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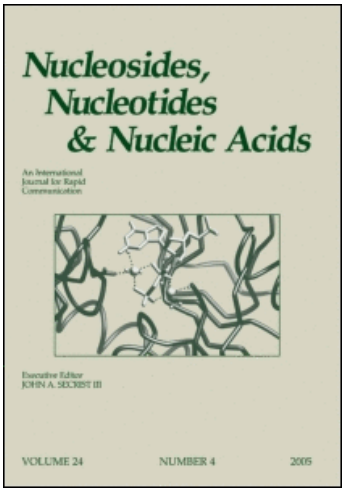

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NUCLEOSIDE H-PHOSPHONATES. IX. POSSIBLE SIDE-REACTIONS DURING HYDROGEN PHOSPHONATE DIESTER FORMATION

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Abstract

Possible side-reactions during hydrogenphosphonate diester formation in the presence of various condensing reagents (e.g. arenesulfonyl derivatives, chlorophosphates and acyl chlorides) have been investigated using ^{31}P NMR spectroscopy.

INTRODUCTION

The reaction of nucleoside 3'-hydrogenphosphonates with suitably protected nucleosides in the presence of a condensing agent offers an experimentally simple and efficient way for the formation of dinucleoside H-phosphonates,[1] which under mild conditions can be converted into the natural phosphorodiesters.[2] This approach has recently been successfully applied to the synthesis of DNA[3,4] and RNA[5] fragments *via* H-phosphonate intermediates.

However, in all methods of oligonucleotide synthesis,[6] which involve activation of a nucleotidic component by a condensing agent, some side-reactions between the coupling agent and the starting materials or products, are observed.[7,8]

This prompted us to investigate the reaction of 5'-O-dimethoxytrityl-thymidine 3'-H-phosphonate with suitably protected nucleosides having a free 3'-hydroxyl group (DMT-dG^{isb}-OH, DMT-dA^{bz}-OH, DMT-dC^{bz}-OH, DMT-T-OH) in the presence of various coupling agents. These condensations should be slower, and therefore competing side-reactions should be more pronounced than in the corresponding, normal condensation of a 3'-hydrogenphosphonate with a 5'-hydroxyl group. To evaluate the stability of the

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hydrogenphosphonate diester bonds under the condensation conditions, model reactions of 5'-O-dimethoxytritylthymidine 3'-H-phosphonate with 3'-O-benzoylthymidine, in the presence of large excesses of condensing agents and during prolonged reaction times, were also investigated.

RESULTS AND DISCUSSION

During oligonucleotide synthesis *via* the phosphorotriester approach, coupling reagents, i. e. aryl sulfonyl derivatives may react not only with the phosphate center of nucleotides but also with other nucleophiles present,[6,7] causing sulfonation of a hydroxyl function in the nucleosidic component[8,9] and/or modification of heterocyclic bases.[7] However, since these side-reactions are much slower than the condensation (usually several hours *versus* 10-60 min condensation time), they can practically be neglected in syntheses of medium size oligonucleotides.[6,10]

In this respect, synthesis of oligonucleotides *via* the hydrogenphosphonate approach seems to be safer because of even shorter condensation time (ca 1-2 min).[1,3-5] This was clearly demonstrated using benzenesulfonyl chloride, which was found to promote fast H-phosphonate diester formation without any noticeable sulfonation of the hydroxylic component.[1]

Thus, the only sites for side-reactions during oligonucleotide synthesis *via* the H-phosphonate approach should be the phosphorus centers, with P-H bonds, and the guanine residues having the rather reactive heteroaromatic lactam systems.[11] The latter source of side-reactions can be eliminated *via* O^6-protection of guanosine;[12,13] however in the present studies O^6-unprotected guanosine derivatives were used in order to evaluate to what extent the lactam function may interfere in oligonucleotide synthesis *via* the H-phosphonate approach.

Aryl sulfonyl derivatives as coupling agents

The condensation of 5'-O-dimethoxytritylthymidine 3'-H-phosphonate **1** with suitably protected nucleoside **2a** in the presence of 3 equiv. of an arenesulfonyl derivative (TPS-Cl, BS-Cl, TPS-Te) in pyridine afforded, after ca 2-3 min (time necessary to record the ^{31}P NMR spectrum), the H-phosphonate diesters **3a** as the sole reaction product (see Table 1 and 2).

The reaction of nucleoside H-phosphonate monoesters with aryl sulfonyl chlorides, reported by Hata et al.[14a] and further investigated

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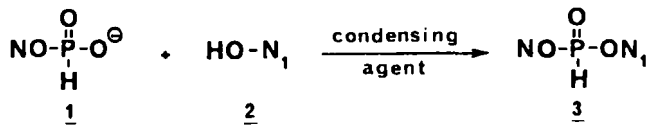
Table 1



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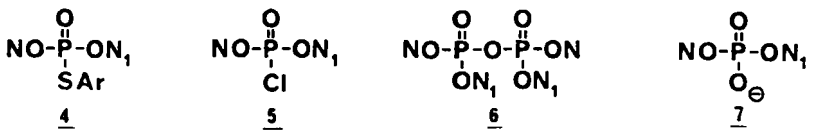

4 5 6 7

1, N=DMT-T-

2a, N_1=-T-OBz

2b, N_1=DMT-T-

2c, N_1=DMT-dG^{isb}-

2d, N_1=DMT-dA^{bz}-

2e, N_1=DMT-dC^{bz}-

3a, 5, 6, 7, N=DMT-T-, N_1=-T-OBz

3b, N=N_1=DMT-T-

3c, N=DMT-T-, N_1=DMT-dG^{isb}-

3d, N=DMT-T-, N_1=DMT-dA^{bz}-

3e, N=DMT-T-, N_1=DMT-dC^{bz}-

3f, N=DMT-T-, N_1=DMT-dG^{isb}(O^6-diphenylphosphoryl)

3g, N=DMT-T-, N_1=DMT-dG^{isb}(O^6-pivaloyl)

4a, N=DMT-T-, N_1=-T-OBz, Ar=2,4,6-triisopropylphenyl

4b, N=DMT-T-, N_1=-T-OBz, Ar=phenyl

4c, N=DMT-T-, N_1=-T-OBz, Ar=4-nitrophenyl

Abbreviations

For protected nucleosides, abbreviations as suggested by UIPAC-IUB (1970), Biochemistry, 9, 4022. For other compounds : 2,4,6-triisopropylbenzenesulfonyl chloride and tetrazolide, TPS-Cl and TPS-Te respectively; benzenesulfonyl chloride and 4-nitro derivative, BS-Cl and NO_2BS-Cl respectively; pivaloyl chloride, PV-Cl; diphenylphosphorochloridate, DPCP; bis-oxazolidonylphosphorochloridate, OXPC

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by us,[14b] has been shown to afford nucleoside 3'-phosphates and nucleoside S-aryl 3'-phosphorothioates as final products. This reaction is, however, apparently much slower than the coupling reaction of H-phosphonate monoesters with nucleosides, and we did not observe any oxidation products from the H-phosphonate monoester 1 in the reaction mixtures, using ^{31}P NMR spectroscopy. However, since we suspected that the product of coupling (i. e. H-phosphonate diesters) may undergo subsequent oxidation by aryl sulfonyl derivatives, we carried out condensations in the presence of 5-10 molar excess of TPS-Cl.

With 5 equiv. of TPS-Cl, the coupling 1 + 2a was as clean as in the reaction with 3 equiv. of the condensing reagent, but after ca 10 min a small signal (ca 5%) in the ^{31}P NMR spectrum at -13.1 ppm (multiplet) appeared. Addition of another 5 equiv. of the condensing reagent resulted in further oxidation, and after 1 h ca 40% of H-phosphonate diester 3a had been oxidized and converted into species with ^{31}P NMR resonances at ca: 24 ppm (two singlets), 4 ppm (two singlets) and -13 ppm (multiplet, six signals). On the basis of chemical shifts, splitting patters and chemical reactivity, we assigned these signals to: 5'-O-dimethoxytritylthymidine(3'-5')-3'-O-benzoylthymidine S-triisopropylphenyl phosphorothioate (two diastereoisomers, 24.4 and 24.2 ppm, compound 4a), 5'-O-dimethoxytritylthymidine(3'-5')-3'-O-benzoylthymidine phosphorochloridate (two diastereoisomers, 4.0 and 3.4 ppm, compound 5) and P^1,P^2 (5'-O-dimethoxytritylthymidine(3'-5')-3'-O-benzoylthymidine) pyrophosphate (multiplet, six signals at -13.2 ppm, compound 6). The structures of the oxidation products were additionally confirmed by independent synthesis of 4b, 5 and 6.

When other aryl sulfonyl chlorides were used as coupling agents, similar patterns of signals were observed in the ^{31}P NMR spectra, with the exception that the signals for 4a were replaced by two singlets at 22.9 and 22.5 ppm (compound 4b, in the reaction with BS-Cl), or by two singlets at 21.2 and 20.9 ppm (compound 4c, in the reaction with pNO_2-BS-Cl). Upon addition of water to these reaction mixtures, the signals at ca 24 ppm (compounds 4a, 4b or 4c) remained unchanged while those at ca 4 and -13 ppm were replaced by a resonance at -1.1 ppm (phosphorodiester 7). The ratio of 4 to 7 in all reaction mixtures after hydrolysis was 1:2.

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Reaction of H-phosphonate 1 with the nucleoside 2a in the presence of TPS-Te (10 equiv.), followed a similar pathway and after 1 h, all H-phosphonate diester 3a had been oxidized and converted into 4a and 6. As expected, we did not observe any ^{31}P NMR signals ca at 4 ppm, which we previously assigned to the chlorophosphate 5.

To evaluate the influence of N-methylimidazole and triethylamine on the rate of condensation and the subsequent oxidation of H-phosphonate diester 3a, we carried out the reaction 1 + 2a with TPS-Cl in the presence of these bases.

Both N-methylimidazole and triethylamine had rather little effect on the rate of condensation, but they accelerated the rate of oxidation substantially. With 5 equiv. of TPS-Cl and 10 equiv. of N-methylimidazole, ca 50% of 3a had been oxidized after 15 min, and converted into 4a and 6. In a similar experiment when N-methylimidazole was replaced by triethylamine, the rates of oxidation of the starting material and of the product 3a were even higher and became comparable to the rate of condensation. The ^{31}P NMR spectrum in that case revealed the presence of 3a together with its oxidation products as well as oxidation products of 1.[14b] In agreement with these findings, oxidation was almost 3 times slower when the coupling was carried out with 5 equiv. of TPS-Cl in the presence of 10 equiv. of pyridinium hydrochloride. With this large excess of pyridinium hydrochloride, we did not observe (^{31}P NMR) any formation of phosphorothioate 4a, but only the chlorophosphate 5 and pyrophosphate 6. After the additon of water only phosphorodiester 7 was detected.

The experiments discussed above indicate that the condensation of H-phosphonate 1 with nucleoside 2a, in the presence of arenesulfonyl derivatives in pyridine, is substantially faster than the oxidation of both starting material and the product of coupling. However, the rates of oxidation may become comparable to the rate of condensation if a strong base is present in the reaction mixture. This indicates that abstraction of the proton from H-phosphonate diesters may be the limiting step during their oxidation. This is in agreement with other studies on the oxidation of H-phosphonates.[15]

The ratio of compounds 4, 5 and 6 in the reaction mixtures varied, depending on the amount of condensing agent, oxidation time, and the reaction conditions. However, the ratio of phosphorothioates 4 to the

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phosphorodiester 7 after hydrolysis was usually 1:2. This seems to indicate that, as it was found previously for the reaction of H-phosphonate monoesters with aryl sulfonyl derivatives,[14b] the sulfonyl derivatives are reduced to sulfinyl and sulfenyl derivatives during the course of oxidation and that these react faster than the sulfonyl compound with the H-phosphonate diester.

Formation of chlorophosphate 5 during the oxidation was expected in light of our studies on the reaction of pyrophosphates with aryl sulfonyl chlorides.[16] However, the reaction pathway that leads to the pyrophosphate 6 is not all together clear and further studies are in progress.

Chlorophosphates as condensing agents

Two types of chlorophosphates were investigated as coupling reagents for the H-phosphonate diester formation: diphenylphosphorochloridate (DPCP)[1] and bis-oxazolidonylphosphorochloridate (OXPC).[17]

The coupling reactions of 1 + 2a-e in pyridine in the presence of DPCP (3 equiv.) proceeded rapidly (reactions were over after recording the first spectra, ca 2 min) and afforded H-phosphonate diesters 3a-e as the sole nucleotidic material (^{31}P NMR and TLC analysis). However, in reactions with the guanosine nucleoside (reaction 1 + 2c --- 3c) additional resonances (8.3 and 8 ppm), downfield from the original signals from 3c (7.9 and 7.4 ppm), appeared after 15 min. Addition of another 3 equiv. of DPCP to the reaction mixture resulted in further increase of the former signals and in the gradual decrease of the resonances from 3c. At the same time an additional signal at -19.5 appeared in the ^{31}P NMR spectrum. After 20 min, ca 75% of 3c was converted into the new intermediate. The ^{31}P NMR spectrum without ^{1}H-heteronuclear decoupling showed that this new compound still had a P-H bond ($^{1}J_{PH}$=715 Hz) and the splitting pattern (four triplets, $^{3}J_{PH}$=8.5 and 7.4 Hz) indicated on the H-phosphonate diester. The singlet at -19.5 ppm remained unchanged in the undecoupled spectrum and thus can be assigned to the phosphorus nuclei in a diphenyl phosphorotriester. We interpreted these findings as an indication for O^{6}-phosphorylation of the guanine residue[18] in 3c by diphenylchlorophosphate which resulted in the formation of 3f. As a support for such an interpretation, we have found that 5',3',N^{2}-triisobutyryldeoxyguanosine reacts with DPCP (1 equiv.) in pyridine yielding presumable the O^{6}-phosphorylated derivative[18] (singlet at -20.0 ppm in

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Table 2. ^{31}P NMR data for compounds 1, 3, 4, 5, 6 and 7.

Compound	Chemical shift (ppm)*	$^{1}J_{PH}$ (Hz)**	$^{3}J_{PH}$ (Hz)**
1	2.5	618	8.6 (two doublets)
3a	8.1	716	8.5 (four quartets)
	9.6	718	
3b	7.3	714	8.6 (two triplets)
3c	7.9	714	7.9 (two triplets)
	7.4	716	8.6 (two triplets)
3d	7.4	715	7.3 (two triplets)
	7.2	714	8.6 (two triplets)
3e	7.3	715	7.3 (two triplets)
	7.2	718	8.6 (two triplets)
3f	8.3	714	8.5 (two triplets)
	8.0	713	7.3 (two triplets)
3g	8.2	714	8.6 (two triplets)
	7.9	713	7.3 (two triplets)
4a	24.4;24.2	-	7.8 (two quartets)
4b	22.9;22.5	-	7.8 (two quartets)
4c	21.2;20.9	-	7.8 (two quartets)
5	3.4;3.9	-	9.3 (two quartets)
6	12.9;13.0; 13.1;13.2; 13.3;13.4	-	(not estimated)
7	-1.1	-	8.1 (quartet)

*In pyridine as solvent. Chemical shifts relative to 2% H_3PO_4 in D_2O.
**Spectra without ^{1}H-heteronuclear decoupling.

the ^{31}P NMR spectrum). After addition of water this compound underwent hydrolysis, which was completed after 1h, and TLC analysis revealed recovery of the starting material, triacylated deoxyguanosine.

When OXPC was used as a condensing agent in the reaction 1 + 2c, the final product of the condensation, 3c, did not undergo any changes during 1 h in the presence of an excess of condensing agent (9 equiv.). This is in agreement with the phosphorylating properties of OXPC. This reagent (1 equiv.) was found to be unreactive towards 5',3',N^2-triisobutyryldeoxyguanosine in pyridine, during 1 h, as revealed by ^{31}P NMR spectroscopy.

From these studies, it seems that bis-oxazolidonylphosphorochloridate is a mild but still efficient condensing reagent which can be used in the H-phosphonate diester synthesis with less risk of heterocyclic base modification. Diphenylphosphorochloridate, on the other hand, being a more reactive coupling agent can cause guanine base modification and thus does not offer any advantage over the former, milder agent.

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Pivaloyl chloride as a condensing agent

Pivaloyl chloride (PV-Cl) was found to be the coupling reagent of choice in the automated solid phase synthesis of DNA[3,4] and RNA[5] fragments via the H-phosphonate approach.

The coupling reactions 1 + 2a-e in the presence of PV-Cl (3 equiv.) were fast and the ^{31}P NMR spectra were indistinguishable from those when arenesulfonyl derivatives or chlorophosphates were used as condensing agents.

As in the reaction with DPCP, we observed a subsequent reaction of the condensing reagent (PV-Cl) with the guanine residue. In a typical experiment, when 1 and 2c were reacted in pyridine in the presence of PV-Cl (3 equiv.), the ^{31}P NMR spectrum revealed (after 2 min) only the presence of H-phosphonate diester 3c. After another 15 min reaction time, however, additional signals (ca 5%), downfield from 3c appeared. These signals (8.2 and 8.0 ppm) became the predominant ones when another 6 equiv. of PV-Cl were added and the reaction was left for another 15 min. Both signals showed a large coupling constant (715 Hz), characteristic for the P-H bond and a small one (four triplets, $^3J_{PH}$=8.0 Hz) indicating on the dinucleoside H-phosphonate diester, probably with a modified guanine residue (compond 3g). Addition of water to such a

reaction mixture showed rather slow conversion into 3c. After 1 h the reaction was almost completed but during that time traces of H-phosphonate internucleotidic bond cleavage were detected.

TLC analysis of a model reaction showed that 5',3',N^2-triisobutyryldeoxyguanosine reacts in pyridine with pivaloyl chloride yielding presumably the O^6-acylated derivative.[18,19] This compound hydrolysed slowly in water-pyridine (ca 1 h) and more rapidly in 2% ammonia in aqueous pyridine (a few minutes) to afford the starting nucleoside (TLC analysis).

These results would indicate that reaction of guanosine with pivaloyl chloride during oligonucleotide synthesis does not pose any serious problem. The acyl groups (most likely O^6-acyl) can be removed from the guanine residues during final deprotection with ammonia, and also this acyl group introduced in situ may serve as a guanosine protecting group during the oligonucleotide synthesis. It remains to be proved if guanosine without initial O^6-protection can be used for the preparation of oligoguanylic nucleotides via the H-phosphonate approach.

We also carried out some experiments to find out if a reactive species generated from H-phosphonate monoester 1 and PV-Cl may react with the P-H bonds present in the H-phosphonate diesters. Thus, H-phosphonate 1 was activated with PV-Cl (3 equiv.) in pyridine in the presence of diethyl hydrogenphosphonate. No reaction between H-phosphonate monoester and H-phosphonate diester was detected by ^{31}P NMR spectroscopy.

We also could not detect, using ^{31}P NMR spectroscopy, any reaction at the phosphorus center of H-phosphonate diesters with PV-Cl durimg 30 min. However, it should be remembered that such a reaction may occur[20] with a large excess of a condensing agent, especially in the presence of strong nucleophilic catalysts. For example, 5'-O-dimethoxytritylthymidine 3'-methyl hydrogenphosphonate was found to be completely resistant towards acetic anhydride (6 equiv.) in pyridine during 1 h, but the addition of 3 equiv. of 4-dimethylaminopyridine (DMAP) caused rapid formation of a compound showing absence of P-H bond (two signals at 8.0 and 6.9 ppm in the ^{31}P NMR spectrum). This compound, presumably acylphosphonate[20], can be converted back into the H-phosphonate diester, however, probably not without partial degradation of internucleotidic bonds.[20] It is particularly important to be aware about such a reaction

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since acetic anhydride/DMAP or acetic anhydride/N-methylimidazole are routinely used as capping reagents in the phosphorotriester and the phosphoroamidite approaches to oligonucleotide synthesis.

CONCLUSIONS

These studies have shown that the rate of H-phosphonate diester formation using arenesulfonyl derivatives, chlorophosphates and acyl chlorides, is high enough to ensure clean and practically quantitative formation of dinucleoside H-phosphonates.

However, to evaluate the utility of various condensing agents, distinction should be made between the "solution" and "solid phase" synthesis of oligonucleotides.

In the former one, equimolar amounts of nucleotidic and nucleosidic components are used together with 2-3 equiv. of coupling reagents.[1] Under such conditions the reaction can be stopped by quenching with aqueous buffers long before any side-products (arising from the subsequent reactions) can be detected. Thus, all coupling agents investigated during these studies seem to be potentially useful in solution synthesis of oligonucleotides.

On the other hand, in the solid phase synthesis, when 10-20 molar excess of a nucleotidic component and 50-100 molar excess of a coupling agent are used,[3-5] the subsequent reactions of H-phosphonate diesters with condensing agents can probably not be completely excluded. For these reasons, aryl sulfonyl derivatives may be unsuitable as condensing agents, because they cause oxidation of H-phosphonate esters with a concomitant formation of phosphorothioates 4.

The modifications of guanine residues, especially that caused by pivaloyl chloride, are not disadvantageous from a synthetic point of view, since they are reversed during the final deprotection. Because PV-Cl does not produce any detectable amount of acylphosphonates (reaction at the P-H center) during the condensation time, this reagent is considered as safe and efficient both in solution and solid phase synthesis of oligonucleotides via the H-phosphonate approach.

Finally, it should be mentioned that bis-oxazolidonylphosphorochloridate also seems to be a promising condensing agent. It was found to be the only one which does not produce any side-products, even when used in large excess. At the same time, it promotes fast and efficient formation of H-phosphonate diesters.

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EXPERIMENTAL PART

Materials and methods

5'-O-dimethoxytritylthymidine 3'-hydrogenphosphonate (1, triethylammonium salt)[1], 2,4,6- triisopropylbenzenesulfonyl tetrazolide[21], bis-oxazolidonylphosphorochloridate[22] and all suitably protected nucleosides[23] were prepared according to published procedures.

Diphenylphosphorochloridate, pivaloyl chloride, benzenesulfonyl chloride, 4-nitrobenzenesulfonyl chloride, 2,4,6-triisopropylbenzenesulfonyl chloride, N-methylimidazole (Aldrich) and acetic anhydride, 4-dimethylaminopyridine (Merck) were commercial grades.

Anhydrous pyridine and triethylamine were purified as described previously[1].

^{31}P NMR spectra were recorded on Varian Associates XL-100 FT (40.48 MHz) or on Jeol JNM GX 400 FT (161.7 MHz) spectrometer. All chemical shifts are reported relative to 2% H_3PO_4 in D_2O (inner tube).

The following compounds were synthesized in order to compare with reaction products or intermediates observed during reactions. 5'-O-dimethoxytritylthymidine(3'-5')-3'-O-benzoylthymidine phosphorodiester was prepared *via* oxidation of 3a with 2% I_2 in pyridine-water (98:2)[2,3] and found to be identical with compound 7. P^1,P^2-(5'-O-dimethoxytritylthymidine(3'-5')-3'-O-benzoylthymidine) pyrophosphate 6 was prepared from 7 in a reaction with 3 equiv. of TPS-Cl. 5'-O-dimethoxytritylthymidine(3'-5')-3'-O-benzoylthymidine S-phenyl phosphorothioate 4b was synthesized from 5'-O-dimethoxytritylthymidine and S-phenylphosphorochloridate (1.1 equiv.) in pyridine, followed by the addition of 3'-O-benzoylthymidine. 5'-O-dimethoxytritylthymidine(3'-5')-3'-O-benzoylthymidine phosphorochloridate 5 was prepared in a analogous way using $POCl_3$ instead of S-phenylphosphorochloridate.

General procedure for condensations (synthesis of 3a-e)

Compound 1 (0.1 mmol) and 2 (0.1 mmol) were rendered anhydrous by repeated evaporations of added pyridine, and the resulting foam was dissolved in pyridine (2.5 ml). An appropriate condensing reagent (3 equiv. or as stated in the text) was added and ^{31}P NMR spectra were recorded directly after mixing of the reagents.

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