248. Nucleosides and Nucleotides. Part 18. *p*-Nitrobenzenesulfonyl Nitroimidazole as a New Condensing Agent in the Triester Synthesis of Oligonucleotides[1])

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Summary

p-Nitrobenzenesulfonyl nitroimidazole (*p*-NBSNI) (**5**) has been shown to be an efficient and convenient condensing agent in the coupling of protected nucleotide fragments. Its utility has been demonstrated by a direct comparison with previously-described condensing agents in the synthesis of various di- and tetradeoxynucleotides.

Introduction. – The current world-wide interest in gene recombination experiments has stimulated an unprecedented demand for synthetic oligonucleotides of defined sequence and high purity. Early attempts at the synthesis of such molecules were based on the so-called diester method [2], in which a phosphate monoester is coupled with an alcohol to give an anionic diester. Subsequently, the triester method was introduced as an alternative [3] [4], in which the phosphate carries a lipophilic protecting group. This approach is superior, as it overcomes some fundamental drawbacks of the diester method, and also allows standard chemical techniques to be applied to the purification of the neutral triester, notably extraction into an organic solvent (mostly chloroform), and chromatography on silica gel. The triester approach has now become the method of choice, despite the need for additional synthetic steps in introducing and removing the phosphate protecting group. However, when the triester method was first introduced, it was immediately obvious that more powerful reagents were needed for the activation of intermediate diesters. Side reactions between the condensing agent and the hydroxy component were also a serious problem. In particular, 2,4,6-triisopropylbenzenesulfonyl chloride (TPS, **1**), although widely used, caused sulfonation of the free 5′-hydroxy group of the incoming nucleotide [5], and of the O(6)-position of guanosine bases [6], and the hydrogen chloride liberated during the reaction destroyed purine bases [7]. Therefore, *Narang* and others introduced a range of arenesulfonamide condensing agents, successively imidazolides [8], triazolides (*e.g.* **2**) [7] [9] [10], tetra-

[1]) Part 17: [1].

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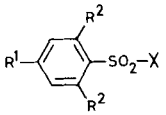
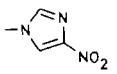


	R^1	R^2	X	Abbreviation
1	*i*-Pr	*i*-Pr	Cl	TPS
2	NO_2	H	**6**	*p*-NBST
3	*i*-Pr	*i*-Pr	**7**	TPSTe
4	CH_3	CH_3	**8**	MSNI
5	NO_2	H	**8**	*p*-NBSNI

X =   

T **6** Te **7** NI **8**

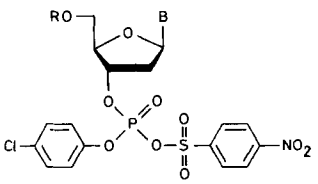
zolides (*e.g.* **3**) [11], nitroimidazolides (*e.g.* **4**) [5] [12], and nitrotriazolides [13] [14]. The reagents first tried gave cleaner reactions than **1** but reaction times were inconveniently long. Triazolides, particularly *p*-NBST (**2**), have been used with considerable success, but are still rather slow. Tetrazolides react very quickly, but are the least stable of the condensing agents so far tested, and must be freshly recrystallized before each reaction. The nitroimidazolides (NI) and nitrotriazolides (NT) were investigated in the search for highly reactive reagents which would be more stable than the tetrazolides, and hence more convenient for regular practical application. TPSNI proved to be rather ineffective for coupling large nucleotide blocks [13], and though nitrotriazolides can give good yields of condensed products in a short time, *Reese* [15] has reported side reactions occurring between these reagents and the O(6)-position of guanine residues; by-products in the reactions of *p*-TSNI had already been reported by *Gough* [12]. Most recently, *Jay* [16] has reported an attempt to overcome the problems associated with older condensing agents by using a mixture of TPS (**1**) and excess tetrazole (**7**) in place of unstable pre-synthesized tetrazolide (**3**).

In the course of a polynucleotide synthesis, we first used *p*-NBST (**2**) and MSNI (**4**), the former particularly when purine nucleotides were involved. Both are well-documented, can be purchased or easily prepared, are stable and have no serious drawbacks, even if they are not the fastest and most efficient reagents. More recently, we have also applied *Jay*'s **1**/tetrazole reagent.

***p*-Nitrobenzenesulfonyl-nitroimidazole** (*p*-NBSNI, **5**). – Throughout the work on arylsulfonylazolide condensing-agents virtually all the modifications and improvements have concentrated on adjusting the pK_a of the azolide leaving group in order to control reactivity [13]. In general, the aryl group has been 2,4,6-triisopropylphenyl or, less commonly, mesityl; these are designed to introduce sufficient steric hindrance to reduce side reactions, such as 5-sulfonation, but add nothing to the reactivity of the reagent. A notable exception is *p*-NBST (**2**), which has an electron-withdrawing nitro group on the aromatic ring. In this case, both halves of the molecule are activated, and capable of acting as good leaving-groups; thus the mixed phosphoric-sulfonic anhydride **9**, which is presumably an intermediate in the condensation, is highly reactive as well as being readily formed. Although unacceptably slow by modern standards, *p*-NBST (**2**) reacted approximately twice as fast as the corresponding mesitylene and triisopropylbenzene derivatives, and performs well in other respects. Our experiences with this reagent

in the synthesis of an oligonucleotide, especially with purine-rich compounds, led us to the view that nitrobenzenesulfonylazolides deserve further study.

These considerations led us to structure **5**, *p*-Nitrobenzenesulfonyl-nitroimidazole as a promising new condensing agent. This was expected to be a stable crystalline compound and more reactive than either **2** or **4**. It was synthesized by reaction of *p*-nitrobenzenesulfonyl chloride with nitroimidazole, in a similar manner to other nitroimidazolides [5] [17]. This simple preparation gave the required compound, stable at RT. and requiring no special protection from atmospheric moisture (a sample stored in a screw-cap bottle was analytically and spectroscopically pure after 6 months). This bis-nitroaromatic compound is highly crystalline and relatively insoluble, but it can be recrystallized from ethyl acetate and is reasonably soluble in polar solvents such as pyridine and DMSO. The ^{1}H-NMR. spectrum is very simple, since the four benzene ring protons coincide, and traces of sulfonic acid impurity are readily detectable as higher-field signals. A sample of *p*-NBSNI (**5**) dissolved in damp DMSO containing approximately 1% absorbed water was *ca.* 20% hydrolyzed after 40 min. In pyridine/water, **5** was completely hydrolyzed after 30 min by 50% water, and after 1 h by 10% water.



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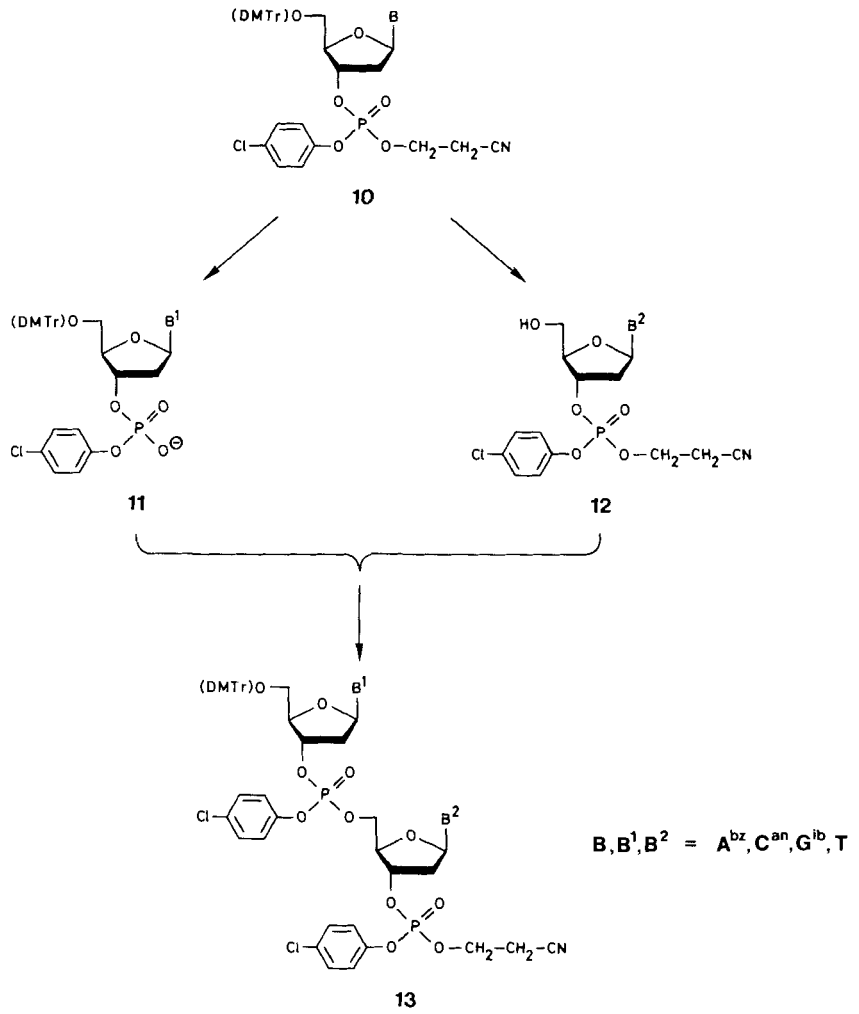
Coupling reactions. – At the beginning of our current work on oligonucleotide synthesis, we naturally based our strategy on established procedures, mainly on those of *Narang* and *Itakura* [3] [10]. Thus, the key intermediates are the fully-protected nucleotides **10**, with standard protecting groups [4]: benzoyl on adenine bases, anisoyl on cytidine, isobutyryl on guanine, monomethoxy- or dimethoxytrityl on the 5′-hydroxy groups, and cyanoethyl and 4-chlorophenyl on the phosphate group. From **10**, the anionic diester **11** can be prepared by treatment with triethylamine [18], and 2% benzenesulfonic acid [19] or zinc bromide [20] detritylates **10** to the 5′-hydroxy-compound **12**. The two deprotected nucleotides can then be coupled in pyridine by a suitable condensing agent to give the triester **13** *(Scheme)*. In practice, it is necessary to use a slight excess of the phosphodiester. To avoid the presence of moisture in solutions, the nucleotides were dried by repeated evaporation from anhydrous pyridine before addition of the condensing agent, and all manipulations and reactions were carried out under argon.

Coupling reactions were in all cases monitored by TLC. In early experiments, *p*-NBST (**2**) carried out condensations in 44 h or longer, MSNI (**4**) in 18 h. As expected, *p*-NBSNI (**5**) was faster than either, the reaction generally being complete within 8–12 h. The *Table* summarizes the results so far obtained. Reaction times up to 15 h are shown for *p*-NBSNI reactions, because it was convenient to carry out the condensation overnight; no extra side-products or loss of yield were ob-

Scheme[3])



served during this extended reaction period. The yields quoted are for chromatographically pure material. Purification was by short column chromatography (40–63 μ silica gel; methanol gradient in dichloromethane). We found this less trivial than other authors appear to have done, and in many cases two columns were needed to purify the product completely. Since the most serious contaminant is unreacted hydroxy-component, purification problems can of course be overcome by using a bigger excess of the diester, but this makes the synthesis more expensive and wasteful, thus replacing one problem by another. In an attempt to compromise,

[3]) Abbreviations: d- = 2′-deoxy, A = adenosine, C = cytidine, G = guanosine, T = thymidine, φ = *p*-chlorophenylphosphate, (CE) = 2-cyanoethyl, (MMTr) = 5′-*O*-monomethoxytrityl, (DMTr) = 5′-*O*-dimethoxytrityl, (bz) = 3′-*O*-benzoyl, an = N^4-anisoyl, bz = N^6-benzoyl, ib = N^2-*i*-butyryl.

Table[3])

Starting Materials			Condensing Agent (Equiv.)	Product	Isolated yield	Reaction time
10	Ratio	**12**				
d-(MMTr)$G^{ib}\varphi$(CE)	1:1	d-$G^{ib}\varphi$(CE)	*p*-NBST (4)	d-(MMTr)$G^{ib}\varphi G^{ib}\varphi$(CE)	65%	44 h
	1,05:1		*p*-NBSNI (2.5)		64%	15 h
d-(DMTr)$C^{an}\varphi$(CE)	1,1:1	d-C^{an}(bz)	MSNI (5)	d-(DMTr)$C^{an}\varphi C^{an}$(bz)	75%	18 h
	1,1:1		*p*-NBSNI (2.5)		83%	10 h
d-(DMTr)$A^{bz}\varphi$(CE)	1,2:1	d-$G^{ib}\varphi$(CE)	*p*-NBSNI (2.5)	d-(DMTr)$A^{bz}\varphi G^{ib}\varphi$(CE)	75%	15 h
			p-NBSNI (1.5)		40%	24 h
			TPS (3) + tetrazole (9)		66%	50 min
d-(DMTr)$G^{ib}\varphi$(CE)	1,3:1	d-$C^{an}\varphi$(CE)	*p*-NBSNI (2.5)	d-(DMTr)$G^{ib}\varphi C^{an}\varphi$(CE)	60%[a])	14.5 h
d-(MMTr)$G^{ib}\varphi G^{ib}\varphi$(CE)	1,2:1	d-$C^{an}\varphi T\varphi$(CE)	*p*-NBST (6.8)	d-(MMTr)$G^{ib}\varphi G^{ib}\varphi C^{an}\varphi T\varphi$(CE)	59%	64 h
			p-NBSNI (3.5)		65%	16 h
d-(DMTr)$A^{bz}\varphi G^{ib}\varphi$(CE)	1,05:1	d-$C^{an}\varphi C^{an}$(bz)	*p*-NBST (5)	d-(DMTr)$A^{bz}\varphi G^{ib}\varphi C^{an}\varphi C^{an}$(bz)	53%	44 h
	1,2:1		*p*-NBSNI (2.5)		71%	10 h
	1,2:1		TPS (3) + tetrazole (9)		71%	1.25 h

[a]) After preparative HPLC. Analytical HPLC. showed that a reaction carried out with TPS/tetrazole gave a very similar product mixture and almost identical yield.

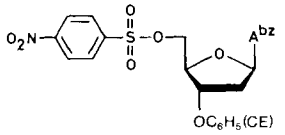
we chose an excess of diester component from 1.05 up to 1.3 mol-equiv., depending on the difficulty of the individual separation. Two purifications were particularly difficult. In the synthesis of d-(DMTr)$G^{ib}\varphi C^{an}\varphi$ (CE)[3]), the product had the same Rf as d-$C^{an}\varphi$ (CE), so after initial purification in the normal way, the product was isolated by preparative HPLC. In the sythesis of d-(MMTr)$G^{ib}\varphi G^{ib}\varphi C^{an}\varphi T\varphi$ (CE) an additional purification by preparative TLC. was necessary to remove some anionic material which could not be separated by column chromatography.

After early experiments with *p*-NBST (**2**) and MSNI (**4**), we carried out some comparative studies. In a direct comparison of **4** and *p*-NBSNI (**5**) in the synthesis of d-CC-dinucleotide under identical conditions, **5** gave a significantly cleaner reaction mixture (no visible sulfonation product as was observed with **4**) and a higher yield[4]). Subsequently, we compared the TPS (**1**)/tetrazole mixture [16] and found this to be similar in performance to **5**, in contrast to the older reagents, which gave darker mixtures and more complex TLC. than either **5** or **1**/tetrazole. The only major difference between the two new reagents is the rate of reaction, **1**/tetrazole reactions being complete in about 1 h, as shown in the *Table*. However, whilst the longer reaction time of **5** would obviously be a serious disadvantage in automatic synthetic procedures, the overnight reaction is quite convenient for carrying out a conventional reaction.

Side reactions. – There have been several reports of side reactions during condensations. Condensing agents can sulfonate the free 5′-hydroxy group of the nucleotide [5] [16] [21], and various side reactions can occur at the O(6)-position of guanine [6] [12] [15]. In the search for more reactive condensing agents, it is therefore necessary to take into account the nature and extent of the possible side reactions.

The majority of previous condensing agents contain bulky alkyl groups on the aromatic ring, intended to hinder such side reactions; *p*-NBSNI (**5**), however, is unsubstituted in the *ortho* position. Despite this, little or no sulfonation was observed during the course of the reactions listed in the *Table*. When **5** is added to a solution of the 5′-deprotected nucleotide **12** in pyridine, sulfonation takes place slowly, but this was not significant in the presence of a phosphodiester **11**.

Since *p*-nitrobenzenesulfonates are clearly much more reactive than the triisopropyl derivatives, it was hoped that it would be possible to hydrolyze any sulfonate which formed during the course of a condensation, in order to recover the starting material (**12**). However, when pure d-$(O_2NC_6H_4SO_2)A^{bz}\varphi$ (CE) (**14**) was dissolved in aqueous pyridine, no hydrolysis of the 5′-sulfonyl group was observed; after 2 days, only starting material and a base-line spot were visible on TLC.



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[4]) We are grateful to Mr. *D. Wallach* for carrying out these experiments in the course of his «Diplomarbeit».

Side reactions between *p*-NBSNI (**5**) and acyl-protected bases appear not to be a problem. Fully protected dG-, dA- and d(AG)-nucleotides were each stirred with 5 mol-equiv. of **5** in pyridine for 24 h; d-(DMTr)$G^{ib}\varphi$ (CE) showed partial decomposition to polar material, but no indication of derivatisation at the O(6)-position, and the other nucleotides appeared essentially unchanged on TLC.

The only reaction in which a significant by-product was observed was the synthesis of d-AG-dinucleotide. In addition to the desired product at Rf 0.50, a TLC. spot was clearly visible at Rf 0.73, exactly the same value as d-(DMTr)$A^{bz}\varphi$ (CE). NMR. showed that some of this starting material was indeed present – surprisingly, as TLC. had previously indicated complete decyanoethylation – but was mixed with another product, as yet unidentified. Significantly, a very similar by-product was formed in comparable amounts when the same reaction was carried out using **1**/tetrazole as the condensing agent, and in this case the compound contained a triisopropylbenzene substituent, clearly visible in the NMR. spectra. This side reaction is the subject of further investigation, but it is nevertheless possible to isolate a good yield of the desired dinucleotide.

Final remarks. – *p*-NBSNI (**5**) can be prepared easily, cheaply, on a large scale and can be stored at RT. for long periods. It can be used to carry out coupling reactions on a convenient time-scale, and gives isolated yields comparable with some of the best modern reagents, even for nucleotides containing purine bases. For a final valuation, further experiments have to be done, in particular with longer oligonucleotides, but on the evidence so far we consider that nitrobenzenesulfonyl derivatives deserve further study.

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Experimental Part

General. See [1], except for the following changes and additions: 4-nitrobenzenesulfonyl chloride and 2,4,6-triisopropylbenzenesulfonyl chloride (**1**) were purchased from *Fluka AG*, Buchs. Tetrazole and 4-nitroimidazole were purchased from *EGA-Chemie*, Steinheim. – Solvent systems for TLC.: A, CH_2Cl_2/CH_3OH 9:1 (for neutral compounds); B, CH_2Cl_2/CH_3OH 75:25 (for anionic compounds). – For preparative TLC. we used precoated silica gel plates 20×20 cm/2 mm from *Merck*. Before application, they were preeluted with CH_2Cl_2/CH_3OH 80:20, dried and equilibrated in air. – To avoid cleavage of the dimethoxytrityl group during column chromatography, columns were usually eluted with solvents containing 0.1% pyridine. – Preparative HPLC. was performed on a *Dupont* model 830 liquid chromatograph fitted with a 2×50 cm column of *LiChrosorb* 10 μ silica gel from *Dupont*. – Evaporation of solutions was performed on the rotatory evaporator at 30–40° and 14 of 0.5 Torr. After evaporation of moisture-sensitive solutions, the rotatory evaporator was filled with argon. – Protected nucleosides were prepared according to published procedures (for a review see [4]). Phosphorylation to give fully-protected nucleotides **10** was performed with *p*-chlorophenylphosphodichloridate [1] in pyridine with an excess of 3-hydroxypropionitrile [22]. Treatment of cyanoethyl compounds with triethylamine was performed according to [18]. Detritylation of 5′-dimethoxytrityl-protected compounds was carried out with 2% benzenesulfonic acid in $CHCl_3/CH_3OH$ 7:3 [19].

Preparation of p-*nitrobenzenesulfonyl-nitroimidazole* (**5**). To a suspension of 4-nitroimidazole (2.28 g, 20.2 mmol, 1.025 mol-equiv.) in dry dioxane (100 ml) cooled to 0°, *p*-nitrobenzenesulfonyl chloride (4.37 g, 19.7 mmol, 1 mol-equiv.) was added, then triethylamine (3 ml, 21.6 mmol, 1.1 mol-

equiv.) added slowly, producing a yellow coloration. Stirring was continued at 0° for 1 h, then at RT. for 14 h. Ethyl acetate (100 ml) was added, the mixture stirred for a further 20 min, then the white precipitate filtered off. This solid was stirred in ethyl acetate (200 ml) at reflux, and the resulting solution filtered, cooled, washed with water, dried (Na_2SO_4), combined with the bulk dioxane solution, and evaporated *i.v.* The crude product was recrystallized from ethyl acetate to give microscopic white or pale yellow needles (4.48 g, 74%), m.p. (sealed tube) 179–185° (dec.) (using a hot-stage microscope, softening of the crystals was observed at around 160°). – UV. (dioxane): 253 nm. – IR. (KBr disc): 1600, 1580, 1560, 1480, 1390, 1370, 1350, 1190 cm^{-1}. – ^{1}H-NMR. (60 MHz, D_6-DMSO): 8.45 (*s*, 4 H); 8.55 (*d*, J=2 Hz, 1 H); 9.10 (*d*, J=2 Hz, 1 H).

$C_9H_6N_4O_6S$ (298.2) Calc. C 36.25 H 2.03 N 18.79% Found C 36.35 H 1.93 N 18.97%

Triester condensations using **5**. a) *General procedure.* The fully protected nucleotide **10** (0.2–1.0 mmol, 1.05–1.3 mol-equiv.) was twice evaporated from dry pyridine at 30°/1 Torr, the rotatory evaporator being filled with argon after each evaporation. The resulting water-free nucleotide was dissolved in dry pyridine (7 ml/mmol nucleotide), an equal volume (*ca.* 50 mol-equiv.) triethylamine added, and the solution stirred under Ar in the dark for 6–15 h, until TLC. showed complete decyanoethylation. The solution was evaporated, 1 mol-equiv. 5′-deprotected nucleotide **12** added, and the mixture dried by twice evaporating from dry pyridine. The gum was dissolved in pyridine (*ca.* 7 ml/mmol **10**), 2.5 mol-equiv. **5** added, and the mixture stirred under Ar in the dark overnight. At this concentration, **5** did not dissolve completely. At the end of the reaction, 50% aq. pyridine (*ca.* 16 ml/mmol **10**) was added, giving a homogeneous solution, which was stirred for a further hour to hydrolyze **5**. After evaporation to a small volume, the solution was poured into aq. $NaHCO_3$-solution (2–5%) and extracted 3–4 times with CH_2Cl_2 (each phase *ca.* 300 ml/mmol **10** in each extraction). Where emulsions occurred, they were broken up by addition of a few percent of 2-propanol. Evaporation of the organic extracts gave the crude product, which was chromatographed on a short silica gel column (4×9 cm, particle size 40–63 μ; if less than 300 mg of product, 3×7 cm), eluted with a stepwise gradient of CH_3OH in CH_2Cl_2. Fraction volume: 50–200 ml. In most cases, the material so obtained was carefully chromatographed on a second column. After repeated evaporation from pure CH_2Cl_2 or $CHCl_3$, the product was isolated as a dry foam.

b) *d-(MMTr)G^{ib}φG^{ib}φ(CE):* 1.05 mol-equiv. of d-(MMTr)G^{ib}φ(CE), reaction time 15 h. The product was eluted TLC.-pure from the column with CH_2Cl_2/CH_3OH (95:5)→(94.5:5.5). Yield 65%.

c) *d-(DMTr)C^{an}φTφ(CE):* [22].

d) *d-(DMTr)C^{an}φC^{an}(bz)*[5]: 1.1 mol-equiv. of d-(DMTr)C^{an}φ(CE), reaction time 10 h. The product was purified by column chromatography (with CH_2Cl_2/CH_3OH 97.5:2.5), or by centrifugally accelerated prep. TLC. (2 mm layer of silica gel, CH_2Cl_2/CH_3OH 97.5:2.5). Yield 83%.

e) *d-(DMTr)A^{bz}φG^{ib}φ(CE):* 1.2 mol-equiv. of d-(DMTr)A^{bz}φ(CE), reaction time 15 h. The product was eluted from the column with CH_2Cl_2/CH_3OH (98:2)→(96:4), after an unidentified, less polar by-product. Yield 75%.

f) *d-(DMTr)G^{ib}φC^{an}φ(CE):* 1.3 mol-equiv. of d-(DMTr)G^{ib}φ(CE), reaction time 14.5 h. The crude product was first chromatographed on a short silica gel column (3–4% methanol), and then further purified by preparative HPLC. on 10 μ silica gel (solvent: CH_2Cl_2/CH_3OH/water 96:4:0.4; conditions: 34.4 bar, 18 ml/min). The peaks of the two pairs of diastereoisomers were collected (retention times: 12.0, 12.8, 15.6, 16.5 min). Yield 60%.

g) *d-(MMTr)G^{ib}φG^{ib}φC^{an}φTφ(CE):* 1.25 mol-equiv. of d-(MMTr)G^{ib}φG^{ib}φ(CE), reaction time 16 h. The product, containing some anionic material, was eluted with CH_2Cl_2/CH_3OH (96:4)→(92:8). After evaporation of solvent, it was applied as a concentrated solution in $CHCl_3$ to a prep. TLC. plate, which was eluted with CH_2Cl_2/CH_3OH 88:12. The pure product was extracted from the silica gel by washing it through a suction filter with CH_2Cl_2/CH_3OH 85:15. Yield 65%.

h) *d-(DMTr)A^{bz}φG^{ib}φC^{an}φC^{an}(bz):* 1.2 mol-equiv. of d-(DMTr)A^{bz}φG^{ib}φ(CE), reaction time 10 h. The product was eluted with 4–5% methanol, after removing some traces of by-products with 2–3% methanol. Yield 71%. After detritylation only a trace of d-C^{an}φC^{an}(bz) was found, with the same Rf as the fully protected tetramer.

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[5]) This experiment was carried out by *D. Wallach.*

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