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Solid-Phase Enzymatic Synthesis of Oligonucleotides†

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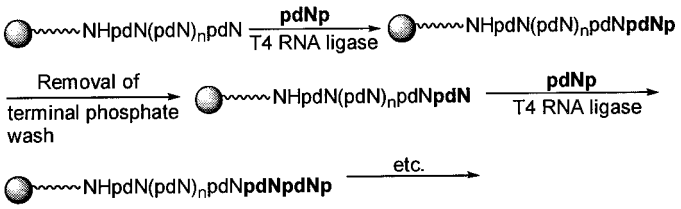
ABSTRACT



The controlled and selective synthesis of oligonucleotides on the solid phase is possible under mild aqueous conditions using the enzyme T4 RNA ligase, the resins being tentagel or kieselguhr/polydimethylacrylamide.

Synthetic oligonucleotides play a key role in molecular biology and in chemistry.[1] Prominent examples pertain to their use as probes for genes, as components in protein−DNA interactions, as tools in molecular cloning of DNA, and as building blocks in pharmaceuticals based on the antisense oligonucleotide concept.[2] Originally, oligonucleotide syntheses were carried out enzymatically,[3] inspite of a number of drawbacks such as laborious purification and the need to prepare protected nucleotides. Early enzymatic syntheses carried out on solid supports were abandoned due to an unacceptably low coupling yield.[4] Following the advent of the phosphoramide methodology, chemical synthesis became the standard protocol.[1,2,5] Nevertheless, a few drawbacks remain, including the use of toxic reagents and solvents, the necessity of synthesis under an inert atmosphere, and purification problems. We now propose an oligonucleotide synthesis on the solid phase using the enzyme T4 RNA ligase,[6] a process which offers a number of advantages by operating in a mild aqueous medium.

The effectiveness of T4 RNA ligase in oligonucleotide synthesis is limited by the fact that it does not work equally well with a variety of acceptor and donor molecules.[3b,7] Indeed, the formation of a 3′,5′-phosphodiester bond requires a trinucleoside biphosphate as a minimal acceptor and a mononucleoside 3′,5′-biphosphate as a donor. We therefore first prepared and purified by HPLC several 3′,5′-biphosphate

† Dedicated to Günther Wulff on the occasion of his 65th birthday.

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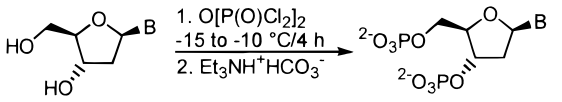
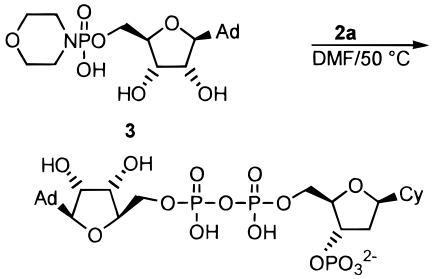
donors **2** from the corresponding nucleosides **1** using the procedure of Barrio.[8]



1a B = deoxycytidine — **2a** 90% (99.7% pure)
b B = deoxyadenine — **b** 52% (92% pure)
c B = deoxyguanine — **c** 80% (94.7% pure)
d B = cytidine — **d** 35% (93% pure)

In the next step, adenylated 3′,5′-deoxycytidine phosphate (**4**), although accessible by the method of Hoffmann and McLaughlin,[9] was prepared more conveniently by reacting the $(n\text{-Oct})_3NH^+$ salt of **2a** with adenosine 5′-monophosphomorpholidate **3**. Compound **4** was formed in 22% yield in addition to some 3′-adenylated deoxycytidine phosphate. Separation of this isomer was not necessary since it is not a substrate for the enzyme.



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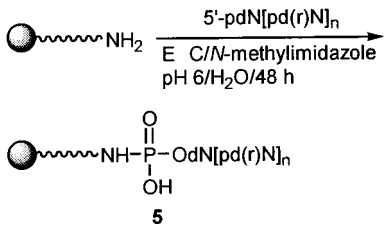
4 (22%)

With these compounds and commercially available[10] primers pdA_5, 5′-pdA$(pdA)_3$prC-3′, and 5′-pdA$(pdA)_9$prC-3′ in hand, we turned our attention to the solid phase, surmising that the choice of the solid support and of the linker could be crucial to the success of the enzymatic oligonucleotide synthesis. Indeed, only a limited number of successful cases of biocatalytic processes on solid supports are known based on the use of other enzymes and substrates.[11] The commercial resins tentagel[12] and kieselguhr/polydimethylacrylamide (PDMA)[13] are available in forms having terminal NH_2 groups, ideal for primer attachment. They appeared to fulfill the requirements with respect to insolubility, inertness to solvents/reagents, polarity, and sufficient surface area. Tentagel is based on a composite of cross-linked polystyrene with poly(ethylene glycol) (MW 3000−4000) and is terminally functionalized with NH_2 groups. The beads swell in water, facilitating biological assays in aqueous systems. Because of their large loading capacity, they have been used for chemical oligonucleotide syntheses.[14] The kieselguhr-derived support consists of fabricated kieselguhr (SiO_2) containing large pores in which cross-linked polydimethylacrylamide resin resides. Because of the macroporous structure of the pores, they are freely permeable for very large molecules and should be suitable for enzymatic synthesis. Our major efforts were therefore directed toward the use of this resin. Several 5′-phosphorylated primers (mainly commercially available) were coupled to the resins at the free amino group by performing the reaction in 0.1 M *N*-methylimidazole buffer (pH 6) in the presence of water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), affording the resin−primer conjugate **5** having a loading of about 2 μmol/g as estimated from the UV analysis of the relevant solution following standard cleavage with NH_2OH/H_2O (pH 4.5)[14] and from analysis of the products by capillary electrophoresis (CE). Higher loading was not striven for, but there is no reason to assume that this is not possible.



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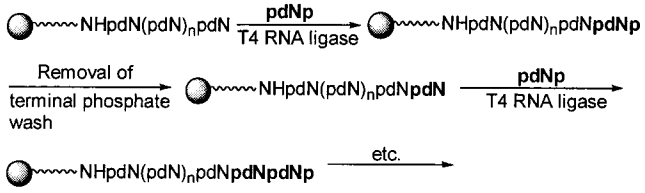
The principle of our solid-phase enzymatic oligonucleotide synthesis is summarized in Scheme 1.

Scheme 1. Principle of Solid-Phase Enzymatic Oligonucleotide Synthesis



The first three steps are repeated as often as desired, the final step being the cleavage from the resin. Specifically, the primer attached with its 5′-end to the solid support has a free 3′-OH group that binds the nucleoside 3′,5′-biphosphate in the presence of T4 RNA ligase. The terminal phosphate blocking group is removed enzymatically by alkaline phosphatase, and all excess reagents are simply washed off from the resin. The next nucleoside 3′,5′-

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biphosphate can then bind to the end of the chain. The ligation reaction was carried out under two different reaction conditions (A and B). First, the process was carried out on a series of resin-supported primers using the ATP regeneration system (condition A) and different biphosphates. Following NH_2OH-induced cleavage from the resins (Scheme 2), the desired products were obtained as shown by CE (Table 1).

Scheme 2. NH_2OH-Induced Selective Cleavage of Phosphoramide Bonds

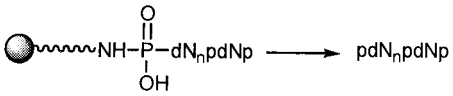

Table 1. Solid-State Enzymatic Oligonucleotide Synthesis under Conditions A[a]

solid phase	primer	pdNp	final product
kieselguhr/PDMA	pdA_4prC	pdCp	$pdA_4prCpdCp$
kieselguhr/PDMA	pdA_4prC	pdAp	$pdA_4prCpdAp$
kieselguhr/PDMA	pdA_4prC	pdGp	$pdA_4prCpdGp$
kieselguhr/PDMA	pdA_4prC	pCp	$pdA_4prCpCp$
kieselguhr/PDMA	$pdA_{10}prC$	pdCp	$pdA_{10}prCpdCp$
tentagel	pdA_4prC	pdCp	$pdA_4prCpdCp$

[a] Tris HCl 50 mM pH 8, $MnCl_2$ 10 mM, DTT 20 mM, ATP 0.4 mM, phosphocreatine 5 mM, spermine 8mM, myokinase 170 U/mL, creatine kinase 175 U/mL, BSA 10 μg/mL, pdNp 8 mM, S-pdN$(pdN)_n$ 0.5 mM, T4 RNA ligase 3300 U/mL, 17 °C/48 h. Cleavage: NH_2OH/H_2O (1:1), pH 4.5; 1 h/37 °C.

The ligation reaction using the pre-adenylated 2′-deoxycytidine phosphate was also carried out, specifically under conditions B which were also applied in the coupling of a second nucleoside 3′,5′-biphosphate (Table 2).

Table 2. Solid-State Enzymatic Oligonucleotide Synthesis under Conditions B[a]

solid phase	primer	AppdNp	reaction time (h)	final product
kieselguhr/PDMA	pdA_5	AppdCp	144	pdA_5pdCp
kieselguhr/PDMA	pdA_4prC	AppdCp	48	$pdA_4prCpdCp$
kieselguhr/PDMA	$pdA_{10}prC$	AppdCp	48	$pdA_{10}prCpdCp$
kieselguhr/PDMA	$pdA_{10}prC$	AppdCp	96	$pdA_{10}prCpdCpdCp$

[a] Tris HCl 50 mM pH 8, $MnCl_2$ 10 mM, DTT 20 mM, BSA 10 μg/mL, AppdNp 5 mM, S-pdN$(pdN)_n$ 0.5 mM, T4 RNA ligase 3300 U/mL, 17 °C. Cleavage: NH_2OH/H_2O (1:1), pH 4.5; 1 h/37 °C.

All reactions proceeded to completion and were monitored by CE, including the cleavage processes. The results show that the composition of the primer has an effect on the reaction rate. For the primer with a ribocytidine at the end, the reaction is complete after 48 h, whereas the use of a pure deoxyribonucleotide primer requires 144 h for completion of reaction. These values are comparable to those in solution and confirm the expectation that ribonucleotides are better acceptors than deoxyribonucleotides. NH_2OH-mediated cleavage sets free the oligonucleotide as detected by CE/MALDI-TOF-MS. At long reaction times, none of the primer was detected, indicating a high coupling efficiency of the ligation. Furthermore, RNase A selectively cleaves the synthesized oligonucleotide at the ribose sugar,[15] in our case the primer remaining on the resin (Scheme 3; Table 3). In this case the products were analyzed by CE and identified by comparison with commercially available nucleoside 3′-phosphates.

Scheme 3. RNase A Catalyzed Cleavage

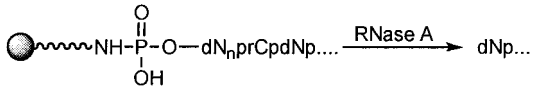

Table 3. Products of RNase A Catalyzed Cleavage from Kieselguhr−PDMA Bound Nucleotides

solid phase	cleavage product
$pdA_4prCpdCp$	dCp
$pdA_4prCpdGp$	dGp
$pdA_4prCpdAp$	dAp
$pdA_{10}prCpdCp$	dCp

Finally, the extended primer attached to the resin can be treated with alkaline phosphatase[16] to give the dephosphorylated product, the free 3′ end group allowing for attachment of a second nucleoside 3′,5′-biphosphate if so desired. This can be conveniently carried out if the enzyme is deactivated by heat treatment (95 °C/5 min) prior to the coupling process.

In summary, our nonoptimized preliminary results clearly show that T4 RNA ligase, although somewhat slow, principally catalyzes under mild conditions the attachment of various 3′,5′-diphosphates and the pre-adenylated 3′-nucleoside phosphate to primers immobilized on solid supports. The relatively low rate is not due to possible immobilization effects. Rather, T4 RNA ligase is known to be a fairly slow enzyme in solution.[3b,7] In view of the possibility of automation and molecular biological optimization of enzymes using directed evolution,[17] the enzymatic synthesis of oligonucleotides on the solid phase may turn out to be a viable alternative to current conventional chemical methods.

Supporting Information Available: Typical experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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