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**Studies on the role of tetrazole in the activation of phosphoramidites**

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**ABSTRACT**

The mechanism of the tetrazole-activated coupling step in the synthesis of oligonucleotides via phosphoramidites is studied with the help of model reactions: Treatment of diethoxydiisopropylaminophosphane with two equivalents of tetrazole resulted in a diethoxy-tetrazolophosphane, whose ( $^{31}\text{P}$ )-NMR shift of 126 ppm is identical with the signal observed during internucleotide bond formation. A series of different related diethoxy-phosphorous-acid derivatives were also synthesized; their ( $^{31}\text{P}$ )-NMR signals between 123.9 and 130.8 ppm are additional evidence for the intermediacy of a tetrazolide species. Further NMR investigations with more basic azoles showed that tetrazole is also active as a proton donor.

**INTRODUCTION**

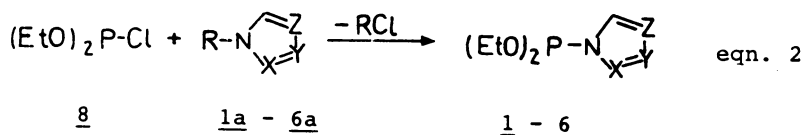
The essential step in the synthesis of deoxyoligonucleotides using phosphoramidite monomers is the tetrazole-activated coupling reaction of the phosphoramidite with the free 5'-hydroxyl-function of an immobilized oligonucleotide (2,3). Among several activating compounds (4), 1H-tetrazole is by far the most commonly used for this reaction. Although this activation process is widely used in deoxy- and ribo-oligonucleotide synthesis, its mechanism still remains to be clarified.

The main question is whether tetrazole, which is a weak acid, serves only as a proton donor yielding as primary product a protonated nucleoside-phosphoramidite (5), or whether it will attack as a nucleophile giving rise to the formation of a "tetrazolide" intermediate (6,7). Arguments in favour of the existence of a tetrazolophosphane intermediate, so far, have come mainly from  $^{31}\text{P}$ -NMR spectroscopy. Thus, the appearance of a new signal at 126 ppm on treatment of a nucleoside-phosphoramidite with an excess of tetrazole has been tentatively assigned to the forma-



The intermediate (eqn. 1) N-tetrazolyl-diethoxyphosphane 6 was prepared and characterized as an authentic substance. A series of several N-azolyl-phosphorus compounds, as shown in Chart 1, were prepared in order to compare their spectroscopic properties with those of the observed intermediate in the model reaction as well as in oligodeoxynucleotide chemistry.

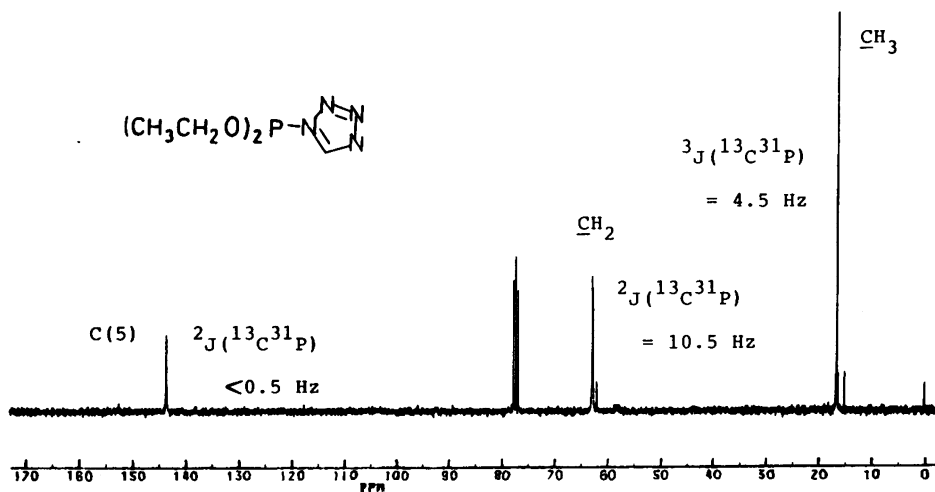
Preparation and characterization of N-azolyl-phosphorus compounds



1a: R = K; 2a - 5a: R = SiMe<sub>3</sub>; 6a: R = Na

Attempts at preparing 1-6 (Chart 1) directly from diethoxychlorophosphane 8 with the corresponding "azoles" did not lead to the desired products. However, when 8 was reacted with N-trimethylsilyl-azoles in benzene at 78°C for 10 min. (13), the azolophosphanes 2-5 were obtained in good yields and purified by distillation in vacuo after removal of trimethylchlorosilane. The reactant N-trimethylsilyl-azoles 2a-5a, in turn, had been obtained by refluxing the corresponding heterocycles for 10-12 h with an excess of hexamethyldisilazane (14) or with trimethylchlorosilane in the presence of a tertiary amine. Unfortunately, the reaction of N-trimethylsilylpyrrole (15) as well as N-trimethylsilyltetrazole (16) with 8 turned out to be unsatisfactory. The pyrrole derivative was too unreactive, whereas in the latter case the reagent decomposed easily on heating with liberation of nitrogen. Compound 1, therefore, was made via the potassium salt of pyrrole (17), while 6 resulted from the reaction of 8 with sodium tetrazolide. Surprisingly, 6 was not decomposed on fractionation in vacuo and was obtained pure in 50 % yield.

Compounds 1-6 were characterized by <sup>1</sup>H, <sup>13</sup>C, <sup>31</sup>P-NMR and elemental analysis. The NMR data for the spectroscopic characterization of the model azolophosphanes are summarized in Table 1. For special reference the <sup>13</sup>C spectrum of the tetrazolophosphane 6 is shown in Figure 1. The <sup>13</sup>C-NMR shifts were determined according to B. Wrackmeyer (18). The <sup>1</sup>H-NMR data were not very



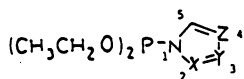
**Figure 1:** 75.5 MHz  $^{13}\text{C}$ -NMR spectrum of 6 in  $\text{CD}_3\text{CN}$ ; internal standard  $\text{Me}_4\text{Si}$

helpful in clarifying the structure of 1-6, since a  $^3\text{J}(^{31}\text{P}^1\text{H}_{\text{azole}})$  coupling could not be observed.

As seen from Table 1 and also from Figure 2b the  $^{31}\text{P}$ -resonance signals of compounds 1-6 are between 123.9 and 130.8 ppm, which is comparable to the shift for the intermediate often observed by us and others (8,10) in the course of nucleoside phosphoramidite activation and suggests that the intermediate is, indeed, a tetrazolophosphane. If this suggestion was correct, the reaction of diethoxydiisopropylamino-phosphane 7 with tetrazole according to eqn.1 should give a product, which is identical with 6 obtained previously. This was in fact the case; after the reaction of 7 with two equivalents of tetrazole, diisopropylammoniumtetrazolide could be crystallized, and after distillation *in vacuo* a colourless liquid identical to 6 ( $^{31}\text{P}$ ,  $^{13}\text{C}$ -NMR, elemental analysis) was obtained. Representative  $^{31}\text{P}$ -NMR spectra taken during the progress of the tetrazole activation of nucleoside phosphoramidites on one hand and diethoxydiisopropylamino-phosphane 7 on the other hand are shown in Figure 2a and 2b.

It is remarkable that in the case of the phosphoramidite activation only one signal, which is unusually broad, is observed, despite of the diastereomeric starting material. This was ex-

**Table 1:**  $^{13}\text{C}$ - and  $^{31}\text{P}$ -NMR data of N-azolyl-diethoxy-phosphorus compounds 1-6



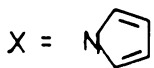
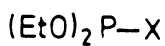
No.	X	Y	Z	CH <sub>3</sub>	CH <sub>2</sub>	C(2)	C(3)	C(4)	C(5)	other solvent	$\delta^{31}\text{P}$
<u>1</u>	CH	CH	CH	16.3 (5.1)	60.0 (10.5)	120.7 (13.2)	110.4 (3.3)	110.4 (3.3)	120.7 (13.2)	DMSO	130.0
<u>2</u>	N	CH	CH	16.6 (5.2)	61.9 (10.9)		143.3 (7.7)	106.3 (-0.5)	131.3 (3.4)	CDCl <sub>3</sub>	126.6
<u>3</u>	CH	N	CH	16.6 (5.2)	60.9 (10.9)	137.8 (-0.5)		117.7*	130.5*	CDCl <sub>3</sub> CD <sub>3</sub> CN	129.6 125.6
<u>4</u>	CCH <sub>3</sub>	N	CH	16.9 (5.2)	61.8 (12.1)	148.2 (-0.5)		123.6*	123.6*	14.9 CDCl <sub>3</sub>	128.5
<u>5</u>	N	CH	N	16.6 (4.5)	61.6 (9.8)		154.0 (6.7)		145.9 (1.9)	CDCl <sub>3</sub> CD <sub>3</sub> CN	127.9 123.9
<u>6</u>	N	N	N	16.6 (4.5)	62.8 (12.1)				143.5 (-0.5)	CDCl <sub>3</sub> CD <sub>3</sub> CN	130.8 126.6

$\delta^{13}\text{C}$  values relative to internal  $\text{Me}_4\text{Si}$ ;  $\delta^{31}\text{P}$  values relative to external 5% aqueous  $\text{H}_3\text{PO}_4$ .  
 $J(^{31}\text{P}^{13}\text{C})$  coupling constants ( $\pm 1\text{Hz}$ ) in parentheses.

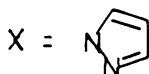
\*  $J(^{31}\text{P}^{13}\text{C})$  not resolved.

plained by an epimerization at phosphorus due to an exchange with the excess tetrazole of the solution (19). In accord with this hypothesis is our observation of broad  $^{31}\text{P}$ -NMR signals for the N-azolophosphanes 1-6 with half band widths of about 30 Hz, if we use deuteriochloroform as solvent. An intermolecular exchange of the azolyl-group at phosphorus could be responsible for this effect.

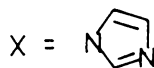
Further reactions should prove whether N-tetrazolyl-diethoxyphosphane 6 is really the active species in our model reaction (eqn. 1). Therefore we added one equivalent ethanol to a solution of 6 in acetonitrile and obtained triethyl-phosphite as a product with a  $^{31}\text{P}$ -NMR shift of 137.9 ppm (Figure 2b) (12). The reaction of 6 with 5'-O-(dimethoxytrityl)thymidine (1:1 in  $\text{CH}_3\text{CN}$ ) resulted, after subsequent oxidation with iodine in the quantitative formation of 5'-O-(dimethoxytrityl)thymidine-3'-diethoxyphosphate 10, which was isolated and characterized by NMR and mass spectrometry. Consequently we used 6 and also 5 successfully as capping reagents (1.5 M solution in  $\text{CH}_3\text{CN}$ ) during automated DNA synthesis.



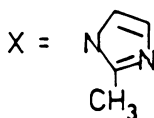
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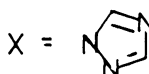
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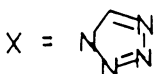
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4



5



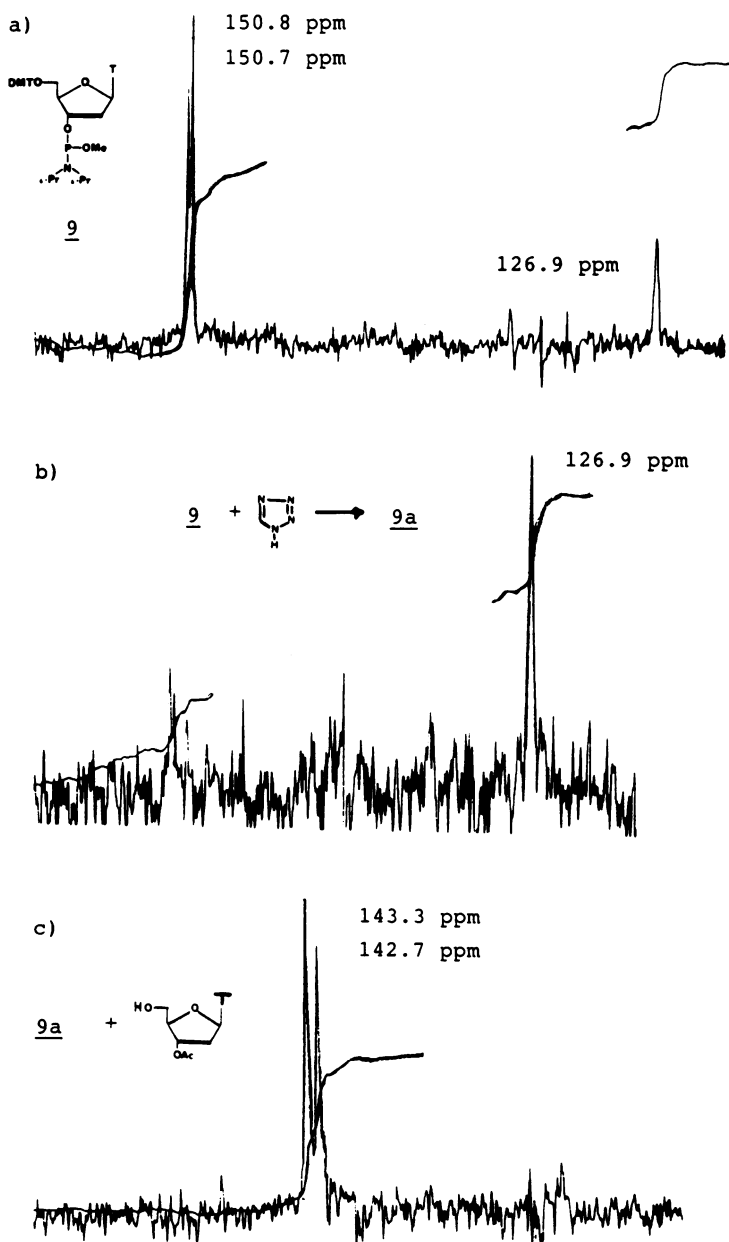
6

Chart 1: N-azolyl-diethoxy-phosphorus compounds 1-6

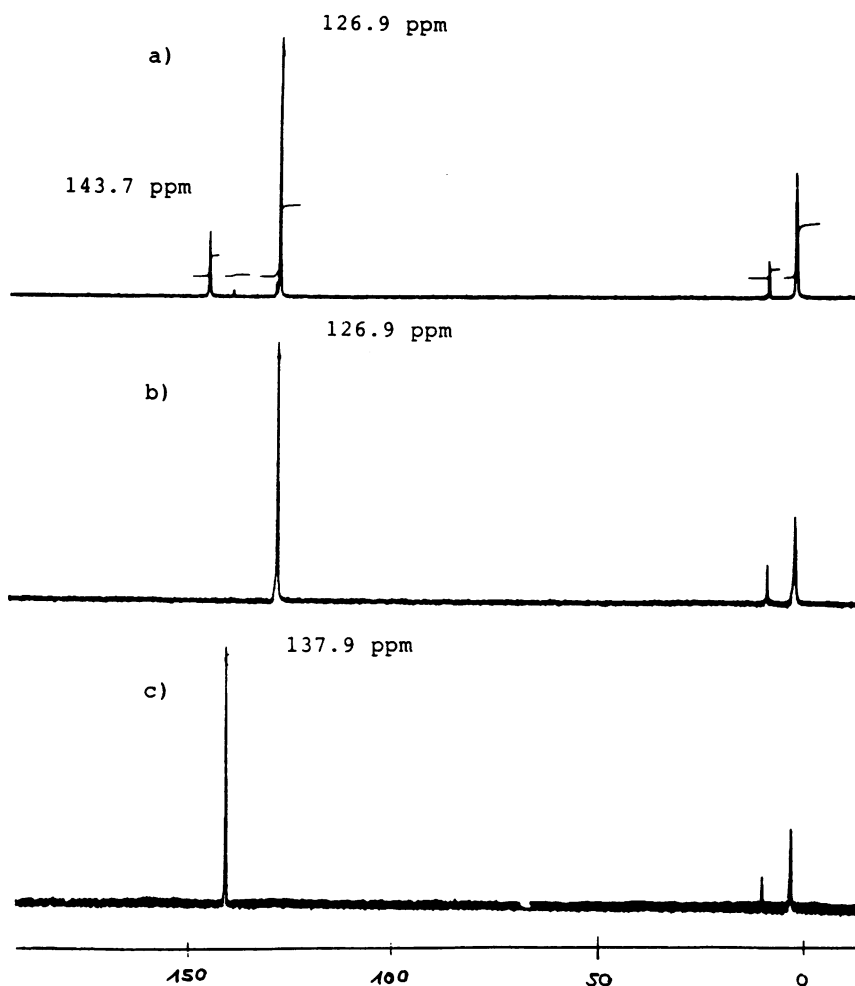
Activation of diethoxydiisopropylaminophosphane 7

All experiments described above indicated that a tetrazolophosphane is the reactive species during the internucleotide bond formation but still the question remained, whether also the acidic strength of tetrazole plays a role in the formation of 6 or whether the mechanism is just that of a nucleophilic substitution. For this purpose, we chose as alternative reactants several heterocyclic compounds with different  $pK_a$ -values like 1,2,4-triazole ( $pK_a = 10.26$ ), imidazole ( $pK_a = 14.0$ ) (20,21) and also acetic acid ( $pK_a = 4.76$ ), which has the same  $pK_a$ -value as tetrazole, to activate 7. The formation of the corresponding N-azolophosphanes or of the mixed anhydride in case of acetic acid were followed by <sup>31</sup>P-NMR spectroscopy. It was shown that two equivalents of tetrazole or acetic acid were enough for a complete formation of 6 or the anhydride within the time which is necessary to monitor the first spectrum, usually 2 minutes, whereas in the case of more basic azoles only 30% yield of the N-triazolyl-diethoxyphosphane 5 and 10% yield of N-imidazolyl-diethoxyphosphane 3 were obtained.

These NMR-investigations prompt us to suggest a mechanism for the formation of the tetrazolophosphane, which consists of three steps: a quick protonation of 7 is followed by a slow formation of the tetrazolophosphane 6 as rate limiting step. The



**Figure 2a:** 81.0 MHz  $^{31}\text{P}$ -NMR spectra ( $\text{CD}_3\text{CN}$ ) of a) 5'-O-(di-methoxytrityl)thymidine-3'-diisopropylaminomethoxy phosphoramidite 9 + tetrazole (1:1); b) 9 + excess tetrazole; c) b) + 3'-O-acetylthymidine



**Figure 2b:** 108 MHz  $^{31}\text{P}$ -NMR spectra ( $\text{CD}_3\text{CN}$ ) of a) diethoxydiisopropylaminophosphane **7** +  $^3\text{tetrazole}$  (1:1); b) **7** +  $^3\text{tetrazole}$  (1:2); c) addition of one equivalent ethanol to b)

reaction is reversible. After addition of diisopropylamine, the starting material is completely recovered. At  $-30^\circ\text{C}$  the rate of formation of **6** is extremely slow. The last step, the formation of the phosphite triester cannot be activated any further.

The results of our studies, which for the first time are based on well-characterized azolophosphanes, prove the formation of a tetrazolophosphane in the activation process of diethoxydi-



isopropylaminophosphane 7 with tetrazole and are strong evidence for an analogous intermediate in nucleoside phosphoramidite activation, in accordance with the mechanism previously described by Dahl (10).

#### EXPERIMENTAL SECTION

NMR spectra were recorded in PFT mode using a Bruker AC-100-SC at 40.53 MHz and a Jeol JNM 270 GX at 108 MHz for  $^{31}\text{P}$ , ( $\delta\text{P}$ ) positive in the lowfield direction. External standard was 5% aqueous  $\text{H}_3\text{PO}_4$ .  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectra were recorded at 300 MHz and 75.5 MHz on a Bruker MSL-300, chemical shifts ( $\delta\text{H}$ ) and ( $\delta\text{C}$ ) are relative to internal tetramethylsilane. All reactions and transfers were carried out under a dry argon atmosphere. Solvents were dried according to standard procedures. The azoles (Fluka) and also diethyl-chlorophosphite (Fluka) were distilled (pyrrole) or recrystallized (pyrazole, imidazole, 2-methylimidazole, 1,2,4-triazole). 1H-Tetrazole (23), diethoxydiisopropylaminophosphane 7 (8) and pyrrole potassium (22) were prepared according to literature procedures.

The synthesis of the N-silylazole compounds is described for one typical example:

N-Trimethylsilyl-(1,2,4)-triazole 5a: a mixture of 3.45g (50 mmol) 1,2,4-triazole and 7.8 ml (37 mmol) hexamethyldisilazane was refluxed for 24 h. The excess of hexamethyldisilazane was removed at  $20^\circ\text{C}/15\text{ mm}$ . Fractional distillation of the residue gave 4.5g (64%) of a colourless liquid (b.p.  $80^\circ\text{C}/15\text{ mm}$ ).  $\delta\text{H}$  ( $\text{CCl}_4$ ): 8.2 (s, 1H, azole), 7.9 (s, 1H, azole), 0.4 (s, 9H, trimethylsilyl).

N-Trimethylsilylpyrazole 2a: yield: 34%, b.p.  $40^\circ\text{C}/15\text{ mm}$ .  $\delta\text{H}$  ( $\text{CCl}_4$ ): 7.8 (d, 1H, azole), 7.65 (d, 1H, azole), 6.4 (t, 1H, azole), 0.5 (s, 9H, trimethylsilyl).

N-Trimethylsilylimidazole 3a: yield 72%, b.p.  $50^\circ\text{C}/15\text{ mm}$ .  $\delta\text{H}$  ( $\text{CCl}_4$ ): 7.6 (1H, azole), 7.1 (2H, azole), 0.45 (s, 9H, trimethylsilyl).

N-Trimethylsilyl-2-methylimidazole 4a: yield: 64%, b.p.  $40^\circ\text{C}/0.4\text{ mm}$ .  $\delta\text{H}$  ( $\text{CCl}_4$ ): 6.7 (s, 2H, azole), 2.3 (s, 3H, methyl), 0.4 (s, 9H, trimethylsilyl).

General procedure for the N-azolyphosphorus compounds 2-5: diethyl-chlorophosphite 8 (3.3 ml, 23 mmol) and 23 mmol of the

corresponding N-trimethylsilyl-azole were heated in 3.5 ml of benzene for 10 min. After removal of the solvent and trimethylchlorosilane in vacuo, the residue was fractionally distilled to give the desired product.

N-Pyrazolyl-diethoxyphosphane 2: yield: 67 %, b.p. 45°C/0.03 mm. Elemental analysis:  $C_7H_{13}N_2O_2P$ , calc: C 44.68 %, H 6.96 %, N 14.90 %. Found: C 44.49 %, H 6.93 %, N 15.12 %.

N-Imidazolyl-diethoxyphosphane 3: yield: 73 %, b.p. 49°C/0.03 mm. Elemental analysis:  $C_7H_{13}N_2O_2P$ , calc: C 44.68 %, H 6.96 %, N 14.90 %. Found: C 44.59 %, H 6.82 %, N 15.32 %.

N-Imidazolyl-2-methyl-diethoxyphosphane 4: yield: 57 %, b.p. 63°C/0.03mm. Elemental analysis:  $C_8H_{15}N_2O_2P$ , calc: C 47.52 %, H 7.43 %, N 13.85 %. Found: C 47.24 %, H 7.47 %, N 14.03 %.

N-Triazolyl-diethoxyphosphane 5 was prepared according to (13), yield: 62 %, b.p. 63°C/0.003 mm.

N-Pyrrolyl-diethoxyphosphane 1: diethyl-chlorophosphite 8 (5.1ml, 36 mmol) in 25 ml benzene were added dropwise to a freshly prepared solution of potassium pyrrole (36 mmol) in benzene (21). The reaction mixture was cooled in an ice bath. After stirring over night at room temperature the precipitated potassium chloride was filtered off. The solvent was removed from the filtrate and the residue distilled in vacuo to yield 2.7 g (40 %) of a colourless liquid at 60-64°C at 0.03 mm. Elemental analysis:  $C_{18}H_{14}NO_2P$ , calc: C 51.33 %, H 7.54 %, N 7.48 %. Found: C 51.55%, H 7.50 %, N 7.75 %.

N-Tetrazolyl-diethoxyphosphane 6: Method A: Ethanol (25 ml) was dropped slowly onto sodium 1.5 g (71 mmol) so that the mixture was allowed to reflux gently. After 20 min. the solution was cooled to room temperature and freshly sublimed tetrazole (4.75g, 65 mmol) was added in three portions. After stirring 12 h at room temperature the solvent was removed and the residue was co-evaporated twice with benzene. To this slurry of sodiumtetrazolide in benzene (25 ml) 8 (9.3 ml, 65 mmol) was added while the mixture was cooled in an ice bath. After stirring for 12 h sodium chloride was filtered off and the filtrate was, after removal of benzene, distilled in vacuo to give 5.75 g (46 %) of 6 at 70°C/0.003 mm. Elemental analysis:  $C_5H_{11}N_4O_2P$ , calc: C 31.58 %, H 5.83 %, N 29.47 %. Found: C 31.90 %, H 5.56 %, N 29.73 %.

Method B: Compound 6 was also obtained in 52 % yield by treating diethoxydiisopropylaminophosphate 7 with two equivalents of tetrazole in acetonitrile. After filtration of diisopropylammoniumtetrazolide, acetonitrile was distilled off at normal pressure and the residue was distilled in vacuo as described above.

5'-O-(dimethoxytrityl)-thymidine-3'-diethoxyphosphate 10:

To a suspension of 5'-O-(dimethoxytrityl)-thymidine (300 mg, 0.6 mmol) in 3 ml CH<sub>3</sub>CN a solution of 6 (0.2 g; 1.2 mmol) in CH<sub>3</sub>CN was added. The reaction was complete as monitored by tlc (silica gel, solvent CH<sub>2</sub>Cl<sub>2</sub>/MeOH = 9 : 1) after 2 min.. 1 ml of iodine solution (0.3 M I<sub>2</sub> in CH<sub>3</sub>CN/Py/H<sub>2</sub>O = 24 : 5 : 1) was added and the iodine colour quenched with 0.1 ml of 40 % NaHSO<sub>3</sub>-solution. The product was extracted with CH<sub>2</sub>Cl<sub>2</sub> and further purified by silica gel chromatography. Precipitation from hexane gave 0.26 g (70 %) pure material.

<sup>31</sup>P-NMR: 4.2 ppm; Fab<sup>-</sup> mass spectrum 679 (M-H)<sup>-</sup>; Fab<sup>+</sup> mass spectrum 703 (M+Na)<sup>+</sup>.

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REFERENCES

1. Synthesis with Nucleic Acid Constituents. 14. Communication no. 13: Seliger, H. and Feger, G., in preparation.
2. Beaucage, S.L. and Caruthers, M.H. (1981) Tetrahedron Lett. 22, 1859-1862.
3. Matteucci, M.D. and Caruthers, M.H. (1981) J. Am. Chem. Soc. 103, 3185-3191.
4. p-nitrophenyltetrazole is also used, but not as commonly as tetrazole.
5. Matteucci, M.D. and Froehler, B.C. (1983) Tetrahedron Lett. 24, 3171-3174.
6. Cao, T.M., Bingham, S.E. and Sung, M.T. (1983) Tetrahedron Lett. 24, 1019-1020.

7. Jayaraman, K. and Mc Clagherty, H. (1982) *Tetrahedron Lett.* 23, 5377-5380.
8. Ogilvie, K.K., Pon, R.T. and Damha, M.J. (1985) *Nucleic Acids Res.* 13, 6447-6465.
9. McBride, L.J. and Caruthers, M.H. (1983) *Tetrahedron Lett.* 24, 245-248.
10. Dahl, O., Nielsen, J. and Dahl, B.H. (1987) *Nucleic Acids Res.* 15, 1729-1742.
11. Seliger, H. and Gupta, K.C. (1985) *Angew. Chem. Int. Ed. Engl.* 24, 685-687.
12. Gorenstein, D., *Phosphorus-31-NMR, Principles and Applications*, Academic Press, Orlando 1984.
13. Kricheldorf, H.R., Fehrle, M. and Kaschig, J. (1976) *Angew. Chem.* 88, 337-338.
14. Birkofer, L., Richter, P. and Ritter, A. (1960) *Chem. Ber.* 93, 2804-2809.
15. Fessenden, R. and Crowe, D.F. (1960) *J. Org. Chem.* 25, 598-603.
16. Birkofer, L., Ritter, A. and Richter, P. (1963) *Chem. Ber.* 96, 2750-2757.
17. Reynolds, E. (1909) *J. Chem. Soc.* 95, 505-508.
18. Wrackmeyer, B. (1984) *Spectrochimica Acta* 40A, 963-977.
19. Stec, W.J. and Zon, G. (1984) *Tetrahedron Lett.* 25, 5279-5282.
20. Hofman, K., *The Chemistry of Heterocyclic Compounds and Derivatives Part I*, Interscience Publishers, London, 1953.
21. Lettau, H. *Chemie der Feterocyclen*, Leipzig 1980, 80-90.
22. Treibs, A. and Dietl, A. (1958) *Liebigs Ann. Chem.*, 619 80-95.
23. Henry, R.A. and Finnegan, W.G. (1954) *J. Am. Chem. Soc.* 76, 290-291.