Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 1285–1290

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

SOLID-PHASE SYNTHESIS OF OLIGOMERIC DEOXYNUCLEIC GUANIDINE (DNG): A POLYCATIONIC ANALOGUE OF DNA

Barry A. Linkletter and Thomas C. Bruice*

Department of Chemistry, University of California, Santa Barbara, CA 93106, U.S.A.

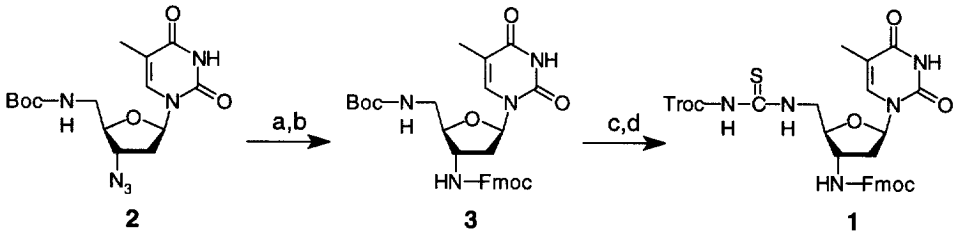
Received 2 March 1998; accepted 30 March 1998

Abstract: DNG is a DNA analogue wherein the negatively charged phosphate backbone linkages have been replaced by achiral positively charged guanidinium linkages and has high affinity for complimentary DNA. The synthesis of these compounds in solution phase has been severely limited due to diminishing yields and solubility limitations. For the first time, an efficient solid-phase synthesis for oligomeric is reported.

In recent years much interest has been expressed in antisense agents for the regulation of gene expression in living cells.[1-3] Many candidates are currently being studied and can be broken down into three broad structural classes: those that retain the nucleoside and phosphorous backbone of DNA and RNA such as phosphorothioates,[3,4] phosphoramidates,[5] etc.; those that replace the phosphate internucleoside linkage with other chemical units such as amides,[6] acetals,[7] heterocycles,[8] carbamates,[9] morpholino phosphoramidates,[10] methylenemethylimino (MMI)[11] or guanidinium groups (DNG);[12-20] and those that eliminate the furanose-phosphate backbone entirely such as PNA[21] or PNAA.[22] In general, oligomers that feature neutral internucleoside linkages have stronger associations with DNA than those with negatively charged linkages because neutral linkages eliminate the charge repulsion between the negatively charged oligomer and the negative phosphate backbone of DNA. DNG oligomers, with a positively charged guanidinium linker between each nucleoside, go one step farther in this regard by exploiting the attraction that exists between positively and negatively charged groups.[16] A 5 unit oligomer of thymidyl DNG [HO-$(Tg)_4$-T-azido] binds to complimentary poly(rA) so tightly that the melting temperature (Tm) for dissociation of the double helix is estimated to be above 100 °C and yet no binding was observed for poly(rC), (rG), (U), (dT) or (I).[17] Due to this strong binding, specificity and resistance to nucleases, DNG represents an extremely interesting putative genetic regulatory agent.

The stepwise synthesis of oligomeric DNG strands in solution[20] proved to be limited by diminishing yields and the need for purification after each step of the synthesis. In order to facilitate the development of longer sequences of DNG, a synthesis that involved stepwise construction on a solid support was deemed necessary. As in the case of solid-phase syntheses (SPS) of oligomeric DNA and peptides, this synthesis required high coupling yields to minimize undesired products and rapid reactions to allow efficient synthesis of multiple desired sequences. In this paper we report the first solid-phase synthesis of a DNG oligomer.

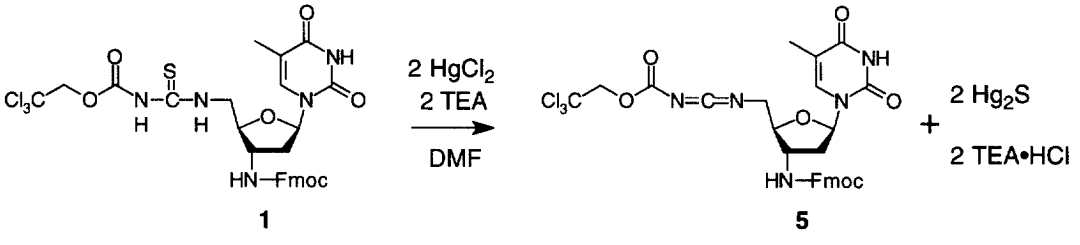
0960-894X/98/$19.00
PII: S0960-894X(98)00228-5



Scheme 1. Synthesis of thymidyl monomer for solid phase synthesis. (a) H_2, 10% Pd/C, 95% EtOH, 3 h, rt, 99%; (b) 1.1 equiv Fmoc-Cl, 1:1 Dioxane:10% Na_2CO_3, 30 min, rt, 83%; (c) 30% TFA/DCM, 5 min, rt, 100%; (d) TrocNCS (**4**), TEA (2 equiv), DCM, 30 min, rt, 85%.

The monomer **1** (Scheme 1) for the SPS of oligothymidyl DNGs is obtained by reducing the 3′-azido group of 3′-azido-5′-*t*-butoxycarbamoyl-3′,5′-deoxythymidine (**2**)[20] by hydrogenation and blocking the resulting 3′-amino group with 9-fluorenylmethylchloroformate (Fmoc-Cl) to give **3**. The *t*-butoxycarbonyl (Boc) protecting group was removed with 30% TFA in dichloromethane (DCM) and the resulting 5′-amine was reacted with trichloroethoxycarbonylisothio-cyanate (**4**)[23] to obtain the trichloroethoxycarbonyl (Troc) protected thiourea (**1**).[24]

2-(2-Aminoethoxy)ethanol 2-chlorotrityl resin was chosen as a convenient commercially available support with a linker that was cleavable by mild acids. The linker arm remained on the 5′-terminus of the DNG oligomer after acid cleavage from the chlorotrityl group and was chosen to provide a convenient starting point for initial resin loading and it may enhance solubility of the oligomer. The synthesis was designed to be compatible with standard Fmoc peptide synthesis techniques to facilitate future synthesis of DNG-peptide conjugates.



Scheme 2. Formation of activated carbodiimide from thiourea by addition of mercury (II) chloride.

The coupling reaction involves the conversion of (**1**) to an electronically activated carbodiimide (**5**) by the addition of mercury(II) chloride[25,26] in DMF (Scheme 2). In a typical synthesis 36 mg of resin were swelled in 0.5 mL DMF in a 3 mL reaction vial. Stock solutions of the protected thiourea (**1**) (25.8 mM, 1.0 mL), mercury(II) chloride (92 mM, 0.56 mL), and triethylamine (92 mM, 0.56 mL) in DMF were added and the vial was agitated for 30 minutes. The formation of the activated carbodiimide is rapid and it is then attacked by the terminal amine on the growing oligomer to produce the trisubstituted protected guanidine group. The addition step was repeated twice to insure a complete reaction and then the resin was washed with copious amounts of DMF. The resulting 3′-Fmoc protected oligomer is deblocked with 20% piperidine in DMF and the cycle began again

(Scheme 3). The addition/deblocking cycle was repeated 7 more times to produce the eight unit oligomer. Cleavage of the completed Troc-protected guanidinium oligomer was accomplished by 5% dichloroacetic acid in DCM. The product was precipitated in ether to give the Troc protected DNG oligomer **6**, which was deprotected by a short treatment of acetic acid and zinc powder.[27] HPLC analysis of the crude DNG product showed the desired product >90% in purity with an estimated coupling yield averaged over the eight additions of 97% (determined by UV analysis of absorbance of thymidine; e = 8700 $M^{-1}cm^{-1}$ at 268 nm[14]). The acetic acid was evaporated and the deprotected DNG oligomer **7** [$HO(CH_2)_2O(CH_2)_2(gT)_8NH_2$] was purified by ion exchange chromatography to give the pure oligomer (Figure 1). Electrospray mass spectroscopic analysis indicates the expected masses for the triply charged [*m/z* = 743.2, calcd for $C_{92}H_{134}N_{41}O_{26}$ $(M + 3H)^{3+}$: 743.4] and quadruply charged [*m/z* = 557.7, calcd for $C_{92}H_{135}N_{41}O_{26}$ $(M + 4H)^{4+}$: 557.8] forms of the oligomeric DNG **7**.

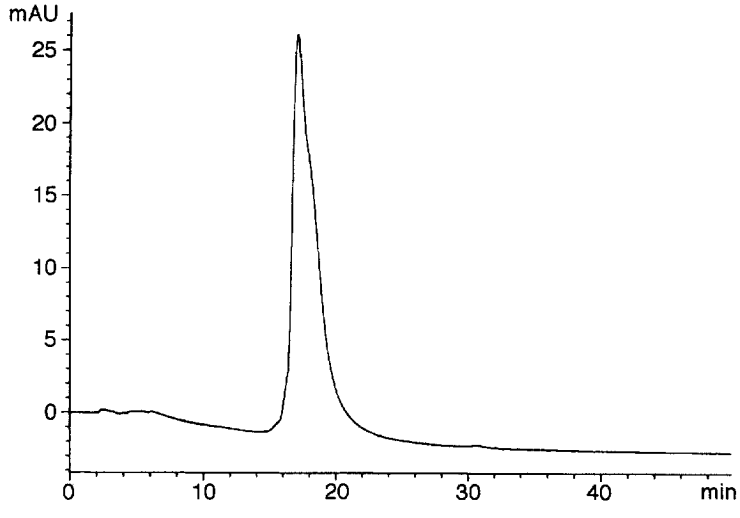
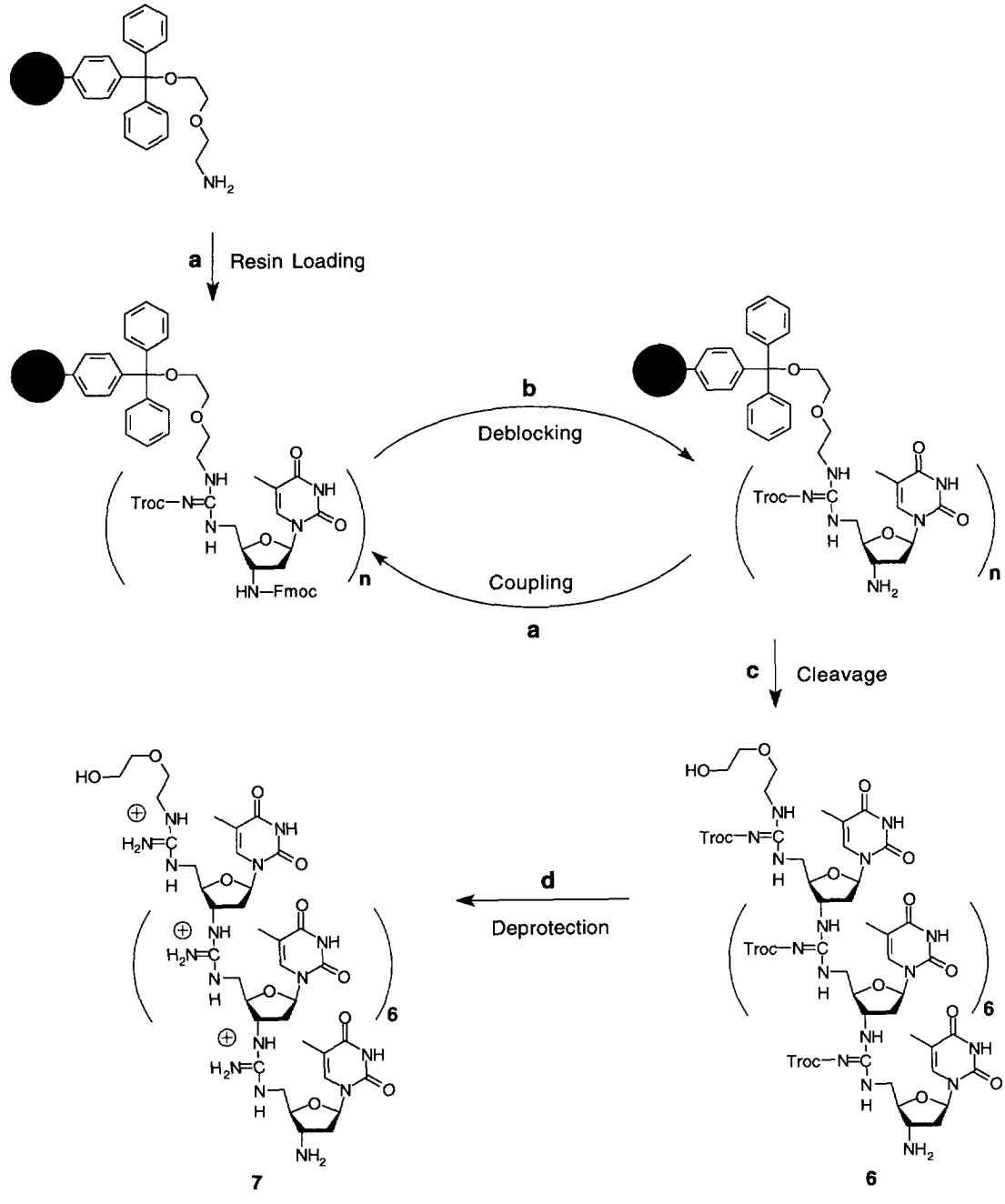


Figure 1. HPLC chromatogram of purified DNG oligomer **7**. Conditions: Alltech macrosphere 300SCX cation exchange column. Buffer a: 50 mM TRIS buffer, pH 6.4; Buffer b: 2 M guanidinium hydrochloride, 50 mM TRIS buffer, pH 6.4. 0 to 100% gradient of b over 45 min. at 1.3 mL/min. HP 1090 HPLC equipped with UV detector, λ = 260 nm. Retention time = 17.1 min.

A Job plot[28] shows that the oligomer **7** associates in a 2:1 ratio with poly(rA) (Figure 2) suggesting a triple helix as has been observed for shorter thymidyl DNG oligomers with adenosine polynucleotides.[16,17] No melting point for this triple helix was observed below 95 °C even at high ionic strength (μ = 0.6 M KCl) when the binding strength of DNG oligomers with DNA is known to weaken.[18,19]



Scheme 3. Solid-phase synthesis of DNG. (a) Coupling: monomer (**1**), $HgCl_2$ (2 equiv), TEA (2 equiv); (b) Deblocking: 20% piperidine in DMF; (c) Cleavage: 3% dichloroacetic acid in DCM; (d) Deprotection: acetic acid/zinc powder.

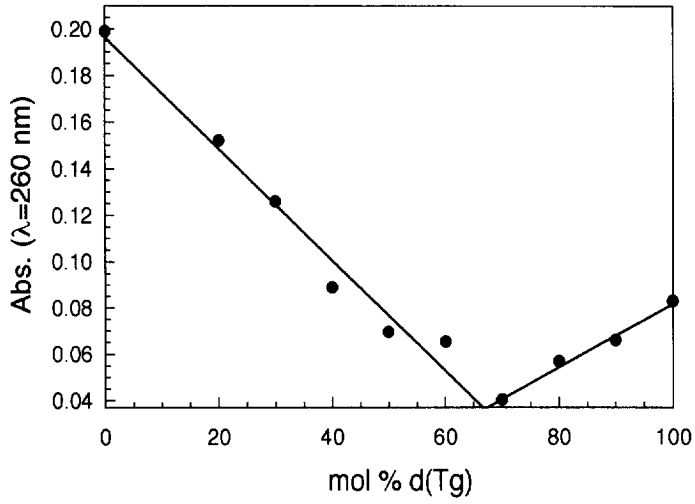


Figure 2. Job plot (continuos variation method) of poly(rA) with **7** at 2.0×10^{-5} M/base total concentration of DNG and RNA, at $\lambda = 260$ nm and 30 °C. Inflection at 67% indicates 2:1 DNG:DNA complex.

In summary, an efficient and rapid solid-phase method for the synthesis of guanidinium linked DNA analogues has been successfully demonstrated. The solid-phase synthesis of the eight unit thymidyl oligomer **7** was easily accomplished in one day. This solid-phase synthesis technique opens the door for the rapid synthesis of DNG oligomers for further binding studies, for combinatorial libraries and the synthesis of DNG-peptide conjugates on solid phase.

Research is currently being conducted towards the synthesis of appropriately protected A, C, and G nucleotide monomers for DNG synthesis. If the nucleotide bases are protected with standard base-labile benzoyl or isobutyryl groups the synthesis can be accomplished as detailed above and the base protecting groups removed by subsequent ammonia treatment after removal of the Troc protecting groups. Alternately, the bases can be protected by the Troc group and deprotected during the guanidinium deprotection step outlined above. All the solid-phase coupling steps are carried out under neutral or basic conditions to which nucleotides are stable and the resin linker is cleaved by mild acids that will reduce the possibility of depurination of any terminal adenine units. The synthesis of interesting mixed sequences is planned and will be reported in the future.

Acknowledgements: This project was supported by the Office of Naval Research (N00014-96-1-01232) and the National Institute of health (3 R37 DK09171-3451).

References and Notes

1. De Mesmaeker, A.; Häner, R.; Martin, P.; Moser, H. E. *Acc. Chem. Res.* **1995**, *28*, 366.
2. Stein, D.; Foster, E.; Huang, S.-B.; Weller, D.; Summerton, J. *Antisense Nucleic Acid Drug Develop.* **1997**, *7*, 151.
3. Stein, C. A. *Chemistry and Biology* **1996**, *3*, 319.
4. Miller, P. S.; Bhan, P.; Cushman, C. D.; Kean, J. M. *Nucleosides Nucleotides* **1991**, *10*, 37.

5. Laurent, A.; Debart, F.; Rayner, B. *Tetrahedron Lett.* **1997**, *38*, 5285.
6. De Mesmaeker, A.; Waldner, A.; Lebreton, J.; Hoffmann, P.; Fritsch, V.; Wolf, R. M.; Freier, S. M. *Angew. Chemie, Int. Ed. Eng.* **1994**, *33*, 226.
7. Wang, J.; Matteucci, M. D. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 229.
8. von Matt, P.; Altmann, K.-H. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1553.
9. Waldner, A.; De Mesmaeker, A.; Lebreton, J.; Fritsch, V.; Wolf, R. M. *Synlett* **1994**, 57.
10. Summerton, J.; Weller, D. *Antisense Nucleic Acid Drug Develop.* **1997**, *7*, 187.
11. Morvan, F.; Sangvhi, Y. S.; Perbost, M.; Vasseur, J.-J.; Bellon, L. *J. Am. Chem. Soc.* **1996**, *118*, 255.
12. Bruice, T. C.; Dempcy, R. O. (University of California), U.S. Patent 5 696 253, **1997**.
13. Dempcy, R. O.; Luo, J.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4326.
14. Dempcy, R. O.; Browne, K. A.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 6097.
15. Dempcy, R. O.; Browne, K. A.; Bruice, T. C. *J. Am. Chem. Soc.* **1995**, *117*, 6140.
16. Luo, J.; Bruice, T. C. *J. Am. Chem. Soc.* **1997**, *119*, 6693.
17. Brown, K. A.; Dempcy, R. O.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7051.
18. Blaskó, A.; Minyat, E. E.; Dempcy, R. O.; Bruice, T. C. *Biochemistry* **1997**, *36*, 1821.
19. Blaskó, A.; Dempcy, R. O.; Minyat, E. E.; Bruice, T. C. *J. Am. Chem. Soc.* **1996**, *118*, 7892.
20. Dempcy, R. O.; Almarsson, Ö.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 7864.
21. Nielsen, P. E.; Haaima, G. *Chem. Soc. Rev.* **1997**, 73.
22. Fujii, M.; Yoshida, K.; Hidaka, J. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 637.
23. Wang, S. S.; Magliocco, L. G. (American Cyanimid Company), U.S. Patent 5 194 673, 1993; *Chem. Abstr.* **1993**, 119, 72382
24. TLC (3:1, EtOAc:Hexanes) R_f= 0.35; ^{1}H NMR (400 Mhz DMSO-d_6): δ (ppm) 1.79 (3 H, s, thymine-CH_3), 2.16 (m, 1 H; 2′-H), 2.30 (m, 1 H; 2′-H), 3.83 (m, 1 H; 5′-H), 3.95 (m, 2 H; 5′-H, 4′-H), 4.10 (p, J = 7 Hz, 1 H; 4′-H), 4.22 (t, J = 7 Hz, 1 H; Fmoc-CH), 4.35 (m, 2 H; Fmoc-CH_2), 4.91 (dd, $J1$ = 12.4 Hz, $J2$ = 16.8 Hz (2 H; Troc-CH_2), 6.13 (t, J = 6.8 Hz, 1 H; 1′-H), 7.32 (t, J = 7 Hz, 2 H; Fmoc-2″-H), 7.40 (t, J = 7 Hz, 2 H; Fmoc-3″-H), 7.52 (s, 1 H; 6-H), 7.69 (m, 2 H; Fmoc-1″-H), 7.75 (d, J = 8 Hz, 1 H; Fmoc-NH), 7.88 (d, J = 7 Hz, 2 H; Fmoc-4″-H), 9.83 (t, J = 5 Hz, 1 H; 5′-NH), 11.35 (s, 1 H; thymine-NH), 11.66 (s, 1 H; Troc-NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ = 12.1, 36.0, 46.7, 51.7, 65.5, 73.8, 80.7, 83.3, 94.9, 109.9, 120.1, 125.1, 127.1, 127.6, 136.2, 140.8, 143.8, 150.4, 152.0, 155.8, 163.7, 179.6; HRMS *m/z*: 696.0844, calcd for $C_{29}H_{29}N_5O_7SCl_3$ $(M + H)^+$ 696.0853
25. Robinson, S.; Roskamp, E. J. *Tetrahedron* **1997**, *53*, 6697.
26. Kim, K. S.; Qian, L. *Tetrahedron Lett.* **1993**, *34*, 7677.
27. Just, G.; Grozinger, K. *Synthesis* **1976**, 457.
28. Job, P. *Ann. Chim.* **1928**, *9*, 113.