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A novel solid support for the synthesis of 3′-aminoalkylated oligonucleotides

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Abstract—A novel improved controlled pore glass (CPG) support based on the 2-(hydroxymethyl)-6-nitrobenzoyl (HMNB) protecting group was developed for the synthesis of 3′-aminoalkylated oligonucleotides. The release of oligonucleotides with free 3′-amino groups from the support is complete within 2 h at 55 °C in concentrated ammonia.
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A number of methods in molecular biology and DNA-based diagnostics to amplify, detect, analyze, and quantify nucleic acids require chemically synthesized and modified oligonucleotides. 3′-Modifications are particularly useful to stabilize oligonucleotides against exonucleolytic degradation[1] and are required for the synthesis of doubly-labeled probes with two different covalently-bound reporters at their 3′- and 5′-termini. Doubly labeled oligonucleotides such as molecular beacons[2] or Taqman[3] hydrolysis probes are widely employed in oligonucleotide-based diagnostic assays, especially in real-time quantitative PCR methods.

Oligonucleotides with 3′-modifications are conveniently prepared through standard solid-phase synthesis on speciality 3′-amino supports, which release the oligonucleotides with an attached 3′-aminoalkyl tail during the deprotection process. Suitable supports must be fully compatible with the chemistry cycle of the solid-phase synthesis and the formation of the oligomers with free 3′-amino groups should be complete under the standard deprotection conditions for oligonucleotides. In addition, the attached amino linker should not introduce chiral centers to the oligomer and should not be prone to side reactions that result in dysfunctional oligonucleotide modifications such as alkylated or acylated 3′-amino groups.

Various solid-phase supports for the synthesis of 3′-amino oligonucleotides have been described,[4] but none of these supports displays all of the desirable features for their routine application in a high-throughput oligonucleotide synthesis laboratory. For instance, the synthesis of 3′-amino oligonucleotides on a support loaded with a cleavable 3-aminopropane-1,2-diol linker[4a] resulted in the formation of 3′-acetamido oligonucleotides as side products.[5] The 3-aminopropane-1,2-diol structure also introduces a chiral center to the oligonucleotides, which complicates their analysis by HPLC. The 3′-amino support based on an immobilized phthalimido protective group[4h] requires long deprotection times in concentrated aqueous ammonia for the formation of the free 3′-amine (17 h at 55 °C still leaves 10–20% of the oligonucleotides attached to the support), which reduces the throughput in oligonucleotide production and makes the support incompatible with base-sensitive modifications.

In this communication we report on the development of an improved 3′-amino CPG support that releases 3′-aminoalkyl oligonucleotides within 2 h at 55 °C in concentrated aqueous ammonia. The novel support is based on the 2-(hydroxymethyl)-6-nitrobenzoyl (HMNB) protecting group for the amino function. A similar group, the 2-(acetoxymethyl)benzoyl (AMB) group has been utilized as a very base-labile 'protected protecting group'[6] and has been employed for the exocyclic amino groups of nucleobases in the synthesis of base-sensitive oligonucleotides, for example, methylphosphonates.[7]

Keywords: 3′-Amino modified oligonucleotides; Amino-ON CPG.
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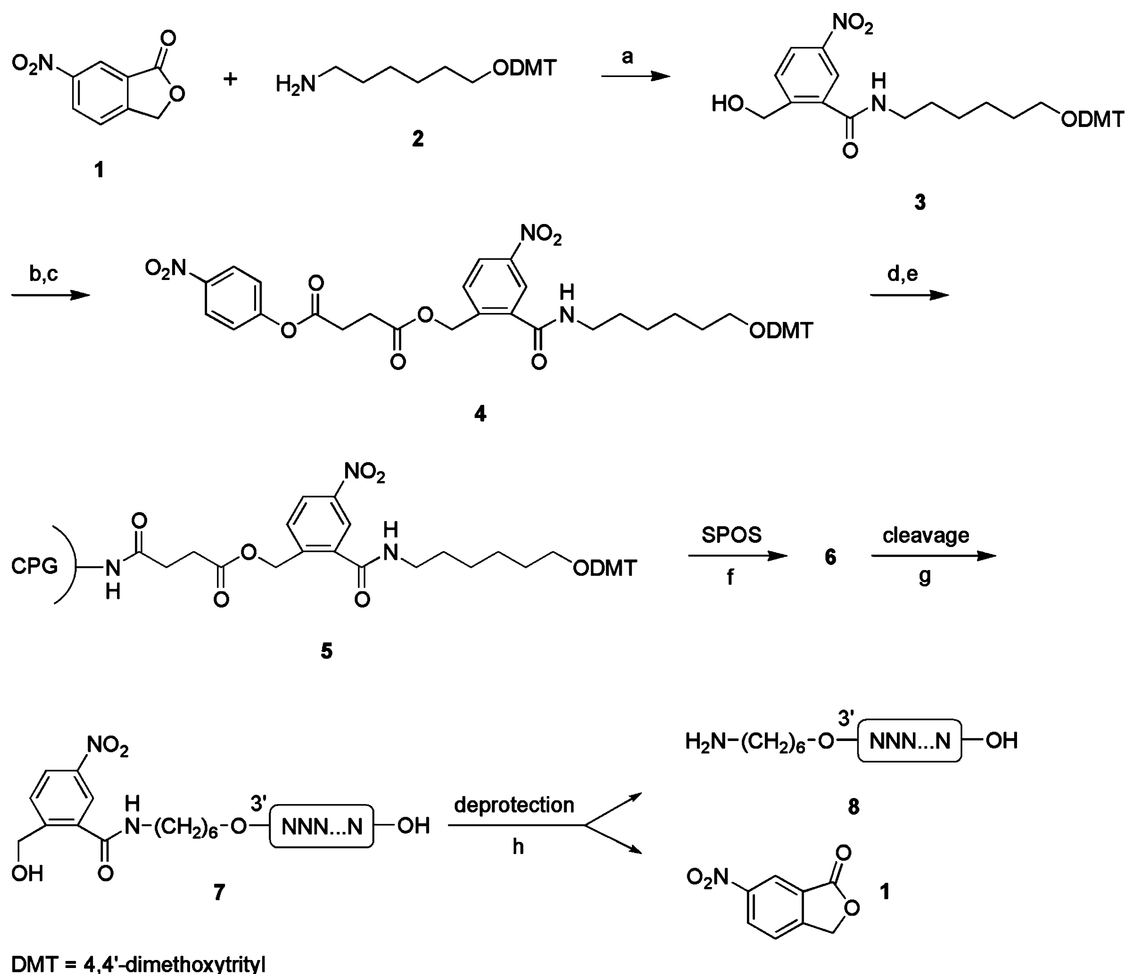

Scheme 1. Synthesis of 3′-amino CPG support **5** and solid-phase oligonucleotide synthesis on **5**. Reagents and conditions: (a) $AlCl_3$, Et_3N, 1,2-dichloroethane, rt, 16 h, 8%;[8] (b) succinic anhydride, Et_3N, DMAP, EtOAc, 50 °C, 2 h; (c) 4-nitrophenol, DCC, 1,4-dioxane, rt, 16 h, 87% over two steps;[9] (d) amino modified CPG, Et_3N, DMF, rt, 16 h; (e) Ac_2O, pyridine, NMI, THF, rt, 2 h;[10] (f) solid-phase oligonucleotide synthesis (SPOS) employing DMT-protected dT-, dA^{bz}-, dC^{bz}-, and dG^{dmf}-phosphoramidites; (g) NH_4OH, rt, 40 min or AMA, rt, 5 min; (h) NH_4OH, 55 °C, 2 h or AMA, 65 °C, 30 min.

The CPG support **5** was prepared in four steps employing 6-nitrophthalide **1** and the DMT-protected amino linker **2**[11] as starting materials (Scheme 1). The synthesis proceeded smoothly despite a rather poor yield in the aminolysis of phthalide **1** with **2**, which can be attributed to the presence of the acidic catalyst $AlCl_3$ in the reaction medium, resulting in partial detritylation. The resulting CPG 500 support **5** had a loading of 36.0 μmol/g.

The novel support **5** releases oligonucleotides with free amino groups **8** in two steps (Scheme 1). First, treatment of support-bound oligonucleotides **6** with concentrated ammonia cleaves HMNB-protected 3′-amino oligonucleotides **7** from the support. In the second step the hydroxymethyl group in the *ortho* position of the aromatic ring removes the HMNB group through an intramolecular attack on the amide bond. 6-Nitrophthalide **1** is formed as a by-product.

The cleavage of oligonucleotides from support **5** and the removal of the HMNB moiety were investigated through 3′-aminoalkyl poly-dT model oligonucleotides. The kinetics under various deprotection conditions were easily monitored by HPLC analysis of the released oligomers and by photometric measurements of the support to determine the amount of uncleaved oligonucleotides. As expected, the HMNB-protected oligonucleotides were completely released into solution within 40 min in concentrated ammonia or within 5 min in AMA reagent[12] (40% aqueous methylamine/concentrated aqueous ammonia 1/1, v/v) at room temperature. The complete removal of the HMNB protecting group from the released oligonucleotides required 2 h in concentrated ammonia at 55 °C or 30 min in AMA reagent at 65 °C. Complete cleavage and deprotection could also be accomplished at room temperature within 24 h in concentrated ammonia. In contrast, the complete removal of the similar 2-(hydroxymethyl)benzoyl group lacking the nitro substituent from 3′-amino oligonucleotides required 6 h in concentrated ammonia at 55 °C.

The utility of support **5** was demonstrated by the synthesis of a poly-dT sequence on a 1:1 mixture of support **5** with a commercially available dT support. The synthesizer was programmed to prepare a dT_{30} oligomer,

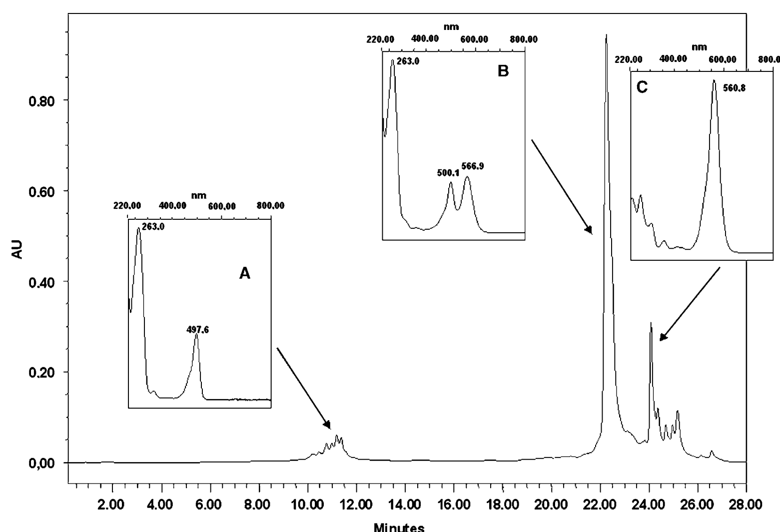

Figure 1. RP-HPLC chromatogram of the crude oligonucleotide **12** after post-synthetic conjugation with QSY7-NHS ester. The UV–vis spectra of the main components are displayed as inserts: (A) nonreacted oligonucleotide with 5′-FAM modification, (B) **12** with 5′-FAM and 3′-QSY7 modifications, (C) oligonucleotide without 5′-FAM, but with 3′-QSY modification.

which gave a mixture of 3′-aminohexyl-d(TTT TTT TTT TTT TTT TTT TTT TTT TTT TT) **9** and d(TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT) **10** after deprotection with concentrated ammonia for 16 h at 55 °C. The ratio of the obtained oligonucleotides **9** and **10** was determined by anion exchange HPLC as 0.96:1.00. The approximately 1:1 ratio of products **9** and **10** demonstrates that the 3′-amino-CPG **5** is compatible with standard solid-phase synthesis conditions applying the phosphoramidite method. The yields and coupling efficiencies are comparable to syntheses on standard CPG supports. A repetition of the experiment with a commercially available phthalimidyl CPG support as a substitute for support **5** gave a ratio of 0.45:1.00 for the oligonucleotide products **9** and **10**. This result can be explained by the stability of the phthalimido group in concentrated ammonia and indicates that the release of the 3′-aminoalkyl-dT_{29} oligonucleotide **9** from the phthalimidyl support is far from being complete under standard deprotection conditions.

Support **5** can be favorably applied in fast deprotection synthetic schemes as exemplified in the preparation of the oligonucleotide 3′-aminohexyl-d(AAC TCC GAG CGA CTC TC)-5′ **11**. A set of dT-, dA^{bz}-, dC^{bz}-, and dG^{dmf}-phosphoramidites[13] was employed in the synthesis to ensure complete base deprotection while applying limited exposure to concentrated ammonia. The crude **11** was obtained after incubation with concentrated ammonia for 2 h at 55 °C in 80% purity (MALDI-TOF MS: calcd 5294.5; found 5294.2).

The 3′-aminoalkylated oligonucleotides synthesized on the CPG support **5** were successfully employed in post-synthetic labeling experiments with dye NHS esters. For instance, the conjugation with TAMRA NHS ester proceeded with more than 90% labeling efficiency under standard conditions, as determined by RP-HPLC. In another example the molecular beacon sequence 5′-FAM-d(GCG AGT TTT TTT TTT TTT TTC TCG C)-QSY7-3′ **12** was synthesized employing dG^{dmf}-phosphoramidite for oligonucleotide synthesis and FAM phosphoramidite for 5′-functionalization. After the deprotection with concentrated ammonia for 2 h at 55 °C the crude oligonucleotide **12** was conjugated at its 3′-terminus with QSY7 NHS ester. The synthesis and the post-synthetic conjugation reaction proceeded with high efficiency as demonstrated in Figure 1. The HPLC-purified molecular beacon **12** (MALDI-TOF MS: calcd 8950.4; found 8939.6) displayed a sharp increase in fluorescence upon incubation with a poly-dA sequence under hybridization conditions.

In conclusion, the novel amino-ON CPG support **5** is compatible with standard solid-phase oligonucleotide synthesis conditions employing the phosphoramidite method, and the observed coupling efficiencies and yields are comparable to those obtained with standard deoxynucleoside-loaded CPG supports. The application of mild cleavage and deprotection conditions allows the use of **5** in high throughput oligonucleotide synthesis and the on-support preparation of labile 5′-modified oligonucleotides with 3′-aminoalkyl chains.

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9. The amide **3** (200 mg, 0.334 mmol) was reacted in 1.5 mL EtOAc with succinic anhydride (40 mg, 0.401 mmol) in the presence of Et_3N (46 µL, 0.327 mmol) and 4-dimethylaminopyridine (10 mg, 0.084 mmol) for 2 h at 50 °C. The mixture was concentrated and redissolved in CH_2Cl_2. The solution was extracted with 10% aqueous citric acid and water, dried over Na_2SO_4, and concentrated to a foam. The residue was redissolved in 1,4-dioxane (3 mL) and pyridine (70 µL) and reacted with 4-nitrophenol (50 mg, 0.358 mmol) and 1,3-dicyclohexylcarbodiimide (94 mg, 0.456 mmol) at room temperature overnight. The reaction mixture was filtered and concentrated to give 250 mg (87%) of the nitrophenylester **4** as a light yellow foam, which was used as a crude product in subsequent reactions. ^1H NMR (300 MHz, CD_3CN) 8.28–8.21 (m, 3H), 7.72–7.70 (m, 1H), 7.46–7.42 (m, 2H), 7.35–7.13 (m, 10H), 6.88–6.84 (m, 4H), 5.40 (s, 2H), 3.76 (s, 6H), 3.33 (q, $J = 6.6$ Hz, 2H), 3.02 (t, $J = 6.4$ Hz, 2H), 2.94–2.90 (m, 2H), 2.83–2.79 (m, 2H), 1.66–1.52 (m, 4H), 1.48–1.32 (m, 4H). MS (FAB) m/z 819 $[M+H]^+$.
10. CPG 500 Å with an aminohexyl spacer (5.0 g) was suspended in DMF (13 mL) and Et_3N (0.5 mL). Nitrophenyl ester **4** (250 mg, 305 µmol) was dissolved in DMF (4.0 mL) and added to the suspension, which was then left overnight on an orbital shaker at room temperature. The CPG was washed with DMF/Et_3N 9/1 (v/v), DMF, EtOH, acetonitrile, and EtOAc in multiple 20 mL portions. The CPG was finally treated with commercially available Cap A (Ac_2O/THF) and Cap B (1-methylimidazole/pyridine/THF) synthesis solutions for 2 h and washed with THF, EtOH/pyridine 9/1 (v/v), EtOH, acetonitrile, and EtOAc. The loading was determined to be 36.0 µmol/g by a photometric (497 nm) DMT loading assay.
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