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A novel synthetic approach to phosphate-methylated DNA oligomers using 9-fluorenylmethoxycarbonyl (Fmoc) as temporary base amino protecting group

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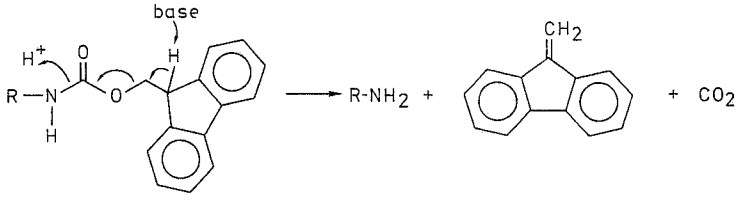
SUMMARY

The phosphate-methylated dinucleotides d(CpC) (**1**) and d(ApT) (**2**) have been synthesized using the 9-fluorenyl-methoxycarbonyl (Fmoc) group for transient protection of the amino group of the bases C and A. In the final stage of the synthesis, the Fmoc group could be removed with preservation of the methylated phosphate group. It is concluded that the Fmoc approach can be used for the synthesis of phosphate-methylated DNA fragments of an arbitrary nucleotide sequence. These systems are of interest because of their inherent conformational properties, and because of their possible utility as inhibitors of DNA replication *in vitro* and *in vivo*.

We have recently studied the structure and stability of a series of oligothymidilic DNA structures dT_n (n = 2, 3, 4, 6, 8), in which the phosphate groups were selectively methylated (Koole et al., 1986; Koole et al., 1987). It was found that these compounds show T – T base pairing, leading to symmetrical parallel double helices with an increasing stability. The preparation of phosphate-methylated dT_n involves treatment of natural dT_n with methyl methane sulfonate (MMS) (Rhaese and Freese, 1969). This reagent transfers a methyl group to the phosphodiester groups, while the T-bases remain essentially unaffected. The use of MMS, however, is strictly confined to oligothymidilic sequences. For other natural DNAs, MMS inevitably leads to methylation at different sites of the aglycons A, C, and G. Herein, we wish to report some preliminary results of a new and potentially versatile method for the solution synthesis of phosphate-methylated DNA analogues of an arbitrary nucleotide sequence. It should be mentioned that these systems are of interest, not only

because of their structural properties, but also because of their possible utility as nuclease resistant inhibitors of DNA replication (vide infra).

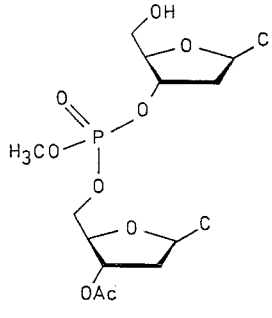
A logical synthetic approach would comprise a number of modifications of the costumary phosphoramidite method (Gait, 1984). Thus, we used 5′-tritylated 2′-deoxyribonucleoside-3′-*methoxy*-phosphoramidites as building blocks. The desired methylated phosphate groups are then formed directly after each tetrazole catalyzed 3′-5′ coupling and subsequent phosphorus oxydation. However, a serious problem is posed by the conventional acyl blocking groups of NH_2 of the bases A, C, and G, since their removal with concentrated ammonia in the final stage of the synthesis will be difficult to accomplish with preservation of the base-labile phosphate triester groups. We have tackled this problem by using the 9-fluorenylmethyloxycarbonyl (Fmoc) group for masking the NH_2 groups (Heikkilä and Chattopadhyaya, 1983). The Fmoc group, relatively common in oligopeptide synthesis (Carpino and Han, 1970), but – hitherto – only scarcely applied in the synthesis of oligonucleotides (Gioeli and Chattopadhyaya, 1982), offers the important advantage that it can be cleaved off via a mild ß-elimination (Scheme I). This reaction can be induced by e.g., triethylamine, which does not affect the methylated phosphate groups in the DNA backbone.



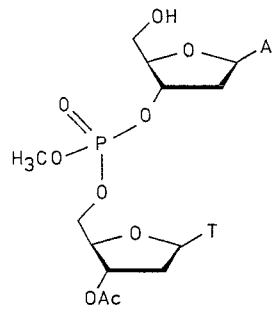
SCHEME I

Base-induced fragmentation of the Fmoc group via a ß-elimination reaction. The reaction is based on the acidic nature of the proton on the ß-carbon atom.

The utility of the Fmoc group was tested in the synthesis of the phosphate-methylated dinucleotides **1** and **2**. 5′-(4-Methoxytriphenylmethyl)-dC-Fmoc (**4**) was the key precursor for **1** (Scheme II). Compound **4** was prepared from dC-



1



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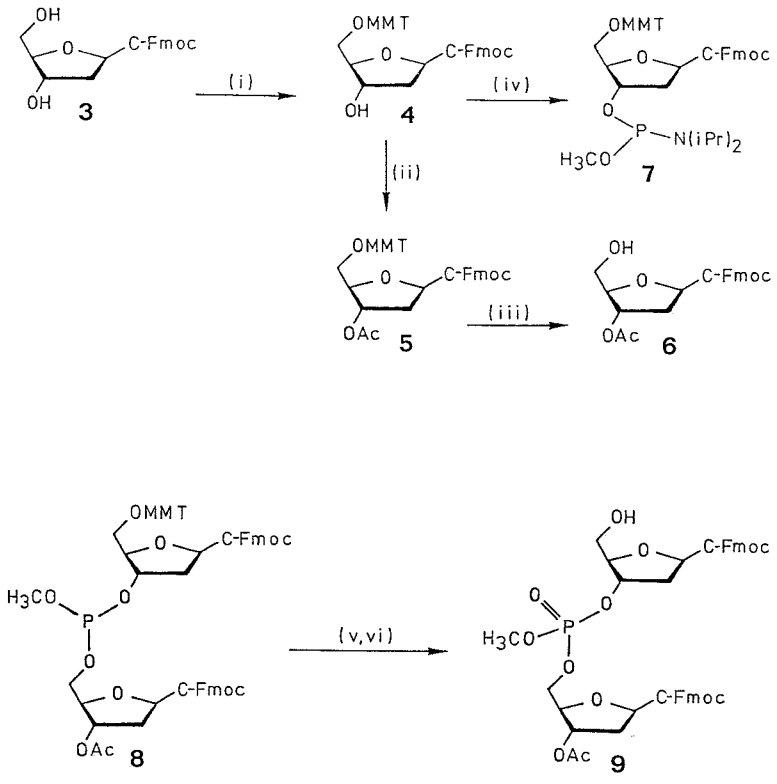

SCHEME II

Synthetic building blocks and intermediates leading to compound **1**. (i): 4-methoxytriphenylmethyl chloride in dry pyridine, (ii): acetic anhydride in dry pyridine, (iii): 80 % acetic acid, (iv): methoxy bis (diisopropylamino) phosphine/1H-tetrazole in dry pyridine, (v): tert-butyl hydroperoxide, (vi): 80 % acetic acid. Removal of the Fmoc group was performed with a 1 : 1 mixture of triethylamine and chloroform (see text).

Fmoc (**3**) and 4-methoxytriphenylmethyl chloride in dry pyridine. Subsequently, **4** was treated with acetic anhydride to form **5**, from which the 3′-terminal building block **6** was obtained through detritylation in 80 % acetic acid. Another portion of **4** was reacted with 1.1 equivalent of methoxy bis (diisopropylamino) phosphine (δ ^{31}P in $CDCl_3$, 131.6 ppm), in the presence of 1H-tetrazole in a strictly water-free pyridine medium, yielding the coupling synthon **7** *in situ* (Marugg et al., 1987). This was evident from the ^{31}P NMR spectrum of a sample of the reaction mixture, showing two peaks at δ 150.0 and 149.4 ppm in a 1 : 1 ratio, due to two diastereomeric forms of **7**. No residual ^{31}p NMR resonance at δ 131.6 ppm was found. The formation of **7** is essentially based on the fact that alkoxy bis (dialkylamino) phosphines are selectively activated by weak acids such as 1H-tetrazole (Nielsen et al., 1986). The solution of **7** was immediately reacted further with a solution of **6** (1.05 equivalent with respect to **7**), and tetrazole in dry pyridine. This readily furnished the diastereomeric phosphite triester **8**, as judged by ^{31}P NMR (δ 140.8 and 140.5

ppm in $CDCl_3$), and thin layer chromatography. The corresponding methyl phosphate triesters (δ 0.4 and 0.3 ppm in $CDCl_3$), were formed quantitatively upon reaction with tert-butyl hydroperoxide. Subsequent detritylation with 80 % acetic acid afforded the partially protected dinucleotide **9**, which was isolated as a white powder after column chromatography using a mixture of chloroform and methanol (9 : 1) as eluent (R_f = 0.65). The two diastereomeric forms of **9** showed ^{31}P NMR resonances at δ 0.1 and -0.4 ppm in $CDCl_3$. Compound **9** was stirred for 14 h in a 1 : 1 mixture of chloroform and triethylamine, which led to deblocking of the C-bases. The target compound **1** precipitated during this reaction. (δ ^{31}P in CD_3OD, 4.0 and 3.4 ppm).

The synthesis of the phosphate-methylated dinucleotide **2** was performed in essentially the same way, using 5′-(4-methoxytriphenylmethyl)-dA-Fmoc (**10**) and 3′-O-acetylthymidine as building blocks. Compound **10** was first reacted with 1.2 equivalent of methoxy bis (diisopropylamino) phosphine in a water-free medium of dichloromethane and acetonitrile in the presence of 1H-tetrazole, exactly according to the procedure of Marugg et al. (1987). This readily afforded the corresponding *in situ* coupling synthon (δ ^{31}P in $CDCl_3$, 149.7 and 149.5 ppm). A solution of 3′-O-acetylthymidine (1.2 equivalent with respect to **10**), and 1H-tetrazole was then added to the reaction mixture, to furnish the dinucleoside phosphite dA-5′-P-3′-dT (δ ^{31}P in $CDCl_3$, 141.3 and 140.4 ppm). After oxydation with tert-butyl hydroperoxide, the corresponding methyl phosphate triester (δ ^{31}P in $CDCl_3$, -0.05 and -0.53 ppm) was obtained. This product was isolated as a white solid after column chromatography, using 2-butanone as eluent (R_f = 0.28). The 4′-methoxytriphenylmethyl group was cleaved in 80 % acetic acid. Finally, the Fmoc group was removed by treatment with a mixture of triethylamine (10 equivalents with respect to the substrate) and pyridine, to yield compound **2**, with ^{31}P NMR signals at -0.4 and -0.7 ppm in $CDCl_3$.

The scope of the Fmoc approach to phosphate-methylated DNA oligomers can evidently be extended to systems of an arbitrary nucleotide sequence.

However, additional work is necessary to optimize the synthesis of 5′-(4-methoxytriphenylmethyl)-dG-Fmoc. A small quantity (30 mg) of this compound has been isolated, and it could be shown that the corresponding phosphoramidite (δ ^{31}P in $CDCl_3$, 150.2 and 149.7 ppm) is formed through reaction with methoxy bis (diisopropylamino) phosphine and 1H-tetrazole. Finally, it should be mentioned that the Fmoc synthesis can be upgraded to millimole scale. This is necessary to investigate the biological activity of the phosphate-methylated oligonucleotides *in vitro* and *in vivo*. Biological studies assessing the inhibitory activity of short phosphate-methylated DNA fragments with respect to DNA replication and protein synthesis are currently under investigation.

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