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Parallel Solid-Phase Synthesis of Nucleoside Phosphoramidate Libraries

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Abstract—Combinatorial chemistry is playing an increasingly prominent role in the process of drug discovery. A nucleic acid-based (NAB^{TM}) scaffold can be engineered to create functional group and topological diversity in a library. Described herein is the parallel solid-phase synthesis of combinatorial libraries of nucleoside phosphoramidates, and the first evaluation of antiviral activity against hepatitis B virus (HBV). © 2001 Elsevier Science Ltd. All rights reserved.

Compounds that have phosphoramidate (PN) functionality have a range of biological activities.[1] Well-known examples include the anticancer drug, cyclophosphamide, and the cardioprotective agent, phosphocreatine. As phosphoric, and carboxylic equivalents, phosphoramidates have been evaluated as analogues of nucleosides and oligonucleotides. Thus, for example, 5′-phosphoramidates have been synthesized as 'prodrug' derivatives of antiviral nucleosides such as 3′-azido-thymidine (AZT) and dideoxyinosine (ddI) and reported to possess anti-HIV activity.[2a–d] Additionally, oligonucleotides with primary, secondary, and tertiary phosphoramidate internucleotidic linkages have been evaluated as antisense agents. However, only a limited number of nucleoside phosphoramidates have been prepared and evaluated for antiviral activity.[2e]

The clinically useful antiviral drugs target mainly viral reverse-transcriptase (RT), DNA polymerase, and protease. However, two critical issues have emerged from their therapeutic use: (a) the rapid development of drug resistance; and (b) side effects such as mitochondrial and bone-marrow toxicity associated with most polymerase and RT inhibitors. Although the use of 'drug cocktail' regimens have been helpful, clearly, newer antiviral strategies are urgently needed to combat viral infections.

Recently, combinatorial synthesis, and high-throughput screening have emerged as powerful drug discovery paradigms.[3] This, in turn, has stimulated efforts to assemble novel libraries of compounds for evaluation against biological targets. A combinatorial approach that employs screening of biologically relevant libraries for their ability to modulate biological pathways, with or without regard to specific molecular targets, is quite appropriate in the context of antiviral lead discovery. This approach would enable simultaneous identification of leads, and the potential discovery of novel molecular targets. Indeed, phosphoramidates derived from NAB^{TM} scaffolds can provide a variable spatial display of functionalities that facilitate hydrophobic, hydrogen bonding, and ionic interactions with critical viral protein and nucleic acid targets. Additionally, one can create libraries with varying degrees of conformational rigidity and flexibility. These attributes make NAB^{TM} phosphoramidates a novel source of biologically relevant chemical diversity.

The synthesis of nucleoside phosphoramidates (PN) has traditionally been accomplished by treatment of a support-bound *H*-phosphonate with a solution of amine in CCl_4.[4] However, the applicability of this method for the parallel assembly of PN library had not been reported.[5] Three challenges had to be met for efficient parallel

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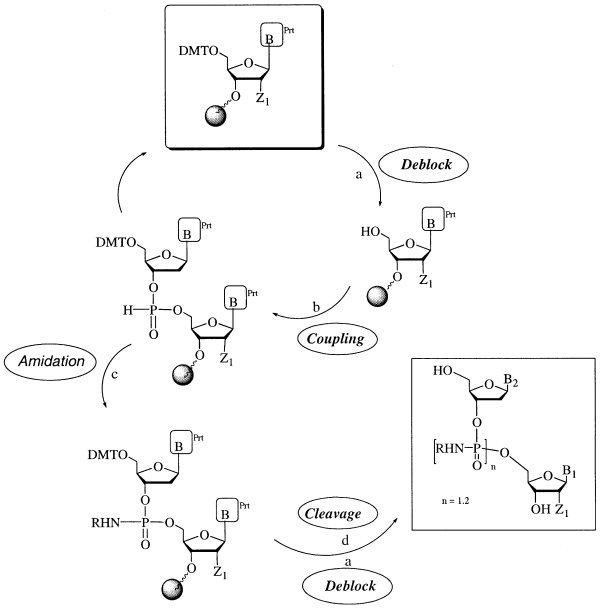
synthesis of PN libraries: (a) The methodology should be adaptable to a variety of primary and secondary amines belonging to acyclic, cyclic, aromatic, and heterocyclic classes having different steric, and electronic environments; (b) the synthesis, work-up, and purification should be practical and provide individual PN compounds of high purity; and (c) the methodology should facilitate the assembly of a chimeric library that has both phosphorothioate (PS) and (PN) internucleotidic linkages.



Scheme 1. (a) 3% Dichloroacetic acid in CH_2Cl_2; (b) *H*-phosphonate, adamantane carbonyl chloride; (c) amine/CCl_4; (d) 28% NH_4OH, 55 °C, 12 h.

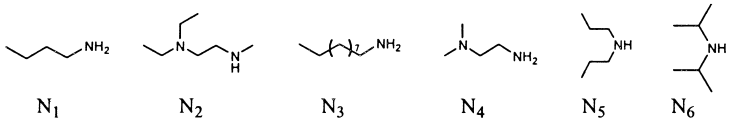
This paper describes improved methodologies that facilitated the parallel synthesis of a representative 600-member library in high yield and purity. Preliminary antiviral evaluation of the libraries against HBV in cell-based assays has helped to validate the biological relevance of the library with respect to: (a) its ability to cross cell membrane; (b) its metabolic stability; and (c) its antiviral activity.

Scheme 1 depicts the *H*-phosphonate methodology for the solid-phase assembly of dinucleoside and trinucleoside phosphoramidate libraries.

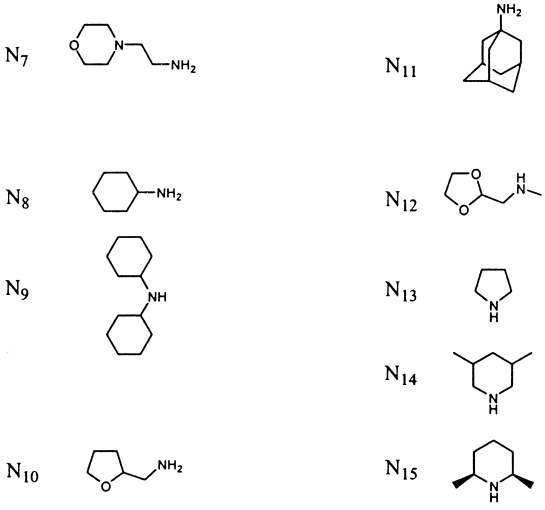
In typical model studies, selected amines (Table 1) were employed. Typically, the requisite solid-support-bound dinucleoside *H*-phosphonates (5′-DMT-off) were assembled on CPG-support using nucleoside *H*-phosphonates in conjunction with 1-adamantane carbonyl chloride as the activator. Each support-bound *H*-phosphonate was treated with a solution of each of the corresponding amines (10% in CCl_4). The resulting phosphoramidates were released from the solid support by treatment with aqueous NH_4OH. Upon analysis, the following salient features emerged: (a) The yield of the library was highly variable, ranging from 10 to 95%, and the purity of the crude library ranged from 50 to

Table 1. Structures of amines used in library assembly

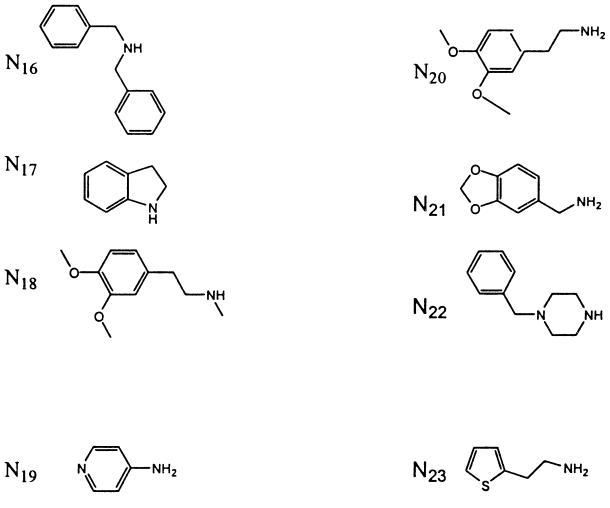
Acyclic amines



Cyclic and heterocyclic amines



Aromatic amines



95% depending on the type of amine employed. Thus, process optimization had to be carried out for improvements in yield and purity. (b) With the hindered amine, adamantamine (N_{11}), the yield of the library was very low (less than 10%). To improve the yields, a variety of oxidative amidation conditions were investigated. When amidation was performed using either pyridine/CCl_4, triethylamine/CCl_4, or collidine/CCl_4, the purity of the library members could be improved to 95% in an overall yield of 50% after purification. However, with the hindered amines, N_6, N_9, and N_{15}, the yield was less than 20%. Variations in solvents and reaction times failed to improve the yield. Consequently, the hinderd amines N_6, N_9, and N_{15} were not used in the assembly of the library. (c) In the case of the phosphoramidates derived from aromatic amines, the yield and purity of the products varied depending on the structure of the aromatic amines. For example, with the aromatic amines N_{16} and N_{17}, the purity of the crude library of dinucleoside phosphoramidates was ca. 50%. However, after reversed-phase HPLC purification, the purity could be improved to 85–95%. Most of the aromatic amines gave the expected phosphoramidates except in the case of dibenzylamine, where partial debenzylation resulted in the formation of the corresponding monobenzylated product.

Based on these model studies, a representative 600-member library (Table 2) was assembled using optimized protocols.

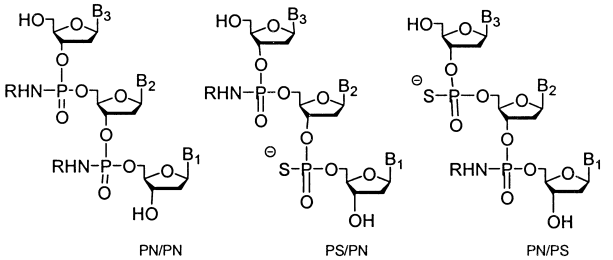
Thus, following their assembly, the solid-support-bound nucleoside *H*-phosphonates were converted to the corresponding phosphoramidates in a Quest 210™ Library Synthesizer.[6]

Members of the library were screened at 10 μM for antiviral activity against HBV in cell-based assays.[7] In preliminary studies, some of the members showed potent antiviral activity in this assay. Lead optimization is in progress and details of these ongoing studies will be presented elsewhere.

Table 2. Di- and tri-nucleoside phosphoramidate library

3′AA-Nx	3′AG-Nx	3′A̲A-Nx	3′A̲G-Nx
3′CA-Nx	3′CG-Nx	3′C̲A-Nx	3′C̲G-Nx
3′GA-Nx	3′GG-Nx	3′G̲A-Nx	3′G̲G-Nx
3′AC-Nx	3′AT-Nx	3′A̲C-Nx	3′A̲T-Nx
3′CC-Nx	3′CT-Nx	3′C̲C-Nx	3′C̲T-Nx
3′GC-Nx	3′GT-Nx	3′G̲C-Nx	3′G̲T-Nx
3′TC-Nx	3′TT-Nx	3′U̲C-Nx	3′U̲T-Nx

Nx corresponds to amines (see Table 1); A, C, G, T correspond to deoxyribonucleosides; A̲, C̲, G̲, U̲ correspond to 2′-OMe ribonucleosides.



NHR = N_1–N_{10}, N_{11}, N_{13}, N_{17}, N_{18}, and N_{20}–N_{23} (see Table 1).

In conclusion, we have achieved efficient parallel synthesis, and preliminary antiviral evaluation of a library of nucleoside phosphoramidate compounds as potentially a new class of anti-HBV agents.

Acknowledgements

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6. The experimental protocols are briefly described below:
Assembly of library

Di- and tri-nucleoside phosphoramidates. The *H*-phosphonates were assembled on a controlled-pore-glass (CPG) support on a 10 μmol scale using standard *H*-phosphonate chemistry. The dry CPG-bound *H*-phosphonate was transferred to 5 mL RV tubes in a Quest 210™ Library Synthesizer and a solution of amine in CCl_4 (10%, 3 mL) was added. In the case of hindered amines, the reaction was performed in the presence of pyridine/CCl_4, triethylamine/CCl_4, or collidine/CCl_4. The reaction mixture was agitated for 20–30 min. After washing, the CPG was transferred to a 5 mL tube and treated with 28% aq NH_4OH (55 °C, overnight). The suspension was cooled and centrifuged. The solution was evaporated to dryness in vacuo, residue dissolved in H_2O (5 mL), and extracted with ethyl acetate (2 mL). The purity of the resulting crude library members ranged from 85 to 95%.

The compounds were purified and desalted by passing through a C18-column (10×1 cm) (Buffer A: H_2O, Buffer B: 20% CH_3CN in H_2O) to give individual library members of 95–99% purity as determined by reversed-phase HPLC.

Post-column synthesis protocols for trinucleoside phosphoramidates were as described for dinucleoside phosphoramidates.

RP-HPLC analysis for crude products showed purity range of 50–80%. The crude product was purified by C18 column as

before to give individual library members of up to 95% purity.

Chimeric trinucleotides (PS-PN and PN-PS). The requisite PS linkage in the trinucleotides was constructed using phosphoramidite chemistry,[8a] in conjunction with 3*H*-1,2-benzodithiole-3-one-1,1-dioxide as the sulfurizing reagent.[8b] The PN-linkage was incorporated using *H*-phosphonate chemistry.

For the 3′PS-PN library, the PS-linked dinucleotides were prepared on a synthesizer using a standard phosphoramidite synthesis cycle (DMT-on). The CPG-column was dried and installed on another DNA synthesizer (BioSearch Model 8700) to establish the *H*-phosphonate linkage. The reverse synthetic sequence was employed for the PN-PS library. In both cases, the conversion of *H*-phosphonates to phosphoramidates was done on the Quest 210™ Synthesizer as previously described.

The crude compounds (50–75% pure) were purified on ion-exchange columns followed by desalting (C18 column) as before to give individual library members of up to 95% purity.

HPLC analysis of library members

RP-HPLC analysis of the libraries was performed on a Waters 600 system equipped with a photodiode-array UV detector 996, 717 autosampler, and Millennium® 2000 software, using a Radial-Pak® cartridge (8 mm I.D., 8NVC18). Mobile phase: Buffer A: 0.1 M NH_4OAc; Buffer B: 20% A/80% CH_3CN, v/v: Gradient: 100% A, 0–3 min; 40% A, 40 min; 100% B, 49 min; 100% B. Product purity ranged from 85 to 95%.

Characterization of library members

Spectral characterization of representative library members was carried out on desalted and purified material. ^{31}P NMR (D_2O, 85% H_3PO_4 external standard, δ ppm) 11–15 (PN linkage) and δ 56–58 (PS linkage). ^{1}H NMR analysis of selected compounds was consistent with the assigned structures. Additionally, the ES-MS of library members gave the expected molecular ions. Typical data are as follows.

3′AG-N_{13}. ^{31}P NMR (D_2O), δ 12.18, 12.06. ES-MS: calcd for 633.2 (M); found: *m*/*z* 634.2 (M + H).

3′UA-N_{18}. ^{31}P NMR (D_2O), δ 14.11, 13.74. ES-MS: Calcd. for 748.3; found: *m*/*z*, 749.1 (M + H).

Anti-HBV activity

The anti-HBV activity and cytotoxicity assays of the compounds were performed at 10 μM concentration using HepG2-derived 2.2.15 cell lines according to published procedures using 3TC (IC_{50} 0.06 μM) as the positive control.[7] For the single-dose antiviral and toxicity analyses, confluent cultures 2.2.15 cells were maintained on 96-well flat-bottomed tissue culture plates in RPMI 1640 medium with 2% fetal bovine serum. Cultures were treated with nine consecutive daily doses of 10 μM of the test compounds. Medium was changed daily with addition of fresh test compounds. Extracellular (virion) HBV DNA levels were measured 24 h after the last treatment.

For the multiple-dose analyses, two separate (replicate) plates were used for each antiviral drug treatment. A total of 3 cultures on each plate were treated with each of four serial 10-fold dilutions of antiviral agents (six cultures per dilution) for the antiviral assays.

Toxicity analyses for the multiple-dose treatments were performed on separate plates than those used for the antiviral assays. Cells for the toxicity analyses were cultured under conditions as used for the antiviral evaluations. Each compound was tested at four concentrations, each in triplicate cultures. Uptake of neutral red dye was used to determine the relative level of toxicity 24 h following the last treatment. The absorbance of internalized dye at 510 nM (A_{510}) was used for the quantitative analysis. Representative antiviral data is as follows.

3′AG-N_{13}. IC_{50} 2.1 ± 0.2 μM; CC_{50} > 300 μM; **3′UA-N_{18}:** IC_{50} 4.4 ± 0.2 μM; CC_{50} > 300 μM.

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