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4-Oxoheptanedioic acid: an orthogonal linker for solid-phase synthesis of base-sensitive oligonucleotides

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ABSTRACT

l,6-Dioxaspiro[4,4]nonane-2,7-dione has been found to react readily with alcohols in the presence of 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU). The 4-oxoheptanedioic acid tether obtained bears (l) a free carboxy group, which enables anchoring to aminoalkylated resins, and (2) a 4-oxobutanoate structural motif, which allows release of the target alcohol by a mild hydrazinium acetate treatment. To demonstrate the applicability of the procedure, nucleosides have been derivatized with this simple difunctional linker arm, anchored to a solid phase and subjected to synthesis and subsequent release of oligonucleotides bearing a base-sensitive biodegradable phosphate protection.

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Synthesis of acid- and base-labile conjugates of oligonucleotides, oligosaccharides or small molecular alcohols on a solid support may require utilization of an orthogonal linker which (1) anchors the molecule via a hydroxyl group, (2) withstands various chemical manipulations, but (3) may still be cleaved under mild conditions. Antisense oligonucleotides bearing biodegradable phosphate protections offer an illustrative example.[1] The biodegradability is usually based on enzymatically hydrolyzable ester linkages which do not withstand conventional ammonolytic deprotection and, hence, an orthogonally cleavable linker and orthogonally removable nucleobase protections have to be used.[2] Unfortunately, there are few such linkers which are readily available. Multistep derivatization of the 3′-terminal nucleoside has to be carried out before attachment to the solid support. In addition, the release is often slow or prone to side reactions. In some cases, a base-labile hydroquinone-*O*,*O*-diacetic acid ('Q-linker')[3] offers a viable alternative, since its cleavage using potassium carbonate in methanol is sufficiently fast to leave the ester functions intact. The Q-linker is commercially available and compatible with the methods generally applied to the attachment of the 3′-terminal nucleoside to solid supports. We herein describe a novel, easily available difunctional handle, which meets the requirements of orthogonality.

The linker precursor, viz. 1,6-dioxaspiro[4,4]nonane-2,7-dione, is a cheap commercially available reagent, which reacts readily with alcohols in the presence of the strong organic base, DBU. The resulting tether bears (1) a free carboxy function, which allows anchoring of the prefabricated terminal unit to the support via an amide linkage and (2) a keto group at the 4 position to the ester linkage, which enables release from the support by mild hydrazinium acetate treatment. The latter reaction is used extensively for removal of levulinoyl protections[4] as 6-methyl-4,5-dihydropyridazin-3(2*H*)-one,[5] but surprisingly, a similar cyclative cleavage has not been applied to linker chemistry. Hydrazinium acetate treatment has only been used for release of oligosaccharides tethered by a succinyl linker arm via the anomeric hydroxyl function,[6] but such a release cannot be extended to succinates of non-anomeric hydroxyl groups. The applicability of the present 4-oxoheptanedioic acid linker arm has been demonstrated by the synthesis of oligonucleotides bearing a base-sensitive biodegradable phosphate protection. The procedure for the derivatization and immobilization of the 3′-terminal nucleoside is exactly the same as with the succinyl linker arm, but the cleavage may be performed under conditions which leave ester functions intact. In contrast to primary succinamides, which may undergo succinimide formation and, hence, result in a premature cleavage,[7] the present linker is stable in the presence of a strong non-nucleophilic Brønsted base.

The reaction between 1,6-dioxaspiro[4,4] nonane-2,7-dione and a nucleoside has been optimized with 5′-*O*-DMTr-thymidine in the presence of various base/solvent systems [DBU/DMF, DBU/dioxane, 4-dimethylaminopyridine (DMAP)/pyridine, DMAP/dioxane, NaH/DMF]. Among these systems, DBU in dioxane turned out to be the method of choice giving the desired tethered nucleoside **2a**[8] in 75% yield (Scheme 1). The reaction occurred readily also in the presence of NaH in DMF, but this treatment cannot be applied to derivatization of nucleosides having an exocyclic amino group. The other organic bases tested (DMAP and pyridine) turned out to be ineffective for the reaction. The prefabricated handle **2a** may be anchored to a long chain alkylamine controlled pore glass (LCAA-CPG, $0.10\ \mathrm{mmol\ g^{-1}}$) in a conventional manner using

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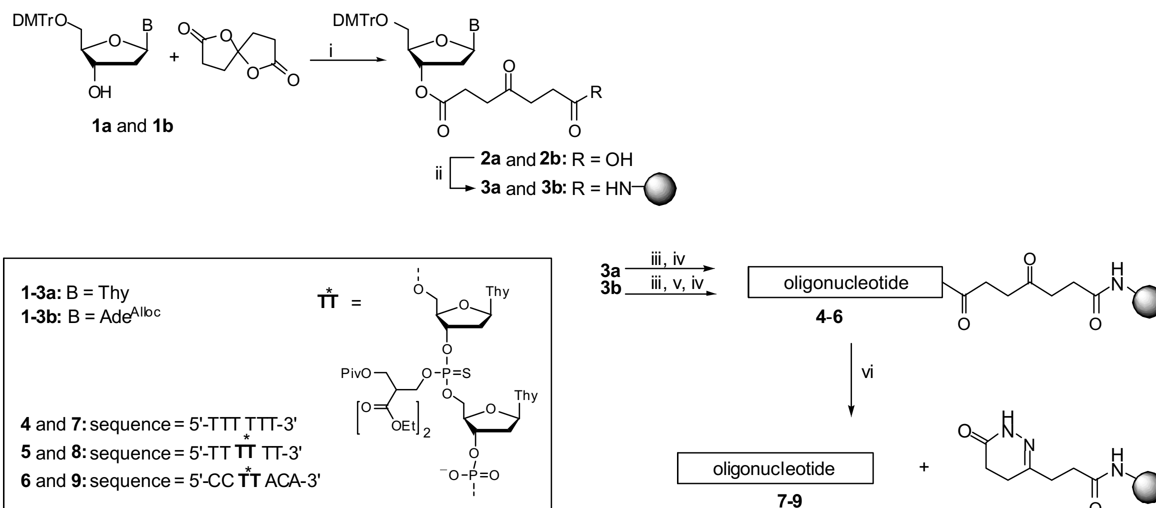

Scheme 1. Reagents and conditions: (i) dioxaspiro[4,4]nonane-2,7-dione (structure also shown, 2 equiv), DBU (2 equiv), dioxane, **2a**: 75%, **2b**: 74%; (ii) LCAA-CPG, **2a** or **2b** (1.0 equiv), HOSu (1.0 equiv), DIC (1.0 equiv), pyridine, 15 h at 25 °C, **3a**: 17 μmol g^{-1}, **3b**: 19 μmol g^{-1}; (iii) phosphoramidite chemistry following the standard RNA coupling protocol; (iv) $(PPh_3)_4Pd^0$ (cat.), 5% phenylsilane in CH_2Cl_2 (v/v), N_2 atm, 1 h at 25 °C; (v) 1.5% DBU in acetonitrile, 1 h at 25 °C; (vi) $NH_2NH_2 \cdot H_2O$/AcOH/pyridine (0.0124/1/4, v/v/v), 1 h at 25 °C.

N-hydroxysuccinimide/diisopropylcarbodiimide (HOSu/DIC)-activation (**3a**, 17 μmol g^{-1}). The unreacted amino groups on the support were capped using acetic anhydride. The more powerful coupling reagents, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]-pyridin-1-ylmethylene]-*N*-methylmethan aminium hexafluorophosphate *N*-oxide (HATU)[9] and 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium tetrafluoroborate 3-oxide (TBTU),[10] were also tested. With these reagents an increased acylation efficiency was obtained, but a high loading (40–60 μmol g^{-1}) was found to retard the DBU-promoted β-elimination used for removal of the cyanoethyl groups (cf. below).

The cleavage of the linker was attempted with various hydrazinium acetate treatments for the release of support-bound DMTr-dT (**3a**) and T_6 (**4**). The progress of the release was monitored by RP HPLC and UV spectroscopy following both the increased amount of released T_6 (**7**) in the mixture and the decreased DMTr cation response on the support (**3a**). The best result was obtained with a mixture of 50 mmol L^{-1} hydrazinium acetate ($NH_2NH_2 \cdot H_2O$/AcOH/pyridine, 0.0124:1:/4, v/v/v, at 25 °C), which provided practically complete cleavage of the linker in 1 h (see • in Fig. 1). The hydrazinium acetate concentration was only one-tenth of that generally used for the removal of the levulinoyl group.[5] It should be noted that a trace amount was released over a longer period. This was attributed to the potential inhomogeneity of the resin. The reaction occurred also in the absence of pyridine (50 mmol L^{-1} $NH_2NH_2 \cdot AcOH$ in water), but with a decreased rate (see ▲ in Fig. 1). Interestingly, an equally slow cleavage rate was obtained in 50 mmol L^{-1} $NH_2NH_2 \cdot H_2O$ in 0.1 mol L^{-1} aqueous triethylammonium acetate. The advantage of these latter procedures is that the cleavage mixtures may be subjected to RP HPLC without workup.

The stability of the linker in a mixture of 1.5% DBU in dry acetonitrile (1 h at 25 °C) was followed by the DMTr-cation assay of **3a** and the solid-supported oligonucleotides (cf. **4**–**6**). Upon this treatment, which may be used for on-resin removal of, for example, *O*-cyanoethyl and *O*-4-nitrophenylethyl protections,[11] no decrease of DMTr-loadings was observed.

The Q-linker and a photo-labile 5-(4-hydroxymethyl-6-methoxy-3-nitrophenoxy)butanoyl linker had previously been used for the solid phase synthesis of oligonucleotides bearing a biodegradable 2,2-bis(ethoxycarbonyl) 3-(pivaloyloxy)propyl phosphate

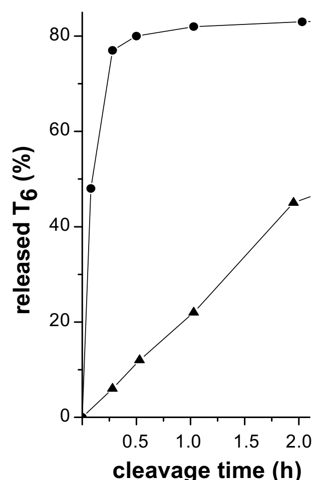

Figure 1. Cleavage of anchored T_6 using different hydrazinium acetate treatments. Notation: 50 mmol L^{-1} $NH_2NH_2 \cdot H_2O$ in AcOH–pyridine (1:4, v/v), at 25 °C (•), 50 mmol L^{-1} $NH_2NH_2 \cdot AcOH$ in H_2O (▲).

protection.[12] The applicability of the present linker for this purpose was demonstrated by the synthesis of oligonucleotides **8** and **9** bearing the same protection. The required dimeric building block, that is, 5′-*O*-(4,4′-dimethoxytrityl)-(*R*P,*S*P-*O*P-[2,2-bis(ethoxycarbonyl)-3-(pivaloyloxy)propyl]-*P*-thiothymidylyl-(3′,5′)-thymidine 3′-[*O*-(2-cyanoethyl)(*N*,*N*-diisopropyl)]phosphoramidite], incorporated into the oligonucleotides (cf. **T*T** in Scheme 1, was prepared as previously described.[12] For the synthesis of the hetero oligonucleotide (**9**), allyloxycarbonyl (Alloc) was used for exocyclic amino group protection.[13] The corresponding Alloc group protected cyanoethyl phosphoramidites were prepared according to the literature.[13] The N^4-Alloc-5′-*O*-DMTr-dA modified resin **3b** was prepared as described for dT (**3a**) above.[7] The prefabricated handle **2b** was obtained in a 74% yield and a loading of 19 μmol g^{-1} was obtained for the resin **3b**. Due to the somewhat low coupling efficiency of the dimeric building block, oligonucleotides **5** and **6** were assembled following standard RNA coupling protocol. The Alloc protections of the hetero oligonucleotide **6** were removed by $(PPh_3)_4Pd^0$ catalyzed treatment in a mixture of 5% phenylsilane

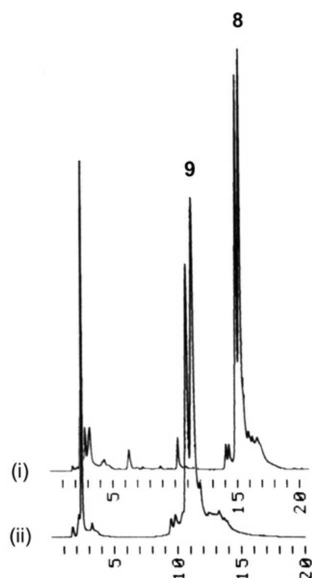

Figure 2. RP HPLC chromatograms of the crude product (**8** and **9**) mixtures, gradient elution from 15% to 35% acetonitrile in 0.1 mol L^{-1} $Et_3N^+OAc^-$ buffer over 20 min.

in dichloromethane (1 h at 25 °C, N_2 atm).[14] The cyanoethyl groups were removed as described above and the oligonucleotides **5** and **6** were released by treatment with a mixture of 50 mmol L^{-1} hydrazinium acetate (1 h at 25 °C).[15] The excess hydrazinium acetate was capped by acetone, and the mixtures were filtered. For solubility reasons, the resins were washed with water, the filtrates were evaporated to dryness and the resulting oligonucleotides **8** and **9** were then subjected to RP HPLC. Crude RP HPLC chromatograms of the product mixtures are outlined in Figure 2 (note R_P and S_P-stereoisomers). Yields of the oligonucleotides **8** and **9** were 70% and 60%, respectively. The authenticity of the products was verified by MS(ESI); **8**: found 1031.2 $[(M-2)/2]^{-2}$ and **9**: found 1170.2 $[(M-2)/2]^{-2}$. For the preparation of these oligonucleotides **8** and **9**, the present linker worked successfully, leaving the 2,2-bis(ethoxycarbonyl) 3-(pivaloyloxy)propyl phosphate protection intact. In this preliminary study the demonstrated compatibility of the linker is limited to the preparation of short oligonucleotides bearing ester groups. Further studies with more biologically relevant longer oligonucleotides would be advisable. It may, however, be noteworthy that the chemistry related to the linker is well known from the levulinoyl protection, the compatibility of which has been studied extensively with a wide range of functional groups and automated oligonucleotide synthesis.

In conclusion a novel and very simple orthogonal linker and its applicability for the synthesis of base-sensitive oligonucleotides have been described. The procedure for the nucleoside derivatization is exactly the same as with the succinyl linker arm, but cleavage may be performed with hydrazinium acetate, which leaves esters intact. In addition, the present linker is orthogonal with the protecting groups, which may be removed by β-elimination.

Acknowledgement

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8. *Procedure for the preparation of tethered nucleosides* (**2a** and **2b**): DMTr-dT (**1a**, 0.50 g, 0.92 mmol), 1.6-diooxaspiro[4.4]nonane-2.7-dione (0.30 g, 1.9 mmol) and DBU (0.28 mL, 1.9 mmol) were dissolved in dioxane (0.5 mL). The mixture was stirred at ambient temperature for 20 h and evaporated to dryness. The residue was purified by silica gel chromatography (1% pyridine and 10% MeOH in CH_2Cl_2) to yield 0.49 g (75%) of the product **2a** as a white foam. Compound **2a**: ^1H NMR (500 MHz, $CDCl_3$) δ 9.50 (br s, 1H), 7.65 (s, 1H), 7.40 (m, 2H), 7.33–7.17 (m, 7H), 6.85 (d, *J* = 8.8 Hz, 4H), 6.42 (dd, 1H, *J* = 8.8 Hz, 5.7 Hz), 5.47 (d, 1H, *J* = 5.9 Hz), 4.16 (br s, 1H), 3.81 (s, 6H), 3.49 (dd, 1H *J* = 10.8 Hz, 2.3 Hz), 3.47 (dd, 1H, *J* = 10.7 Hz, 2.0 Hz), 2.84 (m, 2H), 2.77 (m, 2H), 2.66 (m, 2H), 2.61 (m, 2H), 2.49 (1H, dd, *J* = 13.9 Hz, 5.6 Hz), 2.43 (m, 1H), 1.36 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.3, 164.3, 159.07, 159.05, 151.2, 144.5, 135.9, 135.6, 135.5, 130.44, 130.40, 128.46, 128.35, 127.5, 113.6, 112.3, 87.5, 84.8, 84.2, 76.0, 55.6, 38.1, 37.5, 37.2, 28.6, 11.9; HRMS(ESI): found 739.2258 $[M+K]^+$. Compound **2b** was prepared from **1b** as described for **2a**. Compound **2b** was obtained as a white foam in 74% yield. Compound **2b**: ^1H NMR (500 MHz, $CDCl_3$) δ 8.60 (s, 1H), 8.27 (s, 1H), 7.37 (m, 2H), 7.28–7.18 (m, 7H), 6.78 (m, 4H), 6.49 (dd, 1H *J* = 7.0 Hz, 6.8 Hz), 6.02 (m, 1H), 5.56 (m, 2H), 5.43 (m, 1H), 5.28 (m, 1H), 4.77 (m, 2H), 4.33 (br s, 1H), 3.77 (s, 6H), 3.47 (dd, 1H, *J* = 10.6 Hz, 4.3 Hz), 3.44 (1H, dd, *J* = 10.6 Hz, 4.0 Hz), 3.07 (m, 1H), 2.86 (m, 2H), 2.78 (m, 2H), 2.68 (m, 1H), 2.64 (m, 2H), 2.55 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 208.7, 173.0, 159.2, 152.8, 152.2, 151.4, 150.3, 145.1, 142.4, 136.1, 132.5, 130.6, 128.7, 128.6, 128.0, 127.3, 122.7, 118.2, 113.3, 87.3, 85.5, 85.0, 75.9, 66.9, 64.2, 55.4, 37.9, 37.5, 37.4, 28.5, 28.4; HRMS(ESI): found 832.2567 $[M+K]^+$.
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15. *Procedure for the release of oligonucleotides: Resin* (**4–6**, 30 mg, containing 0.5 μmol of the oligonucleotide) was suspended in a mixture of 50 mmol L^{-1} hydrazinium acetate (0.5 mL, $NH_2NH_2{\cdot}H_2O$/AcOH/pyridine, 0.0124/1/4, *v*/*v*/*v*). After 1 h mixing (at 25 °C), acetone (0.5 mL) was added and the suspension was mixed for an additional 15 min. After capping of hydrazine, the resin was filtered and washed with water (3 × 0.5 mL). The filtrate was evaporated to dryness, the residue was dissolved in water, and then the product (**7–9**) mixture was subjected to RP HPLC.