Synthesis and Properties of Combinatorial Libraries of Phosphoramidates

Reza Fathi,* M. Jonathan Rudolph, Robert G. Gentles,† Rina Patel, Eric W. MacMillan, Michael S. Reitman, David Pelham, and Alan F. Cook

PharmaGenics, Inc., 4 Pearl Court, Allendale, New Jersey 07401

Received January 30, 1996⊗

We have assembled a set of combinatorial libraries of phosphoramidates for pharmacological evaluation. A range of functionalized and unfunctionalized diols, representing a variety of diversity elements, were converted into their corresponding dimethoxytrityl H-phosphonate derivatives which were coupled to each other to produce H-phosphonate dimers and trimers. The H-phosphonate diesters were converted into phosphoramidates by reaction with a wide range of primary and secondary amines. Very large libraries (theoretically, in excess of one million compounds) possessing five sites of diversity were generated for use in our drug discovery program. Smaller libraries with lower molecular weights were also prepared in which only two monomeric units were coupled together and converted into their phosphoramidate derivatives. Methods for the attachment of both radioactive and nonradioactive labels, including ^{32}phosphorus, tritium, and fluorescein, have been developed. Representative single sequences were also prepared and their chemical properties studied.

Introduction

Combinatorial chemistry is playing an increasingly prominent role in the drug discovery process for the identification of lead compounds and optimization of existing drug candidates.[1−4] The intense interest in this area has stimulated efforts to construct novel combinatorial libraries for biological evaluation. Very large libraries of oligonucleotides and L- and D-peptides have been prepared using automated synthesizers, and these have been used to select sequences with high affinities for targets of interest.[5−9] Automated synthesizers have also been used to prepare smaller libraries of peptoids and phosphodiester-containing oligomers. Screening of these libraries has yielded novel, high affinity ligands for biological targets.[10,11] The assembly of large libraries of organic molecules of smaller molecular weight is less well developed, since the repertoire of organic reactions on solid supports needs to be developed on a case by case basis. Nevertheless, the construction of combinatorial libraries of small molecules is rapidly growing, and a number of approaches have been described.[12−17] In general, the numbers of compounds in a given small molecule library are usually much smaller than what has been generated for peptide and oligonucleotide libraries. The most commonly used synthetic method for the assembly of libraries employs the split synthesis procedure of Furka et al.[18] wherein individual reactions are carried out on solid supports which are pooled and divided after each step.

One of the major challenges to the successful application of combinatorial chemistry is to determine which members of the library bind most effectively to the biological target. Several methods for the deconvolution of libraries have been developed to address this need. For small libraries, with high enough concentrations for each individual member, direct identification by mass spectrometry is a viable option, whereas for much larger libraries, indirect methods are usually needed. Techniques which have been used for the deconvolution of large peptide libraries include the iterative analysis of pools and subpools,[5] or recursive deconvolution.[9] For oligonucleotides, procedures have been developed which involve exposure to the target, removal of unbound material, and amplification of the bound material by polymerase chain reaction.[7,8] This amplification step severely restricts the types of libraries which can be employed. Another iterative method for deconvolution (known as SURF) involves the evaluation of a series of sublibraries in which one or more positions are fixed during the synthesis.[19] Tags or "barcodes", including

† Present address: Abbott Laboratories, Abbott Park, IL 60064.

⊗ Abstract published in *Advance ACS Abstracts,* July 15, 1996.

(1) Gordon, E. M.; Barrett, R. W.; Dower, W. J.; Fodor, S. P. A.; Gallop, M. A. *J. Med. Chem.* **1994**, *37*, 1385−1401.
(2) Gallop, M. A.; Barrett, R. W.; Dower, W. J.; Fodor, S. P. A.; Gordon, E. M. *J. Med. Chem.* **1994**, *37*, 1233−1251.
(3) Moos, W. H.; Green, G. D. *Annu. Rep. Med. Chem.* **1993**, *28*, 315−324.
(4) Ecker, D. J.; Crooke, S. T. *Biotechnology* **1995**, *13*, 351−360.
(5) Dooley, C. T.; Chung, N. N.; Schiller, P. W.; Houghten, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10811−10815.
(6) Dooley, C. T.; Chung, N. N.; Wilkes, B. C.; Schiller, P. W.; Bidlack, J. M.; Pasternak, G. W.; Houghten, R. A. *Science* **1994**, *266*, 2019−2022.
(7) Tuerck, C.; Gold, L. *Science* **1990**, *249*, 505−510.
(8) Bock, L. C.; Griffin, L. C.; Latham, J. A.; Vermaas, E. H.; Toole, J. J. *Nature* **1992**, *355*, 564−566.
(9) Erb, E.; Janda, K. D.; Brenner, S. *Proc. Nat. Acad. Sci. U.S.A.* **1994**, *91*, 11422−11426.
(10) Zuckermann, R. N.; Martin, E. J.; Spellmeyer, D. C.; Stauber, G. B.; Shoemaker, K. R.; Kerr, J. M.; Figliozzi, G. M.; Goff, D. A.; Siani, M. A.; Simon, R. J.; Banville, S. C.; Brown, E. G.; Wang, L.; Richter, L. S.; Moos, W. H. *J. Med. Chem.* **1994**, *37*, 2678−2685.
(11) Davis, P. W.; Vickers, T. A.; Wilson-Lingardo, L.; Wyatt, J. R.; Guinosso, C. J.; Sanghvi, Y. S.; DeBaets, E. A.; Acevedo, O. L.; Cook, P. D.; Ecker, D. J. *J. Med. Chem.* **1995**, *38*, 4363−4366.
(12) Chen, C.; Ahlberg Randall, L. A.; Miller, R. B.; Jones, A. D.; Kurth, M. A. *J. Am. Chem. Soc.* **1994**, *116*, 2661−2.
(13) Patek, M.; Drake, B.; Lebl, M. *Tetrahedron Lett.* **1995**, *36*, 2227−2230.
(14) Patek, M.; Drake, B.; Lebl, M. *Tetrahedron Lett.* **1994**, *35*, 9169−9172.
(15) Hutchins, S. M.; Chapman, K. T. *Tetrahedron Lett.* **1995**, *36*, 2583−2586.
(16) Bunin, B. A.; Plunkett, M. J.; Ellman, J. A. *Proc. Nat. Acad. Sci. U.S.A.* **1994**, *91*, 4708−4712.
(17) Gordon, D. W.; Steele, J. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 47−50.
(18) Furka, A.; Sebestyen, F.; Asgedom, M.; Dibo, G. *Int. J. Pept. Protein Res.* **1991**, *37*, 487.
(19) Ecker, D. J.; Vickers, T. A.; Hanecak, R.; Driver, V.; Anderson, K. *Nucleic Acids Res.* **1993**, *21*, 1853−1856.

S0022-3263(96)00192-2 CCC: $12.00

oligonucleotide sequences[20] and polyhalogenated phenyl ethers,[21] have also been used for the deconvolution process.

An indirect method for deconvolution, which we call COMPILE (COMbinatorial Procedure for Indirect Ligand Elucidation), has also been developed for the rapid deconvolution of extremely large libraries. COMPILE is a general procedure that can be used to select and identify high affinity ligands from any combinatorial library. Pools of compounds are synthesized by a strategy whereby one chemical subunit is fixed at a single position in every pool. Each pool is exposed to the target and subsequently, target-bound compounds are separated from unbound compounds by any one of a variety of methods, such as forced dialysis. The stringency of this affinity-based purification (or selection) can be adjusted by manipulating the concentration of the biological target in order to identify the highest affinity ligands. A plot of concentration of library retained, i.e. bound to the target, versus the round of separation of bound and unbound compounds reveals which pools or sublibraries contain high affinity compounds. Those sublibraries in which a higher percentage of the pool was retained after each cycle will exhibit a change in the slope of the curve of such plots. For detection purposes, a label such as fluorescein or tritium is attached to each member of the library. The structures of the compounds with highest affinity can be indirectly deduced from a inspection of the chemical composition of the fixed positions of those pools which contained high affinity compounds. As a demonstration of the power of COMPILE, several peptides which bound with high affinity to an antibody target have been identified from a library of over one million members.[22]

In order to more fully exploit the potential of COMPILE in selecting compounds of interest for our drug development program, large libraries of classes of compounds other than peptides were required. We therefore undertook the synthesis of libraries of non-nucleotide phosphoramidate diesters. The synthesis of these compounds takes advantage of the availability of DNA synthesizers, which can be adapted to couple phosphorus-based non-nucleotide synthons. The synthesis of N-substituted hydroxyprolinol phosphoramidites for use in combinatorial libraries has also been reported.[23] We describe herein the solid-supported synthesis of large combinatorial libraries of non-nucleotide phosphoramidates and a study of their chemical properties.

Results and Discussion

Overall Strategy. Our approach to the synthesis of combinatorial libraries of phosphoramidates is outlined in Figure 1. A set of monomer H-phosphonates is prepared and separately coupled to a suitable solid support, such as thymidine-derivatized polystyrene beads, using a DNA synthesizer. The supports are then pooled and redivided, and a second coupling of each monomer is carried out. After repeating the pool and divide process, the individual mixtures of support bound H-phosphonate derivatives are then converted into phosphoramidates by treatment with primary or secondary amines. At this point, the sublibraries are either handled individually or pooled to produce the master library. The final steps involve introduction of the labeling group and cleavage from the support. Larger libraries can be prepared by performing additional coupling and oxidation steps before labeling and cleavage. The details are described below.

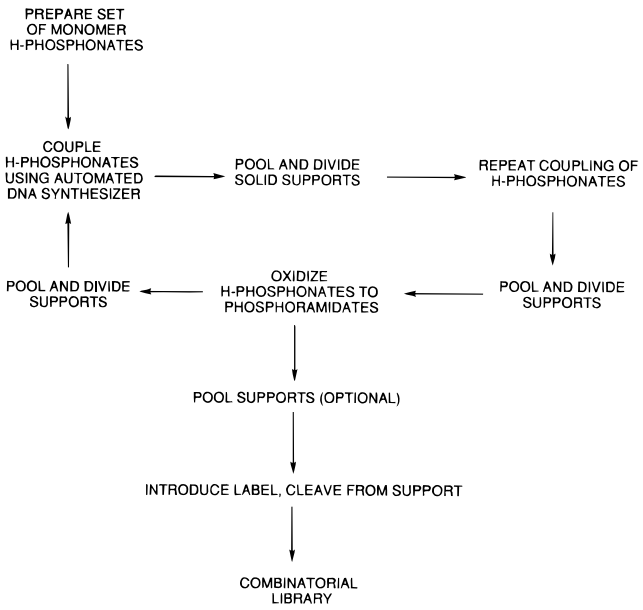

Figure 1. Overall strategy for the synthesis of libraries of phosphoramidates.

Synthetic Approach. DNA synthesizers have primarily been used to prepare oligonucleotides, although non-nucleotide synthons have been used to attach various entities including linker groups, such as triethylene glycol, nonradioactive reporter groups such as biotin, fluorescent dyes for sequencing, and conjugate groups, such as cholesterol. Two general methods are most widely used for the solid-supported synthesis of oligonucleotide phosphodiesters: (a) the phosphoramidite approach,[24] and (b) the H-phosphonate approach.[25] Of these methods, the former is most widely used, due to the higher coupling yields, an important factor for longer oligonucleotides. Although both of these chemistries utilize intermediates which can be oxidized to produce phosphoramidates, we considered the H-phosphonate approach to be the preferred route for the preparation of non-nucleotide phosphoramidates. The reaction of H-phosphonates with amines has been extensively utilized in the oligonucleotide area,[26,27] and reactions of this type have been shown to proceed in high yield on solid supports without the formation of significant amounts of byproducts. The procedure appeared to be general for a wide range of primary and secondary amines.[26] Since the efficiency of formation phosphoramidate linkages on a solid support was an important issue for the assembly of libraries, the H-phosphonate method was used for library synthesis.

(20) Nielsen, J.; Brenner, S.; Janda, K. D. *J. Am. Chem. Soc.* **1993**, *115*, 9812−9813.
(21) Borchardt, A.; Still, W. C. *J. Am. Chem. Soc.* **1994**, *116*, 373−374.
(22) Beutel, B. A.; Bellomy, G. R.; Waldron, J. A.; Voorbach, M. J.; Bertelsen, A. H., manuscript submitted.
(23) Hebert, N.; Davis, P. W.; DeBaets, E. L.; Acevedo, O. L. *Tetrahedron Lett.* **1994**, *35*, 9509−9512.
(24) Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859−1862.
(25) Froehler, B. C.; Matteucci, M. D. *Tetrahedron Lett.* **1986**, *27*, 469−472.
(26) Froehler, B. C. *Tetrahedron Lett.* **1986**, *27*, 5575−5578.
(27) Letsinger, R. L.; Singman, C. N.; Histand, G.; Salunkhe, M. *J. Am. Chem. Soc.* **1988**, *110*, 4470−4471.

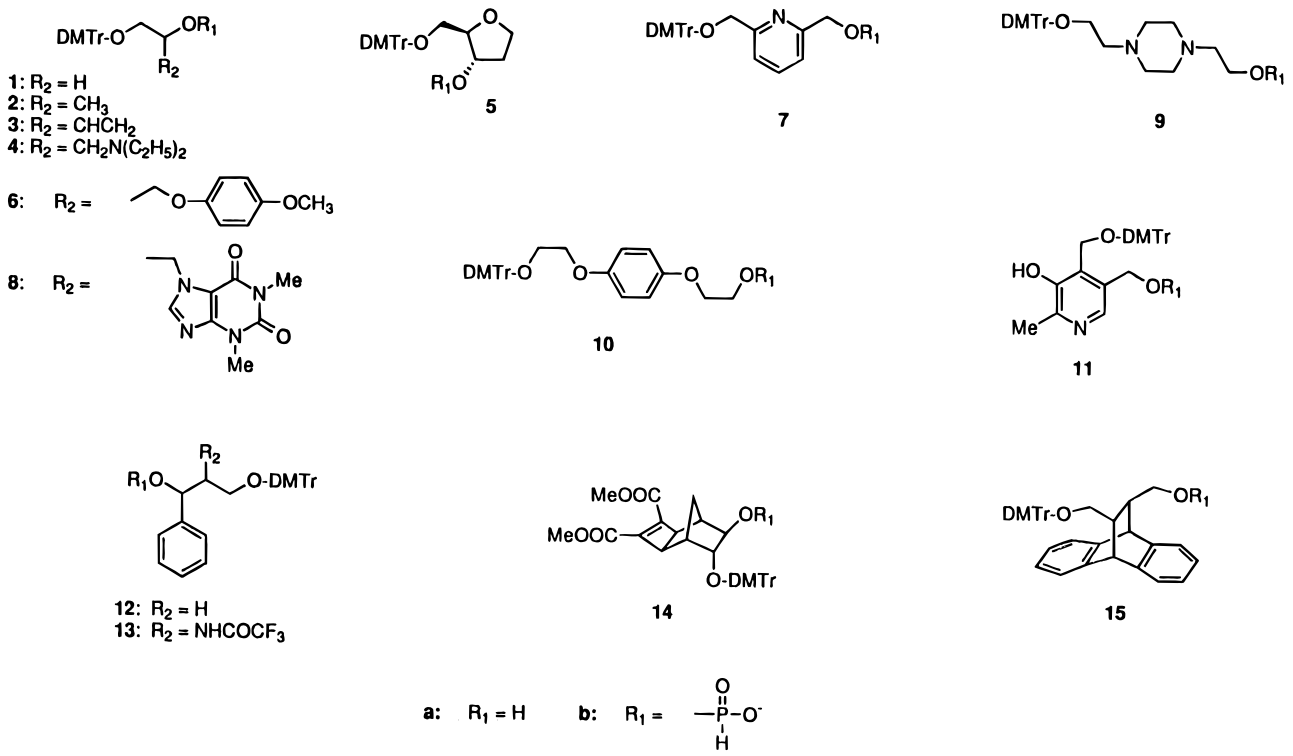

Figure 2. Monomers used for the synthesis of libraries.

Monomer Synthesis. We selected a set of diols that incorporated a wide range of structural variations, including aliphatic and aromatic groups, hydrogen bond donors and acceptors, and diols which provided diversity to the backbone. Reaction of the diols with dimethoxytrityl chloride gave the trityl derivatives **1a**−**15a** (Figure 2), followed by conversion to the H-phosphonates **1b**−**15b** by reaction with phosphorus trichloride/imidazole/triethylamine. For diols possessing hydroxyl groups with similar reactivities, such as those with two primary hydroxyl groups, mixtures of trityl derivatives were produced. These mixtures were separated by conventional silica column chromatography, and one isomer was selected for incorporation into the library. In a few instances, mixtures of isomers were employed. The H-phosphonate derivatives were generally obtained as oils or gums which could not be crystallized, but the presence of the H-phosphonate group could be inferred using phosphorus NMR spectroscopy, which showed a signal in the region of 0−10 ppm, with a J_{P-H} of approximately 600 Hz.

The coupling efficiencies of the H-phosphonate monomers were evaluated by trial couplings using a modified H-phosphonate cycle on the DNA synthesizer. Yields of greater than 90% could be obtained for almost every monomer by varying the coupling time. Diols with a tertiary hydroxyl group, such as pinanediol, gave low coupling yields, and these were excluded from library synthesis. The coupling yields were generally lower than those for conventional oligonucleotides synthesized by the H-phosphonate route. However, the fact that only one or two couplings were needed mitigates the loss of material and the potential skewing of the libraries caused by variable coupling yields. We found that coupling of H-phosphonates is more tolerant of structural variations in the diol, since in several cases a phosphoramidite coupled less efficiently than the corresponding H-phosphonate derivative.

Model Studies on the Formation of Phosphoramidates. Model studies were carried out to confirm that phosphoramidates could be readily produced from a variety of amines using thymidine dinucleotide H-phosphonate as the test system. The reactions of this H-phosphonate with all of the amines used for the construction of libraries were studied, and phosphoramidate formation was found to be very efficient in every case. Reactions with a few of the amines were also studied in more detail, and the compounds were isolated and characterized. The amines selected were 2,6-dimethylmorpholine, 2-(3,4-dimethoxyphenyl)ethylamine, 1-(2-aminoethyl)pyrrolidine, 1-benzylpiperazine, and thiophene-2-methanamine, which are representative of the various types of primary and secondary amines employed, i.e. substituted ethylamines, piperazine derivatives, other cyclic secondary amines, etc. Each amine gave the expected phosphoramidates **16**−**20** (Figure 3) in excellent yield after a reaction time of 10 min, and the purity and identity of the products were confirmed by HPLC, ^{31}P NMR, and electrospray mass spectrometry. In general, the ^{31}P NMR spectra of these compounds showed two signals in the region of δ 7−10 corresponding to the two isomers of the phosphoramidate produced. These experiments provide evidence that the amines selected are capable of producing phosphoramidates from H-phosphonate intermediates and lends support to the proposed structures of the libraries described below.

Assembly of Libraries. Although oligonucleotide synthesizers are capable of producing materials of high molecular weight through many cycles of coupling, our objective was to produce large libraries without unduly increasing the average molecular weight of the compounds. We therefore restricted the number of couplings to produce dimers or trimers connected by one or two phosphorus moieties, respectively, and additional diversity was introduced by reaction of the H-phosphonate groups with a wide variety of amines, to produce phos-

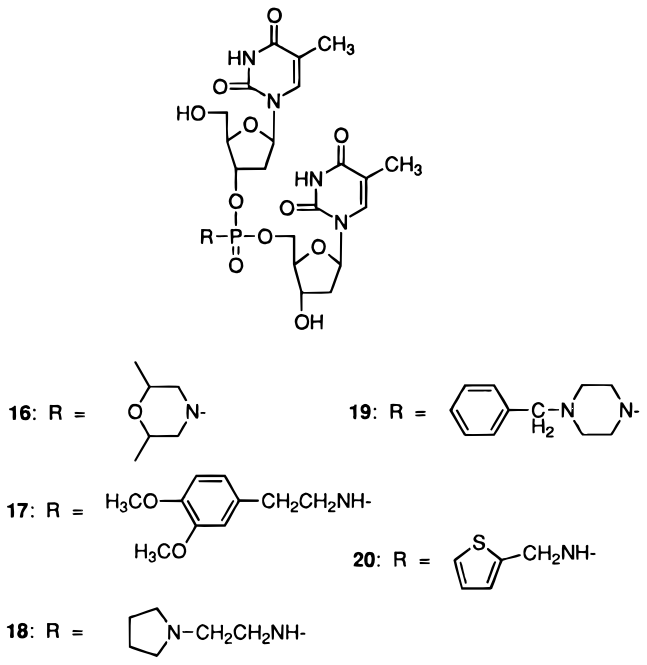

Figure 3. Structures of model phosphoramidates prepared for characterization purposes.

phoramidates. Each H-phosphonate monomer was subjected to trial couplings to determine the efficiency of coupling, and a coupling yield of 90% was considered to be the minimum acceptable for use in the library. Similarly, the reactivities of all of the amines used in this study were evaluated for their ability to produce phosphoramidates from H-phosphonate intermediates using thymidine dinucleotide as the test system. The efficiencies of these reactions were examined by HPLC, and the presence of phosphoramidate linkages was determined by ^{31}P NMR. In general, the yields were found to be uniformly high, although a few amines which did not meet the criterion of almost complete conversion of H-phosphonate to phosphoramidate were excluded from further use in the preparation of libraries. Assembly of the libraries on solid supports was carried out using the standard divide and pool method,[18] and the supports were divided as suspensions in acetonitrile using a liquid handling system. After assembly of the libraries on solid supports, they were labeled using one of the methods described below. Labeling was carried out either on the entire library or on the individual sublibraries obtained after addition of the final diversity element.

Labeling Methods: (a) ^{32}P Labeling. Since the COMPILE procedure requires detection of very small amounts of material, several methods for the attachment of labels to these libraries were evaluated; both radioactive and nonradioactive labels were employed. One of the simplest approaches to radioactive labeling is to introduce a labeled phosphate group using bacteriophage T_4 polynucleotide kinase. This enzyme normally transfers the γ-phosphate of ATP to the 5′-hydroxyl of DNA, RNA, or an oligonucleotide. All of the compounds in the library possess terminal hydroxyl groups, although it is unreasonable to expect that they would all be substrates for the enzyme. Work by Fontanel et al. has shown that the enzymatic ^{32}P labeling of oligomers containing nonnucleoside moieties varied widely, depending upon their structure and stereochemistry.[28] Polynucleotide kinase apparently requires the presence of a terminal nucleoside

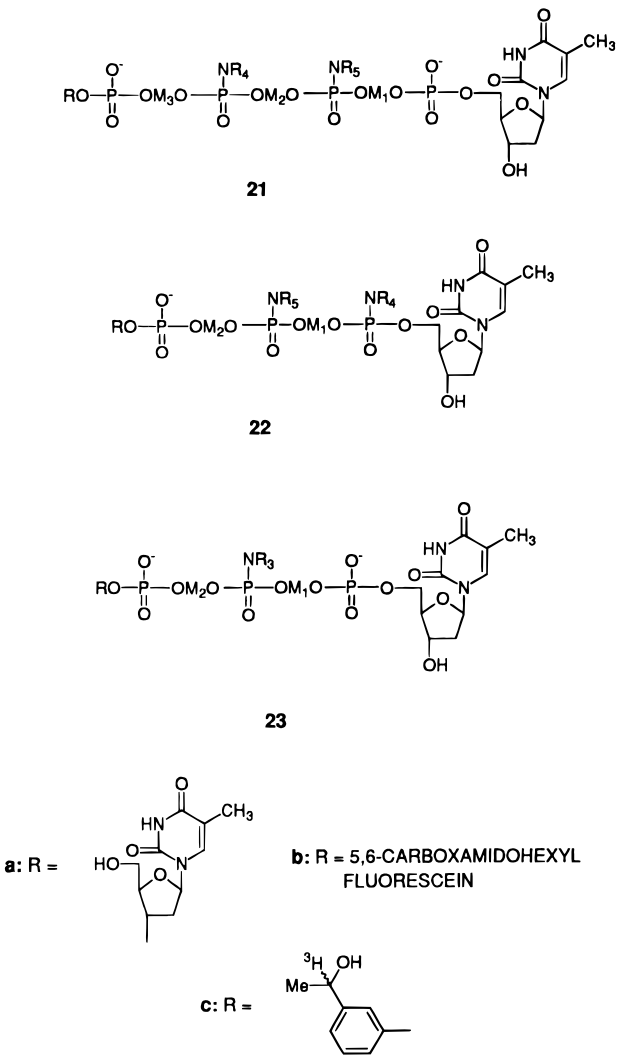

Figure 4. Structures of the libraries of phosphoramidates.

but is tolerant of modifications outside of this position; therefore, a thymidine residue was introduced as the last step in the solid-supported synthesis of the library. Labeling of the 5′-hydroxyl of the thymidine residue using γ-^{32}P-labeled ATP and bacteriophage T_4 polynucleotide kinase was then carried out to produce labeled libraries derived from **21a**−**23a** (Figure 4). Labeling of representative non-nucleotide phosphoramidate single compounds possessing terminal thymidylate residues also confirmed that these compounds were substrates for the enzyme, although labeling was less efficient as compared to oligonucleotides.

(b) Fluorescein Labeling. A second method for labeling employed fluorescein. This group was introduced by reaction of the library attached to the solid support with a commercially available fluorescein phosphoramidite reagent. After coupling, the library was cleaved from the support by reaction with ammonia and lyophilized to give the required libraries **21b**−**23b**, which were used directly.

(c) Tritium Labeling. A third method for labeling employed tritium. A recent report by Tan et al. described a method for the tritium labeling of oligonucleotides by oxidation of the terminal hydroxyls to aldehyde groups followed by reduction with tritium labeled sodium boro-

(28) Fontanel, M.-L.; Bazin, H.; Teoule, R. *Nucleic Acids Res.* **1994**, *22*, 2022−2027.

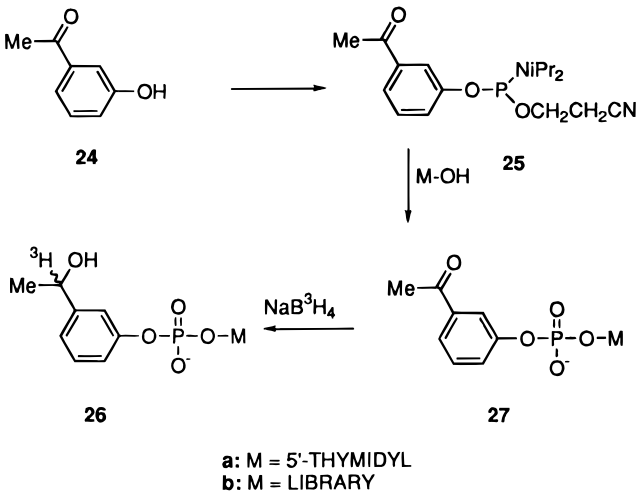

Figure 5. Introduction of tritium by reduction of the *m*-hydroxyacetophenone labeling group.

hydride.[29] We evaluated this method by carrying out some model oxidation/reduction experiments on dimers prepared by coupling of single non-nucleotide monomers to support-bound thymidine. These experiments indicated that the efficiency of the oxidation step varied widely depending upon the nature and environment of the terminal hydroxyl group; some groups oxidized cleanly to the aldehyde whereas others produced a number of byproducts. Due to this variability, an oxidation/reduction method was considered to be unsuitable for the labeling of libraries of this type.

A second approach to tritium labeling employed a "labeling monomer" which introduced a reactive aldehyde or ketone group at a late stage of synthesis, so that reduction with [^{3}H] borohydride would introduce the tritium label without the need for an oxidation step. An important consideration required that the labeling group should also be stable to the alkaline conditions used to cleave the material from the solid support. Simple glycol groups with flexible backbones were therefore ruled out since Fontanel et al. have shown that oligonucleotides terminated with glycol moieties undergo degradation in concentrated ammonia at 55 °C, presumably by attack of the free hydroxyl group on the adjacent phosphate followed by cyclization and elimination of the terminal unit.[28] For these reasons hydroxyacetophenone was selected for the labeling monomer approach, since after reduction, the possibility of attack of the hydroxyl group on the neighboring phosphorus moiety could be ruled out because of the rigidity of the benzene ring. A comparison of reductions of the ketone group of both *p*- and *m*-hydroxyacetophenone indicated that the *meta* isomer was more readily reduced using sodium borohydride. Conversion of *m*-hydroxyacetophenone (**24**, Figure 5) into its H-phosphonate and phosphoramidite derivatives were evaluated, and the phosphoramidite derivative **25**, prepared by a conventional procedure,[24] was more easily isolated in pure form. Trial experiments indicated that **25** could be readily coupled to support-bound thymidine and oxidized to produce the diester **27a**. The latter material was rigorously purified by HPLC and identified by NMR and MS.

The reduction of **27a** with sodium borohydride was investigated to determine the minimum amount of reagent needed for tritiation and to confirm the identity of the product. Reduction was studied both in solution and on the solid support. Complete reduction in solution could be obtained using 1 equiv of borohydride in ethanol, and evidence for the structure of the reduced product **26a** was obtained from electrospray MS, which gave a parent ion at *m*/*z* 441, versus 439 for the ketone **27a**. Upon reduction, ^{1}H NMR indicated the disappearance of signals assigned to the acetophenone methyl group at 2.5 ppm.

Since model studies confirmed that the labeling monomer approach would be useful for the labeling of libraries of this type, we prepared a library of phosphoramidates **(21c)**, based upon the conditions developed for the labeling of **26a**. The efficiency of labeling was somewhat lower than that for the model compound, which is to be expected for a complex mixture in which some components are potentially less reactive.

Table 1

structure	sites of diversity	theoretical number of compounds	average molecular weight
21a	5	1 362 944	1520
22a	4	518 400	1364
23a	3	28 800	1234

Types of Libraries. Several types of libraries were prepared, their differences being in (a) the labeling method, (b) the number of monomeric units which were coupled together, and (c) the number of compounds in the libraries. The largest library **21a** (Figure 4) was assembled using ten H-phosphonate monomers, one of which was a mixture of isomers, which were coupled to produce three sites of diversity together with an additional two sites derived from the introduction of sixteen amines to form phosphoramidates. Structures of all the amines are provided in the supporting information. The introduction of phosphoramidate moieties introduces new sites of chirality so that two isomers are produced at each phosphorus atom. The amines were chosen to introduce a wide range of functionalities, including hydrophilic and hydrophobic groups as well as hydrogen bond donors and acceptors. The regions of diversity produced by the monomers and amines were flanked by thymidine residues, one derived from the solid support and the other for the purpose of introducing a ^{32}P label. The library was theoretically comprised of over one million members, although there is no analytical method to confirm the actual number of compounds in the library. Similar libraries **21b** and **21c** were also prepared using the alternate labeling methods as described above. A second library of similar construction possessed four sites of diversity, two derived from monomers and two derived from amines, shown in structure **22**; this incorporated 15 monomers and 24 amines. The smallest library **23** was constructed by coupling 20 monomers to produce dimers in which one additional site of diversity was derived from 36 amines. A numerical comparison of these libraries is shown in Table 1.

Purification and Analysis. Although very few analytical methods are capable of providing useful information on complex mixtures such as those described above, ^{31}P NMR was used to confirm the presence of P−O and P−N linkages. For libraries of type **21**, two phosphoramidate linkages are formed by the introduction of amines, and these are flanked by two phosphodiester groups to which the thymidine residues are attached. Figure 7 shows the NMR spectrum of one of the sub-

(29) Tan, W. T.; Iyer, R. P.; Yu, D.; Agrawal, S. *Tetrahedron Lett.* **1995**, *36*, 3631−3634.

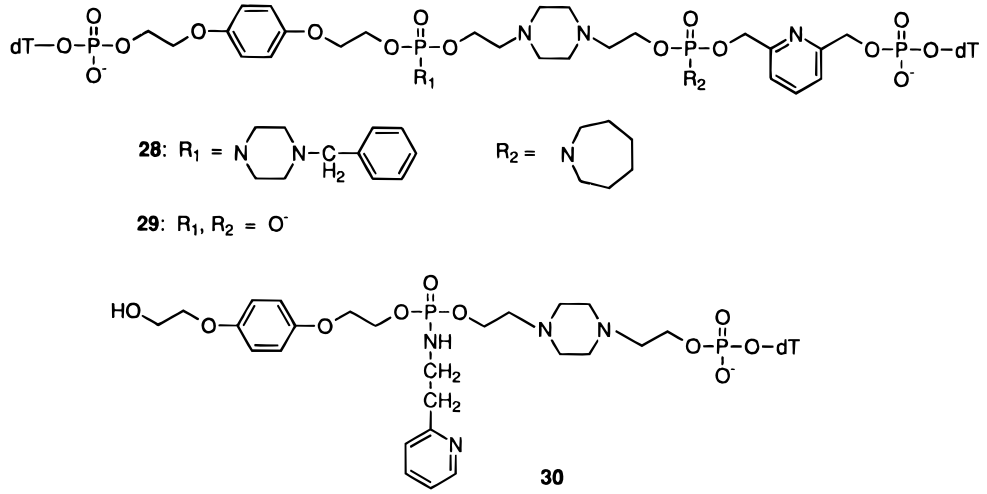

Figure 6. Representative compounds prepared for stability studies.

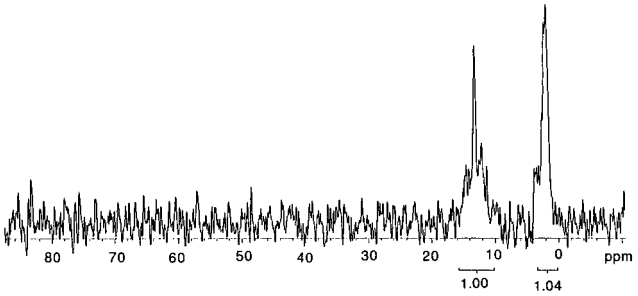

Figure 7. ^{31}P NMR of one of the sublibraries of **21**. P−N resonances are observed at 11−16 ppm and P−O resonances at 1−6 ppm.

libraries of **21**, which shows an envelope of signals in the region of 11−16 ppm corresponding to P−N linkages, together with a second envelope of signals at 1−6 ppm corresponding to P−O linkages. The absence of any other detectable resonances indicates that the phosphorus moieties exist primarily as phosphodiester and phosphoramidate linkages. HPLC analysis showed a multiplicity of peaks as expected for mixtures of this complexity.

Stability Studies. A few compounds were prepared to provide information on stability, since some phosphoramidates are known to be hydrolyzed in aqueous solution, particularly at low pH. Oligonucleotide phosphoramidates, for example, can be hydrolyzed to phosphodiesters under acidic conditions at elevated temperature[26] although compounds of this type have been shown to possess activity in biological systems.[30] Compound **28** (Figure 6), containing three monomer units and two phosphoramidate linkages, was prepared by the method outlined above and purified by HPLC. Since several piperazine derivatives were used for construction of the library, 1-benzylpiperazine was selected as a representative amine component, the other being hexamethyleneimine, representative of the other cyclic secondary amines. Two controls were also prepared and evaluated: (a) the corresponding phosphodiester **29**, and (b) an oligonucleotide pentathymidylate in which the two central phosphorus groups were present as phosphoramidates derived from the same amines as for **28**, with the flanking

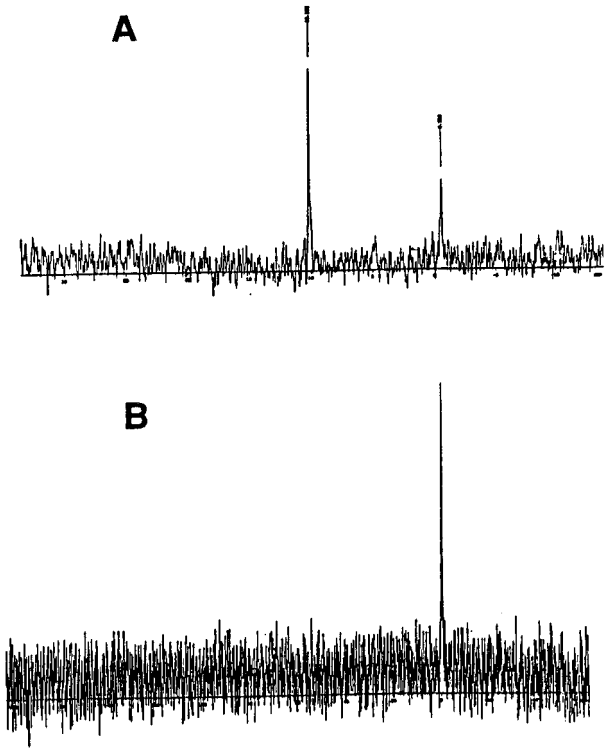

Figure 8. A study of the degradation of **30** using ^{31}P NMR. (a) **30**; (b) after storage for 24 h at 75 °C.

phosphorus groups being phosphodiesters. Each compound was incubated at pH 7 at 37 °C and at 75 °C, and aliquots were examined by HPLC to determine the extent of decomposition. At 75 °C **28** was degraded with a half-life of approximately 45 min while the corresponding phosphodiester **29** and the pentathymidylate were both unchanged under the same conditions. The instability of **28** appears to result from some kind of interaction between monomer groups and amines, since the individual components were shown to be stable, although this issue was not further explored. Both **28**, **29** and the thymidylate control were all found to be stable at 37 °C, so that stability problems are not likely to be encountered when these compounds are used for biological experiments.

^{31}P NMR was used to investigate the hydrolysis of compound **30**, possessing only one phosphoramidate linkage prepared from 2-(2-aminoethyl)pyridine. This

(30) Agrawal, S.; Goodchild, J.; Civeira, M. P.; Thornton, A. H.; Sarin, P. S.; Zamecnik, P. C. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 7079−7083.

was chosen to be representative of the phosphoramidates derived from substituted ethylamines, ten of which were used for library construction. Compound **30** was incubated at pH 7 at both 37 °C and 75 °C over 24 h, and aliquots were removed at intervals and injected onto a C_4-reversed phase HPLC column to determine the extent of decomposition. Hydrolysis was observed at 75 °C, as evidenced by the disappearance of starting material. Upon completion of hydrolysis, the aqueous solution was lyophilized, and the residue was examined by ^{31}P NMR. The spectrum of the starting material **30**, which is shown in Figure 8a, shows two resonances at 0 and 10 ppm corresponding to P−O and P−N bonds, respectively, whereas the degradation product (Figure 8b) shows only P−O bond resonances. This result confirms that breakdown occurs by cleavage of the P−N bond to produce diester linkages. As with **28** and **29**, a sample of **30** stored at 37 °C showed no decomposition.

Experimental Section

General. NMR spectra were obtained as previously described.[31] Mass spectra were obtained from Mass Consortium, San Diego, CA. Tritiated sodium borohydride was obtained from American Radiolabeled Chemicals, Inc., St. Louis, MO. (Dimethoxytrityl)thymidine H-phosphonate was obtained from Glen Research, Sterling, VA.

Dimethoxytrityl Derivatives. 1-*O*-(4,4′-Dimethoxytrityl)-2-hydroxyethanol **(1a)** was prepared by the method of Fontanel et al.[28] 5-*O*-(4,4′-Dimethoxytrityl)-1,2-dideoxy-D-ribose **(5a)** was prepared according to the method of Takeshita et al.[32]

1-*O*-(4,4′-Dimethoxytrityl)-(*S*)-(+)-1,2-propanediol (2a). Dimethoxytrityl (DMT) chloride (5.18 g, 15.3 mmol) was added in portions to a stirred solution of *(S)*-(+)-1,2-propanediol (1.64 g, 15.3 mmol) in anhydrous pyridine (150 mL) containing *p*-(dimethylamino)pyridine (DMAP, 187 mg), and the reaction was stirred at rt overnight. The mixture was evaporated to dryness, and the residue was absorbed onto silica gel and applied to a silica gel flash column. The column was eluted with ethyl acetate/hexane (2:8) containing 0.1% triethylamine, and the homogeneous fractions were combined and evaporated to give **2a** as a pale yellow oil (4.6 g, 79%). ^{1}H NMR δ 7.41−6.85 (13H, m), 4.66 (1H, d, J = 4.9 Hz), 3.78 (1H, m), 3.72 (6H, s), 2.91 (1H, dd, J = 8.8, 5.7 Hz), 2.66 (1H, dd, J = 8.8, 5.7 Hz), 1.05 (3H, d, J = 6.3 Hz).

1-*O*-(4,4′-Dimethoxytrityl)-(*R*)-3-butene-1,2-diol (3a). DMT chloride (8.6 g, 25 mmol) was added in portions to a stirred solution of *(R)*-3-butene-1,2-diol (2.0 g, 23 mmol) in anhydrous pyridine (300 mL) containing DMAP (280 mg). The reaction was left to a stir at rt under nitrogen overnight. The reaction mixture was evaporated, and the residue was adsorbed onto silica gel and applied to a silica gel flash column. The product was eluted with 20% ethyl acetate in hexane containing 0.1% triethylamine, and homogenous fractions were combined and evaporated to give **3a** as a yellow gum (4.4 g, 49%): ^{1}H NMR δ 7.42−6.82 (13H, m), 5.86 (1H, m), 5.23 (1H, m), 5.06 (1H, m), 5.03 (1H, m), 4.13 (1H, m), 3.72 (6H, s), 2.95 (1H, m), 2.79 (1H, m).

1-*O*-(4,4′-Dimethoxytrityl)-3-(diethylamino)-1,2-propanediol (4a). 3-(Diethylamino)-1,2-propanediol (5.0 g, 34 mmol) in dry pyridine (100 mL) was treated with DMT chloride (13.8 g, 41 mmol), triethylamine (4.8 g, 48 mmol), and DMAP (207 mg, 1.7 mmol) and stirred at room temperature for 4 h. The mixture was concentrated by rotary evaporation, dissolved in CH_2Cl_2 (200 mL) and poured into 5% aqueous sodium bicarbonate (250 mL). The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (2 × 200 mL). The combined organic layers were washed with saturated aqueous sodium chloride (250 mL) and dried over anhydrous sodium sulfate. After filtration, the filtrate was evaporated to dryness and coevaporated with toluene, MeOH, hexane, and CH_2Cl_2 to give crude product (20 g) which was purified by column chromatography on silica gel (300 g) using a gradient of MeOH (0−4%) in CH_2Cl_2. The fractions were monitored by TLC, and those containing pure material were combined and evaporated to dryness to give **4a** (14.9 g) as a brown oil. ^{1}H NMR δ (ppm) 7.42−6.85 (m, 13H), 4.47 (br s, 1H), 3.72 (s, 6H), 3.65 (br s, 1H), 2.95−2.88 (m, 2H), 2.40−2.39 (m, 4H), 2.35−2.28 (m, 2H), 0.84 (t, 6H, J = 6.9 Hz). TLC R_f 0.4 (9:1 CH_2Cl_2/MeOH).

1-*O*-(4,4′-Dimethoxytrityl)-3-(4-methoxyphenoxy)-1,2-propanediol (6a). 3-(4-Methoxyphenoxy)-1,2-propanediol (4 g, 20 mmol) in dry pyridine (50 mL) was treated with DMT chloride (8.2 g, 24 mmol), triethylamine (2.9 g, 28 mmol), and DMAP (120 mg, 1 mmol) and stirred at rt for 16 h. The mixture was poured into 5% aqueous sodium bicarbonate (250 mL) and extracted with CH_2Cl_2 (3 × 200 mL). The combined organic layers were washed with saturated aqueous sodium chloride (250 mL) and dried over anhydrous sodium sulfate. After filtration, the filtrate was evaporated to dryness and coevaporated with toluene followed by MeOH, hexane, and CH_2Cl_2 to give the crude product, which was purified by normal phase HPLC with a gradient of hexane (10−0%) in CH_2Cl_2. Fractions were monitored by TLC, and those containing pure material were combined and evaporated to dryness to give **6a** (9.7 g) as a pale yellow oil: ^{1}H NMR δ 7.39−6.80 (m, 13H), 5.09 (d, 1H, J = 4.7, OH), 3.96−3.87 (m, 3H), 3.72 (s, 6H), 3.68 (s, 3H), 3.06−3.03 (m, 2H). TLC R_f 0.16 (9:1 CH_2Cl_2/MeOH).

2-*O*-(4,4′-Dimethoxytrityl)-2,6-bis(hydroxymethyl)-pyridine (7a). 2,6-Bis(hydroxymethyl)pyridine (2.1 g, 15 mmol) in dry pyridine (50 mL) was treated with DMT chloride (1.0 g, 3 mmol), triethylamine (0.37 g, 3.6 mmol), and DMAP (20 mg, 0.15 mmol) and stirred at rt for 16 h. The mixture was isolated by extraction as described above for **6a** and purified by column chromatography on silica gel (300 g) with a gradient of MeOH (0−4%) in CH_2Cl_2 to give **7a** (3.8 g) as an oil: ^{1}H NMR δ (ppm) 7.87−6.90 (m, 16H), 5.29 (m, 1H), 4.44 (m, 2H), 4.06 (s, 2H), 3.73 (s, 6H). TLC R_f 0.68 (9:1 CH_2Cl_2/MeOH).

7-[1-(3-*O*-(4,4′-Dimethoxytrityl)-2,3-dihydroxypropyl)]-theophylline (8a). DMT chloride (22.7 g, 67 mmol) was added in portions to a stirred solution of 7-(2,3-dihydroxypropyl)theophylline (10 g, 61 mmol) in anhydrous pyridine (200 mL) containing DMAP (20 mg). The reaction was stirred at rt under nitrogen overnight. The reaction mixture was evaporated, and the residue adsorbed onto silica gel and applied to a silica gel flash column. The product was eluted using 3% MeOH in CH_2Cl_2, and homogenous fractions were combined and evaporated to give **8a** as an almost colorless oil (14.4 g, 51%): ^{1}H NMR δ 7.91 (1H, s), 7.50−6.80 (13H, m), 5.28 (1H, d), 4.47 (1H, d of d), 4.16 (1H, d of d), 4.04 (1H, m), 3.72 (6H, s), 3.40 (3H, s), 3.22 (3H, s), 3.00 (1H, m), 2.87 (1H, m). R_f = 0.29 (97:3 CH_2Cl_2/MeOH).

***O*-(4,4′-Dimethoxytrityl)-1,4-bis(2-hydroxyethyl)-piperazine (9a).** 1,4-Bis(2-hydroxyethyl)piperazine (10 g, 57.4 mmol) in dry pyridine (200 mL) was treated with DMT chloride (17.5 g, 51.6 mmol), and triethylamine (8.1 g, 80.3 mmol), and DMAP (330 mg, 2.9 mmol) and stirred at rt overnight. The mixture was concentrated and extracted as described for **6a**, and the crude product was purified by column chromatography on silica gel with a gradient of MeOH (0−5 %) in CH_2Cl_2. Homogeneous fractions were combined and evaporated to give **9a** (10.2 g, 37%) as a colorless gum: ^{1}H NMR δ 7.40−6.85 (13H, m), 4.33 (1H, m), 3.72 (6H, s), 3.46 (2H, m), 3.01 (2H, m), 2.49 (4H, m), 2.37 (8H, m).

***O*-(4,4′-Dimethoxytrityl)hydroquinone Bis(2-hydroxyethyl) Ether (10a).** Hydroquinone bis(2-hydroxyethyl) ether (10 g, 50.5 mmol) in dry pyridine (200 mL) was treated with DMT chloride (15.4 g, 45.4 mmol), triethylamine (7.2 g, 71 mmol), and DMAP (308 mg, 2.5 mmol) and stirred at rt overnight. Workup as for **6a** and purification by column chromatography on silica gel eluting using a gradient of MeOH (0−5 %) in CH_2Cl_2 gave **10a** (13.1 g, 52%) as a yellow gum: ^{1}H NMR δ 7.41−6.82 (17H, m), 4.81 (1H, m), 4.08 (2H, m), 3.90 (2H, m), 3.72 (6H, s), 3.68 (2H, m), 3.22 (2H, m).

(31) Fathi, R.; Huang, Q.; Coppola, G.; Delaney, W.; Teasdale, R.; Krieg, A. M.; Cook, A. F. *Nucleic Acids Res.* **1994**, *22*, 5416−5424.

(32) Takeshita, M.; Chang, C.-N.; Johnson, F.; Will, S.; Grollman, A. P. *J. Biol. Chem.* **1987**, *262*, 10171−10179.

3-*O*-(4,4′-Dimethoxytrityl)pyridoxine (11a). Pyridoxine (5.0 g, 29.6 mmol) in dry pyridine (100 mL) was treated with DMT chloride (12 g, 35.5 mmol), triethylamine (4.2 g, 41.4 mmol), and DMAP (181 mg, 1.5 mmol) and stirred at rt overnight. Workup as described for **6a** and purification by column chromatography on silica gel with a gradient of MeOH (0−3%) in CH_2Cl_2 gave **11a** (12.4 g, 89%) as a nearly colorless foam: ^{1}H NMR δ 8.66 (1H, s), 7.97 (1H, s), 7.41−6.87 (13H, m), 4.87 (1H, m), 4.26 (2H, s), 4.14 (2H, s), 3.73 (6H, s), 2.39 (3H, s).

3-*O*-(4,4′-Dimethoxytrityl)-(*R*)-1-phenyl-1,3-propanediol (12a). DMT chloride (4.3 g, 12.8 mmol) was added in portions to a stirred solution of 1-*R*-phenyl-1,3-propanediol (1.95 g, 12.8 mmol) in anhydrous pyridine (150 mL) containing DMAP (156 mg) and stirred at room temperature for 16 h. Additional DMT chloride (1.08 g, 3.2 mmol) was added in portions, and the mixture was stirred for an additional 16 h. The reaction mixture was then isolated as described for **8a** using hexane/ethyl acetate (8:2) containing 1% triethylamine as the solvent for elution of the column. **12a** was isolated as a yellow oil (3.97 g, 68%): ^{1}H NMR δ 7.36−6.82 (18H, m), 5.14 (1H, d, J = 4.9 Hz), 4.69 (1H, m), 3.72 (6H, s), 3.10 (1H, m), 2.90 (1H, m), 1.90 (1H, m), 1.78 (1H, m). TLC R_f 0.27 (2/8 ethyl acetate/hexane/1% triethylamine).

3-*O*-(4,4′-Dimethoxytrityl)-(1*S*,2*S*)-(+)-2-(trifluoroacetamido)-1-phenyl-1,3-propanediol (13a). Trifluoroacetic anhydride (10.6 mL, 65.7 mmol) was added slowly with care to a suspension of (1*S*,2*S*)-2-amino-1-phenyl-1,3-propanediol (5.0 g, 30 mmol) and dry pyridine (7.3 mL, 90 mmol) in dry CH_2Cl_2 (50 mL) with cooling in an ice/salt bath. The mixture was allowed to stir for 20 min during which all the solids dissolved. The ice/salt bath was removed and stirring continued for an additional 70 min. The solvents were evaporated, and the crude solid was coevaporated with toluene, MeOH, hexane, and CH_2Cl_2 and dried under vacuum. This material was dissolved in dry pyridine (250 mL), treated with DMT chloride (14.2 g, 41.9 mmol), triethylamine (8.49 g, 84 mmol), and DMAP (183 mg, 1.5 mmol), and stirred at room temperature for 5.5 h. The mixture was isolated by extraction as described above for **6a** and purified by column chromatography on silica gel (400 g) with CH_2Cl_2/0.1% triethylamine to give **13a** (8.49 g, 50% yield) as a pale yellow foam: ^{1}H NMR δ 9.45 (1H, d, J = 8.7 Hz), 7.35−6.80 (18H, m), 5.50 (1H, d, J = 5.0 Hz), 4.86 (1H, m), 4.10 (1H, m), 3.72 (3H, s), 3.71 (3H, s), 3.03 (1H, m), 2.73 (1H, m). TLC R_f 0.24 (99/1 CH_2Cl_2/MeOH).

7-*O*-(4,4′-Dimethoxytrityl)-(7*R*,8*R* and 7*S*,8*S*)-*trans*-7,8-dihydroxy-3,4-dicarbomethoxy-*exo*-tricyclo[4.2.1.0^{2,5}]non-3-ene (14a). Dimethyl *exo*-tricyclo[4.2.1.0^{2,5}]nona-3,7-diene-3,4-dicarboxylate (1.0 g, 4.3 mmol) was added dropwise to a mixture of 88% formic acid (2.6 mL, 59 mmol) and 30% hydrogen peroxide (0.6 mL, 6 mmol) that had been cooled on an ice bath. The ice bath was removed, and the mixture was stirred for 18 h. The mixture was evaporated to dryness and coevaporated with MeOH, toluene, and CH_2Cl_2. This residue was dissolved in methanol (10 mL), Amberlite IR-120 (H^+) resin (1 g) was added, and the mixture was refluxed for 2 h. The solid was filtered off, and the filtrate was evaporated to give crude *trans*-dimethyl 7,8-dihydroxy-*exo*-tricyclo[4.2.1.0^{2,5}]-non-3-ene-3,4-dicarboxylate (1.4 g) was a brown oil: ^{1}H NMR δ 5.06 (d, 1H, J = 5.6 Hz), 4.39 (d, 1H, J = 7.0 Hz), 3.93 (m, 1H), 3.714 (s, 3H), 3.710 (s, 3H), 3.65 (m, 1H), 3.02 (m, 1H), 2.92 (m, 1H), 2.37 (m, 1H), 2.21 (m, 1H), 1.65 (m, 2H).

This material (1.4 g, 4.3 mmol) in dry pyridine (20 mL) was treated with DMT chloride (1.3 g, 3.9 mmol), triethylamine (290 mg, 5.2 mmol), and DMAP (2 mg, 0.02 mmol) and stirred at room temperature for 4 h. The mixture was isolated by extraction as described above for **6a** and purified by column chromatography on silica gel (100 g) with a gradient of MeOH (0−1%) in CH_2Cl_2 to give **14a** (1.2 g) as a yellow foam: ^{1}H NMR δ 7.36−6.82 (m, 13H), 4.65 (d, 1H, J = 5.0 Hz), 3.76 (m, 1H), 3.724 (s, 3H), 3.721 (s, 3H), 3.63 (s, 3H), 3.47 (m, 1H), 3.38 (s, 3H), 2.90 (m, 1H), 2.77 (m, 1H), 2.09 (m, 1H), 2.02 (m, 1H), 1.44 (m, 1H), 0.82 (m, 1H). TLC R_f 0.28 (CH_2Cl_2).

11-*O*-(4,4′-Dimethoxytrityl)-(11*R*,12*R* and 11*S*,12*S*)-*trans*-9,10-ethano-11,12-anthracenedimethanol (15a). *trans*-9,10-Ethanoanthracene-11,12-dimethanol (2.0 g, 7.5 mmol) in dry pyridine (30 mL) was treated with DMT chloride (2.5 g, 7.4 mmol), triethylamine (0.9 g, 8.9 mmol), and DMAP (45 mg, 0.4 mmol) and stirred at room temperature for 1 h. The mixture was isolated by extraction as described above for **6a** and purified by column chromatography on silica gel (200 g) with a gradient of MeOH (0−3%) in CH_2Cl_2 to give **15a** (2.4 g) as foam: ^{1}H NMR δ (ppm) 7.36−6.82 (m, 21H), 4.59 (d, 1H, J = 5.3 Hz), 4.40 (d, 1H, J = 2.1 Hz), 4.26 (d, 1H, J = 2.0 Hz), 3.713 (s, 3H), 3.707 (s, 3H), 3.01 (m, 1H), 2.83 (m, 1H), 2.68 (m, 1H), 2.29 (m, 1H), 1.39 (m, 1H), 1.16 (m, 1H), 0.92 (m, 1H). TLC R_f 0.5 (99:1 CH_2Cl_2/MeOH).

H-Phosphonate Synthesis. The DMTr derivatives **1a**−**15a** were dissolved in dry CH_2Cl_2 and added over 10 min to a mixture of imidazole (15 equiv), phosphorus trichloride (4.3 equiv), triethylamine (29 equiv), and dry CH_2Cl_2 cooled in an ice bath. The reaction mixture was stirred for 30 min at 0 °C, the ice bath was removed and the mxiture stirred for an additional 30 min. Water was added, and the mixture was stirred for 10 min. The layers were separated, and the aqueous layer was extracted with chloroform. The combined organic layers were evaporated and coevaporated twice with toluene. The crude material was purified by column chromatography on silica gel using a gradient of MeOH (0−30%) in CH_2Cl_2. Fractions containing pure material were combined and evaporated to dryness, and the residue was dissolved in CH_2Cl_2 and washed with 0.1 M triethylammonium bicarbonate. The aqueous layer was backwashed with CH_2Cl_2, and the combined organic layers were evaporated to dryness to give the H-phosphonates **1b**−**15b**.

1b (yellow oil): ^{31}P NMR δ 2.0 (d, J_{P-H} = 580 Hz). TLC R_f 0.09 (95:5 CH_2Cl_2/MeOH/0.5% triethylamine).

2b: ^{31}P NMR δ 2.7 (dd, J_{P-H} = 581 Hz, $J_{P-O-C-H}$ = 9.9 Hz). TLC R_f = 0.04 (95/5 CH_2Cl_2/MeOH/0.5% triethylamine).

3b: ^{31}P NMR δ 8.17 (d, J_{P-H} = 703 Hz). TLC R_f 0.11 (8:2 CH_2Cl_2/MeOH/0.1% triethylamine).

4b: ^{31}P NMR δ 5.2 (dd, J_{P-H} = 601 Hz, $J_{P-O-C-H}$ = 10.7 Hz). TLC R_f 0.26 (9:1 CH_2Cl_2/MeOH).

5b: ^{31}P NMR δ 0.61 (dd, J_{P-H} = 578 Hz, $J_{P-O-C-H}$ = 9.1 Hz). TLC R_f = 0.37 (8:2 CH_2Cl_2/MeOH/0.1% triethylamine).

6b (colorless oil): ^{31}P NMR δ 1.5 (dd, J_{P-H} = 586 Hz, $J_{P-O-C-H}$ = 10.7 Hz). TLC R_f = 0.26 (9/1 CH_2Cl_2/MeOH).

7b (yellow oil): ^{31}P NMR δ 1.65 (dt, J_{P-H} = 584 Hz, $J_{P-O-C-H_2}$ = 9.1 Hz). TLC R_f 0.08 (9:1 CH_2Cl_2/MeOH/0.1% triethylamine).

8b (white foam): ^{31}P NMR δ 0.41 (dd, J_{P-H} = 588 Hz, J_{P-O-CH} = 11.1 Hz). TLC R_f 0.34 (94:6 CH_2Cl_2/MeOH/0.5% triethylamine).

9b: ^{31}P NMR δ 4.28 (dt, J_{P-H} = 596 Hz, J_{P-O-CH_2} = 13.8 Hz). TLC R_f 0.13 (3:7 ethylacetate/hexane/0.5% triethylamine).

10b: ^{31}P NMR δ 1.9 (d, J_{P-H} = 578 Hz). TLC R_f 0.09 (9:1 CH_2Cl_2/MeOH/0.1% triethylamine).

11b: (white foam) ^{31}P NMR δ 1.4 (dt, J_{P-H} = 575 Hz, J_{P-O-CH_2} = 7.7 Hz). TLC R_f 0.33 (8:2 CH_2Cl_2/MeOH/0.1% triethylamine).

12b: (yellow foam) ^{31}P NMR δ 2.0 (d, J_{P-H} = 582 Hz). TLC R_f 0.08 (94:6 CH_2Cl_2/MeOH/0.5% triethylamine).

13b: (white foam) ^{31}P NMR δ 3.86 (dd, J_{P-H} = 594 Hz, J_{P-O-CH_2} = 12.1 Hz). TLC R_f 0.11 (9:1 CH_2Cl_2/MeOH/0.1% triethylamine).

14b: (white foam) ^{31}P NMR δ −0.64 (dd, J_{P-H} = 586 Hz, J_{P-O-CH} = 6.8 Hz). TLC R_f 0.12 (9:1 CH_2Cl_2/MeOH/0.1% triethylamine).

15b: (white foam) ^{31}P NMR δ 1.8 (dd, J_{P-H} = 577 Hz, J_{P-O-CH} = 7.6 Hz). TLC R_f 0.09 (CH_2Cl_2/0.5% triethylamine).

Synthesis of Phosphoramidate Model Compounds. Conventional oligonucleotide H-phosphonate methodology[25] was used to prepare a support-bound thymidine dinucleotide H-phosphonate derivative, which was treated with a 10% solution of cis-2,6-dimethylmorpholine in CCl_4 for 10 min. The dimethoxytrityl group was removed, and the dinucleotide phosphoramidate was cleaved from the support with concentrated NH_4OH. Ammonia was removed under a stream of nitrogen, and the aqueous layer was lyophilized. The residue was analyzed on a C_4 analytical HPLC column with a gradient of 0−80% acetonitrile in 0.1 M TEAA. The desired product

16 (Figure 3) was eluted at 17.71 min (95% yield, as determined by HPLC peak areas). ^{31}P NMR δ 7.4. Electrospray MS *m/z* 644 (M^+), 642 (M^-), calcd for $C_{26}H_{38}O_{12}N_5P$ (M) 643.22.

The following phosphoramidates **17**−**20** were prepared by similar procedures, with substitution of the appropriate amine component in place of *cis*-2,6-dimethylmorpholine.

17: HPLC elution at 19.50 min (96% yield). ^{31}P NMR δ 9.9 (s), 9.7 (s); Electrospray MS *m/z* 710 (M^+), 708 (M^-), calcd for $C_{30}H_{40}O_{13}N_5P$ (M) 709.23.

18: Elution at 13.95 min (90% yield). ^{31}P NMR δ 9.9 (s), 10.1 (s); Electrospray MS *m/z* 643 (M^+), 641 (M^-), calcd for $C_{26}H_{39}O_{11}N_6P$ (M) 642.24.

19: Eluted at 21.19 min (92% yield). ^{31}P NMR δ 8.2 (s), 8.3 (s); Electrospray MS *m/z* 705 (M^+), 703 (M^-), calcd for $C_{31}H_{41}O_{11}N_6P$ (M) 704.25.

20: Elution at 18.26 min (90% yield). ^{31}P NMR δ 9.2 (s), 9.4 (s); Electrospray MS *m/z* 642 (M^+), 640 (M^-), calcd for $C_{25}H_{32}O_{11}N_5PS$ (M) 641.15.

Synthesis of the Phosphoramidate Library 21. Samples of the monomers **1b**−**3b**, **5b**, **7b**−**12b** were separately diluted in 1:1 anhydrous acetonitrile/pyridine to give a 0.12 M solution of each and loaded onto two automated DNA synthesizers. The library was synthesized on 10 columns, each loaded with 60 mg of controlled pore glass (37−70 μm) derivatized with 5′-(dimethoxytrityl)thymidine (44 μmol/g, CPG Inc.), using a modified H-phosphonate cycle with an extended coupling time of 5 min for each monomer. Each of the 10 monomers was coupled to a specific column, monomer one to column one, monomer two to column two, etc., up to monomer 10 to column 10. The efficiency of each coupling was checked by monitoring the intensity of the trityl cation released from each coupling step and was determined to be in excess of 90% for all coupling steps. After addition of the 10 monomers, the supports were removed from the columns, pooled, and divided using the isopycnic slurry method, with acetonitrile as the solvent. An automated liquid handling system was used to redistribute the solid support, and the synthesis columns were weighed to ensure an even division of the resin among the columns. After a second addition of all 10 monomers followed by a second pool-and-divide procedure, the non-nucleotide H-phosphonate linkages were converted to phosphoramidates by reaction with a 10% solution of amine in carbon tetrachloride for 10 min. The following amines were used: 2-(2-aminoethyl)pyridine, 1-(2-aminoethyl)piperidine, 2-(3,4-dimethoxyphenyl)ethylamine, 4-(2-aminoethyl)morpholine, 1-(3-aminopropyl)-2-pyrrolidinone, 2-(3-chlorophenyl)ethylamine, *N*-isopropyl-1-piperazineacetamide, *N*-(3-trifluoromethyl)phenylpiperazine, 3,3-diphenylpropylamine, thiomorpholine, 1-(2-pyridyl)piperazine, hexamethyleneimine, *cis*-2,6-dimethylmorpholine, 1-(4-fluorophenyl)piperazine, 4-fluorophenylethylamine, 3,5-dimethylpiperidine. The amine solutions were flushed from the columns, and the supports were washed extensively with acetonitrile. The supports were removed from the columns, pooled, and divided as described above. A second round of monomer addition followed by pooling, dividing, and amine addition was carried out, followed by addition of the synthon required for introduction of the label (either fluorescein phosphoramidite, thymidine phosphoramidite, or *m*-hydroxyacetophenone phosphoramidite as described above). Labeling was carried out either on the entire library after a final pooling of the supports or on individual sublibraries obtained after addition of the final diversity element to each column. The supports were removed from the columns and treated with concentrated ammonium hydroxide for 5.5 h at 55 °C. The resultant suspensions were filtered and the ammonia removed from the filtrates by bubbling nitrogen through the solutions for 20 min. The sixteen solutions were lyophilized, redissolved in water, and purified by reversed-phase HPLC using a gradient of 5−100% acetonitrile in triethylammonium acetate (TEAA) 0.1 M, pH 7. Portions of the products from these purifications were combined and the mixture was lyophilized to give the phosphoramidate library **21** (Figure 4) or kept as a complete set of sublibraries.

Synthesis of the Phosphoramidate Library 22. The procedure as described for library **21** was followed, except that additional monomers **4b**, **14b**, and **15b** were employed, and the set of amines as used for **21** was expanded to include 1-(3-aminopropyl)imidazole, 3-aminopropiononitrile, 4-benzylpiperidine, furfurylamine, thiophene-2-methanamine, 1-piperonylpiperazine, 3,4-dichlorobenzylamine, and 1-[(pyrrolidinocarbonyl)methyl]piperazine. The individual supports were combined prior to attachment of the labeling group.

Synthesis of Library 23. The procedure as described for library **22** was followed, except that monomers **1b**−**15b** were employed, and the set of amines as used for **22** was expanded to include 1-(2-aminoethyl)pyrrolidine, 1-(3-aminopropyl)-4-methylpiperazine, 1-(4-chlorobenzhydryl)piperazine, 1-benzylpiperazine, *N*-(2,5-dimethylphenyl)piperazine, 2-(1-piperazinyl)pyrimidine, 2-phenoxyethylamine, 3-methylpiperidine, 4-(2-keto-1-benzylimidazolinyl)piperazine, cyclopropanemethanamine, 1-[(morpholinocarbonyl)methyl]piperazine, and 2-methyl-1-(3-methylphenyl)piperazine.

Labeling Methods. (a) ^{32}P. This label was introduced onto the terminal thymidine residue of the compound, library, or sublibrary using γ-^{32}P-labeled adenosine-5′-triphosphoate as the donor source and bacteriophage T_4 polynucleotide kinase as the enzyme. Conditions for labeling approximated those for labeling of an oligonucleotide,[33] and purification was carried out by preparative polyacrylamide gel electrophoresis.

(b) Fluorescein. After introduction of the final phosphoramidate substituents to the library on the solid support, the library or sublibrary was subjected to a phosphoramidite cycle on a DNA synthesizer with a coupling time of 2 × 5 min using fluorescein phosphoramidite (FluoreDite, PerSeptive Biosystems, Framingham, MA) as the reactant. Standard conditions with concentrated ammonia were employed for cleavage, followed by removal of ammonia under a stream of nitrogen and lyophilization to give the fluoresceinated library, which was used directly for biological studies.

(c) Tritium Labeling: 3-Acetylphenyl 2-Cyanoethyl *N*,*N*-Diisopropyl Phosphoramidite (25). To a stirred solution of *m*-hydroxyacetophenone (**24**, Figure 5, 3.0 g, 22 mmol) in anhydrous CH_2Cl_2 (120 mL) and *N*,*N*-diisopropylethylamine (11.4 g, 88.1 mmol) was added (2-cyanoethoxy)-*N*,*N*-(diisopropylamino)chlorophosphine (7.3 g, 30.9 mmol) dropwise over 8 min. The reaction mixture was stirred for 2 h at room temp and evaporated to dryness. The crude oil was partitioned between ethyl acetate (150 mL) and 5% aqueous sodium bicarbonate (150 mL), and the aqueous layer was extracted twice with ethyl acetate (100 mL). The combined organic layers were washed with saturated aqueous sodium chloride solution, dried over anhydrous sodium sulfate, and filtered. The solvents were removed by rotary evaporation, and the crude yellow oil was adsorbed onto silica gel and applied to a silica gel flash column (16 × 10 cm) which was eluted from the column with hexane/ethyl acetate (5:1). Fractions containing pure material were combined and the solvents evaporated to give **25** as a clear liquid (4.8 g, 64%). ^{1}H NMR 7.64 (1H, m), 7.52 (1H, m), 7.45 (1H, m), 7.28 (1H, m), 3.87 (2H, m), 3.71 (2H, m), 2.83 (2H, t, J = 5.7 Hz), 2.55 (3H, s), 1.19 (6H, d, J = 6.7 Hz), 1.11 (6H, d, J = 6.7 Hz). ^{31}P NMR δ 147 (m). R_f = 0.19 (hexane/ethyl acetate 5:1).

Thymidine-5′-(*m*-acetylphenyl phosphate) (27a). This compound was prepared on a DNA synthesizer using four 10 μmol cartridges with a modified 10 μmol phosphoramidite coupling cycle and 2 × 15 min coupling times. A solution of **25** (0.2 M) in anhydrous acetonitrile was used as the coupling reagent. A portion of the solid support (25 μmol) was treated with concd NH_4OH, and the solution was evaporated to give crude **27a** (559 OD_{260}). This material was purified in four portions using a C_4 reversed-phase HPLC column with a gradient of 5−35% acetonitrile in 0.1 M TEAA pH 7 over 6 min and then to 45% acetonitrile over 20 min. Pure fractions were combined and evaporated to give **27a** (424 OD_{260}) as a white powder. ^{1}H NMR 7.72 (1H, m), 7.64 (1H, m), 7.44 (1H, m), 7.40 (1H, m), 7.38 (1H, m), 6.33 (1H, m), 4.57 (1H, m), 4.32 (1H, m), 4.16 (2H, m), 3.18 (6H, q), 2.56 (3H, s), 2.33 (2H,

(33) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning, a Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989; p 5.68.

m), 1.47 (3H, s), 1.26 (9H, t). ^{31}P NMR δ 147 Electrospray MS: M^- 439 (theory 439).

Borohydride Reduction of 27a. Sodium borohydride (3.8 mg, 100 μmol) in 0.02 N aqueous sodium hydroxide solution (500 μL) was added to a solution of **27a** (424 OD_{260}) in water (500 μL), and the mixture was vortexed for 1 h. The reaction mixture was loaded onto a Sep-Pak C_{18} plus cartridge (Waters Corporation, Milford, MA) previously rinsed with acetonitrile (10 mL) and water (10 mL). The cartridge was washed with water (3 × 10 mL) and then eluted with MeOH/H_2O (6:4, 4 × 1 mL), and the aqueous methanolic fractions were evaporated to give unlabeled **26a**. ^{1}H NMR: 7.58 (1H, s), 7.29 (1H, m), 7.14 (1H, m), 7.11 (1H, s), 7.06 (1H, m), 6.32 (1H, m), 4.80 (1H, q, J = 6.7 Hz), 4.51 (1H, m), 4.24−4.21 (1H, m), 4.18−4.13 (2H, m), 3.17 (6H, q), 2.32−2.27 (2H, m), 1.67 (3H, s), 1.385 and 1.380 (3H, 2 × d, J = 6.7 Hz), 1.25 (9H, t). Electrospray MS: M^- 441 (theory 441).

[^{3}H] Sodium Borohydride Reduction of 27a. To 0.5 μmol of **27a** as a lyophilized foam in a screw-cap glass vial was added [^{3}H] sodium borohydride (20 mCi, 0.31 μmol, specific activity 65 Ci/mmol) in 0.01 N aqueous sodium hydroxide solution (200 μL), and the mixture was allowed to react for 2 h. Unlabeled sodium borohydride (0.024 mg, 0.63 μmol) in 0.01 N aqueous sodium hydroxide solution (200 μL of stock solution 2.4 mg/20 mL) was added, and the mixture was allowed to react an additional 2 h. The reaction mixture was loaded onto a Sep-Pak C_{18} plus cartridge previously primed with acetonitrile (10 mL) and water (10 mL). The cartridge was washed with water (5 × 10 mL) and then eluted with MeOH/water (6:4, 4 × 1 mL) followed by MeOH (1 mL). The methanolic fractions were evaporated to give the tritium labeled compound **26a** (20 OD_{260}, 73 nmol, 86 μCi, 4 % radiochemical yield). Specific activity: 1.18 mCi/μmol.

Tritium Labeling of Library 21. Library **21** was prepared as described above, except that the individual sublibraries were not pooled after addition of the final amine. One of these sublibraries, possessing hexamethylenimine as the R_4 diversity element, was selected for tritium labeling, and the acetylphenyl labeling group was introduced by reaction with **25**. The conditions were as described for the preparation of the model compound **27a**, except that 0.56 μmol of support (based on the original loading) was used, and six couplings were employed to ensure complete reaction. After cleavage from the support, the product was lyophilized and treated with [^{3}H] sodium borohydride (20 mCi, 0.26 μmol, specific activity 78 Ci/mmol) in 0.01 N aqueous sodium hydroxide solution (200 μL) for 2 h at room temp. Unlabeled sodium borohydride (0.024 mg, 0.65 μmol) in 0.01 N aqueous sodium hydroxide solution (200 μL of a stock solution containig 2.4 mg/20 mL) was added, and the mixture was allowed to react an additional 2 h to reduce any residual ketone groups. The reaction mixture was loaded onto a Sep-Pak C_{18} plus cartridge previously primed with acetonitrile (10 mL) and water (10 mL). The cartridge was washed with water (5 × 10 mL) and then eluted with MeOH/water (6:4, 4 × 1 mL), followed by MeOH (1 mL). The methanolic fractions were evaporated to give the tritium labeled sublibrary **21c**, where R_4 = hexamethylenimine, 0.38 mCi, 2.3% radiochemical yield, specific activity 0.68 mCi/μmol.

Synthesis and Stability Studies on 28−30. Compounds **28** and **29** were prepared on an automated DNA synthesizer using thymidine-CPG as the support. A 1 μmol H-phosphonate cycle was used with a coupling time of 5 min for non-nucleotide H-phosphonates which were dissolved in acetonitrile/pyridine (1:1, 0.12 M). 5′-(Dimethoxytrityl)thymidine H-phosphonate was used for the synthesis of the dT_5 control sequence and for introduction of the thymidine residues at the termini of **28** and **29**. Solutions of 1-benzylpiperazine and hexamethylenimine (10% in CCl_4 for 14 min) were used for oxidation of the H-phosphonate moieties. After synthesis and ammonia cleavage, the materials were dried in vacuo and the residues were dissolved in water (250 μL each), filtered through a 0.45 μm filter, and loaded onto a C_4 HPLC reversed phase column. The column was eluted with 0.1 M TEAA/acetonitrile using a gradient of 0−80% acetonitrile in 30 min with a flow rate of 2 mL/min. Compound **28** was eluted at 16.3 min and **29** at 13.5 min. The fractions containing pure material were combined and evaporated to dryness for hydrolysis studies.

An amount of 3.5 OD_{260} units of **28** and **29** were separately dissolved in 500 μL of TEAA (0.1 M, pH 7.0) and placed in a heating block at 75 °C. Aliquots were removed at intervals and analyzed by HPLC using the same system. The rate of degradation was determined by measurement of the area remaining under the peak corresponding to starting material.

Compound **30** was synthesized on a 2.75 μmol scale and purified as above. Fractions eluting at 13.6 min were combined and dried to yield 16.4 OD_{260} units of pure material. An amount of 5 OD units were dissolved in TEAA buffer (pH 7.0, 833 μL, 0.1 M) and placed in a heating block at 75 °C. HPLC analysis after approximately 42 h indicated that the sample was almost completely converted to a new compound eluting at 11.4 min. The solution was lyophilized and repurified on a C_4 column as described above to remove a small amount of starting material. Pure fractions were combined and lyophilized for ^{31}P NMR studies.

Conclusions

The use of non-nucleotide phosphoramidates represents an interesting method for the preparation of large, diverse libraries of relatively small molecular weight. Each phosphorus moiety provides three sites for introduction of diversity and large numbers of functionalized and unfunctionalized diols and amines are available as synthons. Coupling of the monomer units using H-phosphonate chemistry was relatively efficient regardless of the monomer employed, and the procedure takes advantage of the availability of automated DNA synthesizers for the formation of phosphodiester linkages. Libraries of over one million compounds can be generated using two phosphorus moieties and five sites of diversity, and many variations on this theme are possible, depending upon the number of phosphorus moieties employed. The use of these libraries to generate lead compounds which bind to biological targets of therapeutic interest using COMPILE technology is in progress, and the results of those studies will be reported elsewhere.

Acknowledgment. We thank Román F. Macaya for help with NMR spectra and Kenneth J. Nawoschik for technical assistance.

Supporting Information Available: NMR spectra of all compounds prepared, mass spectra of **16**−**20**, **26a**, and **27a**, HPLC of **2a**−**4a**, **6a**−**15a**, and a diagram of the structures of the amines employed in the construction of libraries (79 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

JO960192+