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NPE-RESIN, A NEW APPROACH TO THE SOLID-PHASE SYNTHESIS OF PROTECTED PEPTIDES AND OLIGONUCLEOTIDES I : SYNTHESIS OF THE SUPPORTS AND THEIR APPLICATION TO OLIGONUCLEOTIDE SYNTHESIS.

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Summary : The preparation of polymeric supports containing a base labile 2-(2 nitrophenyl) ethyl linkage and the attachment of protected nucleosides is described together with their application to oligonucleotide synthesis.

Solid-phase methodology has become the most used technique for the preparation of peptides and oligonucleotides. Generally, the nucleoside at the 3'-end of the oligonucleotide and the C-terminal aminoacid of the peptide are linked through its 3'-hydroxyl and its carboxyl group to a suitable functionalized support. In oligonucleotide synthesis the most commonly used linkage is the succinate linkage although some alternatives has been proposed (1). Several special linkages have been described to obtain oligonucleotides bearing 3' phosphates (2), 3'-amino and 3' thiol groups (3) and to prepare cyclic oligonucleotides (4).

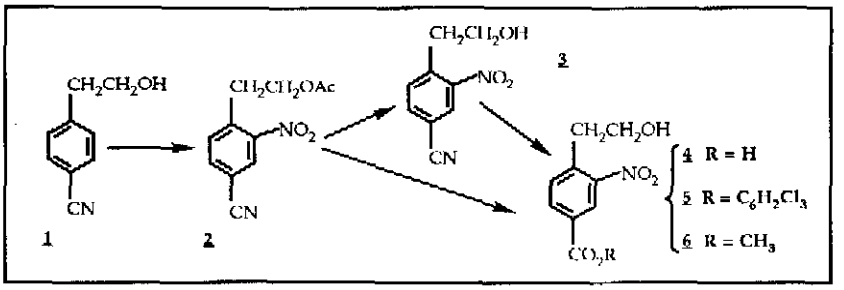
In peptide synthesis, the choice of the linkage between polymeric support and peptide depends on the nature of the peptide and the strategy used during the synthesis for the protection of the α-amino and lateral chain functions (5).

In this and the following paper we describe the preparation of new polymeric supports (NPE-resin) that contain a 2-(2-nitrophenyl)ethyl linkage labile to bases through a β-elimination process and the use of these supports for the synthesis of oligonucleotides and protected peptides.

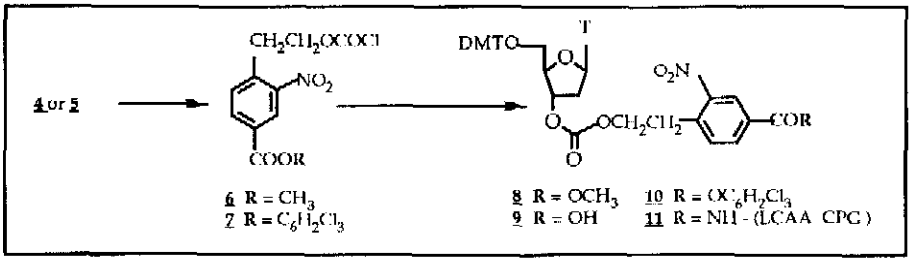
The choice of the o-nitrophenylethyl linkage is based on the work of Pfleiderer who has described the preparation of a variety of substituted phenylethyl groups as amino, alcohol and phosphate protecting groups in oligonucleotide synthesis (6). We were interested in the properties of the (4-cyano-2-nitrophenyl)ethyl protecting group 3. We thought that by converting the cyano function to carboxylic acid 4 we would obtain a compound that could be attached to the standard amino polymeric supports and retain the interesting properties of forming phosphates, esters or carbonate linkages labils to strong non-nucleophilic bases.

Starting from 2-phenylethanol, 4-(2-hydroxyethyl)-3-nitrobenzonitrile 3 was prepared as described in ref. 6a. 4-(2-hydroxyethyl)-3-nitrobenzoic acid 4 was prepared by hydrolysis of compound 3 or directly from 4-(2-acetyloxyethyl)-3-nitrobenzonitrile 2 obtained after nitration of 4-(2-hydroxyethyl)benzonitrile 1. If removal of the acetyl group of compound 2 is done by transesterification with methanol in the presence of anhydrous HCl, the methyl ester 6 is obtained in a 85 % yield (7). Finally, the active ester 5 was prepared by

reaction of the carboxylic acid and 2,4,5-trichlorophenol catalyzed by dicyclohexylcarbodiimide (DCC) (7). The anchoring of 4-(2-hydroxyethyl)-3-nitrobenzoic acid to long chain aminoalkyl controlled pore glass (LCAA-CPG) was achieved by DCC mediated-coupling of compound 4 or directly with the active ester 5 using N,N-dimethylformamide as solvent in the presence of 1 equivalent of 1-hydroxybenzotriazole (HOBt). NPE-CPG support 12 was used directly on a DNA synthesizer and standard 2-cyanoethyl phosphoramidites to prepare oligonucleotides bearing a phosphate in the 3'-end (see below).



A carbonate linkage is needed to prepare oligonucleotides non-phosphorylated at the 3'-end. Reaction of methyl and trichlorophenyl esters 5 and 6 with phosgene affords in quantitative yield the corresponding chloroformates 6 and 7. Subsequent reaction of the chloroformates with 5'-O-dimethoxytrityl thymidine in pyridine gave the carbonates 8 and 10 in moderate yields (45-60 %). The carbonate 10 was attached directly to LCAA-CPG-support. The carbonate 8 was treated with 0.033 M NaOH in dioxane/water/acetonitrile (1:1:1) to selectively hydrolyse the methyl ester, and the resulting carboxylic acid 9 was attached to LCAA-CPG-support by DCC mediated coupling.

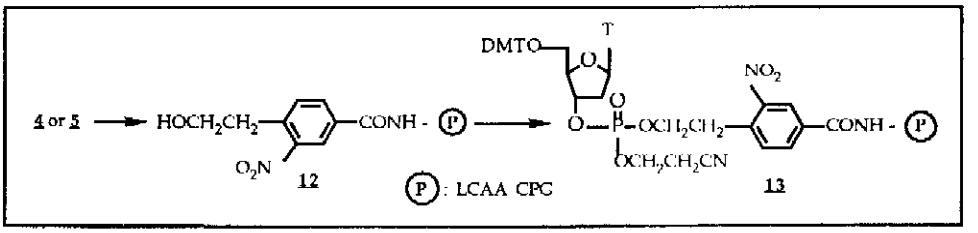


The new carbonate linkage 11 was stable under all the conditions encountered during oligonucleotide synthesis (8), and stable to a solution of 40% triethylamine in pyridine (16 hrs, R.T.). But, on the other hand, the carbonate linkage in DMT-T-NPE-CPG was cleaved in less than 1 hour when it was treated with 0.5 M DBU solution in dioxane or pyridine. When ethanol was used as solvent, 3 hours were needed to break the nucleoside-resin bond. We also found that ammonia (5 hrs, 55 °C) and 20% piperidine/DMF (3hrs, R.T.) were able to cleave the nucleoside from the resin.

To test the utility of the solid support 11 for the synthesis of oligonucleotides two sequences d(CAGACGT) and d(ATGACTGAATATAAACTTGT) were synthesized using a 0.2 µmol scale and standard 2-cyanoethyl

phosphoramidites. The coupling efficiency per cycle based on the absorbance of released 4,4'-dimethoxytrityl cation was found to be greater than 98%. At the end of the syntheses, the supports were treated with conc. ammonia for 16 hrs at 55 °C, and oligonucleotides were purified by reversed-phase HPLC. HPLC profiles were similar to the crudes obtained using standard CPG supports containing 3'-O-succinyl nucleosides.

As mentioned before, polymer 12 can be used as an universal support for the preparation of oligonucleotides bearing a phosphate group at the 3'-end. Reaction of 2-cyanoethyl phosphoramidites with the primary alcohol function on the support and subsequent oxidation give a phosphate triester linkage 13 that is stable during the synthesis and it is cleaved by conc. ammonia. Using this methodology the sequence d(TpTpTpTpTpCp) was prepared on a DNA synthesizer. Coupling yields were greater than 98%. After purification, the pure product was subject to complete digestion with spleen phosphodiesterase giving a mixture of nucleoside 3'-monophosphates Tp and Cp in a 6:1 ratio.



An important application of 2-(2-nitrophenyl)ethyl linkages is that these supports can be used together with p-nitrophenylethyl (NPE)-protected nucleoside 2-cyanoethyl phosphoramidites for the preparation of oligonucleotides without using conc. ammonia, because all protecting groups will be cleaved by DBU. This methodology will be of interest for the preparation of oligonucleotides containing ammonia-sensitive compounds such as base analogues, fluorescent compounds and so on.

To test the utility of this method, the preparation of three sequences d(GACGACTT), d(CAGACGT) and d(CATACGT) was performed using 1 μmol of the derivatized support 11 and NPE-protected nucleoside 2-cyanoethyl phosphoramidites (9, 10) on a home-made manual synthesizer. Coupling efficiency per cycle was about 95%. The supports were treated first with a 40% triethylamine solution in pyridine (3 x 1 hr, R.T.) in order to remove the 2-cyanoethyl phosphate protecting groups (11) and second a 3 hr treatment with a 0.5 M DBU solution in pyridine. The resulting solutions were neutralized with acetic acid, concentrated and desalted on a Sephadex G-10 column. Reversed phase HPLC purification of the crudes gives a major peak that was characterized by snake venom phosphodiesterase and alkaline phosphatase digestion followed by HPLC analysis of the digest.

In conclusion, we have described a base labile linkage that allows the preparation of oligonucleotides and oligonucleotide 3'-phosphates and its use, together with p-nitrophenylethyl base protecting groups, permits the synthesis of oligonucleotides without using conc. ammonia during the final deblocking.

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7) Satisfactory spectroscopic data and elemental analysis were obtained.

8) Small aliquots of DMT-T-carbonate-NPE-CPC were treated at room temperature with the following solutions : a) 0.4 M tetrazole in acetonitrile; b) 5% diisopropylethylamine in dichloromethane; c) 0.1 M iodine in tetrahydrofuran /lutidine /water (30:15:5); d) 0.1 M DMAP / pyridine + acetic anhydride / THF (1:1); e) 0.1 M N-methylimidazole / pyridine + acetic anhydride / THF (1:1); f) 40% triethylamine in pyridine. After 16 hrs, the solution was filtered and the filtrates collected. The amount of the dimethoxytrityl compound was analyzed in the filtrates and the resin. Less than 10% of loss was observed in all the studied conditions except with the DMAP solution that we detected in the filtrates a loss of 15%.

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11) This treatment is important to avoid side reactions between the bases and acrylonitrile liberated from 2 cyanoethyl phosphates (R. Eritja et al. manuscript in preparation).

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