28482-69-1; **15e**, 625-51-4; **16** (isomer 1), 96228-40-9; **16** (isomer 2), 96228-41-0; **18**, 96228-42-1; **19**, 96245-28-2; **20** (isomer 1), 88407-15-2; **20** (isomer 2), 88407-16-3; **21** (isomer 1), 96228-43-2; **21** (isomer 2), 96228-44-3; **22**, 151-18-8; (*R*)-**23**, 96228-45-4; 1-mercapto-2-butanol, 96228-46-5; 1-[(benzamidomethyl)thio]-2-butanol, 96228-47-6; *tert*-butyl acetoacetate, 1694-31-1; propenenitrile, 107-13-1; bis-(2-cyanoethyl)amine, 111-94-4; EC 1.1.1.34, 9028-35-7.

Conceptual Basis of the Selective Activation of Bis(dialkylamino)methoxyphosphines by Weak Acids and Its Application toward the Preparation of Deoxynucleoside Phosphoramidites in Situ

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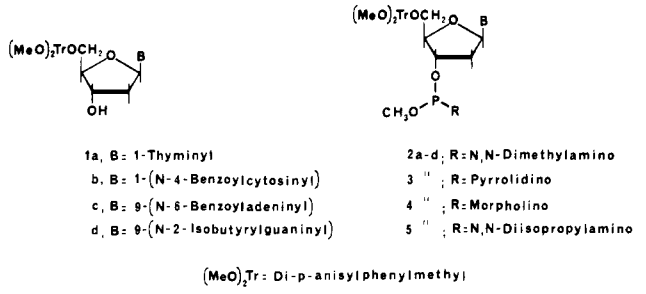
The concept behind the selective activation of bis(dialkylamino)methoxyphosphines is experimentally examined and subsequently used in the preparation of deoxynucleoside phosphoramidites in situ. These intermediates are most efficiently prepared from the reaction of suitably protected deoxynucleosides with dipyrrolidinomethoxyphosphine in the presence of 4,5-dichloroimidazole using 1-methyl-2-pyrrolidinone as solvent. The purity and efficiency of these monomers are evaluated during the solid-phase synthesis of a 22-unit oligomer.

A few years ago, Letsinger and co-workers described the use of a highly reactive 2,2,2-trichloroethyl phosphorochloridite[1,2] as a means of rapidly generating an internucleotidic link. The efficiency of the "phosphite triester" approach was quickly recognized[3-9] and readily adapted to solid-phase oligonucleotide synthesis.[10-19]

Improvements to the methodology were accomplished with the development of the deoxynucleoside phosphoramidites **2a–d** as a new class of monomer units for synthesis.[20] The latter displayed excellent stability toward hydrolysis and produced consistent results during manual solid-phase DNA synthesis.[21-23] However, with the advent of fully automated DNA synthesis, the stability of **2a–d** with prolonged standing in acetonitrile solutions has been questioned. Caruthers[24] and others[25] remedied this problem by using the stable deoxynucleoside phosphoramidite derivatives such as **4a–d** and **5a–d** which can be purified by silica gel chromatography.



1a, B = 1-Thyminyl
b, B = 1-(N-4-Benzoylcytosinyl)
c, B = 9-(N-6-Benzoyladeninyl)
d, B = 9-(N-2-Isobutyrylguaninyl)

2a-d; R = N,N-Dimethylamino
3 " ; R = Pyrrolidino
4 " ; R = Morpholino
5 " ; R = N,N-Diisopropylamino

$(MeO)_2Tr$: Di-p-anisylphenylmethyl

Considering the effort required for large-scale preparation and purification of these key intermediates, a simplification of the procedure was desirable. We briefly reported[26] that the selective activation of dipyrrolidinomethoxyphosphine by 4,5-dichloroimidazole represented a facile and economical approach to the preparation of deoxynucleosides phosphoramidites in situ. Because of our interest in the *stepwise* automated preparation of the latter and their immediate use in a fully automated system for solid-phase DNA synthesis, our investigations were concerned with the highest selectivity of formation of these intermediates within the shortest period of time (less than 10 min) required for such an application.[27]

In this report we wish to describe the conceptual basis involved in the selective activation of bis(dialkylamino)-methoxyphosphines by weak acids. We will also report a detailed study of the reaction conditions (solvent, activator,

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(27) Upon completion of this manuscript, Caruthers and co-workers (Caruthers, M. H.; et al. *Nucleic Acids Res.* **1984**, *12*, 4051) reported a catalytic activation of bis(dialkylamino)phosphines which yielded deoxynucleoside phosphoramidites with high selectivity although some 20 min were required for the formation of these intermediates.

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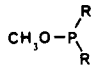
phosphitylating agent, etc.) leading to the optimal preparation of deoxynucleosides phosphoramidites in situ with enhanced reaction kinetics and evaluate their effectiveness in solid-phase DNA synthesis.

Results and Discussion

The basicity and/or nucleophilicity of aminophosphines is highly dependent on the substituents bound to the phosphorus atom and their respective interactions with the vacant d orbital of the phosphorus atom through $p\pi-d\pi$ overlap.[28] Burgada and co-workers elegantly demonstrated this trend by studying chemical exchange between methyl trifluoroacetate and various aminophosphines.[29-31] It was then recognized that a bis(dialkylamino)methoxyphosphine would be more susceptible to protonation than the analogous deoxynucleoside (dialkylamino)methoxyphosphine since it has been demonstrated that an alkoxy group contributes to a lesser extent to $p\pi-d\pi$ interactions than a dialkylamino group.[31] Additionally, it was rationalized that the protonation of a bis(dialkylamino)methoxyphosphine with a weak acid should lower the basicity of the remaining dialkylamino group and inhibit its subsequent protonation. The strong inductive effect generated by the positive charge of the protonated nitrogen atom would act by lowering the energy of the vacant phosphorus d orbital thereby favoring conjugative resonance with the p electrons of the remaining nitrogen and oxygen atom of the dialkylamino and methoxy groups, respectively.[31] The overall effect of this interaction would be a considerable reduction in the basicity and nucleophilicity of the remaining dialkylamino functionality. Consequently, it became clear that a specific class of bifunctional phosphitylating agents such as the bis(dialkylamino)methoxyphosphines could behave like monofunctional phosphitylating agents under appropriate conditions.

Preparation of Bis(dialkylamino)methoxyphosphines. The phosphorodiamidites **6e–h** were chosen



6e, R = N,N-Dimethylamino
f, R = Pyrrolidino
g, R = Morpholino
h, R = N,N-Diisopropylamino

to evaluate the rationale described above since the nature of their respective substituents displays discrete differences in basicity and/or steric hindrance required for such an evaluation. Thus, **6e,f** were prepared in good yields from methoxydichlorophosphine and the corresponding (dialkylamino)trimethylsilane by using a ratio of 1:2.2, respectively. Alternatively, **6g,h** have been prepared by reacting methoxydichlorophosphine with 4.4 molar equiv of the appropriate amine in anhydrous ether. (see Experimental Section.) However, the purification of **6g** by distillation has in our hands been unsuccessful. Since the isolation of pure **6g** is problematic, its use in this study has been withdrawn.

Selective Activation of the Various Phosphorodiamidites and Identification of the Activated Species. Because the activator must selectively protonate **6e,f,h** and ideally should not protonate the deoxynucleoside phosphoramidites subsequently generated in situ, the choice of the specific weak acid necessary for the fulfillment of these requirements is complicated. Previous experience with 1*H*-tetrazole ($pK_a(H_2O) = 4.9$)[32] as an activator for **2a–d** used in solid-phase DNA synthesis[20-23] dictated the use of weaker acids to comply with the above requirements. Thus, representative experiments involving the reaction of **6e,f,h** each with 4 molar equiv of 1,2,4-triazole, 4,5-dichloroimidazole, and 3-chlorotriazole, respectively,[33] using dry THF as solvent were examined by ^{31}P NMR (Figure 1). With 1,2,4-triazole as activator, results were virtually identical regardless of the phosphitylating agent used (spectra A, B, and C). ^{31}P NMR displayed two peaks, the major one representing the unreacted starting material. A minor peak was invariably present at a few parts per million upfield of the peak identified as the starting phosphitylating agent. When 4,5-dichloroimidazole interacted with **6e,f** (spectra D and E), ^{31}P NMR showed that the peak representing the major reaction product appeared a few parts per million upfield of the peak corresponding to the starting material. Additionally, the activation of **6h** with 4,5-dichloroimidazole in dry THF afforded a new species having a ^{31}P NMR chemical shift at 128.3 ppm (spectrum F). The presence of starting material originally observed at 130.6 ppm was totally absent after a reaction time of 10 min at ambient temperature. The protonation of **6f** with 4,5-dichloroimidazole displayed a more complex ^{31}P NMR spectrum. In addition to the small peak identified as **6f** at 133.2 ppm and the major reaction product located at 127.3 ppm, two peaks at 106.9 and 144.0 ppm were also observed (spectrum E). Using 3-chlorotriazole as activator under the above conditions generated results very similar to those obtained with 4,5-dichloroimidazole as protonating agent (spectra G–I). Two additional experiments were carried out involving **6e** with excess 4,5-dichloroimidazole dissolved in dry 1-methyl-2-pyrrolidinone (spectrum J) and dry $CHCl_3$ (spectrum K).

From the above experiments, one can conclude that the protonation kinetics of phosphitylating agents, such as **6e,f,h**, depend upon the acidity of the activator used, which in turn is modulated by the solvent in which the reaction is carried out.

In order to identify the unknown reaction products reported above, a detailed investigation of the reaction of **6e** with 4,5-dichloroimidazole (4 molar equiv) in dry 1-methyl-2-pyrrolidinone (NMP) was monitored by ^{31}P NMR and tentatively illustrated in Scheme I. After 10 min at ambient temperature, two major species were observed at 137.2 and 130.9 ppm respectively in a ratio of 1:0.72. When the starting material that was dissolved in NMP was analyzed by ^{31}P NMR, only one peak was observed at 137.2 ppm. The reaction product represented by the peak at 130.9 ppm has been identified as the 4,5-dichloroimidazolide **9e**. The latter has been prepared by reacting chloro(dimethylamino)methoxyphosphine with a two fold excess of 4,5-dichloroimidazole in the presence of diisopropylethylamine (DIPEA) in dry THF. The ^{31}P NMR chemical shift of the resulting product was identical with that of the product obtained by protonation of **6e** by **7** in a variety of solvents. Attempts to observe the pro-

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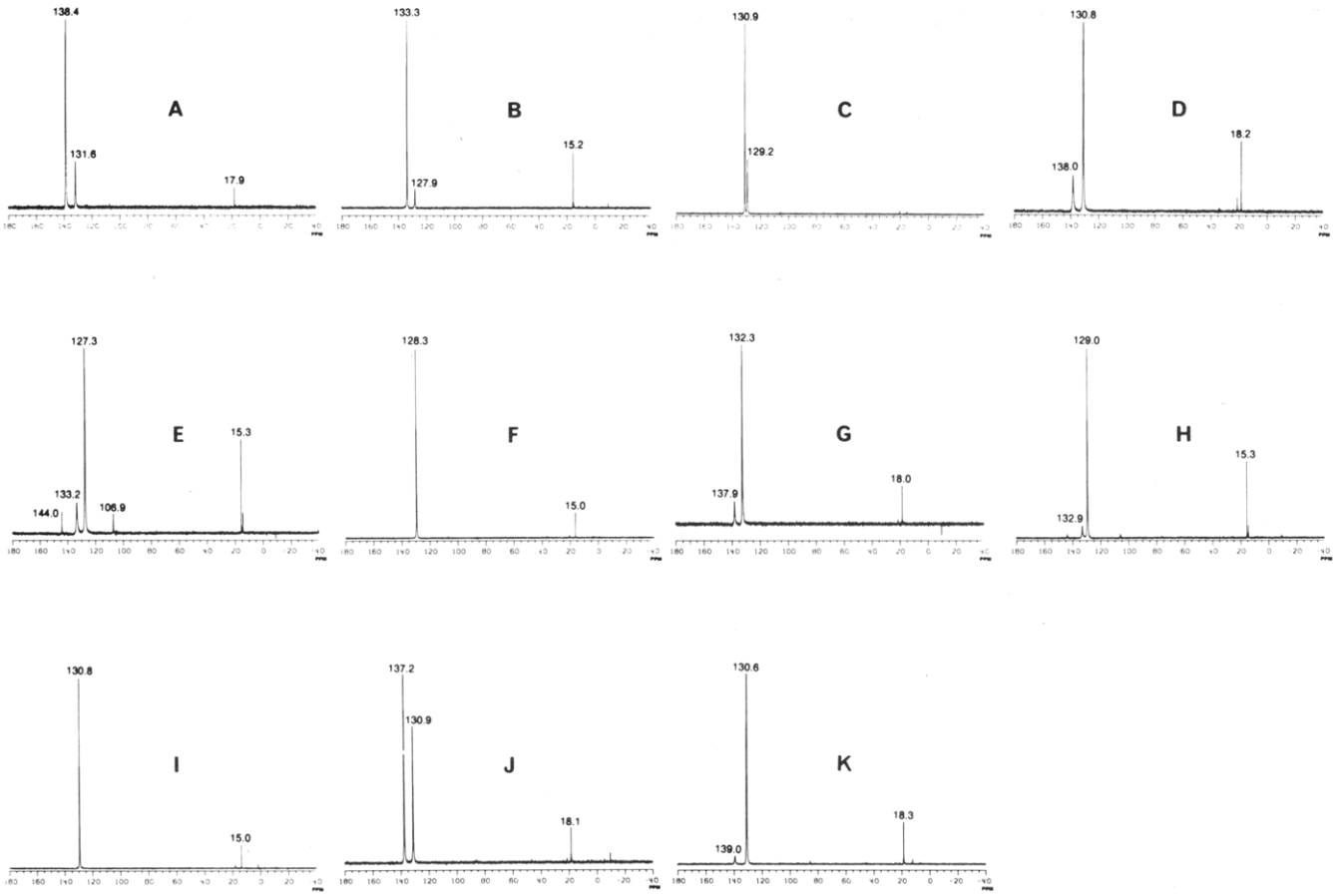
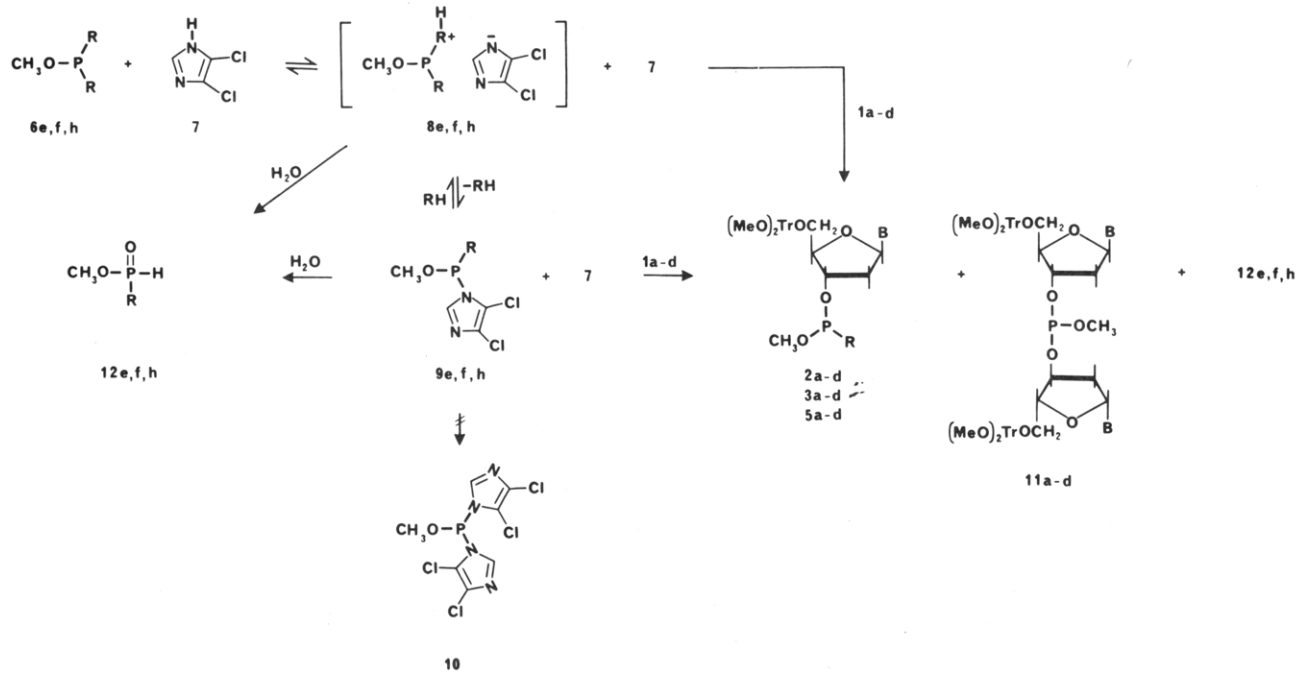


Figure 1. ^{31}P NMR spectra obtained from the selective activation of various phosphitylating agents with weak acids. (A, B, C) Activation of **6e**, **6f**, and **6h**, respectively, with 1,2,4-triazole in dry THF. (D, E, F) Activation of **6e**, **6f**, and **6h** respectively, with 4,5-dichloroimidazole in dry THF. (G, H, I) Activation of **6e**, **6f**, and **6h**, respectively, with 3-chlorotriazole in dry THF. (J, K) Activation of **6e** with 4,5-dichloroimidazole in dry 1-methyl-2-pyrrolidinone and dry chloroform, respectively.

Scheme I. Generation of Deoxynucleoside Phosphoramidites in Situ via Selective Activation of Bis(dialkylamino)methoxyphosphines by 4,5-Dichloroimidazole



tonated species **8e** (shown in Scheme I) by ^{31}P NMR failed even at low temperature (−50 °C), suggesting that **8e** might quickly equilibrate between **6e** and **9e** under these conditions. Even in the presence of excess 4,5-dichloroimidazole, the ratio observed between **6e** and **9e** at ambient temperature did not change even after 1 h. This observation supports the establishment of a rapid equilibrium between the chemical species **6e**, **8e**, and **9e**, which might explain the broader shape of the ^{31}P NMR peaks corresponding to **6e** and **9e** relative to the parent species when

analyzed in the absence of activator. Interestingly, the reaction of **6h** with **7** produced only the corresponding 4,5-dichloroimidazolide **9h** regardless of the solvent used. This behavior could possibly result from the poor nucleophilicity of the diisopropylamine released during the production of **9h**. The more hindered amine would react more readily with the excess 4,5-dichloroimidazole instead of attacking the bulky phosphorus moiety **9h** and generate the chemical equilibrium observed with the less sterically hindered phosphitylating agents.

As shown in Figure 1 (spectrum E), the presence of two additional peaks of unknown identity from the ^{31}P NMR analysis of the reaction of **6f** with **7** in THF suggested that the bis(4,5-dichloroimidazolide) **10** might have been formed. An independent synthesis of **10** was initiated by reacting methoxydichlorophosphine with a twofold excess of 4,5-dichloroimidazole in the presence of DIPEA in dry $CHCl_3$. The reaction mixture was analyzed by ^{31}P NMR. Only one major peak (ca. 95%) was observed at 114.7 ppm. None of the extra peaks resulting from the interaction of **6f** with **7** in THF or in $CHCl_3$ displayed a chemical shift of 114.7 ppm. Since **10** was extremely sensitive to atmospheric moisture, its isolation and characterization were unsuccessful. However, the latter has been indirectly characterized by its reaction with **1b** (1.5 molar equiv) in dry $CHCl_3$. The two major species observed by ^{31}P NMR at 140.2 and 125.9 ppm were produced in a ratio of 1:1.1, respectively. The product represented by the peak at 140.2 ppm has been identified as the dinucleoside monophosphite **11b**, which was alternatively prepared from methoxydichlorophosphine and **1b** (2 molar equiv) in the presence of collidine in dry chloroform. The compound represented by the peak at 125.9 ppm has been tentatively identified as the corresponding deoxynucleoside (4,5-dichloroimidazolyl)methoxyphosphine. Support for this assignment has been provided from the activation of **2a–d** by tetrazole in dry acetonitrile. It has been shown that the chemical shift of the resulting deoxynucleoside (tetrazolyl)methoxyphosphine also appeared at 126 ppm.[24]

No other experiments involving the phosphitylating agents, activators, and solvents covered by the scope of the present investigation succeeded in producing species analogous to **10** even in the presence of an excess of the activator. This trend can be explained in terms of delocalization of the p electrons of the nitrogen atom into the heterocyclic ring in such species as **9e,f,h**. Consequently, the p electrons of the remaining dialkylamino function in **9e,f,h** could participate in a $p\pi-d\pi$ orbital overlap with the adjacent phosphorus atom to such an extent that potential protonation is inhibited.[34]

Practical Application. The reaction of the deoxynucleosides **1a–d** with the selectively activated bis(dialkylamino)methoxyphosphines **8e,f,h** and/or with the monofunctional phosphitylating species **9e,f,h** should generate the corresponding deoxynucleoside phosphoramidites in situ. To evaluate this approach, **6e** was added to a solution of **1a** and 1,2,4-triazole in dry THF. The reaction was examined by ^{31}P NMR. As expected, **2a** was identified as a doublet at 147.3 and 146.5 ppm (ca. 65%) along with **11a** represented as a single line at 139.4 ppm (ca. 5%). Significant amounts of unreacted **6e** and its corresponding triazolide were also present at 137.4 and 130.8 ppm, respectively (ca. 30%). The identities of **2a** and **11a** were unambiguous since they have been prepared by another route and have been fully characterized by elemental analysis.[26]

(A) Activator Selection. In an attempt to improve the reaction kinetics, 1,2,4-triazole was substituted by the more acidic 3-chlorotriazole. Under similar conditions, ^{31}P NMR displayed no traces of unreacted phosphitylating agent but only **2a** (ca. 60%) and **11a** (ca. 40%). Obviously, 3-chlorotriazole was too acidic in THF to selectively produce **2a**. Less acidic activators[35] such as benzotriazole, 1,2,3-triazole, and 4,5-dichloroimidazole were tested in the selective preparation of **2a**. 4,5-Dichloroimidazole exhibited optimal results and the best reaction kinetics according to ^{31}P NMR. **2a** was formed in high yield (ca. 85%) along with some **11a** (ca. 10%). Only minute amounts of unreacted phosphitylating agent (5%) were still observed.

The interaction of **7** with bis(dialkylamino)methoxyphosphine such as **6f,h** was then examined. When **6f** was added to a solution of **1a** and **7** in dry THF, ^{31}P NMR showed complete reaction within 5 min at ambient temperature. **3a** (144.6 and 144.1 ppm) and **11a** (140.1 ppm) were formed in a ratio of 10:1.0, respectively. Repeating the above experiment using **6h** instead of **6f** resulted in an incomplete reaction. According to ^{31}P NMR, **5a** (150.1 and 149.6 ppm) was generated in only ca. 40% yield. The phosphitylating agent **6h** (130.6 ppm, ca. 5%) and its corresponding 4,5-dichloroimidazolide **9h** (128.3 ppm, ca. 55%) were found unreacted after 10 min at ambient temperature, presumably as a consequence of steric factors.

Since **6f** and **7** in the presence of **1a** represent the best combination for rapid generation of the corresponding deoxynucleoside phosphoramidite in situ, it then becomes imperative to verify if the deoxynucleosides **1b–d** would behave similarly in like conditions. Using THF as solvent ^{31}P NMR displayed ratio deoxynucleoside phosphoramidite:3′→3′ dinucleoside monophosphite of 10:1.0 and 10:0.4 when **1b** and **1c** were, respectively, used as starting materials. The reaction involving **1d** gave, however, more 3′→3′ dimer, the ratio **3d**:**11d** being 10:1.5.

(B) Solvent Selection. The variable affect of the solvent was examined by adding **6f** (0.25 mmol) to **1d** (0.28 mmol) and **7** (0.75 mmol) dissolved with 0.5 mL of dry 1,3-dimethyl-2-imidazolidinone (DMI). ^{31}P NMR clearly showed that the reaction kinetics were slower than when THF was used as solvent. After 10 min at ambient temperature, ca. 5% of unreacted phosphitylating agent was still present. On the other hand, the ratio **3d**:**11d** being ca. 10:1.0 was encouraging. The amount of 4,5-dichloroimidazole was increased from 0.75 to 1.00 mmol to further enhance reaction kinetics[36] and tetramethylurea (TMU), 1-methyl-2-pyrrolidinone (NMP), *N,N*-dimethylacetamide (DMA), *N,N*-dimethylformamide (DMF), dioxan, THF, EtOAc, and $CHCl_3$ were tested as solvents for the preparation of **3b** in situ under the conditions described above. The poor solubility of **7** in dioxan, EtOAc, and $CHCl_3$ required at least 1.0 mL of solvent to be used for these experiments. Table I shows the ratio **3b**:**11b** as evaluated by ^{31}P NMR. From these data it is clear that the best solvents for this reaction are DMI, TMU, NMP, and DMA, suggesting that polar solvents "solvate" the activator **7** through hydrogen bonding thereby reducing, to some extent, its acidity. The net effects of this interaction are slower reaction kinetics and improved selectivity of protonation between **6f** and **3b** mediated by 4,5-dichloro-

(34) Burgada and co-workers observed similar behavior with trivalent phosphorus species having a pyrrole substituent; no exchange reaction occurred with methyl trifluoroacetate, implying that the p electrons of the nitrogen atom were strongly engaged in the ring.[31]

(35) Heterocyclic activators in order of increasing acidity: 1,2,4-triazole < 1,2,3-triazole < benzotriazole ≤ 4,5-dichloroimidazole.

(36) Under these conditions, the total volume of the mixture was approximately 0.7 mL implying a nucleosidic concentration of about 0.4 M, the concentration at which optimal reaction kinetics were recorded by ^{31}P NMR.

Table I. Effect of the Solvent on the in Situ Preparation of the Deoxynucleoside Phosphoramidite 3b

SOLVENT	RATIO 3b : 11b
DMI	10 : 1.3
NMP	10 : 0.9
TMU	10 : 1.0
DMA	10 : 1.1
DMF	10 : 1.6
DIOXAN	10 : 2.9
THF	10 : 1.7
EtOAc	10 : 3.0
$CHCl_3$	10 : 5.3

imidazole (**11b** was generated in an average of less than 10%). On the other hand, with less polar solvents such as dioxan, THF, EtOAc, and $CHCl_3$, the formation of **11b** dramatically increases suggesting a poorer "solvation" of the activator by the solvent. The acidity of **7** being relatively greater in these solvents reduced the selectivity of protonation between **6f** and **3b**. Table II displays the ratios between the reaction product, 3′→3′ dinucleoside monophosphite **11a–d**, and unreacted phosphitylating agent **6f** and **9f** during the preparation of **3a–d** in polar solvents under the conditions described above.

(C) Control Experiments. Since 4,5-dichloroimidazole appeared not entirely inert toward the protonation of **3a–d**, the following experiment was done: **1a** (0.28 mmol) and **7** (1.00 mmol) dissolved in dry NMP (0.5 mL) were added to **6f** (0.25 mmol). After 10 min, 3′-*O*-acetyldeoxythymidine (0.35 mmol) dissolved in the same solvent (0.7 mL) was added. After 90 min, ^{31}P NMR showed that ca. 60% of **3a** remained unreacted while the corresponding 3′→5′ dinucleoside monophosphite (140.4, 139.7 ppm) was formed in 40% yield. This observation becomes irrelevant relative to the use of deoxynucleoside phosphoramidites generated in situ in manual and/or fully automatized solid-phase DNA synthesis as long as careful removal of adventitious water from the reagents, the solvent, and the environment is exercised.

A control experiment in which 4,5-dichloroimidazole was omitted in the last reaction gave, as expected, no formation of **3a**. Experiments in which the ratio nucleoside: phosphitylating agent have been 1:0.9, 1:1, and 1:1.1 during the preparation of deoxynucleoside phosphoramidite in situ suggest that the ratio 1:0.9 is recommended for these preparations, since it is occasionally observed that when the deoxynucleoside and activator solution are added to the phosphitylating agent small amount (<5%) of the latter are found unreacted after 10 min. The subsequent 1*H*-tetrazole activation of the corresponding deoxynucleoside phosphoramidite formed (employed in solid-phase DNA synthesis) would also activate unreacted phosphitylating agent. The slight excess of deoxynucleoside used in the reaction should consume any minute amounts of activated phosphitylating agent before reacting with the solid support. In the unlikely event that some activated phosphitylating agent does react with the solid support, excess deoxynucleoside would generate the desired product by reacting with the now immobilized phosphitylating agent in a manner similar to the procedures reported by Cao[19] and Jayaraman.[17]

	REAGENT [VOLUME]	TIME
1.	Sat. $ZnBr_2$ soln. in CH_3NO_2:CH_3OH (95:5) [3 mL]	4 min
2.	CH_3OH [10 mL]	
3.	CH_3NO_2 [10 mL]	
	Go to 1 for two more cycles, then go to 4.	
	OR	
1.	3% Trichloroacetic acid in CH_3NO_2:CH_3OH (99:1) [3 mL]	2 min
2.	CH_3NO_2 [10 mL]	
	Go to 1 for one more cycle, then go to 3.	
3.	10% 2,6-Lutidine in CH_3CN [10 mL]	
4.	CH_3CN [10 mL]	
5.	DRY CH_3CN under N_2 atm. [10 mL]	
6.	DRY CH_3CN under N_2 atm. [5 mL]	
7.	CONDENSATION (3a-d + 1H-Tetrazole)	3 min
8.	DRY CH_3CN under N_2 atm. [10 mL]	
9.	CONDENSATION (3a-d + 1H-Tetrazole)	3 min
10.	0.1M Iodine soln. in THF:2,6-Lutidine:H_2O (40:20:1) [3 mL]	2 min
11.	DRY THF [10 mL]	
12.	Acetic anhydride:2,6-Lutidine:DRY THF (1:1:8) [1.5 mL] + N,N-Dimethylaminopyridine (6.5% in DRY THF) [1.5 mL]	3 min
13.	CH_3NO_2 [10 mL]	
	Go to 1	

Figure 2. Synthesis cycle used during the preparation of d-(GCATCGCCAGTCACTATGGCGT).

(D) Phosphitylating Agent Selection. The ^{31}P NMR data displayed in Table III suggest that deoxynucleoside phosphoramidites can be best prepared in situ using **6f** as

Table II. Summary of the Experiments Related to the Preparation of Deoxynucleoside Phosphoramidites 3a–d in Situ from Dipyrrolidinomethoxyphosphine and the Deoxynucleosides 1a–d in the Presence of 4,5-Dichloroimidazole in Polar Solvents

	RATIO 3a-d : 11a-d : 6f and/or 9f			
SOLVENT	1a	1b	1c	1d
DMI	10 : 0.9 : 0.0	10 : 1.3 : 0.0	10 : 0.8 : 0.0	10 : 1.3 : 0.1
NMP	10 : 0.9 : 0.0	10 : 0.9 : 0.0	10 : 0.8 : 0.2	10 : 1.3 : 0.0
TMU	10 : 0.8 : 0.4	10 : 1.0 : 0.1	10 : 0.5 : 0.7	10 : 1.2 : 0.3
DMA	10 : 1.0 : 0.4	10 : 1.1 : 0.0	10 : 0.5 : 0.8	10 : 1.3 : 0.0
DMF	10 : 1.7 : 0.0	10 : 1.6 : 0.0	10 : 1.0 : 0.5	10 : 1.6 : 0.2

Table III. Analysis of Various Deoxynucleoside Phosphoramidite Preparations Generated from the Phosphitylating Agents 6e,f,h, the Deoxynucleosides 1a–d, and 4,5-Dichloroimidazole in Dry 1-Methyl-2-pyrrolidinone

	RATIO 2a-d / 3a-d / 5a-d : 11a-d : 6e,f,h and/or 9e,f,h.			
PHOSPHITYLATING AGENT	1a	1b	1c	1d
6e	10 : 0.6 : 0.5	10 : 1.0 : 0.0	10 : 0.4 : 0.5	10 : 1.1 : 0.3
6f	10 : 0.9 : 0.0	10 : 0.9 : 0.0	10 : 0.8 : 0.2	10 : 1.3 : 0.0
6h	10 : 0.5 : 2.9	10 : 0.3 : 2.3	10 : 0.3 : 2.8	10 : 0.5 : 2.5

phosphitylating agent[37] under the optimal conditions described in this report (see Experimental Section). Thus, **3a–d** were produced in ca. 86% yield along with **11a–d** in ca. 9% yield. The phosphoramidous acid **12f** was also generated in ca. 5% yield. The identity of the latter has been confirmed by an alternate synthesis involving the careful hydrolysis of chloro(pyrrolidino)methoxyphosphine at low temperature in the presence of DIPEA as an acid scavenger and monitoring the reaction by ^{31}P NMR.[38] The presence of variable amounts of symmetrical 3′→3′ dinucleoside monophosphite **11a–d** was anticipated since, compared to **8f**, the nucleosidic substituent adjacent to the phosphorus atom should contribute as much as the methoxy substituent to the $p\pi$–$d\pi$ interactions, thereby increasing the electron density on the nitrogen atom of the dialkylamino functionality in **3a–d**. In the presence of excess activator and depending upon its acidity in a given solvent, the activation of the latter could be possible.

(E) Solid-Phase DNA Synthesis. It has been shown that the deoxynucleoside phosphoramidites **3a–d** generated in situ were suitable for the synthesis of a variety of dinucleoside monophosphates and for the preparation of d(GCATCGCCAGTCACTATGGCGT) on solid support.[26] The synthesis cycle used for the manual preparation of the 22-mer is illustrated in Figure 2.

Detritylation using the Lewis acid zinc bromide[12,39] was used only to deblock terminal benzoylated deoxyadenosine moieties to minimize depurination that might significantly occur with trichloroacetic acid. Additionally, two condensation steps were performed to ensure optimum yields. During manual solid-phase DNA synthesis, coupling step yields might be reduced due to extraneous moisture. It should be understood that using two condensation steps are not a requirement for the overall synthesis but only a safety measure.

The repetitive coupling yield obtained during the synthesis of the oligomer was spectrophotometrically measured at each cycle from the dimethoxytrityl cation (500 nm, ϵ 72 000) released. An average coupling yield of 95% was attained throughout the preparation of the 22-mer. After full deprotection, the crude oligomer was 5′-end labeled with polynucleotide T-4 kinase in the presence of γ-^{32}P ATP according to standard procedures.[40] The radiolabeled oligomer was then purified by electrophoresis using a denaturating 7 M urea–20% polyacrylamide gel. (Figure 3 exhibits an autoradiogram of the gel). It can be seen that some oligomeric material migrated less rapidly than the 22-mer. This abnormality can be tentatively interpreted as the removal of some heterocyclic base protecting group[41] during the detritylation step mediated by zinc bromide in the presence of protic solvents thereby generating functional groups susceptible to phosphorylations. Consequently, unwanted "ramifications" of the original oligomer contribute to increasing its molecular weight, generating material that is anticipated to move less rapidly than the desired oligomer on electrophoretic gel.

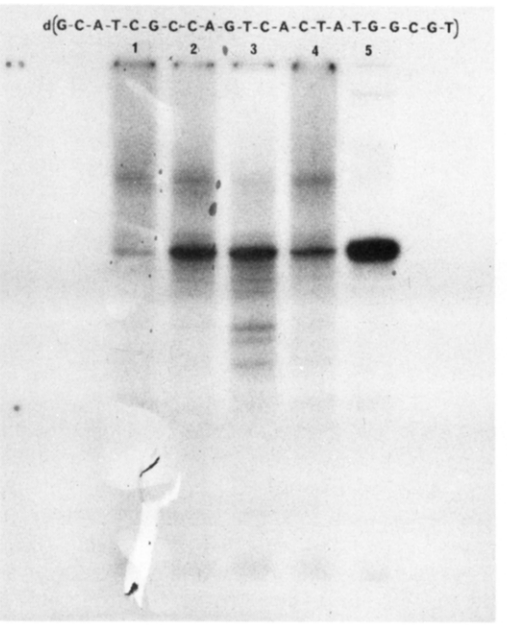


Figure 3. Electrophoretic pattern of the crude 5′-radiolabeled 22-mer. Lanes 1, 2, and 3 exhibit the oligomer distribution from the first three fractions (1.5 mL) eluted from a short (1.5 cm × 7.0 cm) G-50 Sephadex column with 0.2 M TEAB buffer (pH 8.2). Lane 4 displays the oligomer distribution in an equal volume mixture of the three fractions. Lane 5 shows the purified oligomer.

Additionally, each "ramification site" on slow moving material is 5′-end radiolabeled, thereby contributing to apparently elevated concentration of these species (displayed as discrete bands on the autoradiogram) relative to the concentration of the natural oligomer. Ultraviolet detection of an identical oligomeric mixture indicates that the natural oligomer is the only prominent species on the gel,[42] suggesting that multiple radiolabeling of slow-moving species is responsible for their apparently high concentrations. Alternatively, incomplete removal of the methyl phosphate protecting groups may generate oligomers having less Coulombic charge than the expected 22-mer. These "undercharged" species are also expected to migrate less rapidly than the fully deprotected 22-mer on electrophoretic gel. Finally, a combination of the above events might account for the slow-moving material shown on the autoradiogram. The purified oligomer was isolated and submitted for commercial sequence determination. The 22-mer was unambiguously characterized as d-(GCATCGCCAGTCACTATGGCGT).

To conclude, the approach for solid-phase DNA synthesis described in this manuscript represents a logical simplification of the current methodologies used in DNA synthesis. The inherent advantages of the above synthetic techniques as previously reported[26] constitute a valuable asset to simple, efficient, and economical solid-phase DNA synthesis automation.

Experimental Section

Materials and Methods. Dichloromethoxyphosphine, morpholine, and diisopropylamine were purchased from Aldrich Co. and used as received. (Dimethylamino)trimethylsilane and (trimethylsilyl)pyrrolidine were commercially available from Petrarch System and were used without further purification. Solvents such as chloroform, ethyl acetate, tetrahydrofuran, dioxan, acetonitrile, *N*,*N*-dimethylformamide and *N*,*N*-dimethylacetamide were purchased from Burdick & Jackson Laboratories and were carefully dried by using standard procedures.[43]

(37) Additionally, the chemical stability of **6f** was evaluated by ^{31}P NMR. The neat liquid stored in a sealed tube at room temperature showed less than 5% degradation after 1 month and about 11% degradation after 1 year implying that its use in fully automated systems poses no problems.

(38) Alternatively, the phosphoramidous acid has been prepared in greater than 90% yield by adding water to a solution of **6f** and **7** in NMP. Furthermore, similar phosphoramidous acids have been prepared and characterized by Andreev and co-workers.[47]

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(42) Photographic support of this observation is available upon request.

Tetramethylurea, 1,3-dimethyl-2-imidazolidinone, and 1-methyl-2-pyrrolidinone were purchased from Aldrich Co. and were refluxed and distilled over CaH_2. All dry solvents were kept over 4-Å molecular sieves in amber bottles prior to use. 1,2,3-Triazole was purchased from Alfa Ventron and used as received. 4-Nitroimidazole, benzotriazole, 1,2,4-triazole, 1*H*-tetrazole were commercially available from Aldrich Co. 3-Chlorotriazole was prepared according to the procedure of Becker and co-workers.[44] 1,2,4-Triazole, 1*H*-tetrazole, and 3-chlorotriazole were purified and dried by sublimation under reduced pressure prior to use.

The fully protected deoxynucleosides purchased from P. L. Biochemicals were carefully dried by several coevaporations from dry pyridine and were isolated pyridine free by precipitation in large quantities of stirred hexanes. After vacuum drying, the protected nucleosides were stored desiccated at −20 °C.

The solid support used for DNA synthesis was CPG/long-chain alkylamine purchased from Pierce Chemical Co. and derivatized according to the procedure of Chow and co-workers.[45]

The ^{31}P NMR spectra were recorded on a NTC 300 Nicolet wide-bore instrument operating at 120 MHz under a broad-band proton-decoupling mode.

General Procedures for the Preparation of Bis(dialkylamino)methoxyphosphines (6e–h). To 16.8 g of magnetically stirred dichloromethoxyphospine (0.13 mol) cooled at −15 °C in a 200-mL round-bottom flask was added under an inert atmosphere 44 mL of (dimethylamino)trimethylsilane (0.28 mol) dropwise over a period of 30 min. The reaction mixture was then removed from the cold bath and left stirring at ambient temperature for 1 h. Most of the byproduct [(chlorotrimethyl)silane] was removed under reduced pressure (water aspirator) leaving a suspension which was filtered. Fractional distillation of the filtrate afforded 13.0 g of **6e** (86 mmol, 68%) as a cloudy liquid, which was subsequently filtered and redistilled [bp 38–40 °C (13 mmHg)]. The clear and colorless liquid had a density of 0.936 g/mL at 25 °C: ^{31}P NMR ($CDCl_3$) 138.2 ppm (internal H_3PO_4 standard); ^1H NMR ($CDCl_3$) 3.5 (d, OCH_3, J_{PH} = 13 Hz), 2.7 ppm (d, $N(CH_3)_2$, J_{PH} = 9 Hz) (internal Me_4Si standard). Anal. Calcd for $C_5H_{15}N_2OP$: C, 39.98; H, 10.09; N, 18.66; P, 20.62. Found: C, 40.17; H, 9.88; N, 18.58; P, 20.38.

6f was similarly prepared from 54.3 g of *N*-(trimethylsilyl)-pyrrolidine (0.38 mmol) and 22.6 g of methoxydichlorophospine (0.17 mol). The product (27.6 g, 0.14 mol) has been isolated (82%) and purified as above. **6f** [bp 75–77 °C (0.55 mmHg)] had a density of 1.037 g/mL at 25 °C: ^{31}P NMR ($CDCl_3$) 134.0 ppm; ^1H NMR ($CDCl_3$) 3.3 (d, OCH_3, J_{PH} = 13 Hz), 3.0 (m, $N(CH_2)_2$), 1.7 ppm (m, CH_2CH_2). Anal. Calcd for $C_9H_{19}N_2OP$: C, 53.44; H, 9.49; N, 13.85; P, 15.31. Found: C, 53.21; H, 9.61; N, 14.02; P, 15.08.

6h was prepared from the dropwise addition of 15.0 g of chloromethoxyphosphine (0.11 mol) over a period of 30 min to a magnetically stirred solution of 44.5 g of diisopropylamine (0.44 mol) to 100 mL of anhydrous ethyl ether at −15 °C under an inert atmosphere. The resulting suspension was left stirring at ambient temperature for 1 h. The diisopropylamine hydrochloride was filtered and washed with 100 mL of anhydrous ethyl ether. The filtrate was then distilled at atmospheric pressure, leaving a material which was fractionally distilled under reduced pressure. **6h** was isolated (79%) as a clear liquid boiling at 74–75 °C (0.45 mmHg) (22.8 g, 87 mmol). **6h** had a density of 0.911 g/mL at 26 °C: ^{31}P NMR ($CDCl_3$) 131.8 ppm; ^1H NMR ($CDCl_3$) 3.4 (d, OCH_3, J_{PH} = 14 Hz), 3.6 (m, $N(CH)_2$), 1.2 ppm (d, CH_3). Anal. Calcd for $C_{13}H_{31}N_2OP$: C, 59.49; H, 11.93; N, 10.68; P, 11.80. Found: C, 59.29; H, 11.99; N, 10.90; P, 11.87.

6g was similarly prepared using 20.0 g of dichloromethoxyphosphine (0.15 mol) and 54.9 g of morpholine (0.63 mol). In our hands the purification of the product by high-vacuum distillation was troublesome since **6g** was obtained as a semisolid material. ^{31}P NMR of the product displayed a single line at 132 ppm ($CDCl_3$) suggesting that the desired dimorpholinomethoxyphosphine had been produced. The compound was ca. 80% pure according to ^{31}P NMR. Further characterization of the reaction product was not pursued.

Preparation of 4,5-Dichloroimidazole (7). The compound was prepared according to a slight modification of the procedure reported by Lutz and DeLorenzo.[46] The tan precipitate obtained from the acidic neutralization of the reaction mixture was collected by filtration and washed with water. After most of the water was drained, the product was dried overnight under reduced pressure. The material was then purified by repeated sublimation at 105–110 °C (0.2 mmHg). The white crystalline material melted at 179–180 °C. Anal. Calcd for $C_3H_2Cl_2N$: C, 26.31; H, 1.47; N, 20.46; Cl, 51.76. Found: C, 26.17; H, 1.54; N, 20.19; Cl, 52.00.

Optimal Preparation of the Deoxynucleoside Phosphoramidites 3a–d in Situ Required for the Manual Solid-Phase Synthesis of d(GCATCGCCAGTCACTATGGCGT). Dry suitably protected deoxynucleoside **1a–d** (168 μmol) and sublimed 4,5-dichloroimidazole (82 mg, 600 μmol) were dissolved with dry 1-methyl-2-pyrrolidinone (0.3 ml) in a serum capped and flame-dried 4-mL glass vial. The solution was then slowly added by syringe into another dry 4-mL glass vial containing dipyrrolidinomethoxyphosphine (30 μL, 150 μmol). The resulting solution was left undisturbed for ca. 1 min and then shaken occasionally for ca. 9 min prior to use. Since two condensations were performed per cycle, this preparation will last only one cycle. Stock solutions containing up to eight condensations or four cycles were routinely prepared. The above stock solution, 225 μL, followed by 750 μL of 0.5 M 1*H*-tetrazole in dry acetonitrile were added via syringe to a vented (syringe needle) serum-capped glass sintered funnel (medium porosity, 15 mL) containing 91 mg of CPG/long-chain alkylamine derivatized with ca. 3 μmol of deoxythymidine. The suspension in the closed system was shaken for 3 min and filtered and the procedure from step 8 on of Figure 2 was followed. Deoxynucleoside phosphoramidites **3a–d** prepared in situ as above were analyzed by ^{31}P NMR along with their side products **11a–d** and **12f**. Chemical shifts are given in parts per million from 1-methyl-2-pyrrolidinone downfield relative to internal 85% H_3PO_4 standard: **3a**, 143.7, 143.4; **11a**, 139.7; **3b**, 144.0, 143.9; **11b**, 139.8; **3c**, 143.5; **11c**, 140.0; **3d**, 143.8, 143.7; **11d**, 140.7; **12f**, 15.4. Illustration of individual spectrum has been reported elsewhere.[26]

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Registry No. **1a**, 40615-39-2; **1b**, 67219-55-0; **1b** (3′-*o*-[(4,5-dichloroimidazolyl)methoxyphosphino]), 96151-47-2; **1c**, 64325-78-6; **1d**, 68892-41-1; **2a**, 78635-91-3; **2b**, 78635-92-4; **2c**, 78635-93-5; **2d**, 78635-94-6; **3a**, 86030-47-9; **3b**, 89983-16-4; **3c**, 89983-17-5; **3d**, 89983-18-6; **5a**, 84416-85-3; **5b**, 84416-83-1; **5c**, 84416-82-0; **5d**, 84416-84-2; **6e**, 17166-16-4; **6f**, 89983-14-2; **6g**, 93183-35-8; **6h**, 92611-10-4; **7**, 15965-30-7; **9e**, 96151-40-5; **9f**, 96164-21-5; **9h**, 96151-41-6; **10**, 96151-42-7; **11a**, 89983-15-3; **11b**, 96151-43-8; **11c**, 96151-44-9; **11d**, 96151-45-0; **12f**, 96151-46-1; NMP, 872-50-4; d(GCATCGCCAGTCACTATGGCGT), 89962-60-7; dichloromethoxyphosphine, 3279-26-3; (dimethylamino)trimethylsilane, 2083-91-2; *N*-(trimethylsilyl)pyrrolidine, 15097-49-1; diisopropylamine, 108-18-9; morpholine, 110-91-8; 1,2,4-triazole, 288-88-0; 3-chlorotriazole, 6818-99-1; chloro(dimethylamino)methoxyphosphine, 70063-12-6; benzotriazole, 95-14-7; 1,2,3-triazole, 288-36-8; deoxythymidine, 50-89-5.

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