Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 8, pp. 1041-1046, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00152-2

A CONVENIENT SYNTHETIC ROUTE TO OLIGONUCLEOTIDE CONJUGATES

Joseph G. Harrison and Shankar Balasubramanian*

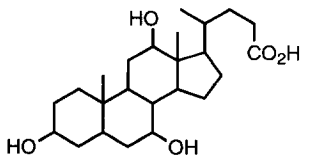
Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW

Abstract: The synthesis of oligodeoxyribonucleotide 13mers conjugated to cholic, folic, lipoic and pantothenic acids through thioether and disulphide bonds is described employing a convenient methodology which involves initial preparation of cysteinyl derivatives of the acids on solid phase.

The use of sequence specific antisense or antigene oligonucleotides to control gene expression is a promising rational drug design approach to treat a range of diseases that include viral infections and cancers.[1] In recent years considerable effort has been invested in the synthesis of chemically modified oligonucleotides[2] to improve nuclease stability,[3] target binding affinity[4] and cellular uptake properties.[5] We have chosen to synthesise conjugates of an oligodeoxyribonucleotide with the following biomolecules: cholic, folic, lipoic and pantothenic acids (Figure 1). We wish to test the hypothesis that the natural receptor mediated uptake mechanisms for biomolecules **a** to **d** may enhance the cellular uptake of the corresponding oligonucleotide conjugate.[6]

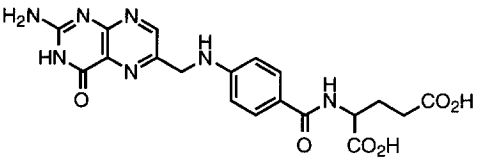
In this paper we describe the preparation of oligonucleotide conjugates of biomolecules **a** to **d** by a simple and effective approach that combines the advantages of solid phase methods with mild solution phase coupling. The target molecules were prepared in >90% purity from common precursor molecules in steps involving only washing or gel filtration as rapid and convenient workup steps.

a



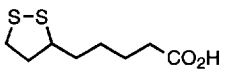
Cholic acid

b



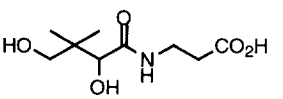
Folic acid

c



Lipoic acid

d



Pantothenic acid

Figure 1

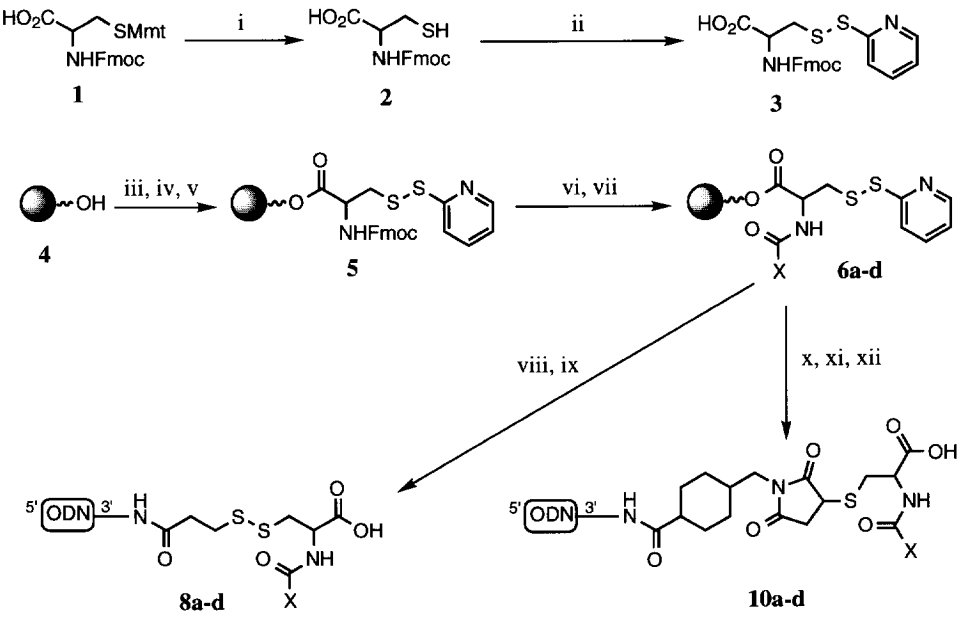
Derivatives of DNA may be generated during oligonucleotide synthesis by the incorporation of modified phosphoramidite monomers[7] or use of derivatised controlled pore glass support.[8] Alternatively, oligonucleotides may be modified postsynthetically by prior incorporation of a reactive functionalised sidechain, e.g. thiol or primary amine, at the 3'-end, 5'-end or in the nucleobase, followed by chemoselective coupling.

Postsynthetic modification of oligonucleotides has the advantage of being able to generate several derivatives of a particular sequence without having to carry out separate oligonucleotide syntheses which can be costly and time consuming. We initially attempted direct coupling of lipoic and folic acids to an alkyl amine modified oligodeoxyribonucleotide *via* peptide coupling conditions using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), which was unsuccessful, and by prior formation of the symmetrical anhydride or N-hydroxysuccinimide ester derivatives. Moderate yields of the lipoic acid derivative were obtained; however, there was no detectable product formation for the reaction with folic acid, so an alternative strategy was sought. Thiol chemistry is a proven approach for the formation of bioconjugate molecules with high chemoselectivity and excellent coupling efficiency under mild conditions.[9] A further advantage being that it is possible to link the molecule of interest to an oligonucleotide through either a biologically stable thioether bond or a disulphide bond that has been shown to be cleavable under the reducing conditions of the intracellular environment.[10]

We have developed an approach that employs postsynthetic oligonucleotide modification in solution having generated the conjugate molecules on solid support. By employing a solid support loaded with a cysteine derivative it is possible to couple any molecule of interest bearing a carboxylic acid group through the primary amine, and a suitably derivatised oligonucleotide through the cysteinyl thiol group, allowing easy access to a range of oligonucleotide conjugates. Thus N-α-Fmoc-S-2-pyridinesulphenyl-L-cysteine (**3**) was prepared as depicted in the Scheme by deprotection of N-α-Fmoc-S-p-methoxytrityl-L-cysteine (**1**) with trifluoroacetic acid to initially give **2**, which was subjected to thiol activation with 2,2'-dipyridyl disulphide affording **3** in 78% overall yield. TentaGel hydroxytrityl resin (**4**, NovaSyn® TGT, load capacity 0.26 mmol/g) was activated to the chlorotrityl derivative with acetyl chloride then coupled to **3** in the presence of diisopropylethylamine to give N-α-Fmoc-S-2-pyridinesulphenyl-L-cysteinyl-trityl TGT resin (**5**). Any excess chlorotrityl groups were capped with methanol. Resin **5** was obtained with a loading of 0.24 mmol/g (92% yield) as determined by quantitation of Fmoc release on treatment with 20% piperidine in dimethylformamide.

Removal of the Fmoc protecting group from **5** with 20% piperidine in dimethylformamide permits coupling to any carboxylic acid-bearing molecule. Folic, cholic and lipoic acids were coupled to the free amine using Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP®) as coupling agent, and pantothenic acid was coupled using 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU).[11] Excess reagents were removed by washing the resin with DMF and DCM. All reactions proceeded to completion within 4 hours, as judged by the Kaiser test for free amines[12] and reverse-phase HPLC analysis of the products **6a-d** after cleavage from the resin with 50% acetic acid. Compounds **6a-d** were further characterised by electrospray mass spectrometry.[13] At this stage the constructs can either be cleaved from the resin and conjugated to a thiol derivatised oligonucleotide, or their thiol groups can be deprotected with dithiothreitol (DTT) followed by cleavage and reaction with a maleimide bearing oligonucleotide.

Scheme



i) DCM/trifluoroacetic acid/triisopropylsilane (18:1:1 v/v), RT, 20 min, 96%; (ii) 2,2'-dipyridyl disulphide (10 eq), RT, 1 hr, 81%; (iii) acetyl chloride (50 eq), toluene, 60°C, 4 hr; (iv) **3** (1.2 eq), diisopropylethylamine (4 eq), DMF, argon, RT, 2 hr; (v) MeOH, diisopropylethylamine (4 eq), RT, 10 min, 92% for steps iii - v; (vi) 20% piperidine in DMF, RT, 10 mins; (vii) XCO_2H (10 eq), PyBOP (10 eq) or TBTU (10 eq), HOBt (10 eq), diisopropylethylamine (20 eq), DMF, argon, RT, 4 hr, >95% by HPLC; (viii) acetic acid/DCM/MeOH (5:4:1 v/v), RT, 2 hr, or trifluoroacetic acid vapour, RT, 1 hr; (ix) 3'-thiol oligonucleotide **7** (10 nmoles), 0.1 M potassium phosphate pH 7.0/MeCN (9:1 v/v), RT, 3 hr, >90% by HPLC; (x) DTT (25 eq), 0.1 M ammonium acetate pH 7.8/DMF (1:1 v/v), argon, RT, 2 hr; (xi) acetic acid/DCM/MeOH (5:4:1 v/v), RT, 2 hr, or trifluoroacetic acid vapour, RT, 1 hr; (xii) 3'-maleimide oligonucleotide **9** (10 nmoles), 0.1 M potassium phosphate pH 7.0/MeCN (9:1 v/v), RT, 3 hr, >90% by HPLC.

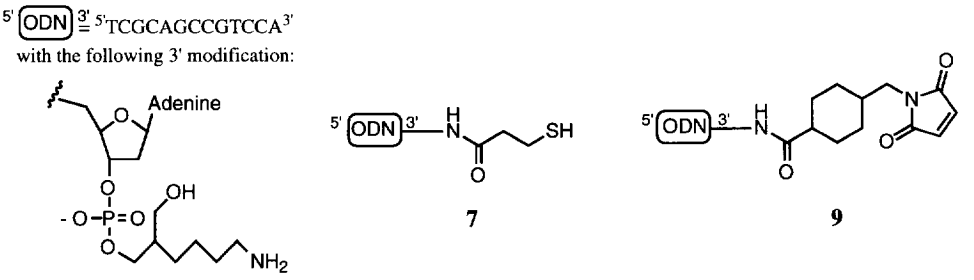
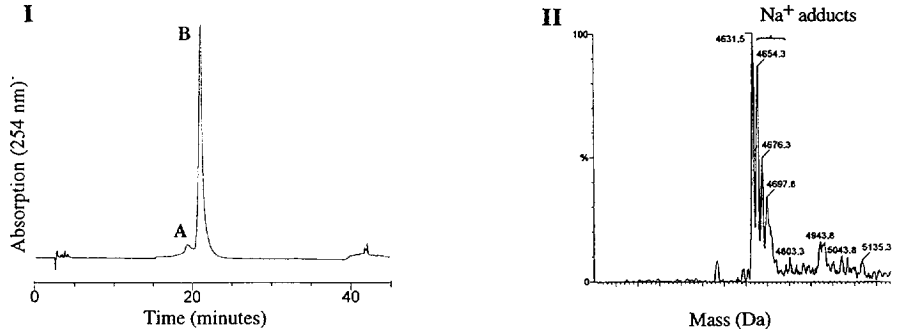


Figure 2

A 3'-amino linked 13mer (Figure 2, 6-amino-2-hydroxymethyl-1-heptanol linker, Oswel DNA Service) of sequence $^{5'}$TCGCAGCCGTCCA$^{3'}$ (100 nmoles, 12.2 AU_{260}) was reacted with >60 equivalents of 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester (SPDP) for 3 hours at room temperature in 4:1 0.1 M HEPES (pH 8.2) : acetonitrile yielding a 3'-dithiopyridyl 13mer in 79% yield after reverse phase HPLC purification. This was converted to the free 3'-thiol oligonucleotide **7** (Figure 2) by reduction with DTT, followed by gel filtration, and was used promptly to avoid formation of disulphide dimers.

Compounds **6a-d** were cleaved off the resin prior to coupling.[14] Approximately 1 mg portions of resin loaded with constructs **6a-d** were cleaved using either mild acid (5:4:1 acetic acid : dichloromethane : methanol), or by exposure to TFA vapour in a sealed glass tank. Cleavage of the hydroxytrityl linker proceeded rapidly and efficiently in either case. Evaporation of residual cleaving agent under reduced pressure was followed by spontaneous coupling of cleaved material with freshly prepared oligonucleotide **7** (10 nmoles, 1.2 AU_{260}) in 9:1 0.1 M potassium phosphate (pH 7.0) : acetonitrile. In practice, cleavage, drying and coupling was carried out without the need to manipulate the beads outside of the original reaction vessel. Gel filtration of the crude product on a NAP-5 column gave the desired products in >90% purity as judged by analytical HPLC. At this stage the oligonucleotide conjugates are probably of sufficient purity for biological testing. Further purification by reverse phase HPLC, however, afforded the disulphide-linked conjugates **8a-d** in recovered yields of 69-80%.

In a slightly modified strategy, the 3'-maleimide derivatised oligonucleotide **9** (Figure 2) was prepared by reacting the 3'-amino modified 13mer (100 nmoles, 12.2 AU_{260}) with >60 equivalents of 4-(maleimidomethyl) cyclohexanecarboxylic acid N-hydroxysuccinimide ester (SMCC) for 3 hours at room temperature in 3:2 0.1 M potassium phosphate, pH 7.5 : acetonitrile to give a 3'-maleimide 13mer in 60% yield after reverse phase HPLC purification. The resin-bound constructs **6a-d** were thiol deprotected on resin using DTT, cleaved, as described above, and reacted directly with **9** (10 nmoles, 1.2 AU_{260}), under reaction conditions identical to the coupling of **6a-d** with **7**, to give the thioether linked oligonucleotide series **10a-d** depicted in the Scheme. Isolated yields ranged from 70-78% following work up as for **8a-d**. Once again, all coupling reactions proceeded to >90% completion as judged by HPLC as shown for product **10c** in Figure 3.



(**I**) Reverse phase HPLC trace of coupling reaction producing oligonucleotide **10c** showing unreacted oligonucleotide **9** (**A**) and conjugate **10c** (**B**). (**II**) Electrospray mass spectrometry trace of conjugate **10c**.

Figure 3

All conjugates were also characterised by electrospray mass spectrometry. The mass spectrum of each conjugate (shown for product **10c** in Figure 3) displayed a parent peak mass in good agreement with calculated values (Table 1). Product **10a** appeared as a double peak at 27.1 and 27.4 minutes by HPLC (Table 1); however, both peaks when collected separately gave the same mass by electrospray. It has been reported that multiple peaks are sometimes observed for derivatised oligonucleotides.[15] Best results were obtained by injecting a small volume (5 µl) of 5 mM ammonium citrate approximately 2 minutes before sample injection to minimise oligonucleotide-sodium adduct formation. Even so, some sodium adducts were still detected for each product as seen for **10c** in Figure 3.

Table 1: Reverse phase HPLC retention times, final collected yields and electrospray mass spectrometry characterisation of oligonucleotides.

Oligonucleotide	3'-amino 13mer	3'-SPDP 13mer	**9**	**8a**	**8b**	**8c**	**8d**	**10a**	**10b**	**10c**	**10d**
Retention time (mins)	16.7	18.5	18.6	25.3	18.9	19.4	16.9	27.1-27.4	19.7	20.9	18.0
Isolated yield (%)	N/A	79	60	79	76	69	80	70	72	78	72
Isolated yield (AU_{260})	N/A	9.6	7.3	0.95	0.91	0.83	0.96	0.84	0.86	0.94	0.86
Calculated mass (Da)	4102.9	4300.3	4322.2	4700.6	4734.0	4498.6	4510.4	4833.5	4866.4	4631.5	4643.3
Electrospray mass (Da)	4103.3	4302.0	4325.5	4703.0	4735.5	4499.8	4513.8	4834.5	4867.0	4631.5	4645.3

A future adaptation of this methodology will be to generate a synthetic library of oligonucleotide conjugates where the variable component, which may be peptide or non-peptide, is generated on solid phase by combinatorial synthetic methodology. The availablity of large, high capacity (~ 5 nmol/bead) resin beads such as TentaGel Macrobeads (Rapp Polymer) should facilitate the formation of a one-bead one-compound library[16] on a suitable scale.

In conclusion, we have developed a convenient approach for the synthesis of oligonucleotide derivatives with excellent efficiency to give products of greater than 90% purity after gel filtration. Once the common precursors **5** and either **7** or **9** have been generated, the approach should be amenable to manual or automated high throughput parallel synthesis of a wide range of oligonucleotide derivatives for biological testing. The potential of this approach was illustrated by the preparation of disulphide and thioether linked conjugates between cholic, folic, lipoic and pantothenic acids and a 13 base oligodeoxyribonucleotide in good yield and purity. The cellular uptake properties of these conjugates are currently under investigation.

Acknowledgements: We thank the MRC for a studentship to J. G. H. and the Royal Society for a University Research Fellowship to S. B. We gratefully acknowledge the helpful advice of Professor Tom Brown of Southampton University.

References and Notes

1. (a) Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 543-584. (b) Stein, C. A.; Cheung, Y.-C. *Science.* **1993**, *261*, 1004-1011. (c) Milligan, J. F.; Matteucci, M. D. and Martin, J. C. *J. Med. Chem.* **1993**, *14*, 1923-1937.
2. Goodchild, J. C. *Bioconjugate Chem.* **1990**, *1*, 165-187.
3. (a) Morvan, F.; Porumb, H.; Degols, G.; Lefebvre, I.; Pompon, A.; Sproat, B. S.; Rayner, B.; Malvy, C.; Lebleu, B.; Imbach, J.-L. *J. Med. Chem.* **1993**, *36*, 280-287. (b) Marshall, W. S.; Caruthers, M. H. *Science.* **1993**, *259*, 1564-1569.
4. (a) Wagner, R. W.; Matteucci, M. D.; Lewis, J. G.; Gutierrez, A. J.; Moulds, C.; Froehler, B. C. *Science* **1993**, *260*, 1510-1513. (b) Dempcy, R. O.; Browne, K. A.; Bruice, T. C. *J. Am. Chem. Soc.* **1995**, *117*, 6140-6141.
5. Krieg, A. M.; Tonkinson, J.; Matson, S.; Zhao, Q.; Saxon, M.; Zhang, L.-M.; Bhanja, U.; Yakubov, L.; Stein, C. A. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1048-1052.
6. Wang, S.; Lee, R. J.; Cauchon, G.; Gorenstein, D. G.; Low, P. S. *Proc. Natl. Acad. Sci. USA.* **1995**, *92*, 3318-3322.
7. Beaucage, S. L.; Iyer, R. P. *Tetrahedron.* **1993**, *49*, 1925-1963.
8. MacKellar, C.; Graham, D.; Will, D. W.; Burgess, S.; Brown, T. *Nucleic Acids Res.* **1992**, *20*, 3411-3417.
9. (a) Corey, D. R.; Pei, D.; Schultz, P. G. *J. Am. Chem. Soc.* **1989**, *111*, 8523-8525. (b) Arar, K.; Aubertin, A.-M.; Roche, A.-C.; Monsigny, M.; Mayer, R. *Bioconjugate Chem.* **1995**, *6*, 573-577. (c) Tung, C.-H.; Rudolph, J.; Stein, S. *Bioconjugate Chem.* **1991**, *2*, 464-465.
10. Oberhauser, B.; Wagner, E. *Nucleic Acids Res.* **1992**, *20*, 533-538.
11. TBTU was used for coupling of pantothenic acid to avoid acylation of free primary hydroxyl groups which has been noted when using PyBOP in the presence of unprotected serine side chains. Yon, S. M.; Groome, N. P.; White, P. Poster 79 at 3rd SPS meeting, **1993**, Oxford.
12. Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595-598.
13. Electrospray mass spectrometry (positive mode) **6a** mass found 621.0 $(M + H)^+$, calculated 621.3; **6b** mass found 654.3 $(M + H)^+$, calculated 654.2; **6c** mass found 418.8 $(M + H)^+$, calculated 419.3; **6d** mass found 432.2 $(M + H)^+$, calculated 431.1.
14. Direct coupling of oligonucleotide **7** to solid phase bound constructs **6a-d** was found to proceed only with low efficiency (<5%) on TentaGel. Investigations to find a solid support suitable for efficient postsynthetic coupling of oligonucleotides to reactive moieties on solid phase are ongoing.
15. Agrawal, S.; Zamecnik, P. C. *Nucleic Acids Res.* **1992**, *18*, 5419-5423.
16. Furka, A.; Salmon, S.; Hersh, E.; Hruby, V.; Kazmierski, W.; Knapp, R. *Nature.* **1991**, *354*, 82-84.

(*Received in Belgium* 24 *January* 1997; *accepted* 17 *March* 1997)