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A VERSATILE ACID-LABILE LINKER FOR MODIFICATION OF SYNTHETIC BIOMOLECULES

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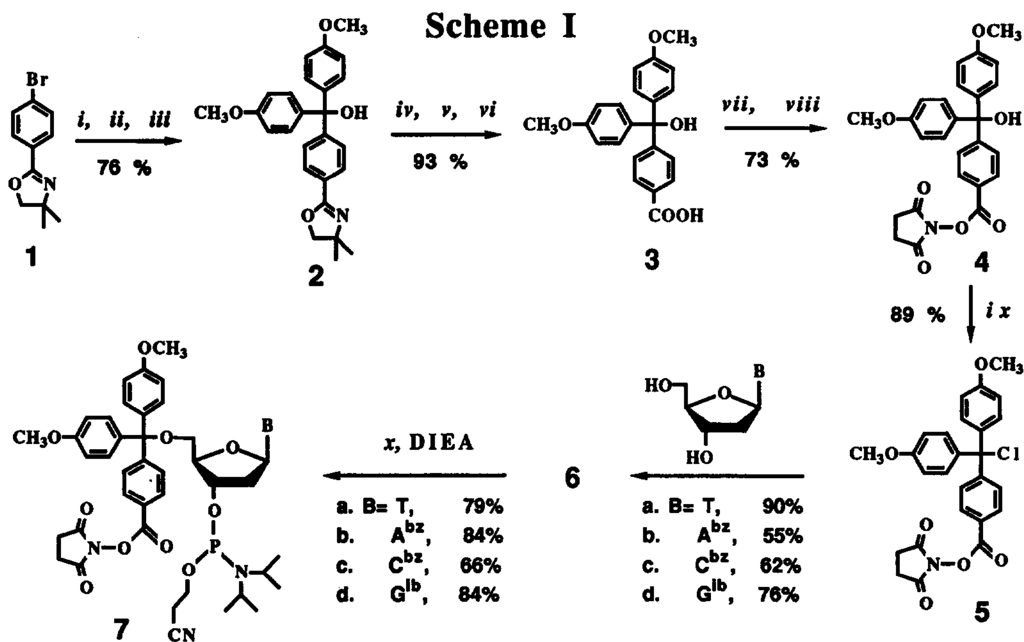
Summary: A triphenylmethyl protecting group/linker suitable for automated labeling of synthetic biopolymers is described. The method is unique since the protecting group/linker may be removed to give the unmodified biomolecule.

In response to intensifying concerns over hazards associated with use and disposal of radionuclides, non-isotopic reporter groups have become increasingly utilized for detection of biopolymers[1-4]. The conventional approach to preparation of a labeled synthetic biopolymer involves introduction of a nucleophilic functional group that is subsequently reacted with an electrophilic reporter moiety[2-11]. Protected amino group containing nucleoside derivatives[2-6], thiol group containing nucleoside derivatives[7] and 5'-terminal linkers[8-11] have been described for DNA labeling applications. All of these can be easily incorporated during chemical synthesis of an oligonucleotide, but in no case can the linker and/or label be selectively removed to generate natural (unmodified) DNA. We reasoned that a cleavable linker might be useful for the manipulation, purification or analysis of chemically and enzymatically synthesized nucleic acids. As the 4,4'-dimethoxytrityl group (DMT) is routinely utilized for the protection of 5'-hydroxyl groups of nucleotide intermediates during chemical assembly of DNA and RNA, we decided that synthesis of a modified DMT derivative containing an exocyclic active ester group would allow us to selectively introduce a reactive electrophilic site at the 5'-termini of synthetic oligonucleotides. To this end, we undertook the synthesis of 5 (Scheme I) which can be considered the combination of a regioselective protecting group and a linking group. Others[12] had suggested that "suitably derivatized trityl moieties" might be attractive candidates for effecting the reversible modification of synthetic DNA, but to our knowledge no such compounds or their application have been previously described.

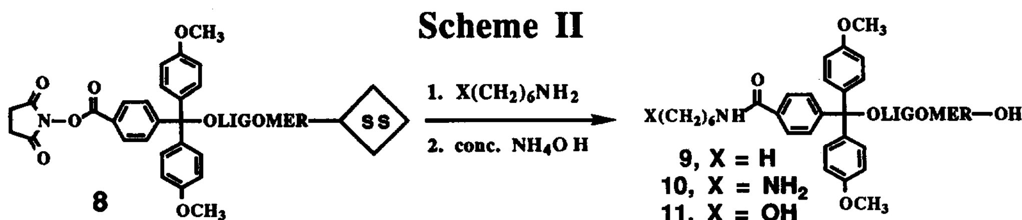
Meyers and Temple[13,14] proposed the 2-oxazoline as a means of protection for the carboxylic acid group during Grignard reactions. This approach appeared well suited to the synthesis of the desired 4-carboxy-4',4"-dimethoxytriphenylhydroxymethane intermediate 3. Consequently, the Grignard reagent formed from 2-(4-bromophenyl)-4,4-dimethyl-1,3-oxazoline (1) was allowed to react with 4,4'-dimethoxybenzophenone (DMBP) to give 2-[4-(bis-4-(methoxyphenyl)-hydroxymethyl)-phenyl]-4,4-dimethyl-1,3-oxazoline (2) as a crystalline solid (mp. 149-151 °C). Hydrolysis of the oxazoline ring produced 4-carboxy-4',4"-dimethoxyphenylhydroxymethane (3) (mp. 106-109 °C)[15]. The active ester 4 was obtained by dicyclohexylcarbodiimide (DCC) mediated condensation of 3 with N-hydroxysuccinimide (NHS) (two crystalline forms: mp. 170-172 °C or 188-190 °C). Thereafter, 4 was converted to N-succinimidyl-4-[bis-(4-methoxyphenyl)-chloromethyl]-benzoate (5) by treatment with acetyl chloride[16]. The product crystallized conveniently from the reaction mixture as a white solid (mp. 209-211 °C). Regioselective tritylation of the 5'-hydroxyl group of the partially protected four common 2'-deoxynucleosides gave 6a-d[17,18] which were converted to their respective 2'-cyanoethylphosphoramidite derivatives (7a-d)[19] in the usual manner[20].



i = Mg, *ii* = DMBP, *iii* = 5% aqueous $KHSO_4$, *iv* = 80% aqueous HOAc, *v* = 20% NaOH, *vi* = HCl, *vii* = DCC, *viii* = NHS, *ix* = acetyl chloride, *x* = 2-cyanoethyldiisopropylaminochlorophosphine

It was subsequently shown that 7a-d could be condensed with the 5'-hydroxyl group of support bound partially protected oligonucleotides using an automated synthesizer and a standard coupling cycle (Method A). For example, tetrazole catalyzed reaction of 7a with resin bound HO-CCCAGTCACGACGT gave NHS-DMT-TCCCAGTCACGACGT (8). Alternatively, 8 was prepared (Method B) by on-resin etherification of HO-TCCCAGTCACGACGT with 5 in the presence of tetra-N-butylammonium perchlorate and collidine[12,21].

The 5'-terminal NHS ester of 8, prepared by methods A and B, was then treated with 1-aminohexane, 1,6-hexanediamine and 6-amino-1-hexanol (Scheme II)[22]. As a control, resin bound DMT-TCCCAGTCACGACGT was prepared and similarly treated. Following removal from the support and partial deprotection, the oligonucleotides were analyzed by reversed-phase HPLC[23]. Retention times for the 5' alkyl (9), aminoalkyl (10) and hydroxyalkyl (11) terminated products, as well as a control oligomer possessing the DMT group, are presented in the Table. Integration of peak areas showed the desired products were prepared in similar yield by either method relative to the control syntheses and that no reaction between the control oligonucleotides and the various amine nucleophiles had occurred. Acid catalyzed hydrolysis of the trityl ether bond[24] of 9, 10, and 11 gave, as expected, deprotected oligonucleotides that coeluted with the detritylated control samples.



TABLE

Sample	Method	Sample Description	Protected: Ret. Time (min)	Protected: Area %	Detritylated: Ret. Time (min)
Control	- -	DMT-TCCCAGTCACGACGT	26.89	50.3	10.82
9	A	Hexyl-DMT-TCCCAGTCACGACGT	31.84	45.8	10.82
	B	Hexyl-DMT-TCCCAGTCACGACGT	31.84	43.6	10.83
10	A	6-Aminohexyl-DMT-TCCCAGTCACGACGT	22.84	42.6	10.82
	B	6-Aminohexyl-DMT-TCCCAGTCACGACGT	22.79	40.6	10.80
11	A	6-Hydroxyhexyl-DMT-TCCCAGTCACGACGT	25.24	51.2	10.81
	B	6-Hydroxyhexyl-DMT-TCCCAGTCACGACGT	25.24	47.8	10.83

Compound 10, containing a 5'-terminal amine group, was of greatest interest as a precursor to labeled oligonucleotides. Thus 10 was reacted in aqueous HEPES buffer, pH 7.7, with the NHS ester of d-biotin to generate a 5'-biotinylated oligonucleotide (12)[8]. The reaction was nearly quantitative in two hours as assayed by reversed-phase HPLC. The biotin label was also introduced prior to removal of the aminoalkyl terminated oligomer from the synthesis support by exposure of the resin to NHS-d-biotin in the presence of diisopropylethylamine[25]. No significant differences in product yield or purity were observed for the two different labeling methods but it was more convenient to label oligomers while resin bound.

To demonstrate the utility of this cleavable linker, the biotin/streptavidin affinity system was used to purify DNA fragments. Initially, 12 was shown to be quantitatively bound by streptavidin agarose (Figure 1) whereas the amino terminated oligomer 10 was not[26]. Treatment of the agarose with aqueous acetic acid liberated 85% of the captured oligomer in its fully deprotected form (Figure 1C)[27]. We then prepared biotinylated primers similar to 12 for amplification of nucleic acids by the polymerase chain reaction[28]. Amplified fragments were rapidly purified and obtained in their unmodified form suitable for direct sequence analysis or cloning.

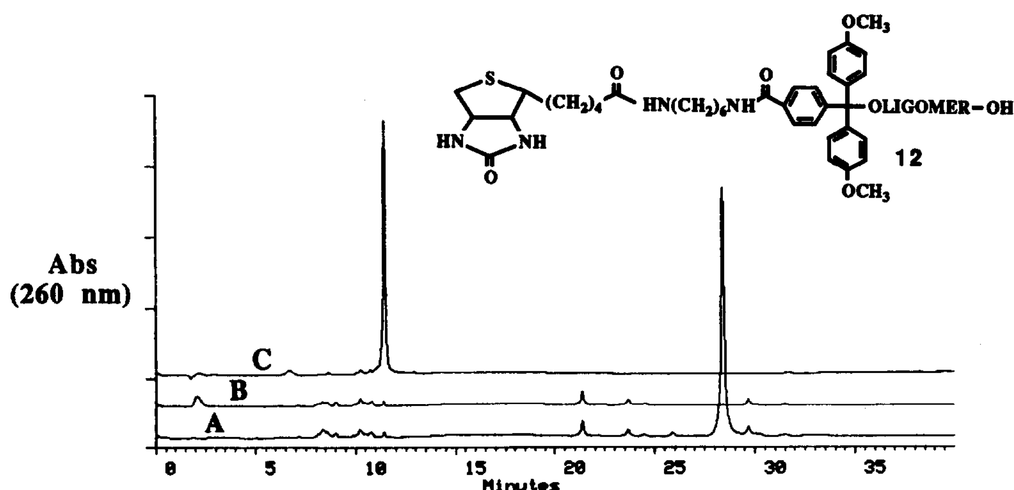


Figure 1. Reversed-phase HPLC[23] analysis of binding of 12 to streptavidin agarose. A) Crude 12 prior to exposure to streptavidin agarose. B) Material recovered from the supernatant upon incubation of 12 with streptavidin agarose. C) The fully deprotected oligomer (HO-TCCCAGTCACGACGT-OH) obtained upon treatment of the support with 80% aqueous acetic acid.

Although developed for applications in the field of nucleic acid chemistry, compound 5 may be employed for protection and derivatization of other synthetic biopolymers such as peptides and oligosaccharides. In contrast to conventional labeling methods, the use of 5 allows one to easily vary the length and composition of the tether between the label and the biomolecule. If desired, the label/linker/protecting group can be removed to generate the unmodified biopolymer and/or facilitate detection of the label. Advantages afforded by the convenient removal of the label/linker/protecting group should find application in life science research.

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References and Notes

1. Beck, S., O'Keefe, T., Coull, J.M. and Köster, H. (1989) Nucl. Acids Res., **17**, 5115-23
2. Ruth, J.L. (1984) DNA, **4**, 327
3. Smith L.M., Fung, S., Hunkapiller, M.W., Hunkapiller, T.J. and Hood, L.E. (1985) Nucl. Acids Res., **13**, 2399-412
4. Haralambidis, J., Chai, M. and Tregear, G.W. (1987) Nucl. Acids Res., **15**, 4857-76
5. Gibson, K.J. and Benkovic, S.J. (1987) Nucl. Acids Res., **15**, 6455-67
6. Sproat, B.S., Beijer, B. and Rider, P. (1987) Nucl. Acids Res., **15**, 6181-96
7. Sproat, B.S., Beijer, B., Rider, P. and Neuner, P. (1987) Nucl. Acids Res., **15**, 4837-48
8. Coull J.M., Weith, H.L. and Bishoff, R. (1986) Tett. Lett., **27**, 3991-94
9. Agrawal, S., Christodoulou, C. and Gait, M.J. (1986) Nucl. Acids Res., **14**, 6227-45
10. Connolly, B.A. (1987) Nucl. Acids Res., **15**, 3131-39
11. Sinha, N.D. and Cook, R.M. (1988) Nucl. Acids Res., **16**, 2659-69
12. Reddy, M.P., Rampal, J.B. and Beaucage, S.L. (1987) Tett., Lett. **28**, 23-26
13. Meyers, A.I. and Temple, D.L. (1970) J. Amer. Chem. Soc., **92**, 6644-6646
14. Meyers, A.I. and Temple, D.L. (1970) J. Amer. Chem. Soc., **92**, 6646-6647
15. Compound 2 was treated with 80% aqueous acetic acid (2 mL/mmol) for 12-14 hours at 55-60 °C. The mixture was concentrated and dissolved in 20% NaOH (w/v) in ethanol/water (1/1,v/v) (2 mL/mmol) at vigorous reflux for 2 hours. The mixture was again concentrated, dissolved in water (2 mL/mmol) and acidified with 6 M HCl. The product was extracted into ethyl acetate and crystallized from benzene.
16. Compound 4 was boiled in acetyl chloride (5 mL/mmol) for 3 hours. The reaction flask was cooled and an equal volume of anhydrous ether was added. After 16 hours, 5 was isolated by filtration.
17. Smith, M., Rammler, D.H., Goldberg, T.H. and Khorana, H.G. (1962) J. Amer. Chem. Soc., **84**, 430
18. Compounds 1-6a-d were characterized by ^{1}H-NMR. The structures of 1-5 were also confirmed by elemental analysis and infrared absorption spectroscopy.
19. The ^{31}P-NMR spectra of 7a-d were as expected for 2-cyanoethylphosphoramidites.
20. Sinha, N.D., Biermat, J., McNanus, J. and Köster, H. (1984) Nucl. Acids Res. **12**, 4539-4557
21. A solution of 5 (0.25 M), tetra-N-butylammonium perchlorate (0.25 M) and sym-collidine (0.38 M) in dichloromethane (600 uL) was delivered to the resin using a MilliGen/Biosearch 7500 DNA synthesizer. After 40 minutes the support was then thoroughly washed with acetonitrile and dried in vacuo.
22. Portions of resin (0.2 mmol) were treated with 1 mL of dioxane/water (9:1 v/v) containing the various amino compounds (1 M) for two minutes. The supports were then washed with 2 mL of dioxane/water (9:1 v/v), 2 mL of acetonitrile and dried in vacuo.
23. Conditions: Buffer A, 100 mM TEA-HOAc, pH 7.1; Buffer B, Acetonitrile/H_2O (95/5, v/v); Linear Gradient; 5-40% B over 40 min; Flow rate: 1 mL/min; Temperature: 40 °C; Detector: 260 nm.
24. Oligonucleotides were treated for 2 hours with 80% aqueous acetic acid at ambient temperature.
25. The support was treated with 1 mL of 0.2 M NHS-d-biotin dissolved in DMF/DIEA/H_2O (8/1/1, v/v/v) for 60 minutes. The resin was washed with 2 mL of warm aqueous DMF (8/2, v/v) and 2 mL of acetonitrile.
26. To 200 uL of streptavidin agarose (BRL) in an Ultrafree spin cartridge (Millipore) was added 100 uL of buffer (9 mM $NaHPO_4$, pH 7.2, 135 mM NaCl and 0.018% NaN_3 in acetonitrile/H_2O (1/9, v/v)) and 100 uL of DNA (0.3 OD_{260} units). After 30 minutes the supernatant was removed by centrifugation and the support was washed twice with buffer. The eluates were combined for analysis by reversed-phase HPLC.
27. To the support was added 100 uL of 0.5 M NaCl in 80% aqueous acetic acid. After 2 hours, the eluent was removed by centrifugation and the resin was washed twice with 0.25 M Tris HCl, pH 9.0, 0.15 M NaCl and 0.05% NaN_3 in acetonitrile/H_2O (2/8, v/v). The eluates were combined for analysis.
28. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988) Science, **239**, 487-491

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