SHORT COMMUNICATION

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Synthesis of Short Oligodeoxyribonucleotides by Phosphotriester Chemistry on a Precipitative Tetrapodal Support

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Short oligodeoxyribonucleotides have been assembled from appropriately protected nucleoside 3′-(benzotriazol-1-yl 2-chlorophenyl phosphate) derivatives on hundred-milligram scales on a soluble tetrakis-*O*-(4-azidomethylphenyl)pentaerythritol support bearing four 3′-*O*-(4-pentynoyl)thymidines. Small-molecule reagents and waste were removed by two precipitations of the support-bound growing oligonucleotides with methanol, the first after removal of the 5′-protecting group and the second after coupling. The building blocks were obtained by one-step phosphorylation of commercially available protected nucleosides with bis(benzotriazol-1-yl) 2-chlorophenyl phosphate. Removal of the phosphate protecting groups from the completed chain with (*E*)-2-nitrobenzaldoxime followed by conventional ammonolysis allowed precipitation of the oligonucleotide as the sodium salt with ethanol.

Introduction

Oligonucleotides are usually prepared on a solid support by the so-called phosphoramidite strategy, which is based on formation of internucleosidic P–O bonds at the P^{III} level, followed by oxidation of the resulting phosphite triester to the phosphate triester immediately after each coupling step.[1–3] Virtually the same chemistry is used in lab-scale synthesis[4] and in large-scale production.[5] For in-house preparation of short oligonucleotides on hundred-milligram scales, which on a lab-scale synthesizer requires dozens of repetitions, the application of a similar strategy in solution on a soluble support has been attempted.[6–15] The need for oxidation after each coupling step unfortunately increases the number of steps in the coupling cycle. Whereas this is not a problem with solid-supported synthesis, for which waste and excess monomeric blocks and activators can be removed by simple washing, it considerably complicates the synthesis in solution. In fact, the "outdated" phosphotriester strategy,[16,17] based on coupling of P^{V} blocks and hence avoiding the oxidation step, might offer a more straightforward procedure for synthesis in solution. This chemistry has previously been applied to the synthesis of oligonucleotides on a soluble poly(ethylene glycol) support[18,19] by using either 1-hydroxybenzotriazole[20–22] or 1-(mesitylsulfonyl)-3-nitro-1*H*-1,2,4-triazole (MSNT)[18] activation. Among these methods, the approach based on coupling of prefabricated 3′-(benzotriazol-1-yl 2-chlorophenyl phosphate) building blocks appears particularly interesting. We have now applied this method to the preparation of short oligodeoxyribonucleotides on our recently reported soluble support,[12] tetrakis-*O*-(4-{4-[3-(thymidin-3′-yl)-3-oxopropyl]-1,2,3-triazol-1-ylmethyl}phenyl)pentaerythritol (**1**; Figure 1). The usefulness of this support stems from its quantitative precipitation with MeOH. Accordingly, each coupling cycle contains only two precipitations, one after removal of the 5′-protecting group and the second after the coupling. All the small-molecule compounds remain in solution, while the support precipitates quantitatively. By ammonolytic release, nearly homogeneous heterotrimers were obtained in 70% isolated yield and a pentamer in 55% yield. In spite of the simplicity of the synthesis, the yields favorably compete with the previously described approaches. Interestingly, a related branched core structure has previously been used for immobilization of prefabricated dimers and trimers as building blocks of nanoscale objects.[23,24]

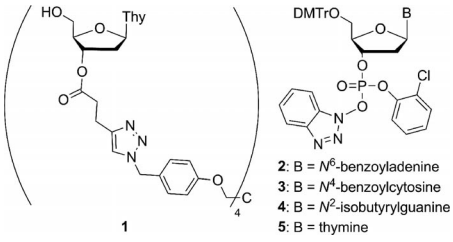

Figure 1. Structure of the soluble support and the building-blocks employed.

Results and Discussion

The tetravalent nucleoside cluster **1** has been shown to be a promising soluble support for the synthesis of oligonu-

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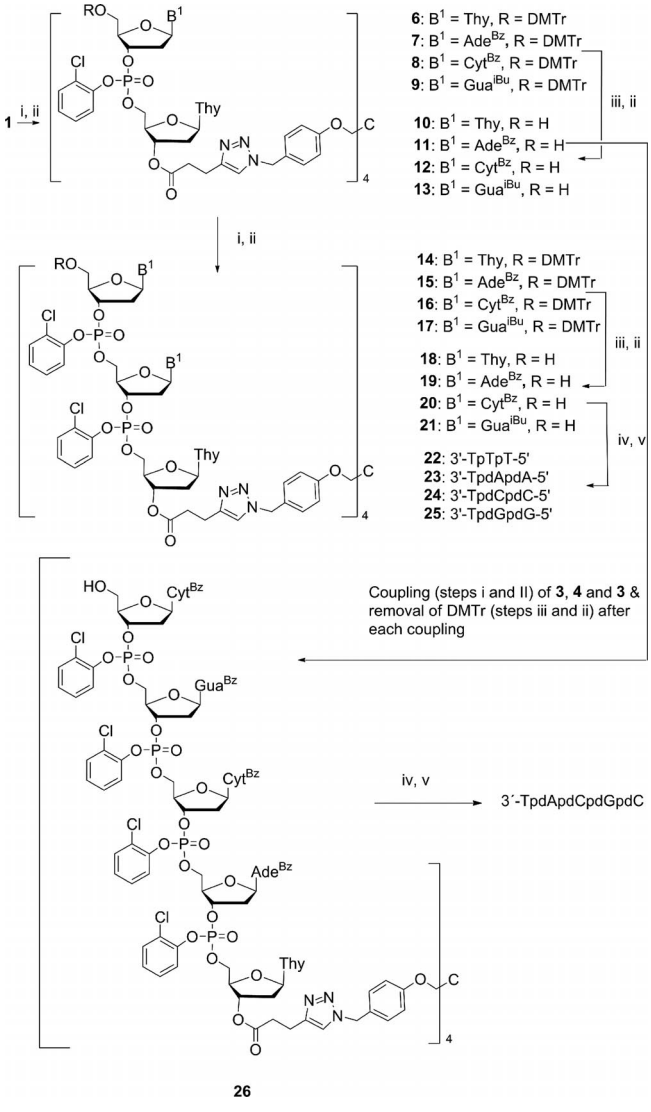
cleotides in solution.[12] It has radial symmetric structure that allows the homogeneity of the products to be verified after each coupling by NMR spectroscopic analysis, in addition to HPLC and mass spectrometry. The structure itself is very stable, allowing storage at ambient temperature for a long time (no sign of degradation was observed after 8 months). Quantitative precipitation of the support-bound oligonucleotide cluster with MeOH allows facile removal of monomeric reagents and waste. Although the application of phosphoramidite chemistry on this support allowed the preparation of a heteropentamer in 45% isolated yield, the protocol employed still suffered from some minor side reactions, including premature cleavage of the 2-cyanoethyl and benzoyl protecting groups and depurination of 2′-deoxyadenosine upon acidolytic removal of the 5′-*O*-(4,4′-dimethoxytrityl) group. For these reasons, and to avoid the oxidation step, the feasibility of applying phosphotriester chemistry on this support by using 3′-(benzotriazol-1-yl 2-chlorophenyl phosphotriesters) as building blocks has been studied. The results are described herein.

Commercially available appropriately protected nucleosides, 5′-*O*-(4,4′-dimethoxytrityl)-N^6-benzoyl-2′-deoxyadenosine, N^4-benzoyl-2′-deoxycytidine, N^2-isobutyryl-2′-deoxyguanosine and thymidine, were converted into their 3′-(benzotriazol-1-yl 2-chlorophenyl phosphates) (**2**–**5**; Figure 1) by treatment with bis(benzotriazol-1-yl)(2-chlorophenyl)phosphate (1.1 equiv.) in 1,4-dioxane.[22] The stock solution of the latter reagent (0.2 mol L^{-1}) was obtained by allowing commercial 2-chlorophenyl phosphorodichloridate to react with 1-hydroxybenzotriazole (2 equiv.) in dioxane in the presence of pyridine (2 equiv.). The resulting pyridinium chloride precipitate was removed by filtration, and the filtrate was divided into small portions and stored at –20 °C. As indicated earlier,[22] the stoichiometry upon preparation of **2**–**5** is of crucial importance: excess nucleoside results in formation of a 3′,3′-dinucleosidephosphate, whereas excess phosphorylating agent may lead to phosphorylation of the 5′-OH of the supported nucleotide. Of these potential side reactions, only dimer formation was sometimes detected. Fortunately, the presence of a 3′,3′-dimer did not markedly affect subsequent coupling. To achieve sufficient accuracy, a gravimetric method was applied to the preparation and dosage of the stock solution.

Four trimers and one pentamer containing all four 2′-deoxynucleosides were prepared (Scheme 1). Support **1**, bearing four thymidine residues, was prepared as described previously.[12] To achieve the first coupling, benzotriazole-activated building block (**2**–**5**; 8 equiv.; i.e., 2 equiv. per support-bound thymidine), and 1-methylimidazole (10 equiv.) were added in dioxane onto the dried support under nitrogen. After 2 h of stirring, the support was precipitated by diluting the reaction mixture with 10 volumes of MeOH. HPLC analysis of the precipitate and the liquid phase verified completeness of the precipitation. An illustrative example is given in Figure 2. The 5′-*O*-DMTr protection was then removed with HCl in a 1:1 (v/v) mixture of MeOH/dichloromethane. Upon completion of the removal, the mixture was neutralized with pyridine and concentrated to an oil. The residue was dissolved in a minimal amount of the mixture of MeOH and dichloromethane and precipitated by diluting with MeOH. As seen from Figure 3, the detritylated support precipitated virtually quantitatively. The coupling and detritylation steps described above were then repeated to obtain support-bound trimers **18**–**21**, still bearing the phosphate and base moiety protecting groups (see Table 1 for ESI-MS data). The 2-chlorophenyl protecting groups were then removed with the tetramethylguanidium salt of (*E*)-2-nitrobenzaldoxime in aqueous dioxane,[25] and the base-moiety protecting groups were removed with 25% aqueous ammonia at 55 °C. The precipitated support was removed by filtration, and the filtrate was concentrated to dryness under reduced pressure. The residue was dissolved in aq. MeCN, aq. NaOAc was added, and the oligonucleotide was precipitated by diluting with EtOH. Trimers



Scheme 1. Synthesis of oligonucleotides. Reagents and conditions: (i) **2**–**5**, *N*-MeIm, dioxane, under N_2; (ii) precipitation with MeOH; (iii) 1. HCl in MeOH/CH_2Cl_2 (1:1); 2. Py, concentration; (iv) 1. (*E*)-2-nitrobenzaldoxime tetramethylguanidinium salt in aq. dioxane; 2. aq. NH_3; (v) aq. MeCN, aq. NaOAc, EtOH, –20 °C.

22–**25** were obtained in 70% isolated yield (determined by UV absorption in comparison to support **1**). The pentameric oligonucleotide **27** was obtained similarly in 55% yield. The coupling efficiency and precipitation properties of the support remained unchanged upon increasing the chain length. However, we have not attempted to assemble oligonucleotides longer than a pentamer on this support. The identities of the products were verified by ESI-MS analysis (Table 1), and their homogeneity was established by HPLC analysis (Figure 4).

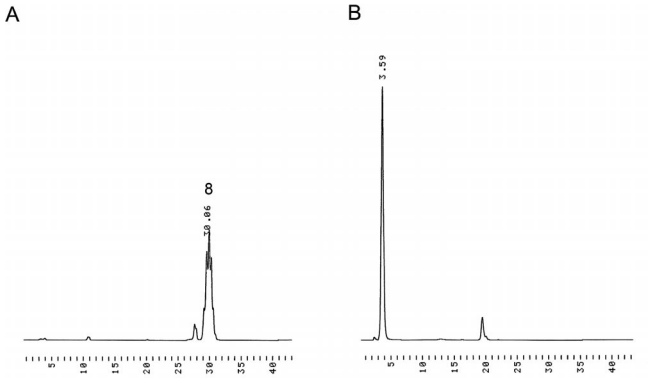


Figure 2. (A) HPLC traces for the precipitated support bearing phosphate-protected 3′-TpdC^{Bz}-5′ dimer as a 5′-*O*-DMTr ether (**8**), and (B) HPLC traces of the filtrate after the precipitation. For the chromatographic conditions, see gradient C in the Exp Sect.

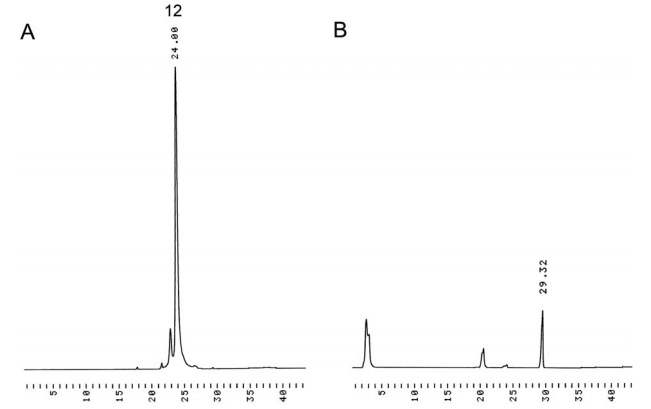


Figure 3. (A) HPLC traces for the precipitated support bearing phosphate-protected 3′-TpdC^{Bz}-5′ dimer having the 5′-*O*-detritylated (**12**), and (B) HPLC traces of the filtrate after precipitation. For the chromatographic conditions, see gradient A in the Exp. Sect.

In addition to 1-hydroxybenzotriazole-activated building blocks **2**–**5**, more conventional coupling of appropriately protected 2′-deoxyribonucleoside 3′-(2-chlorophenyl phosphates) was attempted by using (mesit-2-yl)sulfonyl chloride for activation and 1-methylimidazole as a nucleophilic catalyst.[17] 3′-TpdC-5′ and 3′-TpdA-5′ dimers were obtained in a still reasonable 85% coupling yield, but upon coupling of the 2′-deoxyguanosine block, strong coloration of the reaction mixture was observed (data not shown). In fact, it has been reported that when this kind of approach is used, the O^6 atom of the guanine base should be protected.[3] With the corresponding 1-hydroxybenzotriazole-activated block **4**, this was not necessary.

Table 1. Negative-ion ESI-MS analysis of the support-bound protected (**18**–**21** and **26**) and released deprotected (**22**–**25** and **27**) oligonucleotides.

Compound	Calculated mass	Observed mass
18	1754.9 $[(M - 3\ H)/3]^{3-}$	1754.3 $[(M - 3\ H)/3]^{3-}$
19	2056.6 $[(M - 3\ H)/3]^{3-}$	2056.1 $[(M - 3\ H)/3]^{3-}$
20	1992.5 $[(M - 3\ H)/3]^{3-}$	1992.1 $[(M - 3\ H)/3]^{3-}$
21	2008.6 $[(M - 3\ H)/3]^{3-}$	2008.1 $[(M - 3\ H)/3]^{3-}$
26	3376.0 $[(M - 3\ H)/3]^{3-}$	3375.1 $[(M - 3\ H)/3]^{3-}$
22	849.18 $[M - H]^-$	849.16 $[M - H]^-$
23	867.21 $[M - H]^-$	867.19 $[M - H]^-$
24	819.19 $[M - H]^-$	819.20 $[M - H]^-$
25	899.20 $[M - H]^-$	899.17 $[M - H]^-$
27	730.14 $[(M - 2\ H)/2]^{2-}$	730.12 $[(M - 2\ H)/2]^{2-}$

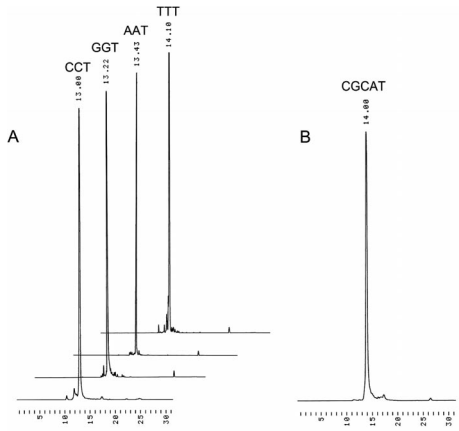


Figure 4. HPLC traces of the trimeric (A) and pentameric (B) oligonucleotides prepared. For the chromatographic conditions, see gradient B in the Exp. Sect.

Conclusions

A convenient phosphotriester approach that allows the preparation of short oligodeoxyribonucleotides on hundred-milligram scales in solution has been described. The method is based on 1-methylimidazole-promoted assembly of appropriately protected nucleoside 3′-(benzotriazol-1-yl 2-chlorophenyl phosphates)[22] on a soluble tetrakis-*O*-(4-azidomethylphenyl)pentaerythritol support bearing four thymidines.[12] All small molecules were removed after each coupling and 5′-*O*-detritylation by precipitating the support with MeOH. On using 2 equiv. of building blocks per 5′-OH, a pentamer containing all four nucleosides was obtained in 55% isolated yield. The advantages of this approach compared to the phosphoramidite method on the same support[12] include avoiding the oxidation step and preventing premature cleavage of phosphate and base-moiety protecting groups. Although depurination is not entirely avoided during acid-catalyzed removal of the 5′-*O*-DMTr group, the coupling efficiency remains high, enabling iso-

lated yields that compare favorably with previously described approaches.

Experimental Section

General: NMR spectra were recorded with a 500 MHz spectrometer. Chemical shifts are given in ppm and referenced relative to the residual solvent signals.[26] RP HPLC conditions: (A) gradient elution from 25% MeCN in 0.1 mol L^{-1} Et_3NHOAc to 100% MeCN in 25 min, then continued with MeCN; (B) gradient elution from 2.5% MeCN in 0.1 mol L^{-1} Et_3NHOAc to 50% MeCN in 0.1 mol L^{-1} Et_3NHOAc in 25 min, then continued with 50% MeCN in 0.1 mol L^{-1} Et_3NHOAc; (C) gradient elution from 50% MeCN in 0.1 mol L^{-1} Et_3NHOAc to 100% MeCN in 25 min, then continued with MeCN. An analytical C-18 RP column (250 × 4.6 mm, 5 μm; flow rate 1.0 mL min^{-1}; λ = 260 nm) was used. Reactions were monitored by TLC (Merck, silica gel 60 F_{254}), using short-wavelength UV or charring with 10% aq. H_2SO_4 for detection (system A: 10% MeOH in CH_2Cl_2; system B: 8% MeOH in CH_2Cl_2). Mass spectra were recorded with a Bruker Daltonics MicrOTOF-Q spectrometer using ESI ionization. Tetrakis(4-{4-[3-(thymidin-3′-*O*-yl)-3-oxoprop-1-yl]-1*H*-1,2,3-triazol-1-yl}methylphenoxymethyl)methane (**1**) was synthesized as reported previously.[12]

Bis(benzotriazol-1-yl) 2-Chlorophenyl Phosphate: A solution of 2-chlorophenyl phosphorodichloridate (7.6 mmol, 1.88 g) in anhydrous dioxane (5.75 mL) was added in one portion to a suspension of 1-hydroxybenzotriazole (15.2 mmol, 2.06 g; dried in vacuo over P_2O_5 at 55 °C for 3 d) and pyridine (15 mmol, 1.2 mL) in anhydrous dioxane (30 mL). The reaction mixture was stirred for 2 h, and the precipitate was filtered off under anhydrous conditions to give a stock solution of bis(benzotriazol-1-yl) 2-chlorophenyl phosphate (0.2 mol L^{-1}) as a clear colorless liquid. The solution (ρ = 1.057 g mL^{-1}) could be stored for several weeks at –20 °C.

General Procedure for the Coupling of 1-Hydroxybenzotriazole-Activated Phosphotriester Building Blocks: N^6-Benzoyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)adenosine (0.41 mmol; 0.27 g) was dried by coevaporation with anhydrous pyridine (3 × 5 mL) and concentrated to a small volume followed by addition of the stock solution of bis(benzotriazol-1-yl) 2-chlorophenyl phosphate in dioxane (0.41 mmol, 0.2 mol L^{-1}, 2.05 mL), giving N^6-benzoyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)adenosine 3′-(benzotrizol-1-yl 2-chlorophenyl phosphate) (**3**) in dioxane. The formation of a product with zero mobility on TLC (system B) indicated that the reaction was complete. In a separate vessel, support **1** (0.051 mmol, 0.10 g) was dried by coevaporation with anhydrous pyridine (3 × 5 mL), and then **3** in dioxane and 1-methylimidazole (2 mmol, 0.170 mL) were added under nitrogen. The reaction mixture was stirred for 2 h to obtain the supported dimer **8**, transferred to stoppered 50 mL plastic tubes, and MeOH (46 mL) was added. The precipitate formed was kept at –20 °C overnight, isolated by centrifugation, and dried to give **8** (0.25 g, 93%) as a white solid. The precipitate and supernatant were analyzed by HPLC to verify the completeness of precipitation.

General Procedure for Detritylation: Support-bound dimer **7** (0.046 mmol, 0.25 g) was dissolved in a mixture of CH_2Cl_2 and MeOH (1:1 v/v, 25 mL), and HCl in MeOH (1.25 mol L^{-1}, 0.14 mL) was added portionwise. The reaction was monitored by TLC (system A). Once complete, the reaction mixture was neutralized with pyridine (1 mL), and the liquid was concentrated. The resulting oil was dissolved in CH_2Cl_2/MeOH (1:1, 3 mL), and MeOH was added (47 mL). The precipitate formed was kept at –20 °C overnight, collected by centrifugation and dried to give the product (0.173 g, 90%) as a white solid. The precipitate and supernatant were analyzed by HPLC and ESI-HRMS.

Assembly of the Support-Bound Oligonucleotides: By using the methodology described above, the following support-bound oligonucleotides were assembled. AAT cluster **19** was obtained in 82% yield (0.215 g) from **1** (0.100 g), corresponding to 91% average yield of a coupling/detritylation cycle. CCT cluster **20** was prepared in 85% yield (0.102 g) from **1** (0.048 g); GGT cluster **21** in 80% yield (0.352 g) from **1** (0.150 g), and TTT cluster **18** in 82% yield (0.345 g) from **1** (0.200 g). CGCAT cluster **26** was obtained in 61% overall yield (0.440 g) starting from support **1** (0.150 g), corresponding to 88% average yield of each coupling/detritylation cycle.

General Procedure for Oligonucleotide Isolation: Support-bound trimer **19** (0.035 mmol, 0.215 g) was mixed with the tetramethylguanidinium salt of (*E*)-2-nitrobenzaldoxime (0.3 mol L^{-1}, dioxane/water, 9:1, v/v, 8 mL), stirred for 2 h, and then volatiles were removed under reduced pressure. The residue was diluted with 25% aq. ammonia (8 mL) and stirred at 55 °C for 4 h. The precipitated support was removed by filtration, and the filtrate was concentrated and dried in vacuo to give a yellow oil.

The oily residue was dissolved in a mixture of MeCN and water (3:7 v/v, 0.5 mL) followed by aqueous sodium acetate (3 mol L^{-1}, 1.2 mL). The resulting mixture was diluted with EtOH (16 mL), vortexed for 20 s, and kept at –20 °C for 1.5 h. The precipitate formed was collected by centrifugation, washed with EtOH (10 mL) and dried to give 108 mg of a yellow powder corresponding to 90% isolated yield and 73% overall yield. The precipitate was dissolved in water (8 mL) and analyzed by UV, HPLC and ESI-HRMS.

Support-bound trimers **18**, **20**, **21** and pentamer **26** were isolated as analytical samples on 1–3 μmol scale. The overall yields starting from **1** were 69% for **24**, 73% for **23**, 69% for **25**, 70% for **22**, and 55% for **27**.

General Procedure for the Coupling of Phosphodiester Building Blocks: Support **1** (0.015 mmol, 30 mg) and the Et_3NH^+ salt of N^4-benzoyl-5′-*O*-(4,4′-dimethoxytrityl-2′-deoxycytidine) 3′-(2-chlorophenyl phosphate) (0.12 mmol, 114 mg) were dissolved in anhydrous pyridine (1 mL), and 2-mesitylsulfonyl chloride (0.22 mmol, 48 mg) and 1-methylimidazole (0.62 mmol, 0.050 mL) were added under nitrogen. The reaction mixture was stirred overnight, and then MeOH (10 mL) was added. The precipitate formed was kept at –20 °C overnight, collected by centrifugation, and dried to give **8** (69 mg, 87%) as a white solid. The precipitate and supernatant were analyzed by HPLC.

Supporting Information (see footnote on the first page of this article): ^{1}H NMR spectra for **18**, **19**, **21**, and **26**, ^{13}C NMR spectra for **19** and **26**, HSQC spectra for **18**, **19**, **21**, and **26**, mass spectra for **18**–**27**.

Acknowledgments

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