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A NEW SOLID PHASE APPROACH FOR RAPID SYNTHESES OF OLIGONUCLEOTIDES BEARING A 3'-TERMINAL PHOSPHATE GROUP

Eduard Felder*, Robert Schwyzer*, Ramamurthy Charubala+, Wolfgang Pfleiderer+ and Bernd Schulz+

* Institute of Molecular Biology, ETH-Hönggerberg, CH-8093 Zürich, Switzerland

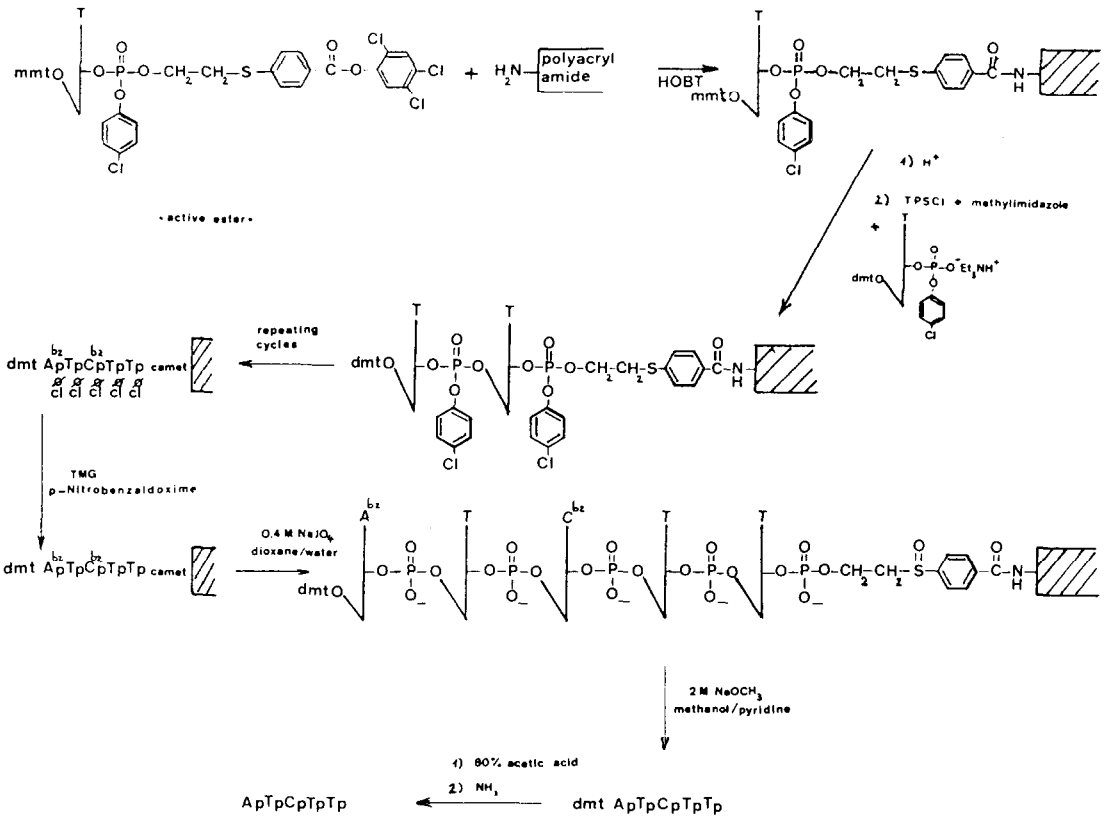
+ Fakultät für Chemie, Universität Konstanz, D-7750 Konstanz, West Germany

Abstract: Synthesis of pentanucleotide ApTpCpTpTp containing a 3'-terminal phosphomonoester group was accomplished with the help of the new phosphate-solid phase link 2-(4-carboxyphenylmercapto)ethanol; a similar molecule, 2-benzylsulfonylethanol, revealed interesting features as a temporary 3'-phosphate protecting group.

Methods for the synthesis of nucleotides with a terminal unprotected phosphate function developed in the last few years from rather laborious and time consuming to increasingly efficient and simple approaches. The crucial problem has to be seen in mild deblocking conditions to form a free phosphomonoester from a fully protected phosphotriester. The use of phosphorothioates (1,2) and phosphoramidates (3,4) was proposed. Furthermore it was demonstrated that the p-nitrophenylethyl group could be β-eliminated from a phosphodiester function giving rise to the synthesis of oligonucleotide 3'-phosphates (5).

So far solid phase synthesis of 3'-phosphate terminated oligomers required chain elongation from 5' to 3' direction. This however implies the lack of an easy coupling rate control (detritylation assay) and leads to partially deprotected products with a 3'-phosphodiester function (6), unless hardly accessible dialkyl nucleoside phosphotriesters are used as chain terminators (7). Since a few years we have been studying a class of versatile phospho- and carboxy-protecting groups containing a thioether function and investigating the influence of different oxidation stages of sulfur on the protecting properties and cleaving conditions. Basing on preliminary experiments in solution and with model amines (8) we developed a new solid phase link, 2-(4-carboxyphenylmercapto)ethanol (=camet), which allows the attachment of a fully protected nucleotide triester to a carrier provided with free primary amino groups (scheme 1). The advantage of including a β-thioether function in the molecule resides in the fact that stability towards cleavage by β-elimination can be influenced with a mild oxidation step. Moreover the lability can be regulated through additional substituents at the adjacent benzene ring on electronic grounds. Simple insertion of a methylene group between the sulfur and the aromatic ring produces a remarkable decrease of the electronic withdrawing effect on the β-methylene hydrogen (9), diminishing the tendency to eliminate. The camet link proved to be extremely stable towards either acid or alkali in the primary stage. This allows clean conversions of triesters to diesters promoted by tetramethylguanidine and 4-nitrobenzaldoxime

SCHEME 1



Functionalization		
step	reagent	time
1	150 mg (=50 μmoles) resin[a] in 6 ml $H_2N-CH_2-CH_2-NH_2$	1 x 16 h
2	DMF wash 10 ml	2 x 5 Min
3	Pyridine wash 10 ml	b
4	250 μmoles active ester(14) in 3.5 ml pyridine + 90 mg HOBT in 1 ml pyridine	1 x 16 h
5	Pyridine wash 10 ml	4 x 5 Min
a	Polydimethylacrylamide resin produced in our laboratory according to (15)	
b	Check absence of ethylene diamine	

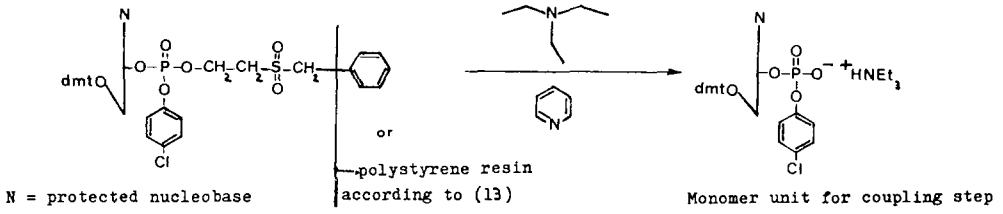
Reaction cycle		
step	reagent	time
1	CH_2Cl_2 + DMF (9+1) wash 10 ml	3 x 3 Min
2	3% BSA in CH_2Cl_2+DMF (9+1) 10 ml	5 Min[a]
3	CH_2Cl_2 + DMF (9+1) wash 10 ml	3 x 3 Min
4	Pyridine wash 10 ml	5 x 3 Min
5	Coupling step in 1.5 ml pyridine	25 Min[b]
6	Pyridine wash 10 ml	5 x 3 Min
7	Ac_2O in pyridine+methylimidazole (95+5) 10 ml	10 Min
8	Pyridine wash 10 ml	3 x 3 Min
a	According to the optimized procedure of Patel et al.(16)	
b	Coupling mixture contains diester component as 0.1 M solution in pyridine in a 3-4 fold excess compared to the actual resin capacity. Additives of 1.5-2 equiv. TPSCl and 3-4 equiv. of methylimidazole are forseen. Coupling rates superior to 85% are standard and were measured spectrophotometrically	

Abbreviations:

A^{bz}	= N^6-Benzoyladenine
BSA	= Benzenesulfonic acid
C^{bz}	= N^4-Benzoylcytosine
DMF	= N,N-Dimethylformamide
dmt	= Dimethoxytrityl
HOBT	= 1-Hydroxybenzotriazole
mmt	= Monomethoxytrityl
T	= Thymine
TMG	= Tetramethylguanidine
TPSCl	= 2,4,6-Triisopropyl-benzenesulfonyl chloride
⌀ Cl	= p-Chlorophenyl

in dioxane/water (1); no losses of resin-bound material were observed during this step. Release from the solid carrier is accomplished with the help of a two-step manipulation: a mild oxidative step (sodium metaperiodate) during which no oxidative damage of nucleotides arises and a final alcali treatment with sodium methoxide. Oxidation to the sulfone stage with stronger reagents is unnecessary, since the carbamide group in para position to the sulfoxide function enhances lability. A similar linker molecule used years ago in the diester approach (10) lacking this feature was judged unsuitable later on (11).

Our studies about the behaviour of arylthioethyl groups as phosphate protections led us to investigate also a new temporary protecting group for phosphotriesters that shall be deprotected to diester level for further condensations (an analogy to the β-cyanoethyl group). We chose the 2-benzylsulfonylethyl group for this purpose. Balgobin et al. (12) used 2-phenylsulfonylethanol instead of β-cyanoethanol pointing out that cleavage conditions were more favourable (2 equiv. of triethylamine in pyridine instead of 15 equiv. needed for β-cyanoethanol). In our opinion a slight decrease of β-elimination tendency is more desirable in order to avoid inappropriate degradations during longer chain syntheses. We therefore introduced the stabilizing methylene group between the aromatic ring and the sulfonyl group. Conversion to diesters still remained easy and effective (15 equiv. triethylamine in pyridine remove the benzylsulfonylethyl group in less than two hours at 20° C).



Very recently the same benzylsulfonylethyl functional unit was used by Efimov et al. (13) as an integral solid phase link. Its use is however limited to the assembly of partially protected oligomers for further condensations in solution due to the fact that the chlorophenyl group cannot be cleaved from the resulting diester. Contrastingly the "camet approach" opens the way to both possibilities: smooth release of protected fragments with a terminal phospnodiester function (treatment with excess tetramethylguanidine in pyridine of the sulfoxide form) but also synthesis of completely deprotected terminal phosphomonoesters (scheme 1).

In order to remove the oligomers from the resin the following treatment is most recommended: cleavage of p-chlorophenyl group with 0.4 M p-nitrobenzaldoxime in dioxane+water (1+1) containing 240 µl of TMG (16h) followed by oxidation with 0.4 M $NaIO_4$ in the same solvent (16h) and final reaction with 2 M $NaOCH_3$ in abs. methanol+pyridine (1+1) (16h). Appropriate washing steps have to be included. Subsequent treatments with acetic acid 80% and ammonia 25% are performed after neutralization of the reaction mixture with an acidic cation exchange resin (i.e. Dowex H^+-form). Crude ApTpCpTpTp was purified through a Sephadex A-25 anion exchange column with a linear gradient of 0.001 M to 0.8 M triethylammonium bicarbonate buffer pH 7.5.

Purity was checked by C_{18} reversed phase hplc eluting with 12% acetonitrile in 0.1 M triethylammonium acetate. The pure product was subjected to complete digestion with spleen phosphodiesterase (E.C.3.1.16.1) according to (17) and converted to a mixture of the nucleoside 3'-monophosphates Ap, Cp and Tp in a ratio of 1:1:3. This analysis was done by ion exchange hplc on a Nucleosil 10 SB column with 0.6 M KH_2PO_4 pH 4.5 and comparison with reference substances (Böhringer) as well as computing the areas of elution diagrams with the help of an integrator (HP 3380A).

Literature and footnotes

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   The preparation of the educt camet can be performed as followed: a solution of 4-mercaptobenzoic acid in dioxane is treated with an excess of ethylene oxide and then with an equal amount of triethylamine. The mixture is kept in a pressure flask at room temperature for 40 h during which a brown, oily precipitate is formed. After evaporation the residue is dissolved in water and acidified to pH 2 with 1 N HCl. The solid product is gathered by filtration and purified by recristallization.
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14. Active ester: 2,7 equiv. of triazole were treated with 1,3 equiv. of p-chlorophenylphosphodichloridate with pyridine as a solvent. A solution of 1 equiv. of 5'-O-(4-methoxytrityl)thymidine in pyridine was then added dropwise. After 40 min the solution was complemented with the 2,4,5-trichlorophenyl ester of camet (2 equiv.). The reaction was stopped after 4 hours and work up included silica gel column chromatography with $CHCl_3$ as eluent. Elementary analysis and ^{1}H-NMR confirmed identity and purity.
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