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Supplementary Material Available: Physical and spectroscopic characterizations of **9** and **10** and complete, tabulated data from the NOE experiments performed on **5** and its C-1 diastereomer (1 page). Ordering information is given on any current masthead.

A Study on the Alkylsilyl Groups in Oligoribonucleotide Synthesis

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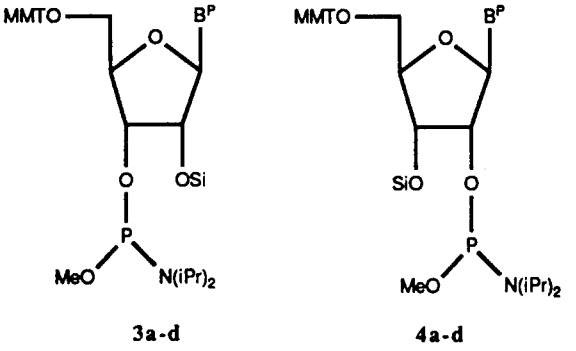
A detailed study has been carried out to show that the fidelity of the 3'-5' phosphate linkage is preserved during oligoribonucleotide synthesis when alkylsilyl groups are used to protect the 2'-hydroxyl groups. The isomeric purity of the 2'-silylated ribonucleoside 3'-phosphoramidites (**3a–d**), the key intermediates in oligoribonucleotide synthesis, have been established by comparing them with the 3'-silylated ribonucleoside 2'-phosphoramidites (**4a–d**) using ^1H and ^{31}P NMR spectroscopy. Using these 3'-amidites, a series of natural dinucleotides (A_pU, C_pU, G_pU, U_pU) were synthesized in solution. Isomeric dinucleotides with 2'-5' phosphate linkages (A_pU, C_pU, G_pU, U_pU) were prepared using the 3'-silylated nucleoside 2'-phosphoramidites. The intermediates during the syntheses and the final products were characterized by ^1H and ^{31}P NMR spectroscopy and HPLC. Comparison of the data from these two series of compounds provided unambiguous evidence for the fidelity of phosphate linkages in both the intermediates and the final products. To complete the comparison, a dinucleotide (U_pU) was prepared on a solid support.

Introduction

Some time ago, we introduced the alkylsilyl groups, principally *tert*-butyldimethylsilyl (TBDMS) and triisopropylsilyl (TIPS) groups, as 2'-hydroxyl protecting groups in oligoribonucleotide synthesis.[1] We extensively investigated the alkylsilyl groups in oligoribonucleotide synthesis both in solution and on the solid support using the phosphite triester coupling procedures.[2,3] We recently adapted the phosphoramidite coupling procedure, originally developed for the oligodeoxyribonucleotide synthesis,[4] to oligoribonucleotide synthesis on solid supports using the alkylsilyl groups as the 2'-protecting group.[5] Following this strategy, we were able to achieve the total chemical synthesis of an RNA molecule whose sequence corresponded to that of a 77-unit *E. coli* tRNA.[6]

To this point synthetic RNAs have been characterized in a number of ways. Enzymatic sequencing of synthetic RNA molecules indicated the correct ribonucleotide sequences.[5a,6] Synthetic RNAs were found to have the distinctive biological activity of natural RNAs. For example, the synthetic analogue of a tRNA retains some amino acid acceptance activity[6] even without the incorporation of modified bases common to the natural tRNA. Recently, several ribozymes have been synthesized.[7] These catalytic RNAs do not have rare bases in their sequences. Synthetic ribozymes have an activity comparable to those made by biochemical methods.[8] These examples have clearly demonstrated the success of the silyl protecting groups in oligoribonucleotide synthesis. Others have also described the successful use of the silyl protecting groups in oligoribonucleotide synthesis using the H-phosphonate coupling procedure.[9]

Scheme I. Ribonucleoside 3'-Phosphoramidites and 2'-Phosphoramidites



3a-d **4a-d**

a: B^P = N^6-benzoyladenine, Si = TBDMS
b: B^P = N^4-benzoylcytosine, Si = TBDMS
c: B^P = N^2-phenoxyacetylguanine, Si = TIPS
d: B^P = uracil, Si = TBDMS

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Table I. ^{1}H NMR Data[a] and R_f Values[b] of 2′-Silylated Ribonucleosides (1a–d) and 3′-Silylated Ribonucleosides (2a–d)

compd	H1′ (J, Hz)	H2(5)	H8(6)	OCH_3	t-Bu	MeSi	SiMe	R_f
1a	6.08 (d, 5.4)	8.23	8.71	3.78	0.82	−0.03	−0.17	0.60
2a	6.05 (d, 5.0)	8.25	8.77	3.77	0.88	0.08	−0.01	0.36
1b	5.94 (s)	8.48	7.88	3.82	0.93	0.32	0.20	0.56
2b	6.06 (d, 2.6)	8.42	7.87	3.81	0.81	0.02	−0.10	0.14
1c	5.94 (d, 5.8)		7.93	3.76	0.99	0.92	0.90	0.52
2c	5.92 (d, 4.3)		7.95	3.77	0.97	0.97	0.97	0.24
1d	5.92 (d, 3.1)	5.25	7.92	3.80	0.92	0.17	0.15	0.70
2d	5.93 (d, 4.1)	5.35	7.50	3.79	0.84	0.05	−0.06	0.46

[a] Signals are referenced to $CDCl_3$. [b] TLC solvent: solvent D.

Despite these facts, there have been some arguments in the literature about the stability of the silyl groups as 2′-protecting groups in the oligoribonucleotide synthesis.[10-12] As a result, we provide further evidence that silyl groups are effective as the 2′-hydroxyl protecting group in the phosphoramidite coupling procedure in oligoribonucleotide synthesis. We have synthesized a series of 3′-5′ dinucleotides (A_pU, C_pU, G_pU, U_pU) using 2′-silylated nucleoside 3′-*O*-methylphosphoramidites in solution. Isomeric dinucleotides with 2′-5′ phosphate linkages (A^pU, C^pU, G^pU, U^pU) were also synthesized using 3′-silylated nucleoside 2′-*O*-methylphosphoramidites for comparison. The intermediates in the synthesis of these two series of compounds were analyzed by ^{1}H NMR and ^{31}P NMR spectroscopy and HPLC. A uridine 3′-5′ dinucleotide was also prepared on a solid support, and this was compared with the other samples. The results confirm the stability (lack of migration) of alkylsilyl protecting groups during oligoribonucleotide synthesis.

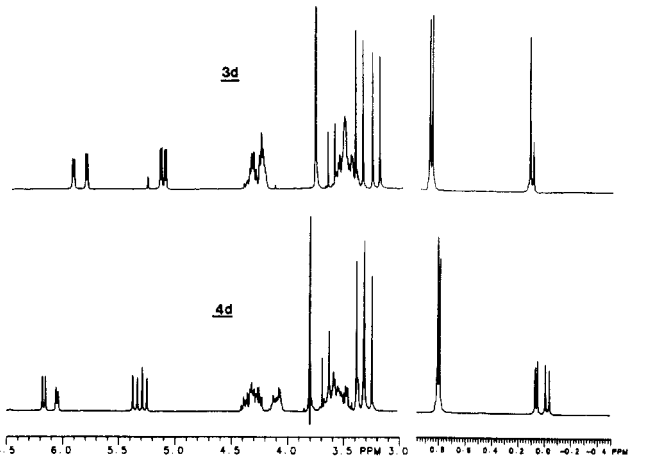

Figure 1. ^{1}H NMR spectra of uridine nucleoside 3′-phosphoramidite (**3d**) and uridine nucleoside 2′-phosphoramidite (**4d**).

Results and Discussion

Isomeric Purity of 2′-Silylated Nucleoside 3′-*O*-Phosphoramidites (3a–d). The key intermediates in our oligoribonucleotide synthesis are the 2′-silylated ribonucleoside 3′-phosphoramidites (**3a–d**, Scheme I) which were prepared by adding a dry THF solution of N-protected 5′-monomethoxytritylated 2′-silylated nucleosides (**1a–d**) to a dry THF solution of chloro(diisopropylamino)methoxyphosphine following the standard literature procedure.[5,13] The isomeric purity of the 3′-amidites is crucial to ensuring that only the 3′-5′ phosphate linkages will be introduced in the coupling step; they must, therefore, be free of 3′-silylated ribonucleoside 2′-phosphoramidites (**4a–d**). Obviously, this first requires that the protected nucleosides **1a–d** are isomericly pure, i.e. free of their corresponding isomers, the N-protected 5′-tritylated 3′-silylated ribonucleosides (**2a–d**). Compounds **1a–d** were prepared following previously described procedures.[2b,13] The silylation of N-protected 5′-tritylated nucleosides gives a mixture of **1a–d** and **2a–d** which are separated using routine silica gel column chromatography.[2b,13] All 2′-silylated nucleosides have higher R_f values than the corresponding 3′-silylated isomers in Solvent D on TLC (Table I). The purified (column chromatography) samples contain no trace of the undesired isomer on either TLC or HPLC. The ^{1}H NMR data of **1a–d** and **2a–d** are shown in Table I. The data suggest that the two isomers can be distinguished by their proton chemical shifts. The signal of the anomeric proton for each isomer is distinctive. The two isomers are also easily distinguishable through the chemical shifts of the silyl protons. No signal belonging to **2a–d** was observed in the ^{1}H NMR spectrum of **1a–d** and vice versa.

The 2′-silyl group in **1a–d** has to be stable under phosphorylation conditions in order to prepare the isomerically pure **3a–d**. The stability of the silyl groups in **1a–d** under various conditions has been extensively discussed.[14] We and others have found that the 2′-silyl group isomerizes in neutral protic solvents such as methanol and ethanol[15] and in wet pyridine or wet DMF.[16] On the other hand, the 2′-silyl group is stable in dry aprotic solvents such as chloroform or DMF.[14]

To establish the isomeric purity of the nucleoside 3′-phosphoramidites prepared under the phosphorylation conditions, the isomeric 3′-silylated nucleoside 2′-phosphoramidites (**4**) of the four nucleosides were prepared and compared to **3**. The preparation of these 2′-amidites (**4a–d**, Scheme I) is similar to that of the 3′-amidites. The precursors are the 3′-silylated nucleosides (**2a–d**) prepared above. Thus, a dry THF solution of **2a–d** was added to a dry THF solution containing a slight excess of chloro(diisopropylamino)methoxyphosphine in the presence of diisopropylethylamine and a catalytic amount of DMAP at room temperature. The phosphorylation is generally completed in 4–6 h with **2c** requiring a longer period (12 h). The products **4a–d** were isolated after silica gel column chromatography in yields of 70–80%.

The ^{1}H and ^{31}P NMR data for the diastereomeric pairs **3a–d** and **4a–d** are shown in Table II. Examination of the data in Table II suggests several interesting features. ^{1}H NMR indicates that the ribose protons and silyl protons

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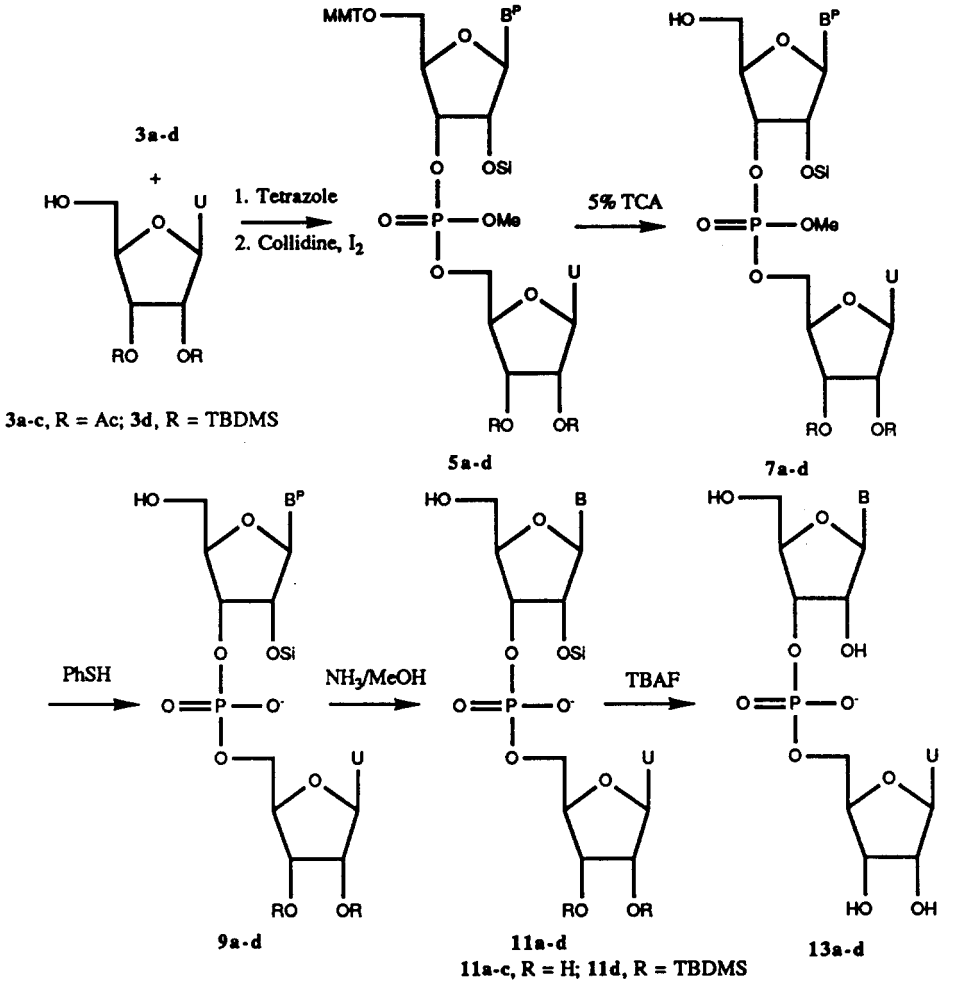
Scheme II. Preparation of 3′-5′ Dinucleotides



of a 3′-amidite have different chemical shifts from those of its corresponding 2′-isomer. In particular, the anomeric proton and the silyl protons in each amidite are distinctive. The anomeric proton of the 2′-amidite is shifted downfield compared to that of the corresponding 3′-amidite. This is probably related to the inductive effect of the 2′-phosphoramidite on the anomeric position. The ^{31}P NMR spectrum of a 3′-amidite is clearly distinguishable and, like that of the 2′-amidite, shows two peaks corresponding to a pair of diastereomers.

The ^{1}H NMR (Figure 1) and the ^{31}P NMR (Figure 2) spectra of the uridine nucleoside 2′-amidite (**4d**) and its 3′-amidite (**3d**) are presented. In addition to the above features, the proton spectra indicate that no silyl or anomeric proton signals corresponding to the 2′-amidite were detected in the spectrum of the 3′-amidite and vice versa. ^{31}P NMR analysis of an artificial mixture of **3d** and **4d** (Figure 2) prepared by mixing the two pure amidites shows the expected signals corresponding to the two isomers. The 3′-amidite is clearly free of isomeric contamination. The preparation of the isomericly pure 3′-amidite proves that the 2′-silyl group is stable under the phosphorylation conditions used.

In order to establish the fidelity of the phosphate linkage in the synthetic nucleotides, we synthesized and characterized a series of natural dinucleotides A_pU, C_pU, G_pU, and U_pU with 3′-5′ phosphate linkages (**13a–d**) using the nucleoside 3′-amidites. The synthesis of **13a–d** involves the same steps as those used in the solid-phase synthesis (Scheme II), i.e., assembling the chain to obtain the protected dinucleotide (**7a–d**); removal of phosphate and base protecting groups to produce the 2′-silylated dinucleotide (**11a–d**); and finally, removal of the 2′-silyl group to give

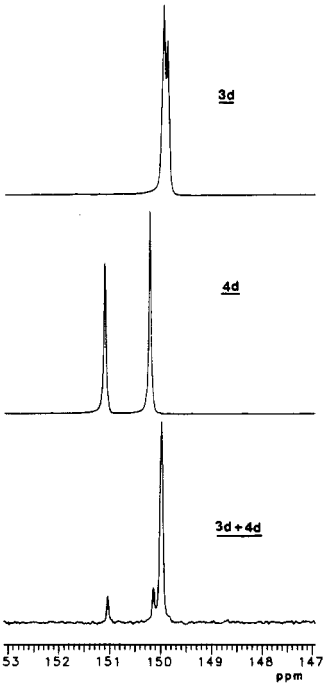


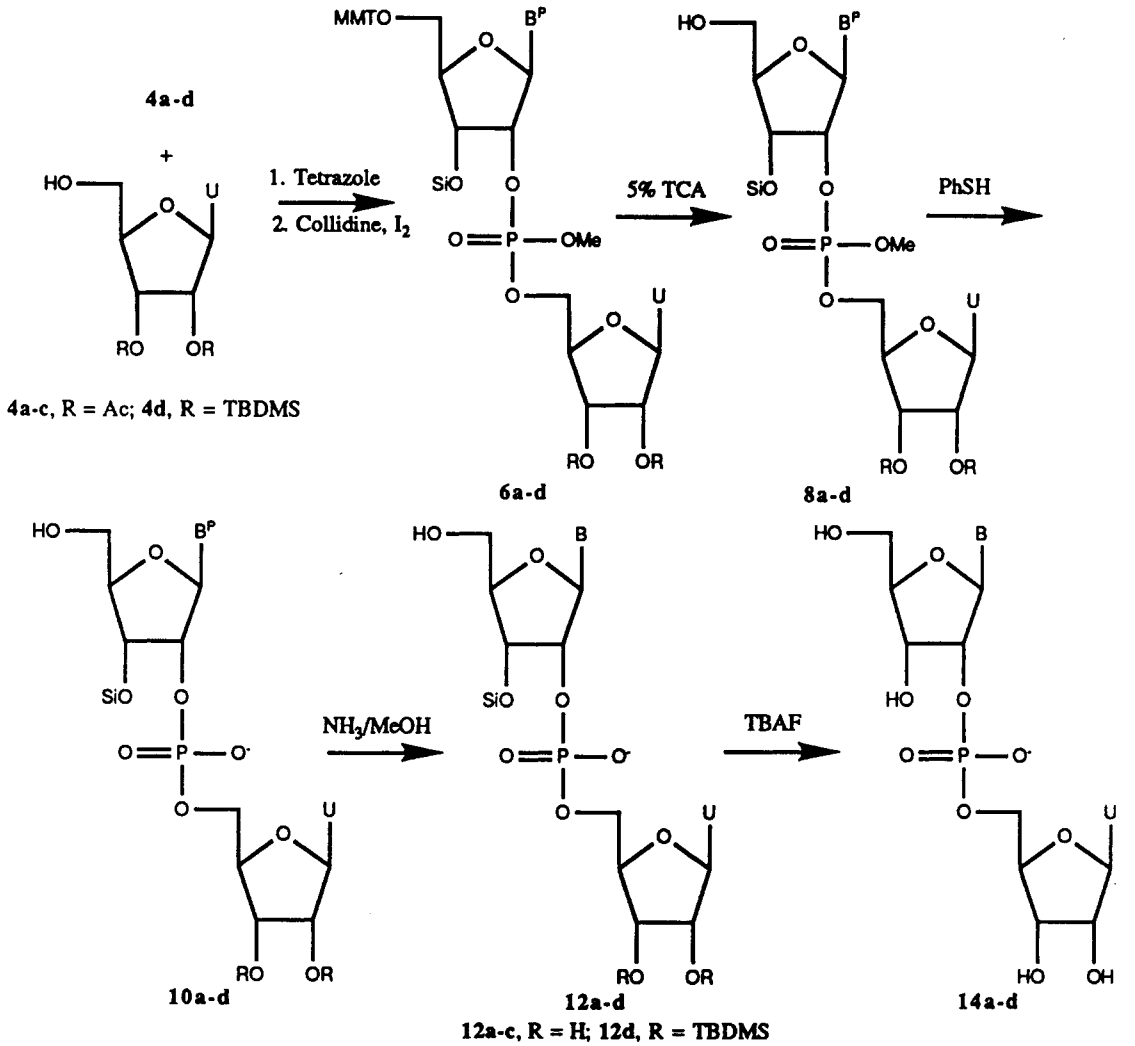
Figure 2. ^{31}P NMR spectra of uridine nucleoside 3′-phosphoramidite (**3d**), 2′-phosphoramidite (**4d**), and an artificial mixture of the two amidites (**4d** + **3d**).

Table II. ^1H and ^{31}P NMR Data of 2′-Silylated Nucleoside 3′-Phosphoramidites (3a–d) and 3′-Silylated Nucleoside 2′-Phosphoramidites (4a–d)[a]

	^1H NMR chemical shifts								^{31}P
entry	H1′ ($J_{1'-2'}$)	H2(5)	H6(8)	$POCH_3$(J_{P-H})	OCH_3(MMT)	t-Bu	MeSi	SiMe	
3a	6.10 (d, 6.1)	8.23	8.71	3.21 (d, 13.1)	3.77	0.76	−0.01	−0.20	149.8
	6.03 (d, 6.0)	8.23	8.67	4.42 (d, 13.2)	3.77	0.75	−0.03	−0.24	151.7
4a	6.22 (d, 5.4)	8.21	8.73	3.30 (d, 13.1)	3.78	0.87	0.12	0.04	150.8
	6.17 (d, 5.5)	8.20	8.70	3.00 (d, 13.0)	3.77	0.85	0.08	0.03	150.3
3b	5.92 (d, 1.2)	7.84	7.84	3.23 (d, 13.1)	3.82	0.91	0.26	0.16	150.1
	5.84 (s)	7.84	7.84	3.33 (d, 13.1)	3.82	0.90	0.25	0.14	149.1
4b	6.26 (d, 3.5)	7.87	7.87	3.40 (d, 13.2)	3.82	0.77	0.03	−0.06	151.2
	6.08 (d, 1.4)	7.87	7.87	3.33 (d, 13.1)	3.81	0.74	0.02	−0.11	150.2
3c	6.03 (d, 6.0)		7.96	3.40 (d, 13.1)	3.77	0.97	0.97	0.97	152.3
	5.98 (d, 5.4)		7.94	3.14 (d, 13.0)	3.77	0.97	0.97	0.97	150.4
4c	6.11 (d, 6.4)		7.93	3.29 (d, 13.1)	3.77	1.09	1.06	1.02	152.2
	6.05 (t)		7.93	3.07 (d, 13.1)	3.77	1.09	1.06	1.02	150.9
3d	5.94 (d, 3.7)	5.16	8.07	3.40 (d, 13.1)	3.79	0.90	0.13	0.13	150.4
	5.83 (d, 3.1)	5.12	8.04	3.25 (d, 13.1)	3.79	0.88	0.13	0.11	150.3
4d	6.16 (d, 5.4)	5.34	8.01	3.34 (d, 13.0)	3.79	0.81	0.07	−0.01	151.1
	6.03 (t)	5.26	7.82	3.27 (d, 13.0)	3.79	0.79	0.05	−0.04	150.2

[a] Note that each compound exists as a pair of diastereoisomers.

Scheme III. Preparation of 2′-5′ Dinucleotides



the fully deprotected diribonucleotide (**13a–d**). In solution synthesis, unlike solid-phase synthesis, the product must be isolated by chromatography or precipitation at each step. The fully protected dinucleotides containing the 2′-5′ phosphate linkage (**14a–d**) were synthesized in the same manner but using **4a–d** (Scheme III).

Preparation of Protected 3′-5′ Dinucleotides (7a–d). The condensation of activated **3a–d** with 2′,3′-protected uridine, followed by the aqueous oxidation with I_2 gave the fully protected dinucleotides (**5a–d**). Similarly, the fully protected dinucleotides with 2′-5′ phosphate linkages (**6a–d**) were synthesized using **4a–d**. The ^{31}P and ^1H NMR data of **5a–d** and **6a–d** are shown in Table III. The ^{31}P NMR spectrum shows that each dinucleotide has two phosphorus signals corresponding to a pair of diastereomers. The 3′-5′ dinucleotides have phosphorus chemical

Table III. ^{1}H NMR and ^{31}P NMR Data of Fully Protected Dinucleotides (5a–d, 6a–d)[a]

	^{1}H NMR Chemical Shifts,[b] ppm													
	X[c]			U			POMe	OMe						
	H1′	H2(5)	H8(6)	H1′	H6	H5	(J_{P-H}, Hz)	(MMT)	OAc	OAc	*t*-Bu	MeSi	SiMe	^{31}P
5a	6.04–	8.23	8.67	6.04–	8.01	5.76	3.82 (d, 11.4)	3.78	2.14	2.07	0.72	–0.06	–0.28	0.1
	6.18	8.28	8.63	6.18	8.04	5.78	3.76 (d, 11.4)	3.78	2.12	2.07	0.69	–0.08	–0.29	–0.5
6b	6.28–	8.26	8.72	5.95–	8.04	5.75–	3.76 (d, 11.4)	3.77	2.07	2.02	0.86	0.12	0.03	0.0
	6.32	8.25	8.70	6.40	8.03	5.71	3.58 (d, 11.4)	3.76	2.07	2.03	0.85	0.11	0.03	–0.03
5b	5.92–	7.18	8.20	5.92	8.20	5.62–	3.73 (d, 11.3)	3.82	2.10	2.07	0.90	0.22	0.13	0.1
	5.99	7.18	8.20	5.99	8.20	5.70	3.60 (d, 11.2)	3.81	2.09	2.07	0.89	0.23	0.13	0.2
6b	6.14–	7.62	8.43–	6.14–	7.86	5.73–	3.84 (d, 11.4)	3.83	2.10	2.05	0.75	0.07	–0.11	0.3
	6.20	7.79	8.56	6.20	7.86	5.82	3.89 (d, 11.4)	3.82	2.09	2.03	0.73	0.05	–0.09	–0.6
5c	6.11 (d)		7.93	5.85–	7.52	5.85–	3.80 (d, 11.4)	3.76	2.11	2.07	0.97	0.88	0.86	1.4
	6.16 (d)		7.93	5.91	7.60	5.91	3.63 (d, 11.3)	3.75	2.10	2.07	0.97	0.88	0.86	0.4
3c	6.15 (d)		7.91	5.80–	7.20–	5.66	3.55 (d, 11.3)	3.77	2.09	2.07	1.02	1.00	0.99	0.4
	5.85		7.91	5.89	7.40	5.70	3.70 (d, 11.4)	3.75	2.08	2.07	1.02	1.00	0.99	0.2
5d	5.94–	5.65–	7.81	5.94–	7.50–	5.65–	3.62 (d, 11.3)	3.79			0.87–		0.05–	0.6
	6.00	5.70	7.95	6.00	7.60	5.70	3.65 (d, 12.3)	3.65			0.89		0.13	0.3
6d	6.09–	5.75–	7.68	6.09–	8.00	5.75	3.81 (d, 11.4)	3.80			0.77–		–0.03	0.8
	6.13	5.80	7.68	6.13	8.00	5.80	3.79 (d, 11.3)	3.80			0.89		0.07	0.3

[a] Note that each compound exists as a pair of diastereoisomers. [b] – indicates a range (e.g. for **5a** the H1′ for both diastereomers is found in the range 6.04–6.18 ppm). [c] The number in the parentheses indicates the proton for X on a pyrimidine base, the unbracketed number refers to purine proton.

Table IV. ^{1}H NMR and ^{31}P NMR Data of Detritylated Dinucleotides (7a–d, 8a–d)[a]

	^{1}H NMR chemical shifts,[b] ppm												
	X[c]			U									
	H1′	H2(5)	H8(6)	H1′	H6	H5	POMe, $J_{(P-H}$, Hz)	OAc	OAc	*t*-Bu	MeSi	SiMe	^{31}P
7a	6.00–	8.35	8.79	6.00–	8.02	5.99	3.86 (d, 11.4)	2.15	2.05	0.67	–0.21	–0.4	0.4
	6.18	8.20	8.79	6.18	8.02	5.95	3.86 (d, 11.4)	2.13	2.05	–0.69	–0.18	–0.45	–0.9
8a	6.03–	8.17	8.76	5.73–	8.09	5.92	3.60 (d, 11.4)	2.06	2.02	0.93	0.15	0.15	–0.3
	6.16	8.22	8.74	5.78	8.07	5.89	3.60 (d, 11.4)	2.02	1.98	0.93	0.14	0.14	–0.7
7b	5.79–	7.49	8.31	6.03–	7.89	5.68	3.81 (d, 11.3)	2.12	2.07	0.89	0.16	0.08	1.2
	5.77	7.49	8.43	5.72	7.89	5.69	3.81 (d, 11.4)	2.09	2.03	0.87	0.11	0.08	0.1
8b	5.98–	7.67	8.23	5.98–	7.89	5.78	3.90 (d, 11.3)	2.08	2.03	0.90	0.13	0.11	–0.3
	6.11	7.67	8.32	6.11	7.89	5.78	3.78 (d, 11.4)	2.06	2.00	0.89	0.11	0.10	–0.4
7c	5.75–		8.00	5.75–	7.32–	5.75–	3.86 (d, 11.4)	2.13	2.09	0.84–			0.3
	5.99		8.00	5.99	7.48	5.79	3.86 (d, 11.4)	2.13	2.08	0.89			0.1
8c	5.96–		7.80	5.11–	7.45	5.61–	3.65 (d, 11.4)	2.12	2.07	1.09–			0.3
	6.02		7.80	5.84	7.97	5.84	3.52 (d, 11.3)	2.09	0.08	1.11			0.3
7d	5.63–	5.52–	7.58	5.63–	7.88	5.52–	3.81 (d, 11.4)			0.87–	0.05–		1.7
	5.75	5.59	7.58	5.75	7.88	5.75	3.81 (d, 11.4)			0.88	0.12		1.6
8d	5.83–	5.64–	7.65	5.83–	7.75	6.64–	3.79 (d, 11.3)			0.86–	0.04		0.6
	5.99	5.87	7.65	5.99	7.75	5.87	3.77 (d, 11.3)			0.91	0.16		0.0

[a,b,c] Same as for Table III.

shifts different from those of the corresponding 2′-5′ linked dinucleotides. The proton chemical shifts of the silyl group are also diagnostic in distinguishing the two isomeric nucleotides. The 2′-silyl protons of the purine nucleoside in a 3′-5′ linked dinucleotide (**5a**, **5c**) have chemical shifts at a higher field than the 3′-silyl protons of the purine nucleoside in the corresponding 2′-5′ linked nucleotide (**6a**, **6c**). For the pyrimidine nucleotides (**5b**, **5d** vs **6b**, **6d**) the shifts are reversed. The ^{1}H NMR and ^{31}P NMR data of each dinucleotide are free from any trace of its isomer.

Compounds **5a–d** and **6a–d** were then detritylated to give **7a–d** and **8a–d**, respectively, by treatment with an excess of 5% trichloroacetic acid in CH_2Cl_2 for half an hour at room temperature. Excess acid was then neutralized by aqueous sodium bicarbonate solution, and the product was purified either by silica gel chromatography or simply by precipitation in ether. **7a–d** and **8a–d** were also characterized by ^{1}H NMR and ^{31}P NMR spectroscopy (Table IV). The ^{31}P chemical shifts and the proton chemical shifts of the silyl group, of each trityl-free dinucleotide, are diagnostic in differentiating isomeric nucleotides. It is worth noting that in a detritylated dinucleotide the 2′-silyl protons in the 3′-5′ dinucleotide appear at higher field compared to the 3′-silyl protons in the corresponding 2′-5′ dinucleotides. The chemical shifts of 2′-silyl protons in the detritylated 3′-5′ dinucleotide are at a higher field compared to those of the corresponding tritylated dinucleotide (Tables III and IV). On the other hand, the chemical shifts of 3′-silyl protons in a detritylated 2′-5′ dinucleotide are at a lower field as compared to the corresponding tritylated dinucleotide. Apparently the 5′-trityl group has a shielding effect on the 3′-silyl group in the 2′-5′ dinucleotide (**6a–d**) and has a deshielding effect on the 2′-silyl group in the 3′-5′ dinucleotide (**5a–d**). The ^{31}P NMR spectra of **7a** and **8a** are shown in Figure 3. It is obvious that **7a** is isomerically pure as no signal belonging to **8a** was found. These results further confirm that the oligoribonucleotides assembled using 2′-silylated nucleoside 3′-*O*-phosphoramidites have the correct phosphate linkage.

Isomeric Purity of 2′-O-Silylated Dinucleotides (11a–d). We next analyzed the intermediates in the deprotection of the assembled nucleotides. The first step is to remove the methyl phosphate protecting group. Thus compounds **7a–d** and **8a–d**, typically 10–50 mg, were treated with excess thiophenoxide in an Eppendorf tube to give N-protected 2′-silylated 3′-5′ dinucleotides (**9a–d**) and 2′-5′ dinucleotides (**10a–d**), respectively. TLC of the reaction mixture in solvent B indicated that the starting material was converted to a very polar compound. Excess

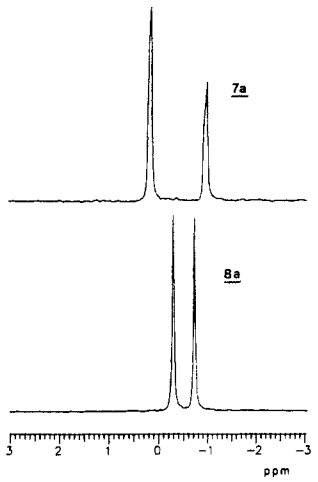


Figure 3. ^{31}P NMR spectra of **7a** and **8a**.

Table V. ^{31}P Chemical Shifts and HPLC Retention Times of N-Acylated 2′-O-Silylated Dinucleotides (9a–d, 10a–d)[a]

entry	^{31}P, ppm	t_R, min	HPLC solvent[b] (% CH_3CN in TEAA)
9a	−0.3	18.2	33
10a	−1.4	19.6	33
9b	0.3	11.4	30
10b	−0.9	9.8	30
9c	0.1	8.0	38
10c	−1.0	12.4	38
9d	0.7	19.8	45–57
10d	−0.7	18.8	45–57

[a] ^{31}P chemical shifts are reported as downfield positive to the external 85% H_3PO_4. HPLC conditions are described in experimental. [b] Either isocratic or linear gradient mode.

thiophenol was removed by precipitation of the crude product in hexane.

In contrast to all the ^{31}P spectra of previous triester intermediates (diastereomers), the ^{31}P NMR spectra of methyl deprotected dinucleotides all showed only one peak (Table V) as expected. It is further noted that the ^{31}P chemical shift of a 3′-5′ dinculeotide is downfield compared that of the corresponding 2′-5′ dinucleotide. No phosphorus signal (^{31}P NMR) belonging to the 2′-5′ dinucleotide was observed in the sample of a synthetic 3′-5′ dinucleotide.

Compounds **9a–d** and **10a–d** were also analyzed by HPLC on a reverse-phase C8 column. For each isomeric pair, an artificial mixture of the two was prepared by mixing the two individual samples. This was analyzed on HPLC to determine the HPLC conditions under which the two isomers could be cleanly separated. The retention time for each dinucleotide is shown in Table V. The isomeric dinucleotides have different retention times on HPLC. Each sample of either **9a–d** or **10a–d** appeared as one major elution peak on HPLC, and no peak corresponding to its isomer was detected. HPLC data further confirm the result from the ^{31}P NMR data that **9a–d** do not have any isomeric contamination.

Compounds **9a–c** and **10a–c** were next N-deacylated with methanolic ammonia to give the 2′-silylated 3′-5′ dinucleotides (**11a–c**) and 3′-silylated 2′-5′ dinucleotides (**12a–c**), respectively. This procedure also removed the terminal 2′,3′-acetyl protecting groups on the uridine nucleoside. This step is not required in the case of **9d** and **10d** since no base protecting group is used and the TBDMS group is used for the terminal 2′,3′-hydroxyl protection. The crude samples from the treatment were directly analyzed on a C8 reverse-phase column using isocratic acetonitrile in aqueous triethylammonium acetate buffer (pH 7) as the HPLC solvent. The retention times for all the samples are shown in Table VI. A 2′-silylated 3′-5′ dinucleotide and the corresponding 3′-silylated 2′-5′ dinucleotide have different retention times on HPLC and can be separated.

Table VI. HPLC Retention Times of 2′-Silylated Dinucleotides (11a–c, 12a–c)[a]

entry	t_R, min	HPLC solvent[b] (% CH_3CN in TEAA)
11a	13.7	17
12a	35.5	17
11b	10.6	20
12b	16.7	20
11c	6.5	26
12c	15.3	26

[a] HPLC conditions are described in the Experimental Section. [b] Isocratic elution.

Table VII. HPLC Retention Times of Fully Deprotected Dinucleotides (13a–d, 14a–d)[a]

entry	t_R, min	HPLc solvent[b] (% CH_3CN in TEAA)
13a	21.6	6
14a	17.0	6
13b	11.6	4
14b	8.9	4
13c	12.0	3
14c	7.5	3
13d	12.5	5
14d	10.4	5

[a] HPLC conditions are described in the Experimental Section. [b] Isocratic elution.

This part of the study has shown that the 3′-5′ phosphate linkages on the assembled nucleotide chain remain intact during removal of the phosphate and N-protecting groups. The 2′-O-silylated oligoribonucleotide, the key intermediate in the chemical synthesis of oligoribonucleotides, contains the correct 3′-5′ phosphate linkages.

Removal of 2′-Silyl Protecting Groups. Deprotecting the 2′-protecting group is always the last step in the synthesis of oligoribonucleotides. The natural oligoribonucleotide unit released during the deprotection of the 2′-position is so sensitive to general acid and base catalyzed hydrolysis that few protecting groups meet all of the criteria for synthesis, yet can be successfully removed during the final step. For example, in addition to the possible isomerization during the phosphorylation, another drawback of the acyl group as the 2′-protecting group is that its final removal under basic conditions may cause cleavage of the assembled nucleotide chain.[17] The TBDMS and TIPS groups can be easily removed by the fluoride ion under neutral conditions.[18] We use a THF solution (1 M) of tetrabutylammonium fluoride (TBAF) for deprotection.[3,5a] Thus dinucleotides **11a–d** and **12a–d** were dissolved in TBAF for 10–12 h to give the fully deprotected dinucleotides **13a–d** and **14a–d**, respectively. In the case of **11d** and **12d**, this also removed the terminal 2′,3′-hydroxyl protecting groups. The crude reaction mixture from this treatment was analyzed without purification on a Whatman C8 reverse-phase column, and the HPLC results are shown in Table VII.

The 3′,5′ dinucleotide has a longer retention time than the corresponding 2′-5′ dinucleotide (Table VII) in each

(17) Griffin, B. E.; Jarman, M.; Reese, C. B.; Sulston, J. E.; Trentham, D. E. *Biochemistry* **1966**, *5*, 3638–3649.
(18) Corey, E. J.; Venkateswarlu, A. *J. Am. Chem. Soc.* **1972**, *94*, 6190–6191.

isomeric pair. A sample of **13d** (U_pU) from the synthesis was also compared with a sample of U_pU (3′-5′) from Sigma Chemical Co. by HPLC, and the HPLC profile of the synthetic sample was found to be superimposable on that of the commercial sample.

Solid-Phase Synthesis of a Uridine Dinucleotide. The solid-phase synthesis of oligoribonucleotides using nucleoside 3′-phosphoramidites follows the same steps as the solution synthesis[3,5,6] except that an extra capping step is needed in solid-phase synthesis to deactivate the unreacted 5′-hydroxyl group at the end of each cycle. The procedures for deprotection are the same as those in solution synthesis. A uridine 3′-5′ dinucleotide was assembled using the uridine 3′-amidite and deprotected using the deprotection conditions as previously described.[5] The HPLC profile of the crude reaction product showed the 3′-5′ linked dinucleotide as the only nucleotide product. The HPLC of the sample from the solid-phase synthesis was also superimposable on the commercial sample of 3′-5′ U_pU from Sigma.

Conclusion

This study of a series of dinucleotides has provided rigorous proof that oligoribonucleotide synthesis using *tert*-butyldimethylsilyl or triisopropylsilyl group as the 2′-protecting group is reliable within the limits (<0.1%) of ^{31}P NMR, ^{1}H NMR, and HPLC detection. The 2′-silylated nucleoside 3′-*O*-phosphoramidite, the key intermediate for ribonucleotide synthesis, is prepared free of the isomeric 3′-silylated nucleoside 2′-*O*-phosphoramidite. The 2′-silyl groups are thus stable under the conditions used in the preparation of the nucleoside phosphoramidites. The nucleotide intermediate at each stage of the synthesis has the correct phosphate linkage. The present study, together with previous biochemical results, fully establishes the fidelity of the alkylsilyl groups as 2′-hydroxyl protecting groups in the chemical synthesis of RNA.

Experimental Section

Materials and Methods. ^{1}H NMR spectra were recorded on a Varian XL-200 spectrometer in $CDCl_3$. Proton chemical shifts were reported with reference to the internal residual solvent signal at 7.26 ppm. ^{31}P NMR spectroscopy was performed on a Varian XL-300 spectrometer in $CDCl_3$. Phosphorus signals were referenced to external 85% phosphoric acid (downfield positive). UV spectra were recorded on a HP 8451A spectrometer. Melting points were taken on a Fisher-Johns melting point apparatus and reported uncorrected.

HPLC analysis was carried out on a Spectra Physics 8000 chromatographic system equipped with a single wavelength UV detector (254 nm). The system can be operated in either an isocratic or a gradient mode. The instrument was run at 22 °C with a flow of 1 mL/min. A Whatman Partisil 5 C8 (4.6 × 250 mm) column was used for all the analyses. HPLC-grade acetonitrile (Caledon Lab., Gorgetown, Ontario) and double-distilled water were used. HPLC buffer (triethylammonium acetate, 0.1 M, pH 7) was prepared by diluting the stock solution of triethylammonium acetate (1 M, 100 mL) with double distilled water to 1 L. The pH of the solution was adjusted with glacial acetic acid or triethylamine on a Corning 125 pH meter. The solution was filtered through Millapore 0.45 μm filter paper before use. Stock triethylammonium acetate (1 M) was prepared by slowly adding triethylamine (139 mL, distilled) to a magnetically stirred, ice-cold aqueous solution (500 mL) of glacial acetic acid (57 mL). The resulting solution was diluted to 1 L as stock (1 M).

Thin-layer chromatography (TLC) was performed on Merck Kieselgel 60 F_{254} analytical sheets. TLC plates were developed in 5% methanol in methylene chloride (solvent A), 10% methanol in methylene chloride (solvent B), 20% ethyl ether in methylene chloride (solvent C), or 50% ethyl ether in methylene chloride (solvent D) and visualized under a single-wavelength (254-nm) UV source. Flash preparative silica gel column chromatography was performed on columns packed with E. M. Kieselgel 60 (230–400 mesh) silica gel (20 g per gram of sample).

Pyridine, diisopropylethylamine, dioxane, and collidine were distilled over calcium hydride. THF used for silylation, phosphorylation, and condensation was dried over molecular sieves (12 h) before it was refluxed over sodium and benzophenone to generate a distinct purple solution. It was freshly distilled before use. Hexane, ethyl acetate, and triethylamine used for preparative silica gel column chromatographic solvents were distilled. Methylene chloride, methanol, and ethyl ether were used as reagent grade from commercial sources. *tert*-Butyldimethylsilyl chloride, triisopropylsilyl chloride, and chloro(diisopropylamino)methoxylphosphine were purchased from Aldrich and used as they were. Tetrazole was obtained from Aldrich and kept over P_2O_5 in a desiccator. Standard 3′-5′ diuridine nucleotide was obtained from Sigma.

A detritylation solution (5% Cl_3CCOOH in CH_2Cl_2) was prepared by dissolving trichloroacetic acid (25 g, Aldrich) in methylene chloride (500 mL, dried over 4A molecular sieve). The oxidation solution (0.1 M I_2) was prepared by dissolving the solid I_2 (10.2 g) in water (134 mL) and THF (266 mL). Dioxane, triethylamine and thiophenol (Aldrich) were mixed in a ratio of 2/2/1 (volume) for methyl phosphate deprotection. Methanolic ammonia was prepared by bubbling ammonia in ice-cold methanol (20 mL) for 15–20 min and was used immediately. Tetra-*n*-butylammonium fluoride (TBAF) was obtained as a 1 M solution in THF (Aldrich) and used as is. N-Protected 5′-tritylated nucleosides used for the preparation of **1a–d** and **2a–d** were prepared following the described procedure.[2a,b,13] The silylation reaction was carried out as described.[2b,13] The 3′-phosphoramidites (**3a–d**) were prepared as previously described.[5,13] The 2′-phosphoramidites (**4a–d**) were prepared by the same procedures: **4a** (76%, mp 90–92 °C), **4b** (74%, mp 87–90 °C), **4c** (77%, mp 79–81 °C), and **4d** (88%, 81–83 °C). Solvents used for flash chromatographic isolation of the products were hexane/EtOAc/Et_3N (65/35/3) for **4a** and (70/30/3) for **4b**; CH_2Cl_2/EtOAc/Et_3N (65/30/5) for **4c** and (50/48/2) for **4d**. NMR data for series **3** and **4** are compiled in Table II.

The phosphorylation and condensation reactions (scale: ~1.25 g of nucleoside **1** or **2**) were carried out in Hypovials (Pierce). The vials were dried in the oven (120 °C, 12 h) and cooled with Argon. The nucleoside or nucleotide intermediates were dried on the vacuum line (4 mmHg) at room temperature for >12 h. The aqueous nucleotide solution was lyophilized in a Speed-Vac concentrator (Savant Instruments).

Synthesis of Protected Dinucleotides (5a–d, 6a–d). Compounds **5** and **6** were prepared by the standard procedure as illustrated for **5a**: 2′,3′-Bis-*O*-(acetyl)uridine (131 mg, prepared by detritylation of 5′-*O*-(monomethoxytrityl)-2′,3′-bis-*O*-(acetyl)uridine), **3a** (440 mg, 1.2 equiv), and tetrazole (134 mg, 4 equiv to **3a**) were transferred to a dry hypovial. Dry THF (3 mL) was introduced into the vial. After the reaction was stirred at ambient temperature for 3 h, collidine (250 μL) was added to the solution, followed by the dropwise addition of an aqueous iodine solution (0.1 M, 7/3 water/THF) until a dark brown color solution persisted. The solution was stirred for another 10 min and then transferred to CH_2Cl_2 (50 mL). The organic solution was extracted with saturated sodium chloride solution (50 mL) containing 5% sodium bisulfite (5 mL), dried with sodium sulfate, and evaporated under reduced pressure. The crude product was purified on a flash silica gel column eluted with 4% MeOH in CH_2Cl_2 to give 0.34 g of pure **5a** (73%; mp 118–121 °C; UV (95% EtOH) λ_{max} (nm) 210, 278; R_f 0.24 in solvent A).

Compound **6a** was obtained in 71% yield (mp 111–112 °C; UV (95% EtOH) λ max (nm) 210, 278; R_f 0.33 in solvent A).

Compound **5b** was obtained in 63% yield after flash chromatography using 50% EtOAc in CH_2Cl_2 and 4% methanol in CH_2Cl_2 (mp 119–122 °C; UV (95% EtOH) λ max (nm) 208, 263, 306; R_f 0.31 in solvent A).

Compound **6b** was obtained in 60% yield after flash chromatography using 5% methanol in CH_2Cl_2 (mp 123–125 °C; UV (95% EtOH) λ max (nm) 206, 263, 306; R_f 0.26 in solvent A).

Compound **5c** was obtained in 56% yield after flash chromatography using 3% methanol in CH_2Cl_2 (mp 119–121 °C; UV (95%

EtOH) λ max (nm) 210, 263; R_f 0.29 in solvent A).

Compound **6c** was obtained in 54% yield after flash chromatography using 4% methanol in CH_2Cl_2 (mp 115–117 °C; UV (95% EtOH) λ max (nm) 210, 263; R_f 0.26 in solvent A).

Compound **5d** was obtained in 66% yield after flash chromatography using successively 2%, 3%, and 4% methanol in CH_2Cl_2 (mp 103–106 °C; UV (95% EtOH) λ max (nm) 208, 264; R_f 0.37 in solvent A).

Compound **6d** was obtained in 76% yield in the same manner as for **5d** (mp 120–123 °C; UV (95% EtOH) λ max (nm) 208, 263; R_f 0.27 in solvent A).

Preparation of Detritylated Dinucleotides (7a–d, 8a–d). A standard procedure was used for the detritylation reactions and is illustrated for **7a**. A solution of trichloroacetic acid in CH_2Cl_2 (5%, 25 mL) was transferred to a round-bottom flask containing **5a** (300 mg). After stirring for 20 min at room temperature the solution was poured into CH_2Cl_2 (25 mL). The organic solution was then extracted with 5% aqueous sodium bicarbonate solution (2 × 50 mL) and dried over Na_2SO_4, and the solvents were removed at reduced pressure. The residue was redissolved in minimum amount of CH_2Cl_2 and precipitated in Et_2O (50 mL) to give pure **7a** as a white powder in 61% yield (140 mg; mp 130–133 °C; UV (95% EtOH) λ max (nm) 210, 278; R_f 0.65 in solvent B).

Compound **8a** (52% yield; mp 126–129 °C; UV (95% EtOH) λ max (nm) 210, 278; R_f 0.65 in solvent B).

Compound **7b** (precipitated in hexane/Et_2O (2/1, 30 mL); 55% yield, mp 130–133 °C; UV (95% EtOH) λ max (nm) 208, 263, 306; R_f 0.59 in solvent B).

Compound **8b** (precipitated in hexane/Et_2O (1/1, 30 mL); 60% yield; mp 132–135 °C; UV (95% EtOH) λmax (nm) 206, 263, 306; R_f 0.65 in solvent B).

Compound **7c** (isolated by silica gel column chromatography using 5% methanol in CH_2Cl_2; 65% yield; mp 119–121 °C; UV (95% EtOH) λ max (nm) 210, 263; R_f 0.59 in solvent B).

Compound **8c** (precipitated in Et_2O (50 mL); 51%; mp 117–120 °C; UV (95% EtOH) λ max (nm) 210, 278; R_f 0.55 in solvent B).

Compound **7d** (isolated by silica gel column chromatography eluting first with EtOAc/Et_2O/CH_2Cl_2 (4/1/2) followed by 5% methanol in CH_2Cl_2; 82%; mp 122–125 °C; UV (95% EtOH) λ max (nm) 208, 264; R_f 0.59 in solvent B).

Compound **8d** (isolated by silica gel chromatography using 4% methanol in CH_2Cl_2; 87%; mp 108–110 °C; UV (95% EtOH) λ max (nm) 208, 264; R_f 0.59 in solvent B).

HPLC Analysis of Intermediates during the Deprotection. The general procedure is illustrated by the deprotection of **7a** to give **13a**.

(a) Removal of Methyl Phosphate Protection (Preparation of 9a–d, 10a–d). A solution of thiophenoxide (dioxane/Et_3N/thiophenol, 2/2/1, 1.2 mL) was transferred to an Eppendorf tube containing **7a** (45 mg). After the solution was allowed to stand for 1 h at room temperature, TLC indicated that a very polar compound had formed (R_f 0.11 in 20% methanol in CH_2Cl_2). The reaction was quenched by the addition of 95% ethanol (1 mL). The solution was then concentrated under the reduced pressure, and the residue was redissolved in a minimum amount of CH_2Cl_2 and precipitated in hexane (50 mL). The precipitate was filtered to give **9a** (35 mg) as a white powder. This was used directly for ^{31}P measurement, HPLC analysis, and the next deprotection step without further purification. A portion (0.2 mg) of the sample was dissolved in 50% CH_3CN in triethylammonium acetate (100 μL, 0.1 M, pH 7) and used for HPLC analysis (10 μL was used for each analysis; HPLC conditions: column, Whatman C8 Partisil 5 (4.6 × 250 mm); solvent, isocratic or gradient CH_3CN in TEAA (0.1 M, pH 7); flow = 1 mL/min (Table V)). For ^{31}P NMR measurement, the sample (ca. 15 mg) was dissolved in $CDCl_3$ (see Table V).

(b) Removal of the *N*-Acyl Protecting Group (Preparation of 11a–c, 12a–c). Compound **9** (ca. 0.5 mg) was weighed into a 5-mL plastic tube. Methanolic ammonia (4 mL) was introduced to the tube. The tube was then capped with rubber septum (Aldrich) and sealed tightly with tape. After the mixture was allowed to stand at room temperature for 12 h, a needle was inserted to release the pressure inside the tube before it was exposed to the air. The solvent was evaporated by blowing Argon over it and then further lyophilized on a Speed-Vac concentrator. The residue was redissolved in water (1 mL) and divided into two Eppendorf tubes (0.5 mL each). Part of these was used for the next step. Another part was further diluted with water to 1 mL to give a solution of **11** for HPLC analysis. HPLC conditions: column, Whatman C8 Partisil 5 (4.6 × 250 mm); solvent, isocratic CH_3CN in TEAA (0.1 M, pH 7); flow = 1 mL/min (see Table VI).

(c) Removal of the 2'-Silyl Protecting Group (Preparation of 13a–d, 14a–d). A solution of **11** in one of the Eppendorf tubes described above was lyophilized on Speed-Vac. TBAF (100 μL) was added. After the solution was allowed to stand at room temperature for 4 h, the reaction was quenched with sterile water (0.5 mL). The solution was lyophilized and then redissolved in water (1 mL) to give a solution of **13** for HPLC analysis (10 μL was used for each injection; HPLC conditions: column, Whatman C8 Partisil 5 (4.6 × 250 mm); solvent, isocratic CH_3CN in TEAA (0.1 M, pH 7); flow = 1 mL/min (see Table VII)).

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Iminophosphorane-Mediated Synthesis of 2*H*-Indazole Derivatives: Preparation of 2,3-Diamino-2*H*-indazoles by Intramolecular Trapping of Phosphazides and 1*H*-1,2,4-Triazolo[2,3-*b*]indazoles by a Tandem Aza-Wittig/Heterocumulene-Mediated Strategy

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Treatment of *o*-azidobenzaldimines **2** with tertiary phosphines in methylene chloride at 0 °C leads to the corresponding 2,3-diamino-2*H*-indazole derivatives **3** by cyclization of the intermediate phosphazide. Compounds **3** react with isocyanates, carbon dioxide, and carbon disulfide to give the 1*H*-1,2,4-triazolo[2,3-*b*]indazoles **8**, **9**, and **10**, respectively. Compounds **8** can also be prepared from **2** in a one-pot reaction by sequential treatment with polystyryldiphenylphosphine and isocyanates. In tetrahydrofuran at room temperature, iminophosphoranes **3** react with acyl chlorides to form *N*-acyliminophosphoranes **11**, which, under acid catalysis, undergo cyclization to yield the fused indazoles **12**.

The reaction of a tertiary phosphine with an organic azide to produce an iminophosphorane after nitrogen evolution is known as the Staudinger reaction.[1] The primary imination products, phosphazides, are sometimes isolable

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