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High-Yield Immobilization of a Puromycin Analogue for the Solid Support Synthesis of Aminoacyl-tRNA Fragments

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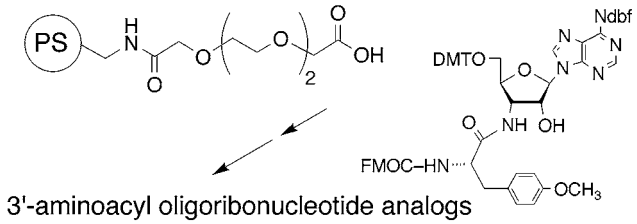
ABSTRACT



An efficient procedure for the immobilization of 3′-deoxy-3′-(*O*-methyltyrosyl)aminoadenosine was developed. A poly(ethylene glycol)-derived diacid linker/spacer was attached to aminomethyl polystyrene. Coupling of the 2′-hydroxy instead of the 2′-*O*-succinylated ribonucleoside resulted in high immmobilization yields (over 80%) and allowed for the recovery of valuable unreacted material. This specific procedure should be applicable to other ribonucleosides containing a bulky modification at the 3′-position and can be used for the stepwise construction of 3′-aminoacyl- or 3′-peptidyl-RNA conjugates.

We recently published the synthesis of structural analogues of puromycin,[1] a natural nucleoside antibiotic broadly used as a tool for molecular biologists. An innovative application of puromycin was developed previously for the in vitro formation of peptidyl-RNA "fusions", which allows for the in vitro selection of peptides[2] and ribosomal peptidyl transfer studies.[3] To realize this fusion between peptide and RNA, it was necessary to synthesize oligoribonucleotides containing puromycin at their 3′-end and thus adapt and anchor puromycin to a solid support for the solid support synthesis of oligonucleotides. Puromycyl-CPG can now be obtained commercially; however, it is of great interest to develop this original approach by synthesizing new molecules, analogues of puromycin having, for instance, a better peptidyl transfer ability. On that account a good immobilization procedure for this class of molecules is needed. We synthesized some analogues of puromycin and after fruitless attempts, as cited in the literature,[4,5] to attach our analogues in good yield on the solid support, we developed a high-yield immobilization procedure. This method enables the recovery of the unreacted nucleosides, which is for us a very important point.

3′-Deoxy-3′-(L-*p*-methoxyphenylalanyl)aminoadenosine derivative **1** is obtained on the synthetic pathway of puromycin analogues.[1] Modification of **1** was necessary to attach it onto the solid support for the oligonucleotide synthesis. We first

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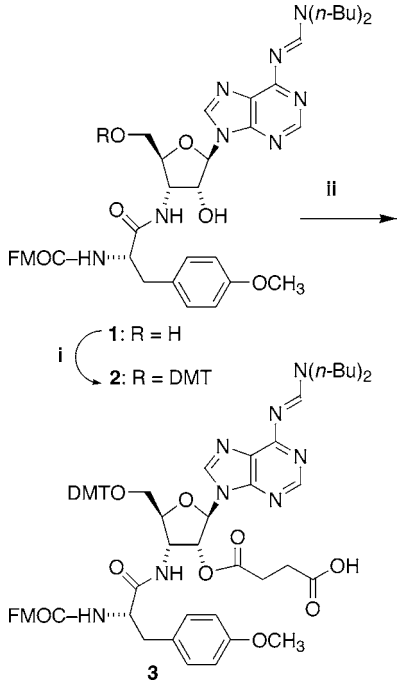
decided to follow the current methodology for the immobilization of nucleosides, which utilizes nucleosides anchored on Long Chain Amino Alkyl-Controlled Pore Glass (LCAA-CPG) by a succinyl linkage.[4−8] So compound **1** was protected at the 5′-position with dimethoxytrityl chloride and the 2′-hydroxy group, in our case, was succinylated in good yield to obtain the product **3** (Scheme 1).

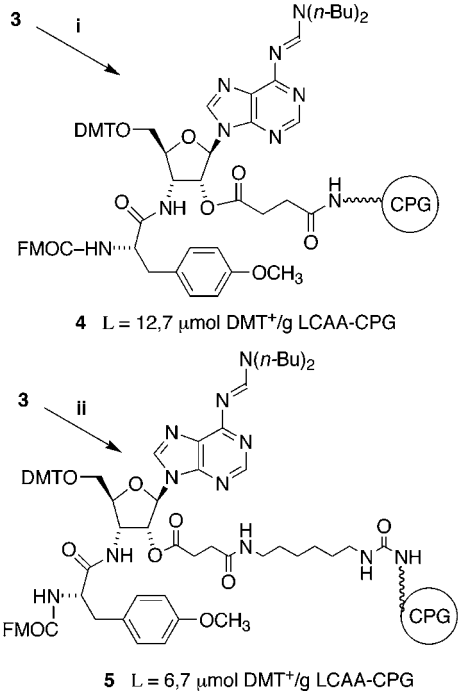
Scheme 1. 5′-*O*-Protection and 2′-*O*-Succinylation of **1**[a]



[a] Reagents and conditions: (i) DMTCl (1.5 + 0.5 equiv), N(*n*-Bu)$_4$NO$_3$ (0.3 equiv), C_5H_5N, rt/1 + 1 h; MeOH quench; $NaHCO_3$ extract; SiO_2; 81%. (ii) succinic anhydride (1.5 equiv), (^{i}Pr)$_2$NEt (1.0 equiv), DMAP (0.5 equiv), 1,2-$EtCl_2$, 50 °C/0.5 h; 10% citric acid extract; precipitation from Et_2O/hexane (1:1); quant.

Succinylated derivative **3** was activated by using DCC and *p*-nitrophenol and then reacted with LCAA-CPG (1000 Å pore size). All immobilizations were carried out manually with use of a Teflon syringe equipped with a polyethylene filter. To obtain the best immobilization yield, 2 equiv of **3** were used with regard to the initial amino loading of LCAA-CPG. The determination of the amino loading was performed by using the ninhydrin test[9] on untreated and on acid-activated[8] LCAA-CPG, which resulted in no significant difference in loading (100−130 μmol NH_2/g). Following the established literature procedure (Scheme 2, i),[4] the best nucleoside loading we obtained was only 12.7 μmol/g, as determined by DMT$^+$ cleavage with 3% Cl_3CCOOH/CH_2Cl_2 and optical detection at 504 nm (ϵ_{504} = 72 678 μM·cm; reproducibility within 2−3%). Compared to the initial amino loading value, the immobilization yield was about 10−12%. But the steric hindrance of the Fmoc-aminoacyl group at the 3′-position might be at the origin of the low yield, so intercalating a spacer between the succinylated ribonucleoside and the solid support should improve the reaction yield. Gupta and co-workers[5] developed an approach using alkyl and aryl diisocyanates as spacers. In this procedure, succinylate **3** is attached to one isocyanate function through the action of DMAP and decarboxylation. Commercial hexamethylene-1,6-diisocyanate was chosen, which reacted within 10 min. LCAA-CPG was added for the attachment of the primary amino groups of the solid support to the second isocyanate function (Scheme 2, ii).

Scheme 2. Immobilization of **3** on LCAA-CPG[a]



[a] Reagents and conditions: (i) DCC, *p*-nitrophenol, dioxane, DMF, LCAA-CPG. (ii) (a) DMAP (1.0 equiv), $OCN(CH_2)_6NCO$ (1.0 equiv), CH_2Cl_2, rt, 10 min; (b) (^{i}Pr)$_2$NEt (1.0 equiv), LCAA-CPG (0.5 NH_2), 16 h; Et_2O (wash); (c) H_2O/C_5H_5N (2:8 v/v), 2 h; (d) Ac_2O/Et_3N/NMI/CH_2Cl_2, 0.5 h.

The results we obtained were even less successful, since a nucleoside loading of 6.7 μmol/g resulted. In addition to the low immobilization yields, whatever the method we used, we were confronted with the loss of **3**. In fact compound **3** is obtained after an 11-step synthesis and for each immobilization attempt 2 equiv of **3** were used and all the unreacted material could not be recovered. To our knowledge, no references referred to this problem. Although the succinate linkage remains the most favored for solid support oligonucleotide synthesis, some other activation procedures have been developed; notably the activation of the solid support,[8] which can be directly condensed with the 2′ or 3′ hydroxy group of an appropriately protected ribonucleoside.

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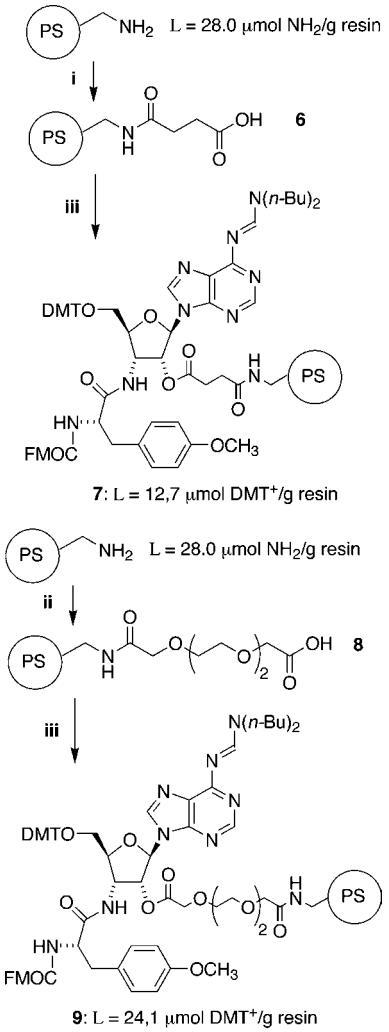
In this way the unreacted material could be recovered and the loss of the precious ribonucleoside was limited. Earlier works showed that this method applied to ribonucleosides resulted mostly in low to medium yield[7,10] and require longer reaction times.[8]

We decided to explore this strategy with some modifications. The LCAA-CPG solid support was replaced by a 50% DVB-cross-linked aminomethyl polystyrene (AMP), whose superiority has been shown by McCollum and Andrus.[11] In addition to the succinate linkage, we also used a commercially available poly(ethylene glycol)-derived linker/spacer: 3,6,9-trioxaundecanoic diacid. We have shown[12] that a similar spacer used for the solid support of oligonucleotides proved to be superior in terms of both coupling yields and homogeneity of the final product, when compared to several other tested spacer molecules of up to double its length.[13]

AMP with an amino loading of 28 μmol/g as determined by the ninhydrin test (cf. Supporting Information) was quantitatively derivatized, on one hand, with succinic anhydride in the presence of (dimethylamino)pyridine (DMAP) in pyridine (Scheme 3: i) and, on the other hand, with 3,6,9-trioxaundecanoic diacid in the presence of HBTU ((*O*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate) and *N*-methylmorpholine (NMM) in DMF (Scheme 3: ii). The carboxylic functions of the derivatized solid supports **6** and **8** were activated with oxalyl chloride within 1 h at room temperature. After being washed with absolute CH_2Cl_2, the polymers were dried under high vacuum for half an hour and submitted to the reaction with **2** in the presence of DMAP in absolute CH_2Cl_2 for 12 h, followed by a capping procedure with acetic anhydride/*N*-methyl imidazole (NMI)/pyridine in DMF (Scheme 3: iii). The ribonucleoside immobilization yield was determined by the trityl analysis and resulted in nucleoside loadings of 12.7 μmol/g for the succinate linkage and 24.1 μmol/g for the poly(ethylene glycol) linkage, respectively. This procedure was carried out several times and the lowest ribonucleoside loading we obtained for the polyethylene linkage was 20 μmol/g, corresponding to immobilization yields of 80−87% with respect to the initial amino loading. An excess of compound **2** was worked up and chromatographed allowing the recovery of pure starting material **2**.

To establish the usefulness of our immobilization method, the charged polymer **9** was subjected to standard oligoribonucleotide chain assembly on an automated DNA/RNA synthesizer, using a phosphoramidite coupling protocol and 5-ethylthiotetrazole activation, a commercial 5′-*O*-phosphitylating reagent, and 2′-*O*-TBDMS ribonucleoside monomers. We synthesized, on a 1-μmol scale with average stepwise yields of >98%, fragments of the invariant 3′-terminal ACCA sequence of tRNA: pA*, CpA*, pCpA*, CpCpA*, and ApCpCpA*, where p stands for a 5′-*O*-phosphate and A* corresponds to 3′-deoxy-3′-(L-*p*-methoxyphenylalanyl)amino-β-D-adenosine. After the partial deprotection and cleavage from the solid support with 33% ethanolic methylamine and evaporation, the crude compounds were desilylated with $Et_3N{\cdot}3HF$/DMF (3.3:1, 55 °C/1.5 h), evaporated, and purified by reverse-phase HPLC (C_{18}; buffer A, H_2O; buffer B, 90% CH_3CN/10% 0.1 M NH_4OAc). The identity, purity, and assignments of the downfield ^1H NMR signals of the 3′-aminoacyl-tRNA analogues were established by electrospray mass spectroscopy, ^1H NMR (600 MHz/inverse detection, in H_2O/5% D_2O, pH 7.5), and ^{31}P NMR (cf. Supporting Information).

Scheme 3. Immobilization of **2** on AMP[a]



[a] Abbreviation: PS = polystyrene. Reagents and conditions: (i) succinic anhydride (50 equiv), DMAP (3.0 equiv), C_5H_5N, AMP (1.0 NH_2), rt/16 h; wash: C_5H_5N, CH_2Cl_2; (ii) 3,6,9-trioxaundecanoic diacid (37 equiv), HBTU (18.5 equiv), NMM (18.5 equiv), C_5H_5N, rt/15 min; AMP (1.0 NH_2), rt, 16 h; wash: DMF, CH_2Cl_2; (iii) (a) oxalyl chloride (100 equiv), CH_2Cl_2, rt/1 h; wash: CH_2Cl_2; (b) **2** (2.0 equiv), DMAP (10 equiv), CH_2Cl_2, rt/12 h; (c) Ac_2O/NMI/C_5H_5N (each 1 M in DMF), rt/5 min; wash: CH_2Cl_2; (d) regeneration of excess **2**: extract solution from (b) with $NaHCO_3$/CH_2Cl_2; SiO_2 (CH_2Cl_2/MeOH 0−5%); 40−54%.

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In conclusion, we developed a high-yielding 2′-*O*-immobilization method for 3′-L-α-aminoacylamino-3′-deoxyadenosines. We obtained over 80% immobilization yield despite the presence of a bulky group in the 3′-position. This procedure should be applicable to other ribonucleosides for an anchorage through either the 2′- or the 3′-hydroxy group. We also showed that the efficient recovery of the unreacted ribonucleoside was possible. We demonstrated the applicability of our charged solid support **9** to an automated oligoribonucleotide synthesis in preparing a number of short 3′-aminoacylated RNA strands. We did not yet explore the construction of 3′-peptidyl-RNA conjugates on **9**, although we are convinced that it is possible to achieve, since we synthesized several 3′-oligoalanyl-RNA conjugates on AMP following a similar immobilization methodology using a slightly longer spacer.[14]

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Supporting Information Available: Procedures for the synthesis of **2** and **3**, the automatized oligonucleotide synthesis, ^{1}H NMR spectra and, where applicable, ^{31}P NMR of **2**, **3**, and the (oligo)ribonucleotides. This material is available free of charge via the Internet at http://pubs.acs.org.

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