesized, which contains the photolabile (7-diethylaminocoumarin-4-yl)methyl moiety. The caged CDP **1** triggers the release of CDP when irradiated at wavelengths between 365 and 436 nm. The rate constant of the formation of alcohol **2** and cytidine 5′-diphosphate **3** is 2×10^8 s^{-1} and the quantum efficiency for the disappearance of caged CDP **1** is 2.9%.

Introduction

Ribonucleotide reductases (RNRs) are essential in living cells because they catalyze all de novo syntheses of deoxyribonucleotides prior to DNA synthesis. The replacement of the 2′-OH group in ribonucleotides by a hydrogen atom is catalyzed by RNRs using radical chemistry.[1] The *Escherichia coli* enzyme consists of two non-identical homodimeric subunits R1 and R2. The R1 protein binds the ribonucleotide substrate while R2 contains a stable tyrosyl radical which initiates catalysis in the R1/R2 holoenzyme by generating a cysteinyl radical in the active site of the R1 subunit.[2] The radical transfer over 35 Å from the tyrosyl to the cysteinyl radical is triggered by the binding of the substrate cytidine 5′-diphosphate (CDP) **3**.[3] In order to study this long distance radical transfer, or the conformational changes that precede this reaction, one has to synthesize a caged CDP that can be activated within nano- to microseconds and at wavelengths, where the extinction coefficient of the RNR[4] is low. Until now, 2-nitrobenzyl derivatives have been used as photocleavable protecting groups at the terminal phosphate of nucleotide diphosphates[5] but they release their substrate in the second to millisecond timescale which is far too slow.[6] Other photolabile precursors such as phenacyl esters and benzoin derivatives, which show shorter releasing times require photolysis at wavelengths between 300 and 350 nm.[6] This is not appropriate for our application because the native RNR has a large extension coefficient in this UV-region.[7] The recent work of Furuta et al.[8] as well as Bendig and Hagen et al.[9] on cyclic nucleotide monophosphates has shown that coumarin systems might be suitable precursors. We therefore worked out a synthesis of (7-diethylaminocoumarin-4-yl)methyl cytidin 5′-diphosphate **1** and measured its photocleavage to coumarin derivate **2** and CDP **3** (Scheme 1).

Results and Discussion

Synthesis

The synthesis of caged CDP **1** starts from 4-methyl coumarin **4**, which gave alcohol **2** in 50% yield after oxidation with selenium dioxide[10] and subsequent reduction with sodium borohydride. Reaction of alcohol **2** with a phosphoramidite and oxidation of the resulting phosphorous(III) with *tert*-butyl hydroperoxide yielded 80% of phosphate **5**. Deprotection with trifluoroacetic acid and coupling with cytidine

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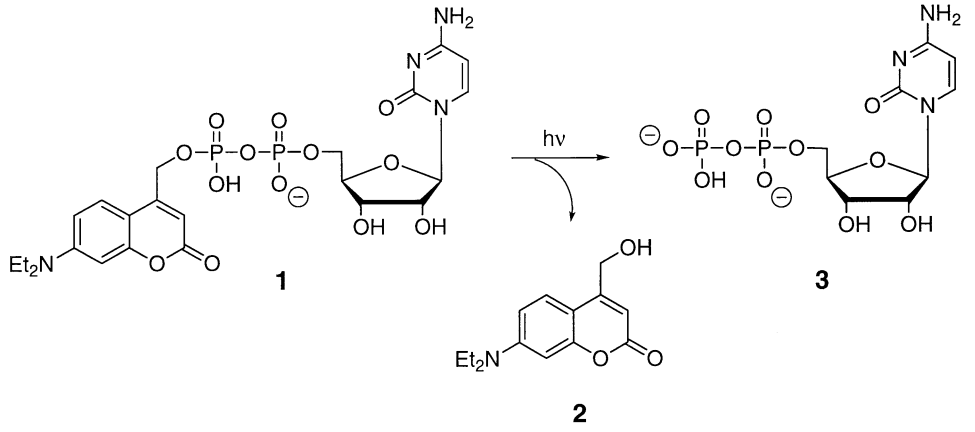
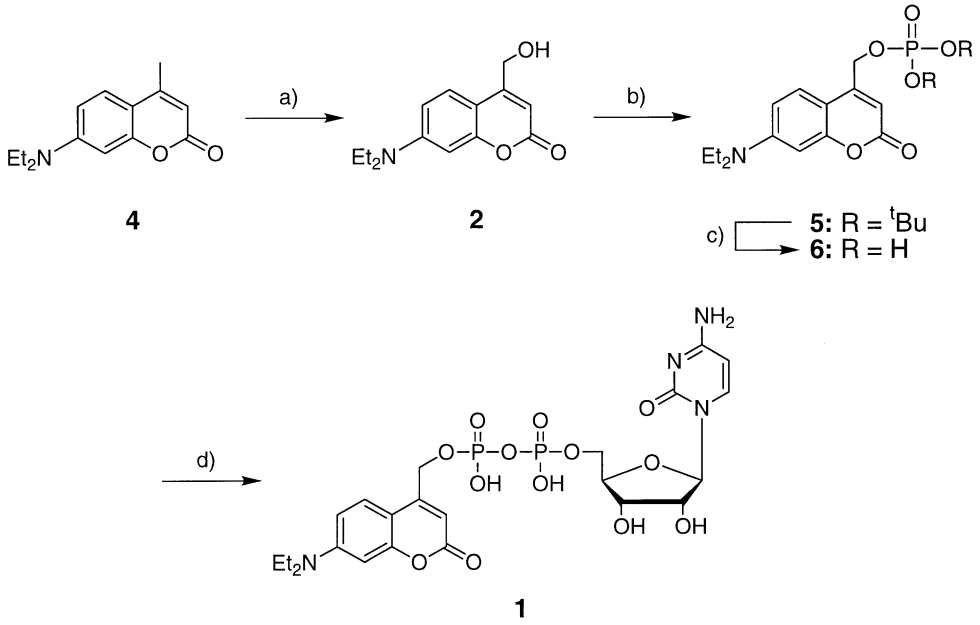


Scheme 1. Release of CDP **3** by photolysis of caged CDP **1**.



Scheme 2. Synthesis of caged CDP **1**. Reagents: (a) SeO_2, *p*-xylene, then $NaBH_4$, EtOH, 50%; (b) $(t\text{BuO})_2\text{PNEt}_2$, THF, 1*H*-tetrazole, then *t*BuOOH, Et_3N, THF, 80%; (c) TFA, CH_2Cl_2, 95%; (d) Im_2CO, CMP, DMF, 34%.

5′-monophosphate (CMP)[11] led to diphosphate **1** in 34% yield (Scheme 2).

Photolytic properties

The caged compound **1** is a bichromophoric system (Fig. 1), which is characterized by two intensive maxima: the S_0–S_1 absorption band of the coumarin chromophor at 392 nm (Table 1), and the absorption band of the pyrimidine base which is at 250 nm, overlapped by a higher transition in the coumarin unit. As expected from the UV curves of Figure 1 even the g-line of the high pressure mercury lamp spectrum at 436 nm, which is far from the absorption bands of the RNR, activates caged CDP **1**.

On photolysis, caged CDP **1** underwent cleavage via a photochemical S_N1 mechanism,[9a] forming CDP **3** and alcohol **2** in at least 95% yield, based on conversion (Scheme 1). The absorption spectra of the caged compound **1** show a slight bathochromic shift of the long-

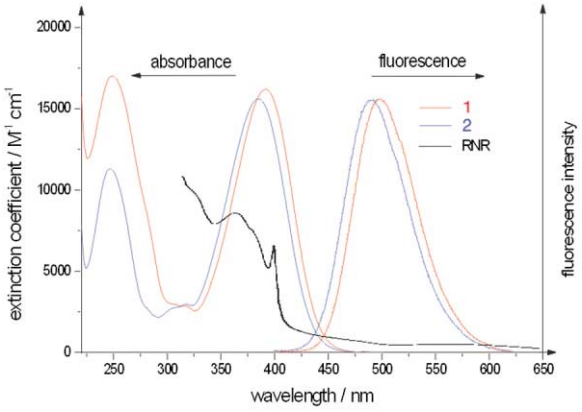


Figure 1. Absorption and fluorescence spectra of the caged CDP **1**, the photolytically liberated alcohol **2** and the R2 subunit of RNR from *E. coli*.

wavelenth absorption compared to the corresponding alcohols **2** (Table 1, Fig. 1). This can be used to determine the concentration of the caged compounds and released alcohols in partly photolyzed solutions. The

Table 1. Photophysical and photochemical data of caged CDP **1** and the corresponding alkohol **2**

Compound	λ_{abs}^{max} (nm)	ε^{max} (M^{-1} cm^{-1})	φ_{chem} (%)	λ_f^{max} (nm)	ϕ_f (%)	τ_f (ns)
1[a]	392	16200	2.9	497	22	1.4
2[b]	385	15600	—	492	48	3.0

λ_{abs}^{max} maxima of the absorption spectra; ε^{max} extinction coefficient in the absorption maxima; φ_{chem} quantum yields for the disappearance of the caged compound; λ_f^{max} maxima in the fluorescence spectra; φ_f fluorescence quantum yields; τ_f fluorescence life times.
[a]In HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer.
[b]In HEPES/MeOH (1:1).

quantum yield φ_{chem} for the disappearance of the caged CDP **1** is 2.9% (Table 1).[12]

Also the fluorescence behavior for caged compound **1** and the released alcohol **2** differ from each other. The fluorescence quantum yields φ_f are in the range of 22–48% (Table 1). The fluorescence life times τ_f were 1.4 ns for caged CDP **1**, and 3.0 for the alcohol **2** (Table 1). The fluorescence curve of the released alcohol is strictly monoexponential, whereas the caged compound **1** shows a more complex multiexponential fluorescence decay.

In order to measure the formation rate of alcohol **2** during photolysis of caged CDP **1**, time-resolved fluorescence spectroscopy[9a] was used. Analysis of the fluorescence decay (N_2-laser, $\lambda_{exc} = 337$ nm, pulse duration about 2.5 ns) showed a multiexponential curve, shaped by the decay of the caged compound **1** and of the formed alcohol **2**. After mathematical deconvolution of the experimental decay curve[13] the rate constant for the formation of alcohol **2** was estimated to be 2×10^8 s^{-1}. This value is comparable to those of the caged cyclic nucleotides.[9a,b]

Conclusion

The synthesis of caged cytidine 5′-diphosphate **1** is described, which leads under photolysis to CDP with a rate constant of 2×10^8 s^{-1}. Because of a λ_{abs}^{max} of 392 nm the photoactivation can occur at wavelengths where the ribonucleotide reductase (RNR) has a very low extinction coefficient.

Experimental

General

Materials and reagents were of the highest grade available commercially and used without further purification. THF was distilled from sodium/benzophenon before use. The reactions were carried out in carefully dried apparatus and under argon. Thin-layer chromatography was performed on E. Merck silica gels 60 F_{254} plates. Flash chromatography (FC) was performed using Merck silica gel 60, particle size 40–63 μm. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on Varian Gemini-300, Bruker DPX-400, DRX-500 and -600 spectrometers. Fast atom bombardment (FAB) mass spectra were recorded on a VG70-250 instrument using *m*-nitrobenzyl alcohol as a matrix. Finnigan MAT LCQ instrument was used for electrospray ionization (ESI) mass spectrometry. UV spectra were recorded with a U-3410 spectrophotometer (Hitachi, Japan).

7-Diethylamino-4-hydroxymethylcoumarin (2). Selenium dioxide (3.33 g, 30.0 mmol) was added to a solution of 4-methyl-7-diethylaminocoumarin **4** (4.63 g, 20.0 mmol) in *p*-xylene (120 mL), and heated under reflux with vigorous stirring. After 24 h, the mixture was filtered and concentrated under reduced pressure. The dark brown residual oil was dissolved in ethanol (130 mL), sodium borohydride (380 mg, 10.0 mmol) was added, and the solution was stirred for 4 h at room temperature. Thereafter the suspension was carefully hydrolyzed with 1 M HCl (20 mL), diluted with H_2O and extracted three times with CH_2Cl_2. The organic phase was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo. By FC (CH_2Cl_2/acetone 5:1) 2.46 g (9.95 mmol, 50%) of alcohol **2** were obtained as a yellow solid: R_f 0.27 (hexane/EtOAc 1:2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.30 (d, $J = 9.0$ Hz, 1H), 6.55 (dd, $J = 9.0$, 2.6 Hz, 1H), 6.46 (d, $J = 2.6$ Hz, 1H),6.27 (t, $J = 1.3$ Hz, 1H), 4.82 (d, $J = 1.3$ Hz, 2H), 3.39 (q, $J = 7.1$ Hz, 4H), 3.12 (s, 1H), 1.19 (t, $J = 7.1$ Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.0, 155.9, 155.5, 150.4, 124.3, 108.6, 106.3, 105.0, 97.5, 60.6, 44.6, 12.4; MS (FAB): *m*/*z* (%) 248 (100) $[M\text{H}]^+$, 232 (22) $[M\cdot\text{Me}]^+$; MS (FAB, + KCl): *m*/*z* (%) 286 (17) $[M + \text{K}]^+$, 248 (100) $[M\text{H}]^+$; HR-MS (EI): calcd for $C_{14}H_{17}NO_3^+$ $[M^+]$: 247.1208; found: 247.1208.

(7-Diethylaminocoumarin-4-yl)methyl di-*tert*-butyl phosphate (5). Alcohol **2** (124 mg, 0.501 mmol) and 1*H*-tetrazole (141 mg, 2.01 mmol) were dissolved in anhydrous THF (10 mL), the solution was cooled to −20 °C and di-*tert*-butyl *N,N*-diethylphosphoramidite (188 mg, 0.752 mmol) was added dropwise. After 10 min at −20 °C the reaction mixture was allowed to warm to 4 °C, stirred for 1 h at this temperature and for 40 min at room temperature. The orange solution was treated with triethylamine (507 mg, 0.70 mL, 5.01 mmol) and *tert*-butyl hydroperoxide (70% in water, 0.345 mL, 2.51 mmol) at 0 °C and allowed to warm up to room temperature. After an additional 4 h, the excess of oxidant was destroyed by adding concd sodium thiosulfate solution (15 mL), diluted with EtOAc (20 mL), the phases were separated and the aqueous phase was extracted with EtOAc (2×10 mL). The combined organic phases were washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was subjected to FC (Et_2O), and 177 mg (0.403 mmol, 80%) of the protected phosphate **5** were obtained: R_f 0.22 (Et_2O), 0.31 (hexane/EtOAc 1:2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.31 (d, $J = 9.0$ Hz, 1H), 6.58 (dd, $J = 2.6$, 9.0 Hz, 1H), 6.51 (d, $J = 2.6$ Hz, 1H), 6.25 (t, $J = 1.3$ Hz, 1H), 5.11 (dd, $J = 1.3$, 6.3 Hz, 2H), 3.42 (q, $J = 7.1$ Hz, 4H), 1.52 (s, 18H), 1.21 (t, $J = 7.1$ Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.9, 156.1, 150.5, 150.0 (d, $J = 9.7$ Hz), 124.3, 108.5, 106.2, 105.7, 97.7, 83.1 (d, $J = 7.3$ Hz), 63.7 (d, $J = 4.6$ Hz), 44.6, 29.8 (d, $J = 4.3$ Hz), 12.3; ^{31}P NMR

(122 MHz, $CDCl_3$) δ −9.5 (t, $J=6.2$ Hz); MS (FAB): m/z (%) 439 (50) $[M\mathrm{H}]^+$; MS (FAB, +KCl): m/z (%) 478 (23) $[M+\mathrm{K}]^+$, 439 (44) $[M\mathrm{H}]^+$; elemental analysis calcd (%) for $C_{22}H_{34}NO_6P$ (439.49) C 60.12, H 7.80, N 3.19, O 21.84; found C 59.93, H 7.79, N 3.01, O 21.56.

(7-Diethylaminocoumarin-4-yl)methyl phosphate (6). A solution of the di-*tert*-butyl protected phosphate **5** (150 mg, 341 μmol) in anhydrous CH_2Cl_2 (2 mL) was cooled to 4 °C. Trifluoroacetic acid (214 mg, 144 μL, 1.88 mmol) was added under stirring and the mixture was agitated for 6 h. The mixture was washed with hexane (3×2 mL) and concentrated in vacuo (for this compound no accurate combustion analysis or HR-MS could be obtained), yielding 106 mg (324 μmol, 95%): R_f 0 (Et_2O); ^{1}H NMR (500 MHz, D_2O) δ 7.80 (d, $J=8.7$ Hz, 1H), 7.43 (d, $J=2.3$ Hz, 1H), 7.35 (dd, $J=2.3$, 8.7 Hz, 1H), 6.59 (t, $J=1.4$ Hz, 1H), 5.08 (dd, $J=1.5$, 7.4 Hz, 2H), 3.58 (q, $J=7.2$ Hz, 4H), 1.05 (t, $J=7.2$ Hz, 6H); ^{13}C NMR (126 MHz, D_2O) δ 164.5, 154.9, 153.7 (d, $J=7.1$ Hz), 144.2, 126.8, 116.2, 114.9, 110.6, 107.2, 63.3 (d, $J=3.8$ Hz), 51.3, 11.0; ^{31}P NMR (203 MHz, D_2O) δ 0.7 (t, $J=7.3$ Hz); MS (ESI, +): m/z (%) 328 (85) $[M\mathrm{H}]^+$, 655 (100) $[M_2\mathrm{H}]^+$; MS (ESI, —): m/z (%) 326 (21) $[M-\mathrm{H}]^-$, 653 (100) $[M_2-\mathrm{H}]^-$.

P^2-(7-Diethylaminocoumarin-4-yl)methyl cytidin 5′-diphosphate (1). The free acid of CMP (441 mg, 1.36 mmol) was dried over P_2O_5 in vacuo (3×10^{-2} mbar) for 2 h at 50 °C. The resulting white powder was dissolved in anhydrous DMF (1.4 mL), treated with tri-*n*-octylamine (333 μL, 1.36 mmol) for a few min at 100 °C and allowed to cool to room temperature. Carbonyldiimidazole (441 mg, 2.72 mmol) was added and the mixture was stirred overnight at room temperature. The phosphate **6** (106 mg, 324 μmol) was dissolved in MeOH/EtOH (1:1, 3.4 mL) and tri-*n*-butylamine (81 μL, 340 μmol) was added. After evaporation of the solvent in vacuo and coevaporation with anhydrous pyridine (2×2 mL), the residue was dissolved in anhydrous DMF, combined with the imidazolide solution and stirred for 11 days at room temperature. The mixture was concentrated in vacuo, dissolved in H_2O and purified first by preparative HPLC, that was performed with a Waters HPLC system using a Lichrospher 100 RP 18 (310×16 mm, 5 μm) column from Knauer and UV detection (254×400 nm). The flow was 8 mL/min and the gradient used for elution is as follows: 0–60 min, 10–35% acetonitrile in 0.1 M sodium acetate buffer (pH 5). The fraction with the retention time of 23.2 min was collected, lyophilized and the product was finally purified on Dowex 50W-X5 (H^+) to give 72.6 mg of **1** as dihydrate (109 μmol, 34%): ^{1}H NMR (600 MHz, D_2O) δ 8.14 (d, $J=7.9$ Hz, 1H), 7.85 (d, $J=8.7$ Hz, 1H), 7.45 (s, 1H), 7.38 (d, $J=8.7$ Hz, 1H), 6.64 (s, 1H), 6.20 (d, $J=7.9$ Hz, 1H), 5.73 (d, $J=3.3$ Hz, 1H), 5.27 (s, 2H), 4.34 (d, $J=11.7$ Hz, 1H), 4.28 (m, 1H), 4.25 (m, 1H), 4.24 (m, 1H), 4.18 (d, $J=11.7$ Hz, 1H), 3.68 (q, $J=7.1$ Hz, 4H), 1.18 (t, $J=7.1$ Hz, 6H); ^{13}C NMR (101 MHz, D_2O) δ 163.5, 159.4, 154.3, 152.9, 148.5, 144.3, 142.3, 127.0, 117.4, 116.4, 111.8, 109.0, 95.4, 90.0, 83.3, 74.7, 69.1, 64.5, 63.7, 52.4, 10.5; ^{31}P NMR (243 MHz, D_2O) δ −14.5; MS (ESI, +): m/z (%): 633 (100) $[M\mathrm{H}]^+$, 655 (34) $[M+\mathrm{Na}]^+$; MS (ESI, —): m/z (%) 631 (100) $[M-\mathrm{H}]^-$, 1263 (5) $[M_2-\mathrm{H}]^-$; elemental analysis calcd (%) for $C_{23}H_{30}N_4O_{13}P_2\times2H_2O$ (668.49) C 41.33, H 5.13, N 8.38; found C 41.20, H 5.17, N 8.26.

Photolysis and quantum yield measurements

The UV-spectra were recorded with a U-3410 spectrophotometer (Hitachi, Japan). Photolysis was carried out using a high-pressure mercury lamp (HBO 500, Oriel) with controlled light intensity and metal interference filter of 365, 405 and 436 nm (Schott, Germany). For quantum yield determination the irradiated solutions were analyzed using HPLC and, additional, using the matrix analysis[14] of the irradiation spectra. The results of both methods were identical. The photochemical quantum yield was defined as the ratio of caged molecules converted to the amount of photons absorbed using the potassium ferrioxalate actinometer.[15] Further details are described by Schade et al.[9c]

Fluorescence measurements

The fluorescence spectra were measured using a MPF-2A fluorescence spectrophotometer (Hitachi-Perkin–Elmer). In the case of the photosensitive caged compounds the excitation intensity was very low to minimize photolysis. The excitation wavelength was $\lambda_{exc}=375$–390 nm. The fluorescence quantum yields were determined at 298 K by the relative method[16] using quinine sulfate as a standard. The time-resolved fluorescence decay measurements were performed using the pulse sampling method.[17]

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