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Tuning the Solubility of Soluble Support Constructs in Liquid Phase Oligonucleotide Synthesis

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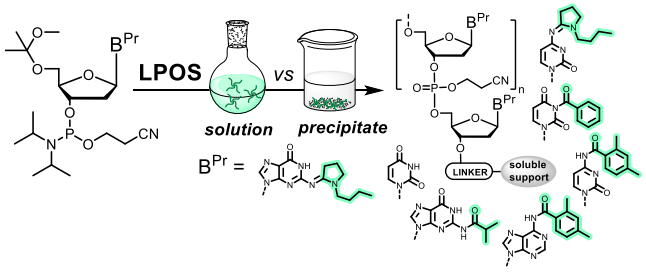


ABSTRACT: Solubility of the growing oligonucleotide-soluble support constructs in the liquid phase oligonucleotide synthesis (LPOS) is a critical parameter, which affects coupling efficiency, purity and recovery of the growing oligonucleotides during the chain elongation. In the present study, oligonucleotides have been assembled on a 4-oxoheptanedioic acid (OHDA) linker-derived tetrapodal soluble support using 5'-*O*-(2-methoxyprop-2-yl)-protected 2'-deoxyribonucleotide phosphoroamidite building blocks with different nucleobase protecting groups [isobutyryl (Gua), 1-butylpyrrolidin-2-ylidene (Gua, Cyt), 2,4-dimethylbenzoyl (Ade, Cyt) and Bz (Thy)]. The solubility of the oligonucleotide-soluble support constructs (molecular mass varying between 3-10 kDa) as models of protected tetra-, octa-, dodeca-, hexadeca- and eicosanucleotides was measured in different solvent systems and in potential anti-solvents. By tuning the nucleobase protecting group scheme, the solubility can be improved in aprotic organic solvent systems, while the recovery of the constructs in the precipitation, used for the isolation and purification of the growing oligonucleotide intermediates in a protic antisolvent (2-propanol), remained near quantitative. The precipitation-based yield of the protected tetrapodal oligonucleotides varied from quantitative to 90% yield. Overall yield (for di-:95%, tri-: 79-96%, tetra-: 82-88% and pentanucleotides: 68-75%) and purity of the LPOS were evaluated by RP HPLC and MS-spectroscopy of the released oligonucleotide aliquots. In addition, orthogonality of the OHDA linker was applied to release authentic protected nucleotides from the soluble supports.

INTRODUCTION

The growing demand for synthetic oligonucleotides, driven by their expanding application into large therapeutic fields, like cardiovascular diseases[1,2], has challenged current industrial manufacturing[3-5] that is reliant on the automated solid phase synthesis[6] with limited scalability and sustainability. To make oligonucleotide synthesis more reagent efficient and process-compatible, liquid phase oligonucleotide synthesis (LPOS)[7,8] has received growing attention[9], including nowadays advanced technologies for the large-scale production of therapeutically relevant oligonucleotides.[10-13] The LPOS technologies aim to harness reactivity features of classical solution phase synthesis, applicable for real-time batch-like optimized process of the reactions, but also for facile quantitative physical or mechanical isolation of the intermediate products from the reactant media. Appropriate soluble supports are utilized in LPOS, facilitating separation of the growing oligonucleotides from the reactants by precipitation, [10,14,15] liquid-liquid extraction[12] or membrane filtration[16]. Soluble supports are consisted of solubility tags,[10-12,17,18] appropriate branching units (star-like dendrimers),[13,15,16] or their combination, in which the protected oligonucleotide themselves become dominant at later steps of the synthesis. It should be realized that a significant mass portion of the protected oligonucleotides comes from the protecting groups (about 30% of the total mass in the standard DNA protecting group scheme), contributing markedly to the solubility of the construct, solvent volume of the reactions and, consequently, to the coupling and 5'-*O*-deprotection

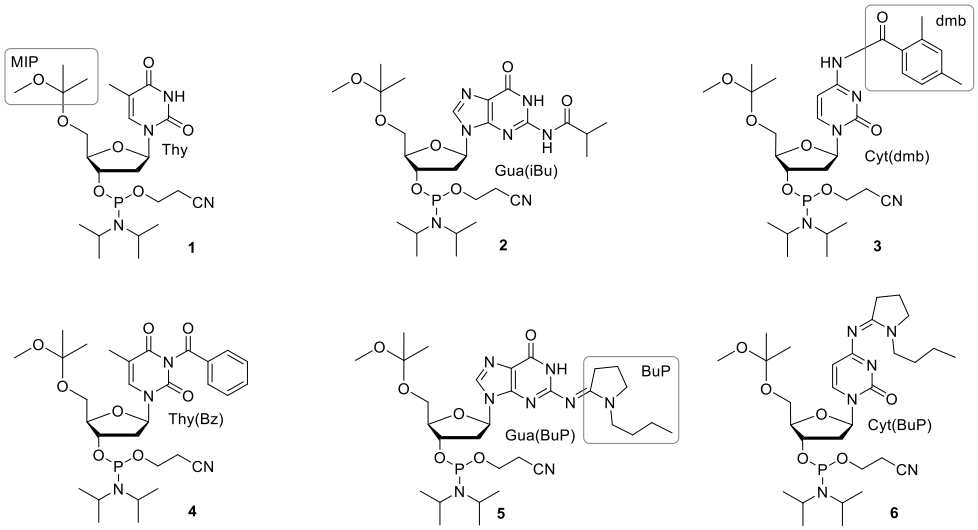


Figure 1. The 5'-*O*-MIP-protected phosphoramidite building blocks used for the LPOS (non-standard protecting groups emphasized)

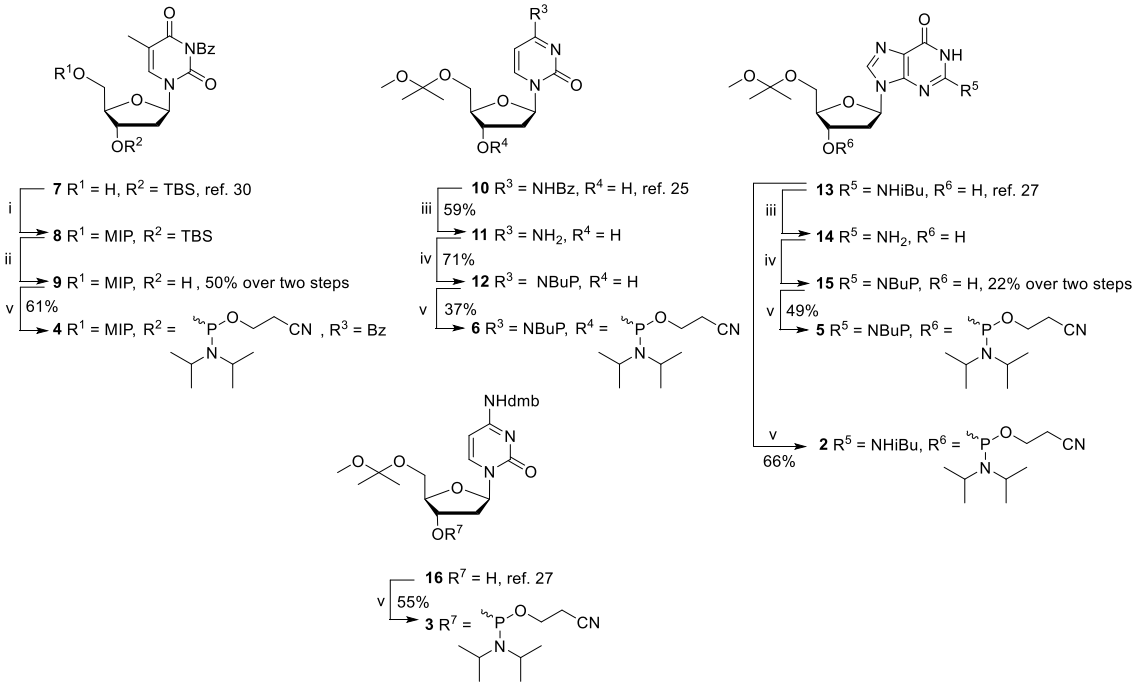
efficiency on these supports. This problem setting resembles swelling properties and suspension volumes of solid supports, which can be drastically different in the beginning and in the end of the biopolymer assembly due to the growing biopolymer payload. This and its role for the synthetic efficiency has extensively been studied in solid phase peptide synthesis (SPPS),[19,20] but to a lesser extent in solid phase oligonucleotide synthesis (SPOS).[21,22] Altered swelling is not an issue with controlled pore glass, but may affect synthetic efficiency on polystyrene supports used for SPOS.[23] Solubility plays a marked role in the LPOS technologies under development, including the convergent ones,[10] which may need further consideration of the protecting group scheme. At the same time, precipitation, filtration or extraction properties should result in quantitative isolation and purification of the growing oligonucleotide products from the reactant media.

In the present study, a set of 5'-*O*-(2-methoxyprop-2-yl = MIP)-protected 2'-deoxyribonucleotide phosphoroamidite building blocks (**1**-**6**, Figure 1) were synthesized (Scheme 1) and used for LPOS on a 4-oxoheptanedioic acid (OHDA)[24] linker-derived tetrapodal precipitative soluble support (Scheme 2 and 3). The solubility of the oligonucleotide-soluble support constructs (**17** – **27**) as models of protected tetra-, octa-, dodeca-, hexadeca- and eicosa-nucleotides was measured after each coupling cycle (including the coupling, oxidation and 5'-*O*-deprotection) in different solvent systems and in potential anti-solvents. The role of nucleobase protecting group scheme [isobutyryl (Gua), 1-butylpyrrolidin-2-ylidene (Gua, Cyt), 2,4-dimethylbenzoyl (Ade, Cyt) and Bz (Thy)] in the solubility and precipitation efficiency of the oligonucleotide-soluble support constructs was examined. Aliquots of the oligonucleotides were released from the precipitated soluble supports by concentrated ammonia to verify yield, purity and authenticity of the oligonucleotide content. In addition, hydrazine acetate was used for the orthogonal cleavage of the OHDA linker that released authentic protected nucleotides from the soluble support.

RESULTS AND DISCUSSION

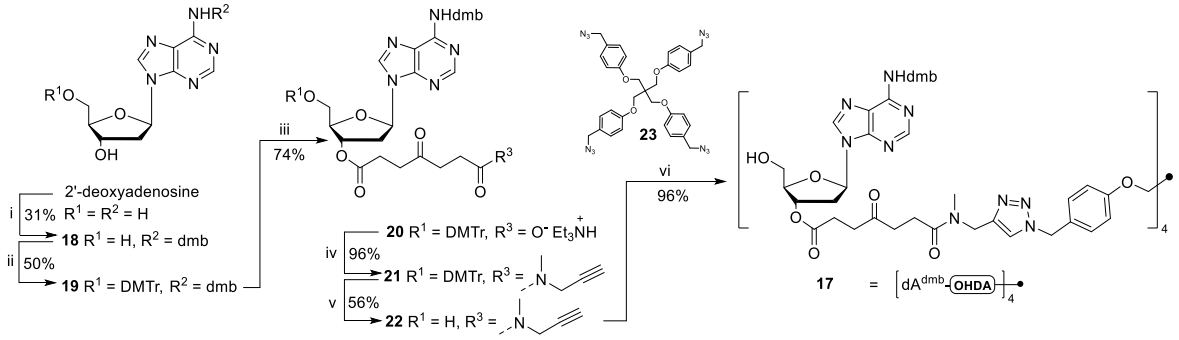
Synthesis of the 5'-O-MIP-protected phosphoramidite building blocks. The phosphoramidite building blocks (**1**-**6**) used in this study are described in Figure 1. MIP was selected as an alternative 5'-*O*-protecting group.[25,26] In comparison to 4,4'-dimethoxytrityl (DMTr) group, the fast acid-catalyzed pseudo irreversible removal and the resultant volatile byproducts (acetone and methanol) make MIP an attractive protecting group for LPOS[27], and especially for the one-pot coupling-deprotection synthesis cycle[28] applied in the present study. N3-Bz-thymidine (**4**) and 1-butylpyrrolidin-2-ylidene (BuP) protected 2'-deoxyguanosine (**5**) and 2'-deoxycytidine (**6**) represent more lipophilic alternatives for thymidine (**1**)[25], *N*-isobutyryl(iBu) 2'-deoxyguanosine (**2**) and *N*-2,4-dimethylbenzoyl (dmb) 2'-deoxycytidine (**3**) building blocks. dmb is used for Ade and Cyt due to its higher hydrolytic stability[29] compared to Bz, which is beneficial for LPOS,[27] and for the hydrazine acetate-mediated orthogonal cleavage of the OHDA linker (cf. below). **1** has been described previously,[25] whereas syntheses of **2** - **6** are outlined in Scheme 1. As previously reported, 3'-*O*-*tert*-butyldimethylsilyl (TBS)-protected nucleosides were used as key intermediates to introduce MIP selectively to the 5'-OH group of each nucleoside. As an example, typical procedure for N3-benzoyl-3'-*O*-TBS thymidine (**7**)[30]: The 3'-*O*-TBS-protected nucleoside (**7**) is exposed to a mixture of 2,2-methoxypropane in THF in the presence of a catalytic amount of p-TsOH. The obtained 5'-O-MIP-3'-O-TBS-protected intermediate (**8**)

Scheme 1. Synthesis of the 5'-*O*-MIP-protected phosphoramidite building blocks **2**-**6**



Conditions: i) 2,2-dimethoxypropane, THF, TsOH·H_2O, for 2h at r.t.; ii) TBAF, THF, for 2h at r.t.; iii) NH_3, H_2O, THF, for 16h at 40°C; iv) 1-butyl-2,2-dimethoxypyrrolidine, MeOH, for 1h at r.t.; v) 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphoramidite, tetrazole, DCM, for 2h at r.t.

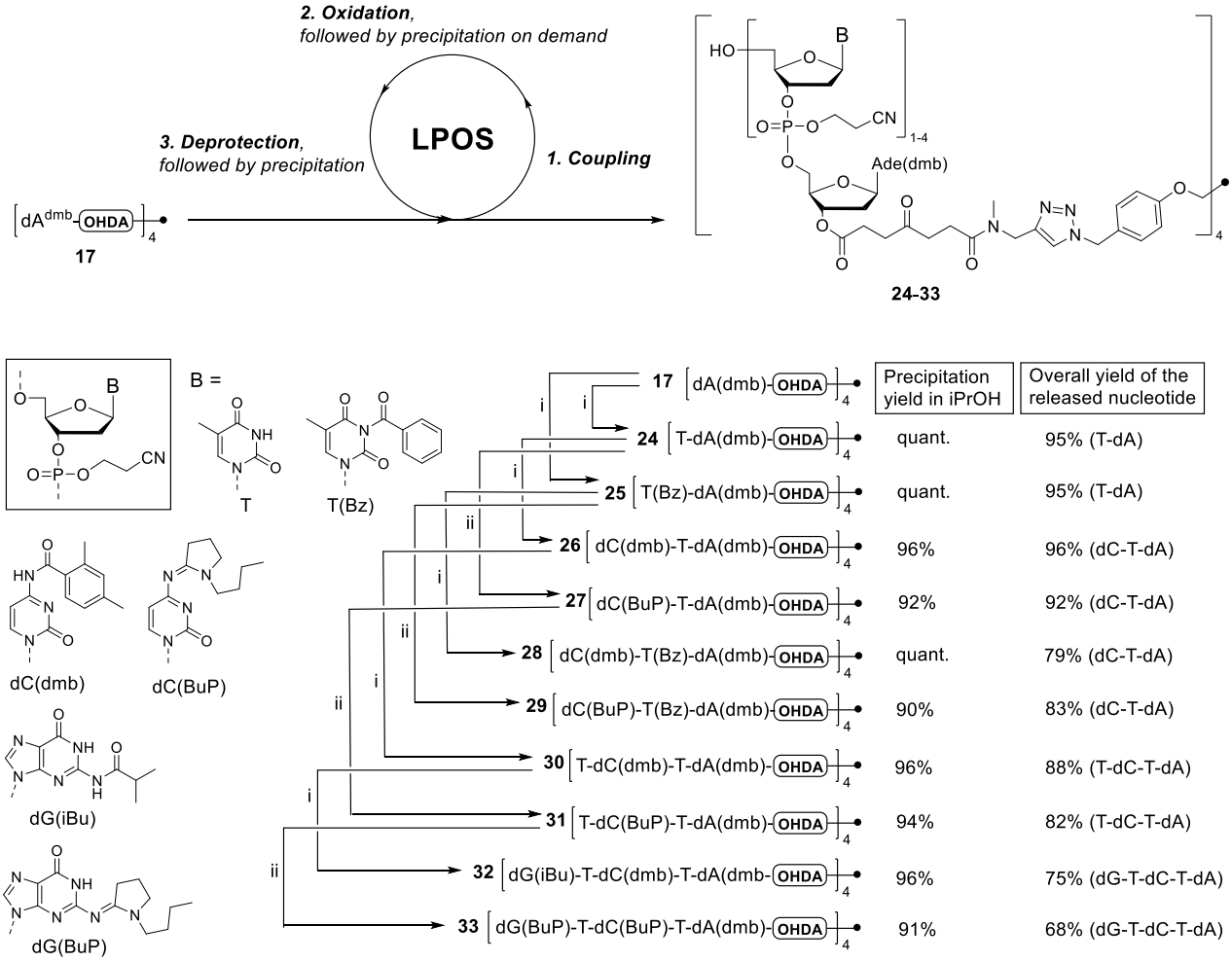
Scheme 2. Synthesis of the tetrapodal soluble support (**17**)



Conditions: i) 1: Me_3SiCl, Et_3N, DCM, for 1h at 0 → r.t.; 2: 2,4-dimethyl benzoylchloride added to the former mixture, at 0 → r.t. overnight; 3: N-methylpiperazine, Et_3N added to the former mixture, r.t. overnight; 4: pyridinium fluoride added to the former mixture, for 3h at r.t.; ii) DMTrCl, Py, for 2h at r.t.; iii) 1: 4-oxoheptanedioic acid, DCC, DMAP, THF, for 16h at r.t.; 2: **19** and DBU added, and then continued overnight at r.t.; iv) N-methyl propynylamine, EDC, HOBt, DCM, for for 12h at r.t.; v) TFA, 1-dodecanethiol, DCM, for 2h at 0°C; vi) CuI, sodium ascorbate, dimethylacetamide, for 48h at r.t.

was treated with TBAF to give the desired 5'-*O*-MIP protected nucleoside (**9**). 5'-*O*-MIP protected nucleosides (**10** and **13**) were used as precursors for BuP-protected guanosine and cytosine. The amide nucleobase protecting groups (Bz or iBu) were removed by concentrated ammonia and the nucleosides (**11** and **14**) with the exposed exocyclic amino group were then treated with 1-butyl-2,2-dimethoxypyrrolidine, following the procedure described for 1-methyl-2,2-dimethoxypyrrolidine-protected nucleosides by Caruthers et al.,[31] to give the desired amidine protected nucleosides (**12** and **15**). The same amidine protection led to stability issues in case of adenine,[31] and the corresponding building block could not be offered. Phosphitylation of the 3'-OH group (**9**, **12**, **13**[27], **15** and **16**[27]) using 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite in the presence of tetrazol gave the desired phosphoramidites **2**-**6**.

Scheme 3. Liquid phase oligonucleotide synthesis (LPOS) and yields of the oligonucleotide products



Conditions: *One precipitation/synthesis cycle:* i) 1: Coupling: 0.095 mol L^{-1} of phosphoramidite (**1-4**, 2 eq/5'-OH group), tetrazole (2 eq/5'-OH group) in MeCN-DMF (1:1, *v*/*v*), 2h at r.t., 2: Oxidation: m-CPBA (3.8 eq / 5'-OH group), 5 min at r.t., 3: Deprotection: DCA (15% of the total volume of the reaction mixture), 15 min at r.t.; 4: Precipitation in 2-propanol. *Two precipitations / synthesis cycle:* ii) 1: Coupling: 0.095 mol L^{-1} of phosphoramidite (**5** or **6**, 2 eq/5'-OH group), tetrazole (2 eq/5'-OH group) in MeCN-DMF (1:1, *v*/*v*), 2h at r.t., 2: Oxidation: I_2 in THF/Py/H_2O added to the reaction mixture, instant at r.t.; 3: Precipitation in 2-propanol; 4: Deprotection: DCA-MeOH-DCM (5:18:27, v/*v*/*v*), 15 min, at r.t.; 5: Precipitation in 2-propanol.

Table 1. Physical parameters of soluble support-oligonucleotide constructs **17, 24-33**

Soluble support construct	Molecular mass / g mol^{-1}	Protecting group content, including the support / m%	tetrapodal support / m%	nucleobase protecting group content / m%	mass concentration[a] used for LPOS /g L^{-1}
17	3023.3	67	49	18	36
24	4452.3	50	34	12	53
25	4868.7	55	31	20	58
26	6349.9	47	24	17	75
27	6314.1	47	24	16	75
28	6766.4	50	22	22	80
29	6730.5	50	22	22	80
30	7779.0	41	19	14	92
31	7743.1	41	19	13	92
32	9588.4	39	16	14	114
33	9765.0	40	15	16	116

[a]Corresponds to 0.012 mol L^{-1} solutions of **17-27**. A mixture of MeCN-DMF (1:1, *v*/*v*) as a solvent.

Table 2 Solubility of soluble support-oligonucleotide constructs **17, 24-33**

Soluble support construct	MeCN / g L^{-1}	MeCN/DCM (1:1) / g L^{-1}	DCM / g L^{-1}	DCM-MeOH (1:1) / g L^{-1}	MeCN-DMF (1:1) / g L^{-1}	MeCN-Py (4:1) / g L^{-1}	MeOH / g L^{-1}	2-propanol / g L^{-1}	MTBE / g L^{-1}
17	0.12	29 ± 2	>300	>300	> 300	16 ± 2	0.5	≈ 0	≈ 0
24	0.007	1.1	0.5	>300	> 300	13 ± 1	0.06	≈ 0	≈ 0
25	1.2 ± 0.4	>300	>300	>300	> 300	>300	0.02	≈ 0	≈ 0
26	0.004	0.8	0.3	>300	> 300	10 ± 1	≈ 0	≈ 0	≈ 0
27	4.4 ± 0.4	13 ± 1	0.11	>300	>300	>300	3.0 ± 0.1	≈ 0	≈ 0
28	26 ± 1	>300	>300	>300	>300	>300	18 ± 1	≈ 0	≈ 0
29	48 ± 2	>300	>300	>300	> 300	>300	32 ± 1	≈ 0	≈ 0
30	0.004	0.15	0.04	>300	> 300	7.0 ± 0.2	≈ 0	≈ 0	≈ 0
31	1.7 ± 0.4	5.8 ± 0.9	0.06	>300	>300	130 ± 20	5.0 ± 0.9	0.003	≈ 0
32	0.006	0.3	0.003	>300	> 300	3.0 ± 0.3	0.01	≈ 0	≈ 0
33	0.5	2.3 ± 0.4	0.07	>300	>300	14 ± 4	1.1 ± 0.4	0.003	≈ 0

Note: the solubility values less than 1g L-1 and more than 300 g L^{-1} are given with one-digit precision without error limits. For the values more than 1 g L-1 (and less than 300 g L^{-1}), two digits and error limits (extracted from the fitted calibration curves of the solubilities) are given.

4-oxoheptanedioic acid linker-derived tetrapodal soluble support. Codon-based SPOS[32,33] and convergent LPOS[10] using protected tri- and tetranucleotides as building blocks may offer analytical benefits and quality improvement for oligonucleotide end products. The key structure for the synthesis of these blockmers is an orthogonal 3'-*O*-protection/linker moiety that should be selectively cleavable in the presence of other protecting groups. We have previously utilized a disulfide-linker[34] and Q-linker[35] on soluble supports for the preparation of protected trinucleotide synthons in solution. In the present study, 4-oxoheptanedioic acid (OHDA) linker[24] was introduced to the tetrapodal soluble support. The levulinoyl ester moiety of this linker undergoes orthogonal cleavage by hydrazine acetate, which may be applied for the release of protected nucleotides on demand, or it can be cleaved by concentrated ammonia upon the global deprotection as regular ester linker structures. Synthesis of the soluble support **17,** preloaded with *N*-dmb 2'-deoxyadenosine, is described in Scheme 2. The exocyclic amino group of 2'-deoxyadenosine was N-dmb-protected (**18**), DMTr was temporarily introduced to the 5′-OH group (**19**) and the 3'-OH group of **19** was acylated with 4-oxoheptanedioic acid (**20**). Amide coupling with *N*-methyl propargylamine (**21**) and removal of the DMTr group gave alkyne precursor **22**, which was attached to tetrapodal azide core **23** in 96% yield using Cu(I)-catalyzed click reaction. It may worth of noting that OHDA was attached to the pentaerythritol core via a secondary amide (cf. the *N*-methyl group) to prevent intramolecular hemiaminal formation and subsequent premature cleavage of the linker.

Liquid Phase Oligonucleotide Synthesis. We have introduced pentaerythritol-derived soluble supports for the precipitation-based LPOS of short DNA and RNA sequences, applying phosphoramidite,[15,36,37] phosphotriester,[38] and more recently stereo-controlled limonene-based P(V) chemistry[27,39,40]. In each case two precipitations were used to isolate the growing soluble support-bound nucleic acid intermediates: one after the coupling and the other after the 5'-deprotection. The synthesis cycle on **17** (Scheme 3) follows practically the same procedure as previously. In addition, one precipitation/coupling cycle approach was applied as follows: 2 equiv. / 5'-OH group and 0.095 mol L^{-1} solutions of phosphoramidites (**1**-**4**) in the presence of stoichiometric amount of tetrazole in MeCN-DMF (1:1, *v*/*v*) were used for the phosphoramidite coupling (2h, at r.t.), followed by oxidation with *m*-chloroperbenzoic acid (mCPBA, 3.8 equiv / 5'-OH group, 5 min, at r.t.). After coupling, dichloro acetic acid (DCA) was added to the reaction mixture (15% DCA of the total volume, for 15 min at r.t.) to deprotect the 5'-*O*-MIP group and the oligonucleotide-soluble support construct (**24**, **26**, **28**, **30** and **32**) was precipitated in 2-propanol. Precipitation yields of **24**, **26**, **28**, **30** and **32** varied from 96% to quantitative (Scheme 3). Recently, a careful impurity analysis has been done in convergent LPOS using phosphoramidite chemistry, in which by-products of partial premature cyanoethyl removal (de-CE) have been observed (without an obvious exposure to a base) and considered as noncritical impurities.[10] MS(ESI-TOF) analysis of the precipitated constructs (**24**, **26**, **28**, **30**, **32**) verified their authenticity in virtually intact form, although partial de-CE could be detected in the end of the assembly (cf. Figure S44). Characterization of the released nucleotides afforded more detailed analysis of the synthesis. Overall yield (for di-: 95%, tri-: 79 and 96%, tetra-: 88% and pentanucleotide: 75%, Scheme 3), purity (97%, 89%, 93%, 87% and 73% for di-, tri-, tetra- and pentanucleotides, respectively) and authenticity of the nucleotides, released from aliquots of the precipitates by concentrated ammonia, were determined by HPLC (Scheme S1), UV-absorbance at λ = 260 nm and MS-spectroscopy (Figures S45, S46, S48, S49). In case of phosphoramidites **5** and **6**, and soluble support-oligonucleotide constructs **27**, **29**, **31** and **33**, two-precipitation / coupling cycle and iodine-oxidation (due to the susceptibility of PuB to mCPBA) were applied. After coupling, 0.2 M I_2 in THF/Py/H_2O (5:4:1 *v*/*v*/*v*) was added to the mixture to oxidize the phosphite triester intermediate and the product (5'-*O*-MIP protected **27**, **29**, **31** and **33**) was precipitated in 2-propanol. The precipitate was exposed to a mixture of DCA-MeOH-DCM (5:18:27, *v*/*v*/*v*, 15 min, at r.t.) to remove MIP and the product (**27**, **29**, **31** or **33**) was precipitated in 2-propanol. Yields of the oligonucleotide-soluble support constructs over two precipitations varied from 90 to 94% (Scheme 3). Overall yield (for tri-: 83% and 92%, tetra-: 82% and pentanucleotide: 68%, Scheme 3), purity (for tri-: 91% and 85%, for tetra-: 81% and for pentanucleotide: 70%) and authenticity (Scheme S1, Figures S45-S47 and S50) of the released oligonucleotides were determined as above.

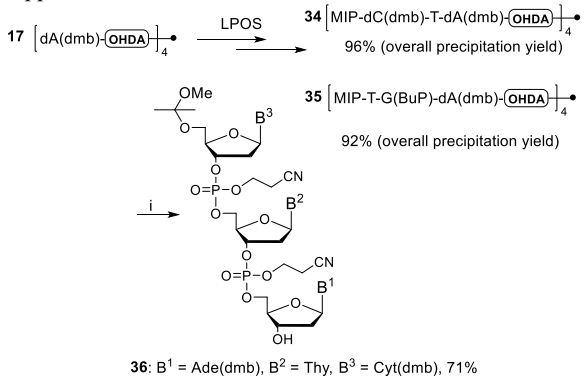
The one-pot synthesis cycle, consisted of sequential coupling, oxidation, and deprotection, followed by a single precipitation, may make the synthesis operationally simpler and improve the overall yield of the oligonucleotides.[28] The two precipitations /synthesis cycle-technique is more labor intensive and may cause unnecessary product losses due to the extra precipitations. However, the compatibility of the reagents (the latter steps should accept the remaining reagent traces left from the previous ones) used for LPOS and isolation and precipitation/purification efficiency of the of the growing oligonucleotides in the reactant media may affect the superiority of these techniques.

Solubility of the soluble support-oligonucleotide constructs. The molecular mass of the soluble support-oligonucleotide constructs (**17, 24-33**) increases from 3023 g mol^{-1} (**17**) to 9765 g mol^{-1} (**33**) during the LPOS (Table 1). At the same time, the total mass content (m%) of the protecting groups decreases from 67 m% to 40 m%, and the content of the tetrapodal support from 49 m% to 15 m% (Figure S51). The nucleobase protecting group content varies between 12 m% and 22 m%. When the length of the oligonucleotide increases, the solubility of the constructs **17, 24-33** decreases, as expected. The mass concentration of **17, 24-33** used for LPOS had to increase from 36 g L^{-1} to 116 g L^{-1} as the oligonucleotide chain grew (corresponds to 0.012 mol L^{-1} solutions of the constructs **17, 24-33,** needed for 0.095 mol L^{-1} solutions of phosphoramidite building blocks **1-6**, 2 equiv/5'-OH group). The solubilities of the constructs (**17, 24-33**) in various solvents and solvent mixtures are presented in table 2. The solubilities were measured in aprotic solvents acetonitrile (MeCN) and dichloromethane (DCM), in solvent mixtures of MeCN/DCM (1:1, *v/v*), MeCN/DMF (1:1, *v/v*) and pyridine/MeCN (1:1, *v/v*), as well as in potential antisolvents methanol, 2-propanol and methyl *tert*-butyl ether (MTBE). A solubility of 300 g L^{-1} has been set to represent an upper limit value (a value that is clearly more than needed for synthetic purposes). This was obtained for all constructs (**17, 24-33**) in mixtures of DCM-MeOH (1:1, v/v) and MeCN-DMF (1:1, *v/v*), used as deprotection and coupling solvent systems in the present study.

Each of the constructs (**17, 24-33**) had modest solubility in MeCN,[41,42] the common solvent choice for standard phosphoramidite coupling. In general, with dmb- and iBu-protected nucleobases and without N3-protection of Thy (**24**, **26**, **30** and **32**), modest solubility can be observed also in DCM and in a mixture of MeCN-DCM (1:1, *v/v*), used frequently to dissolve oligonucleotide segments and trinucleotide blockmers for the coupling.[10,35,43-] Examination of the results show the relationship between the nucleobase protecting groups and the solubility. By introducing of more lipophilic 1-butylpyrrolidin-2-ylidene (BuP) protection for dC (**27, 31**) and dG (**33**), solubility in aforementioned aprotic solvent systems increased, but remained still low, to potentially hamper the reactivity on the soluble support-oligonucleotide constructs in these solutions (cf. the required mass concentration of the constructs **17, 24-33** in Table 1). Interestingly, **27** and **31** showed good solubility (Figure S52), but **33** did not sufficient one (14 g L^{-1}, 0.014 mol L^{-1}), in a mixture of MeCN-Py (4:1, *v/v*), which is a readily volatile and non-halogenated solvent alternative for the phosphoramidite coupling (compared to mixtures of DMF and DCM, respectively). The N3- protection of Thy had the strongest impact, resulting in full solubility of constructs **28** and **29** in DCM, MeCN-DCM (1:1, *v/v*) and MeCN-Py (4:1, *v/v*)(Figure S53). Benzoyl at N3 however, is susceptible to premature cleavage, which dilutes its value for further applicability. Further research could evaluate N3-pivaloyloxymethyl (Pom) protected thymine[40] as a solubility improving group. Favourably, the solubility in antisolvents 2-propanol and MTBE remained negligible in all cases. Solubility in MeOH increased when the more lipophilic protecting groups were introduced to the oligonucleotides (tetrapodal dodecanucleotides **26-29**: from 0 to 32 g L^{-1}), but similar trend cannot be seen in 2-propanol and MTBE. As we have previously noticed[27], 2-propanol is the superior antisolvent in LPOS, resulting in quantitative precipitation and efficient removal of reagent traces.

Orthogonal cleavage of protected nucleotides from the soluble supports. To demonstrate the applicability of hydrazine acetate-mediated orthogonal cleavage of the OHDA linker, two protected trinucleotides 5′-*O*-MIP-dC(dmb)-T-dA(dmb)-OH (**34**) and 5'-*O*-MIP-T-dG(BuP)-dA(dmb)-OH (**35**) were synthesized following the LPOS protocols above (Scheme 4). The trinucleotide-soluble support constructs (**34** and **35**) were obtained in 96% and 92% overall yields, respectively. The precipitates were exposed to a mixture of 0.1 mol L^{-1} hydrazine acetate [5 eq, hydrazine hydrate-acetic acid-pyridine (0.025:1:4, *v/v/v*), 1h at r.t.] (RP HPLC monitoring of the orthogonal cleavage-shown in Figure S54), followed by extraction between DCM and saturated $NaHCO_3$ and elution through a short silica gel column. Protected trinucleotides were obtained in 71% (**36**) and 65% (**37**) yields, corresponding to 68% an 60% overall yields from **17**, respectively.

Scheme 4. Cleavage of protected trinucleotides from the soluble support.



36: B^1 = Ade(dmb), B^2 = Thy, B^3 = Cyt(dmb), 71%
37: B^1 = Ade(dmb), B^2 = Gua(Bup), B^3 = Thy, 65%

Conditions: i) $NH_2NH_2{\cdot}H_2O$-AcOH-Py (0.025:1:4, *v/v/v*), 1h at r.t.

CONCLUSION

5'-*O*-(2-methoxyprop-2-yl = MIP)-protected 2'-deoxyribonucleotide phosphoroamidite building blocks, with different nucleobase protecting groups, were synthesized and used for LPOS on a 4-oxoheptanedioic acid (OHDA)[24] linker-derived tetrapodal soluble support. The solubility of the oligonucleotide-soluble support constructs was measured in different solvent systems and in potential anti-solvents. The solubility of the constructs (molecular mass varying between 3-10 kDa) decreased as the oligonucleotide chain grew, and became in some cases low in most common solvent choices (MeCN and mixtures of MeCN and DCM) used for the phosphoramidite-based chain elongation of oligonucleotides. The nucleobase protecting group scheme [isobutyryl (Gua), 1-butylpyrrolidin-2-ylidene (Gua, Cyt), 2,4-dimethylbenzoyl (Ade, Cyt) and Bz (Thy)] may be tuned to improve the solubility of the constructs in organic solvent systems, including also alternative solvent choices suitable for the phosphoramidite coupling (a mixture of MeCN and pyridine). At the same time, the solubility of the constructs in 2-propanol remained unchanged and negligible to achieve efficient precipitation and purification of the constructs. The results obtained here highlights the importance of the solubility of the growing protected oligonucleotides in coupling conditions, which can be challenging at the later stage of the chain elongation, and that LPOS strategies can benefit from further protecting group optimization. With an appropriate choice of the nucleobase protecting groups, alternative potentially greener reaction solvents can be accessed that will further increase the attractiveness of the LPOS methodologies. The described challenges in solubility (note the decreasing trend when the length of the oligonucleotide increases) also implies that the branched or star-like soluble supports, such as the pentaerythritol-derived one used in our studies, may be suitable for the preparation of short protected oligonucleotide synthons, but efficient synthesis of longer oligonucleotides in solution would favour linear assembly of these synthons applying for example additional solubility determining groups (SDGs).[10] The applicability of the OHDA-linker[24] in LPOS for the preparation of protected trinucleotides, as precursors for such synthons[10,32,33] has been demonstrated and proven to be competitive with our previously published procedures, applying Q- and disulfide linkers.[34,35]

EXPERIMENTAL SECTION

General Methods. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on Bruker Avance 500MHz and 600 MHz instruments. For the RP HPLC analysis, an analytical C18 column (4.6 × 250 mm, 5 µm, flow rate 1 mL/min, detection at λ = 260 nm) and a gradient elution of an aqueous 50 mM triethylammonium acetate and MeCN were used. For the synthesis, DCM and DMF were dried over 4 Å molecular sieves and MeCN and MeOH over 3 Å molecular sieves. 1-butyl-2,2-dimethoxypyrrolidine was prepared from 1-butylpyrrolidin-2-one following the procedure described for 1-methyl-2,2-dimethoxypyrrolidine by Caruthers et al.[31]: 1-butylpyrrolidin-2-one (50 g, 350 mmol) was added to dimethyl sulfate (67 g, 533.1 mmol, 1.5 eq.) at 25 °C. After addition, the reaction was warmed to 90 °C on an oil bath and stirred for 2 hours. Then the mixture was cooled down to -10 °C. A solution of sodium methoxide (25 g, 0.46 mmol, 1.3 eq.) in MeOH (250 mL) was added at -10 °C and the mixture was stirred at 25 °C for one hour. The solution was filtered and the filtrate was concentrated to dryness under reduced pressure. Then MTBE (250 mL) was added to the condensate, the mixture was stirred at 25 °C for 2 hours, filtered and evaporated to give crude 1-butyl-2,2-dimethoxypyrrolidine (50 g) as brown yellow oil which was used as such. Compounds **1**, **7**, **10**, **13** and **16** were prepared by previously reported methods affording products with spectroscopic data matching those described in literature. [25,27,30]

Caution! Hydrazine hydrate is acutely toxic and it has long lasting effects to aquatic life. All manipulations were performed on the smallest practical scale. 1-Hydroxybenzotriazole show explosive properties when heated or mechanically stimulated and should be handled with care. Dichloroacetic acid is corrosive and show reproductive toxicity and should be handled with caution. Tetrazole presents a risk of explosion by shock, friction, or fire which was controlled using dilute commercial solutions.

*N^3-Benzoyl-5'-O-(2-methoxypropane-2-yl)-2'-deoxythymidine (**9**):* To a solution of **7**[30] (100 g, 217 mmol) in 2,2-dimethoxypropane (267 mL) and THF (1000 mL), $TsOH{\cdot}H_2O$ (8.3 g, 43 mmol, 0.2 eq.) was added. The reaction was stirred 2 hours at r.t., quenched by addition of Et_3N (50 mL) and concentrated to dryness. The residue (**8**) was dissolved in THF (290 mL) and TBAF (114 g, 434 mmol, 2.0 eq.) was added. The mixture was stirred for 2 hours at r.t., concentrated to dryness and re-dissolved in DCM (500 mL). The mixture was washed with water three times, dried over Na_2SO_4, and concentrated to dryness to give a crude product. The crude was purified by silica gel chromatography (ethyl acetate/n-heptane, 1:8 → 1:4, *v*/*v*) to give **9** as white solid (45 g, yield: 50%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.92 (dd, J = 8.4, 1.2 Hz, 2H), 7.69 (d, J = 1.1 Hz, 1H), 7.67 – 7.60 (m, 1H), 7.52 – 7.45 (m, 2H), 6.34 (t, J = 6.6 Hz, 1H), 4.48 (dd, J = 5.7, 2.9 Hz, 1H), 4.09 (q, J = 3.0 Hz, 1H), 3.70 (dd, J = 10.8, 3.1 Hz, 1H), 3.65 (dd, J = 10.8, 3.0 Hz, 1H), 3.24 (s, 3H), 2.35 (ddd, J = 13.5, 6.1, 3.4 Hz, 1H), 2.21 (dd, J = 13.8, 6.7 Hz, 2H), 1.97 (d, J = 1.1 Hz, 3H), 1.42 (s, 3H), 1.41 (s, 3H). ^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$): δ 169.0, 163.0, 149.3, 136.0, 135.1, 131.5, 130.4 (2 × C), 129.2 (2 × C), 110.5, 100.4, 86.1, 85.2, 71.9, 60.8, 48.7, 40.9, 24.4, 24.3, 12.4. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{21}H_{27}N_2O_7^+$ 419.1818, found 419.1776.

*N^3-Benzoyl-5'-O-(2-methoxypropane)-2'-deoxythymidine-3'-O-yl cyanoethyl N,N-diisopropylaminophosphoramidite (**4**):* To a solution of **9** (50 g, 120 mmol) in DCM (500 mL), 2-Cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite (43 g, 140 mmol, 1.2 eq.) and tetrazole (6.7 g, 96 mmol, 0.8 eq.) were added successively. The reaction was stirred for 2 hours at r.t. and quenched by addition of saturated aqueous $NaHCO_3$ (400 mL). The organic phase was separated, dried over Na_2SO_4, and evaporated to dryness. The residue was purified by silica gel chromatography (ethyl acetate/n-heptane, 1:8 → 1:2, *v*/*v*) to give **4** as white

solid (45 g, yield: 61%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.96 – 7.83 (m, 2H), 7.70 (d, J = 1.0 Hz, 0.55H), 7.66 (d, J = 1.0 Hz, 0.45H), 7.59 (q, J = 7.3 Hz, 1H), 7.44 (m, 2H), 6.34 (dd, J = 10.0, 3.6 Hz, 1H), δ 4.57 – 4.50 (m, 1H), 4.25 (d, J = 2.1 Hz, 0.55H), 4.16 (d, J = 2.3 Hz, 0.45H), 3.79 (m, 1H), 3.72 – 3.52 (m, 5H), 3.21 (m, 3H), 2.57 (m, 2H), 2.47 (ddd, J = 13.5, 5.9, 2.8 Hz, 0.45H), 2.41 (ddd, J = 13.2, 5.7, 2.4 Hz, 0.55H), 2.26 – 2.13 (m, 1H), 1.93 (s, 3H), 1.38 (t, J = 3.5 Hz, 6H), 1.13 (d, J = 6.9 Hz, 12H); ^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$) (note: diastereomers are observed): δ 169.0, 162.8, 149.3 (d, $J_{C\text{-}P}$ = 3.8 Hz), 135.6, 135.5, 134.9, 131.6, 130.4 (2 × C), 129.1 (2 × C), 117.6 (d, $J_{C\text{-}P}$ = 11.3 Hz), 110.7 (d, $J_{C\text{-}P}$ = 7.6 Hz), 100.4, 85.6 (d, $J_{C\text{-}P}$ = 3.8 Hz), 85.2 (d, $J_{C\text{-}P}$ = 6.3 Hz), 74.1 (d, $J_{C\text{-}P}$ = 8.8 Hz), 60.8, 58.0 (d, $J_{C\text{-}P}$ = 18.9 Hz), 48.8 (d, $J_{C\text{-}P}$ = 2.5 Hz), 43.3 (d, $J_{C\text{-}P}$ = 12.6 Hz), 40.0 (d, $J_{C\text{-}P}$ = 7.6 Hz), 24.5, 24.5, 24.4, 24.4, 24.4, 24.4, 20.4 (d, $J_{C\text{-}P}$ = 7.6 Hz) & 20.3 (d, $J_{C\text{-}P}$ = 7.6 Hz), 12.4. ^{31}P NMR (203 MHz, $CDCl_3$): δ 148.55, 148.38. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{30}H_{44}N_4O_8P^+$ 619.2897, found 619.2912.

N^4-[(1-butylpyrrolidine-2-ylidene)amino]-5′-O-(2-methoxypropane)-2′-deoxycytidine (**12**): To a stirring solution of **10**[25] (189 g, 469 mmol) in THF (950 mL), aqueous 25% ammonia (0.95 L) was added. The solution was stirred at 40 °C on an oil bath for 16 hours and concentrated under reduced pressure. The residue was precipitated in acetonitrile to yield **11** as white solid (83 g, 59%, HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{13}H_{22}N_3O_5^+$ 300.1559, found 300.1558 M+H+). The precipitate (**11**, 83 g, 280 mmol) was dissolved in MeOH (830 mL) and 1-butyl-2,2-dimethoxypyrrolidine (125 g, 666 mmol, 2.4 eq.) was added in one portion. After stirring for 1 hour at r.t., the mixture was concentrated to dryness. The residue was purified by silica gel chromatography (DCM/*n*-heptane, 2:1, *v/v* → DCM-MeOH, 20:1, *v/v*) to give **12** as white solid (84 g, 71%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.04 (d, J = 7.3 Hz, 1H), 6.38 (t, J = 6.1 Hz, 1H), 5.97 (d, J = 7.3 Hz, 1H), 4.59 (s, 1H), 4.43 (dd, J = 10.1, 4.6 Hz, 1H), 4.16 (dd, J = 7.2, 3.3 Hz, 1H), 3.71 (dd, J = 10.6, 3.1 Hz, 1H), 3.63 (dd, J = 10.6, 3.3 Hz, 1H), 3.54 – 3.41 (m, 4H), 3.21 (s, 3H), 3.20 – 3.05 (m, 2H), 2.61 (ddd, J = 13.5, 6.1, 4.8 Hz, 1H), 2.14 (m, 1H), 2.05 – 1.97 (m, 2H), 1.56 (m, 2H), 1.39 – 1.27 (m, 8H), 0.93 (t, J = 7.4 Hz, 3H). ^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$): δ 172.2, 168.8, 156.8, 140.5, 103.1, 100.3, 86.6, 85.9, 71.3, 60.7, 49.4, 48.8, 44.4, 42.0, 31.0, 29.1, 24.4, 20.1 (2 × C), 19.9, 13.8. HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{21}H_{35}N_4O_5^+$ 423.2607, found 423.2599.

N^4-[(1-butylpyrrolidine-2-ylidene)amino]-5′-*O*-(2-methoxypropane)-2′-deoxycytidine-3′-*O*-yl cyanoethyl *N,N*-diisopropylphosphoramidite (**6**): To a stirring solution of **12** (55 g, 130 mmol) in DCM (550 mL), tetrazole (7.3 g, 104 mmol, 0.8 eq.) and 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite (45 g, 150 mmol, 1.2 eq.) were added. After stirring for 2 hours at r.t., the mixture was washed twice with 5% aqueous $NaHCO_3$ and brine. The organic phase was separated, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by silica gel chromatography (DCM) and preparative isocratic RP HPLC (acetonitrile-H_2O, 95:5, *v/v*) to give **6** as colorless oil (30 g, 37%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.90 (d, J = 7.3 Hz, 0.55H), 7.86 (d, J = 7.3 Hz, 0.45H), 6.33 – 6.13 (m, 1H), 5.83 (d, J = 7.3 Hz, 1H), 4.53 – 4.31 (m, 1H), 4.08 (d, J = 3.5 Hz, 0.55H), 4.04 (d, J = 3.4 Hz, 0.45H), 3.75 – 3.69 (m, 1H), 3.67 – 3.61 (m, 1.55H), 3.58 (dd, J = 10.7, 3.1 Hz, 0.45H), 3.54 – 3.43 (m, 3H), 3.36 (t, J = 7.4 Hz, 2H), 3.31 (t, J = 7.1 Hz, 2H), 3.09 (d, J = 7.1 Hz, 3H), 3.03 (t, J = 7.9 Hz, 2H), 2.54 – 2.50 (m, 2H), 2.50 – 2.43 (m, 1H), 2.11 – 2.02 (m, 1H), 1.93 – 1.85 (m, 3H), 1.48 – 1.39 (m, 2H), 1.27 – 1.23 (m, 6H), 1.22 – 1.17 (m, 2H), 1.09 – 1.03 (m, 12H), 0.80 (t, J = 7.4 Hz, 3H). ^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$) (note: diastereomers are observed): δ 172.0, 168.2 (d, $J_{C\text{-}P}$ = 3.8 Hz), 156.2 (d, $J_{C\text{-}P}$ = 1.3 Hz), 140.0 (d, $J_{C\text{-}P}$ = 3.8 Hz), 117.4 (d, $J_{C\text{-}P}$ = 5.0 Hz), 102.7 (d, $J_{C\text{-}P}$ = 8.8 Hz), 100.1 (d, $J_{C\text{-}P}$ = 1.3 Hz), 86.2, 84.9 (d, $J_{C\text{-}P}$ = 3.8 Hz) & 84.7, 84.7 (d, $J_{C\text{-}P}$ = 3.8 Hz), 72.9 (d, $J_{C\text{-}P}$ = 3.8 Hz), 72.3 (d, $J_{C\text{-}P}$ = 7.6 Hz), 60.0 (d, $J_{C\text{-}P}$ = 6.3 Hz), 58.2 (d, $J_{C\text{-}P}$ = 5.0 Hz), 58.1 (d, $J_{C\text{-}P}$ = 6.3 Hz), 49.1, 48.6 (d, $J_{C\text{-}P}$ = 5.0 Hz), 44.1, 43.1 (d, $J_{C\text{-}P}$ = 3.8 Hz) & 43.0 (d, $J_{C\text{-}P}$ = 3.8 Hz), 40.7 (d, $J_{C\text{-}P}$ = 5.0 Hz) & 40.6 (d, $J_{C\text{-}P}$ = 5.0 Hz), 30.6 (d, $J_{C\text{-}P}$ = 5.0 Hz), 28.9, 24.4, 24.3, 24.2, 24.1, 24.1, 24.0, 20.2, 20.2 (d, $J_{C\text{-}P}$ = 2.5 Hz), 20.1, 19.9, 19.6, 13.5. ^{31}P NMR (203 MHz, $CDCl_3$): δ 148.32 (s), 148.14 (s). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{30}H_{52}N_6O_6P^+$ 623.3686, found 623.3655.

N^2-[(1-butylpyrrolidine-2-ylidene)amino]-5′-O-(2-methoxypropane-2-yl)-2′-deoxyguanosine (**15**): To a solution of **13**[27] (80 g, 200 mmol) in MeOH (800 mL), aqueous 25% ammonia (800 mL) was added . The mixture was stirred at 45 °C on an oil bath for 20 hours. The reaction solution was concentrated under reduced pressure to give crude **14** (45 g, HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{14}H_{22}N_5O_5^+$ 340.1621, found 340.1588) as a white solid which was used for next step directly. To a solution of crude **14** (45 g, 130 mmol) in MeOH (450 mL), 1-butyl-2,2-dimethoxypyrrolidine (50 g) was added and the reaction was stirred at r.t. for 30 min. DCM (450 mL) was added and the mixture was washed with water. The organic phase was separated, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by preparative RP-HPLC (CH_3CN/H_2O, 65/35 → 85/15, *v/v*) to give **15** as white solid (20 g, yield: 22% over two steps). ^{1}H NMR (500 MHz, DMSO-*d6*): δ 11.10 (s, 1H), 7.98 (s, 1H), 6.20 (t, J = 6.7 Hz, 1H), 5.38 (d, J = 4.1 Hz, 1H), 4.36 (m, 1H), 3.91 (dd, J = 8.6, 4.4 Hz, 1H), 3.55-3.38 (m, 6H), 3.06-2.96 (m, 5H), 2.66 (m, 1H), 2.27 (m, 1H), 2.05-1.93 (m, 2H), 1.60-1.48 (m, 2H), 1.30 (m, 2H), 1.25 (s, 6H), 0.91 (t, J = 7.4 Hz, 3H). ^{13}C{^{1}H} NMR (126 MHz, DMSO-*d6*): δ 168.0, 157.8, 156.7, 149.8, 136.4, 119.2, 99.6, 85.6, 82.8, 70.9, 61.1, 48.6, 47.8, 43.6, 40.0 (overlap), 30.7, 28.4, 24.1, 24.1, 19.6, 19.4, 13.6. HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{22}H_{35}N_6O_5^+$ 463.2669, found 463.2662.

N²-[(1-butylpyrrolidine-2-ylidene)amino]-5′-O-(2-methoxypropane-2-yl)-2′-deoxyguanosine-3’-O-yl cyanoethyl N,N-diisopropylphosphoramidite (**5**): To a solution of **15** (20 g, 43 mmol) in DCM (200 mL), 2-cyanoethyl N,N,N′,N′-tetraisopropylphosphorodiamidite (16 g, 52 mmol) and tetrazole (2.4 g, 34 mmol) were added under nitrogen. After stirring for 2 hours at r.t., the reaction was quenched with saturated $NaHCO_3$ (200 mL) and the organic phase was separated, dried and evaporated. The crude product was purified by silica gel column chromatography (EtOAc/Heptane, 1:2 → 1:1, v/v) to give **5** as white solid (14 g, yield: 49%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 9.19 (s, 0.5H), 9.18 (s, 0.5H), 7.83 (s, 0.5H), 7.82 (s, 0.5H), 6.23 (t, J = 6.6

Hz, 1H), 4.60-4.52 (m, 1H), 4.20 (d, J = 2.4 Hz, 0.5H), 4.13 (d, J = 2.7 Hz, 0.5H), 3.82-3.74 (m, 1H), 3.73-3.64 (m, 1H), 3.60-3.45 (m, 4H), 3.44-3.33 (m, 4H), 3.15-3.01 (m, 5H), 2.62-2.54 (m, 2H), 2.54-2.39 (m, 2H), 2.05-1.90 (m, 2H), 1.51-1.43 (m, 2H), 1.35-1.18 (m, 8H), 1.11 (s, 6H), 1.10 (s, 6H), 0.83 (t, J = 7.4 Hz, 3H); $^{13}C\{^1H\}$ NMR (126 MHz, $CDCl_3$) (note: diastereomers are observed): δ 169.2 (d, J_{C-P} = 5.0 Hz), 158.0, 156.3, 150.3, 136.0 (d, J_{C-P} = 6.3 Hz), 119.9, 117.5, 100.5 (d, J_{C-P} = 1.3 Hz), 85.6 (d, J_{C-P} = 7.6 Hz) & 85.6 (d, J_{C-P} = 7.6 Hz), 83.5, 74.1 (d, J_{C-P} = 16.4 Hz), 60.8 (d, J_{C-P} = 13.9 Hz), 58.6 (d, J_{C-P} = 12.6 Hz), 58.4(d, J_{C-P} = 12.6 Hz), 49.3, 48.9 (d, J_{C-P} = 1.3 Hz), 44.5, 43.5(d, J_{C-P} = 12.6 Hz) & 43.4 (d, J_{C-P} = 12.6 Hz), 40.8 (d, J_{C-P} = 16.4 Hz) & 40.7 (d, J_{C-P} = 16.4 Hz), 31.7, 29.1, 24.8, 24.7, 24.6, 24.6, 24.5, 24.4, 20.6 (d, J_{C-P} = 16.4 Hz), 20.2, 19.9 (d, J_{C-P} = 16.4 Hz), 13.9; ^{31}P NMR (243 MHz, $CDCl_3$): δ 148.46 (s), 148.31 (s). HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{31}H_{52}N_8O_6P^+$ 663.3747, found 663.3712.

N^2-isobutyryl-5'-O-(2-methoxypropane)-2'-deoxyguanosine-3'-O-yl cyanoethyl N,N-diisopropylphosphoramidite (**2**). To a solution of **13** (30 g, 73.3 mmol) in DCM (300 mL), 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (26.5 g, 87.9 mmol, 1.2 eq.) and tetrazole (4.11 g, 58.6 mmol, 0.8 eq.) were added under nitrogen. After stirring for 60 minutes at r.t., the reaction was quenched by addition of saturated aqueous $NaHCO_3$ (200 mL). The organic phase was separated, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by silica gel column chromatography (EtOAc/Heptane, 1:2 → 3:1, v/v) to give **2** as white solid (29.3 g, yield: 65.6 %). ^1H NMR (500 MHz, $CDCl_3$): δ 12.18 (s, 1H), 9.82 (s, 0.5H), 9.76 (s, 0.5H), 7.98 (s, 0.5H), 7.95 (s, 0.5H), 6.25-6.21 (m, 1H), 4.78-4.73 (m, 0.5H), 4.69-4.64 (m, 0.5H), 4.26-4.21 (m, 1H), 3.94-3.84 (m, 1.5H), 3.81-3.71 (m, 1.5H), 3.67-3.55 (m, 3H), 3.16(s, 1.5H), 3.15 (s, 1.5H), 2.82-2.68(m, 4.5H), 2.57-2.52 (m, 0.5H), 1.37 (s, 3H), 1.34 (d, J = 2.4 Hz, 3H), 1.24-1.17 (m, 18H). $^{13}C\{^1H\}$ NMR (126 MHz, $CDCl_3$): δ 179.5 & 179.4, 155.8 & 155.8, 148.0 (d, *J* = 11.3 Hz), 147.8 (d, *J* = 10.8 Hz), 137.6 & 137.2, 121.5 & 121.3, 117.8 (d, *J* = 5.4 Hz), 100.5 & 100.4, 85.5 (d, *J* = 11.3 Hz) & 85.5, 84.7 & 84.2, 74.1 (d, *J* = 17.6 Hz) & 73.3 (d, *J* = 15.1 Hz), 60.8 & 60.6, 58.0 (d, *J* = 10.1 Hz) & 57.9 (d, *J* = 10.1 Hz), 48.6, 48.6, 43.3 & 43.2, 40.1 (d, *J* = 2.5 Hz) & 39.7 (d, *J* = 3.8 Hz), 36.0 & 35.9, 24.6, 24.5, 24.5, 24.4, 24.3, 24.2, 20.5 (d, *J* = 2.5 Hz) & 20.4 (d, *J* = 3.8 Hz), 19.0, 18.9 (d, *J* = 3.8 Hz). ^{31}P NMR (203 MHz, $CDCl_3$): δ 148.52 (s), 147.79 (s). HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{27}H_{45}N_7O_7P^+$ 610.3118, found 610.3083.

N^4-(2,4-dimethylbenzoyl)-5'-O-(2-methoxypropane)-2'-deoxycytidine-3'-O-yl cyanoethyl N,N-diisopropylphosphoramidite (**3**): To a solution of **16** (70 g, 160 mmol) in DCM (700 mL), 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (59 g, 190 mmol, 1.2 eq.) and tetrazole (9.1 g, 130 mmol, 0.8 eq.) were added. After stirring for 2 hours at r.t., the reaction mixture was washed with 5% $NaHCO_3$ aqueous solution and 10% NaCl aqueous solution successively. The organic phase was separated, dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified by silica gel chromatography (EtOAc/n-heptane, 1:4 → 1:1, *v/v*) to give **3** as white solid (56 g, yield: 54.6%). ^1H NMR (500 MHz, $CDCl_3$): *δ* 8.73 (s, 1H), 8.46 (dd, *J* = 12.1, 7.5 Hz, 1H), 7.50 (d, *J* = 7.3 Hz, 1H), 7.42 (d, *J* = 7.8 Hz, 1H), 7.08 (s, 1H), 7.05 (d, *J* = 7.9 Hz, 1H), 6.27 (m, 1H), 4.57 – 4.47 (m, 1H), 4.31 (d, *J* = 2.9 Hz, 0.5H), 4.25 (d, *J* = 3.0 Hz, 0.5H), 3.91 – 3.82 (m, 1H), 3.79 – 3.73 (m, 2H), 3.71 – 3.55 (m, 3H), 3.24 (d, *J* = 5.6 Hz, 3H), 2.77 – 2.68 (m, 1H), 2.65 (t, *J* = 6.2 Hz, 2H), 2.49 (s, 3H), 2.35 (s, 3H), 2.26 – 2.18 (m, 1H), 1.48 – 1.33 (m, 6H), 1.32 – 1.08 (m, 12H). $^{13}C\{^1H\}$ NMR (126 MHz, $CDCl_3$) (note: diastereomers are observed): *δ* 168.6, 162.1 (d, J_{C-P} = 3.8 Hz), 154.9, 144.7, 142.0, 137.7, 132.6, 131.1, 127.2, 126.7, 117.5 (d, J_{C-P} = 6.3 Hz), 100.3, 95.6, 87.1 (d, J_{C-P} = 5.0 Hz), 85.7 (d, J_{C-P} = 3.8 Hz) & 85.5 (d, J_{C-P} = 3.8 Hz), 72.9 (d, J_{C-P} = 6.3 Hz) & 72.5 (d, J_{C-P} = 16.4 Hz), 59.8, 58.3 (d, J_{C-P} = 3.8 Hz) & 58.1 (d, J_{C-P} = 3.8 Hz), 48.9 (d, J_{C-P} = 1.3 Hz), 43.3 (d, J_{C-P} = 2.5 Hz) & 43.2 (d, J_{C-P} = 2.5 Hz), 41.2 (d, J_{C-P} = 7.6 Hz) & 41.0 (d, J_{C-P} = 7.6 Hz), 24.6, 24.5, 24.5, 24.5, 24.4, 24.3, 21.3, 20.3 (d, J_{C-P} = 7.6 Hz), 20.1. ^{31}P NMR (203MHz, $CDCl_3$): *δ* 148.63 (s), 148.55 (s). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{31}H_{47}N_5O_7P^+$ 632.3213, found 632.3210.

7-[N^6-(2,4-dimethylbenzoyl)-2'-deoxyadenosine (**18**). To a solution of 2'-deoxyadenosine (89 g, 350 mmol) in DCM (890 mL), triethylamine (254 g, 2.51 mol, 7.1 eq.) and chlorotrimethylsilane (121 g, 1,11 mol, 3.1 eq.) were added at 0 °C. After stirring at 25 °C for one hour, 2,4-dimethylbenzoyl chloride (149 g, 886 mmol, 2.5 eq.) was added at 0 °C, and the mixture was stirred at 25 °C for 12 hours. Then, the mixture was washed with water (twice), the organic phase was separated, dried over Na_2SO_4, and filtered to give a solution which was used for the next step directly. *N*-methyl piperazine (35 g, 0.34 mol, 0.97 eq.) and triethylamine (65 g, 0.64 mol, 1.8 eq.) were added and the mixture was stirred at 25 °C overnight. Pyridinium fluoride (70%, 32 mL) was added, and the mixture was stirred at 25 °C for 3 hours. The mixture was concentrated and the crude oil was purified by silica gel column chromatography (DCM/MeOH, 50:1, *v/v*) to give 42.0 g of **18** (31%) as yellow solid. ^1H NMR (500 MHz, DMSO-*d6*) δ 10.99 (s, 1H), 8.69 (s, 1H), 8.68 (s, 1H), 7.47 (d, *J* = 7.7 Hz, 1H), 7.13 (s, 1H), 7.10 (d, *J* = 8.0 Hz, 1H), 6.48 (t, *J* = 6.8 Hz, 1H), 5.36 (d, *J* = 4.2 Hz, 1H), 5.02 (t, *J* = 5.6 Hz, 1H), 4.53 – 4.38 (m, 1H), 3.91 (dd, *J* = 7.5, 4.5 Hz, 1H), 3.68 – 3.61 (m, 1H), 3.57 – 3.52 (m, 1H), 2.84 – 2.75 (m, 1H), 2.42 (s, 3H), 2.39 – 2.34 (m, 1H), 2.33 (s, 3H). $^{13}C\{^1H\}$ NMR (126 MHz, DMSO-*d6*) *δ* 167.82, 151.78, 151.54, 150.04, 142.93, 140.11, 136.60, 132.50, 131.43, 128.32, 126.05, 125.36, 88.00, 83.75, 70.70, 61.63, 39.52 (overlap), 20.84, 19.71. HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{19}H_{22}N_5O_4^+$ 384.1672, found 384.1662.

7-[N^6-(2,4-dimethylbenzoyl)-5'-O-(4,4'dimethoxytrityl)-2'-deoxyadenosine-3'-O-yl]-4,7-dioxoheptanoic acid, triethylammonium salt (**20**): To a solution of **18** (42 g, 110 mmol) in pyridine (450 mL), DMTrCl (47 g, 140 mmol, 1.25 eq.) was added in five equal portions with an interval of 5 mins at 0 °C. After stirring for 2 hours at 0°C → r.t., MeOH (7 mL), $NaHCO_3$ (20 g) and water (5 mL) were added successively to quench the reaction. After quenching, the mixture was concentrated to dryness. The residue was dissolved in DCM (450 mL) and washed twice with water. The organic phase was separated, dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash silica gel column chromatography (ethyl acetate/n-heptane, 1:2 → 2:1, v/v) to give **19** as white solid (37 g, yield: 50 %, HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{40}H_{40}N_5O_6^+$ 686.2979, found

686.2990). To a solution of 4-oxoheptanedioic acid (20 g, 115 mmol, 2.0 eq) in anhydrous THF (300 mL), DMAP (2.8 g, 11 mmol, 0.2 eq.) and DCC (36 g, 170 mmol, 3.0 eq.) were added. The mixture was stirred for 16 hours at r.t. and filtered. A solution of **19** (37 g, 57 mmol) in THF (300 mL) and DBU (52 g, 340 mmol, 6 eq.) were added to the above filtrate successively. After stirring overnight at r.t., the mixture was evaporated to dryness. The residue was purified by silica gel column chromatography (ethyl acetate/n-heptane, 1:1, v/v → DCM/MeOH/TEA, 100/2/1, v/v) to give **20** as white solid (38 g, yield: 74%). ^{1}H NMR (500 MHz, DMSO-d6): δ 11.01 (s, 1H), 8.60 (s, 1H), 8.56 (s, 1H), 7.49 (d, *J* = 7.7 Hz, 1H), 7.35 (d, *J* = 7.5 Hz, 2H), 7.23 (d, *J* = 8.6 Hz, 6H), 7.20 – 7.15 (m, 1H), 7.12 (s, 1H), 7.09 (d, J = 8.0 Hz, 1H), 6.82 (t, *J* = 9.5 Hz, 4H), 6.51 (t, *J* = 7.0 Hz, 1H), 5.47 – 5.43 (m, 1H), 4.23 (s, 1H), 3.72 (s, 6H), 3.37 – 3.25 (m, 3H), 2.79 (t, *J* = 6.4 Hz, 2H), 2.70 – 2.61 (m, 5H), 2.55 (t, *J* = 6.4 Hz, 3H), 2.43 (s, 3H), 2.39 (t, *J* = 6.6 Hz, 2H), 2.32 (s, 3H), 1.00 (t, *J* = 7.2 Hz, 4.5H). ^{13}C{^{1}H} NMR (126 MHz, DMSO-d6): δ 207.8, 174.2, 171.9, 167.8, 158.1 (2 × C),6 151.8, 151.5, 150.2, 144.8, 143.4, 140.2, 136.7, 135.5, 135.5,9 132.5, 131.5, 129.7 (2 × C), 129.7 (2 × C), 128.4, 127.8 (2 × C), 127.7 (2 × C), 126.7, 126.1, 125.5, 113.1 13 (4C),32 85.7, 84.2, 83.5, 74.6, 63.8, 55.0 (2 × C), 45.3 (3 × C), 37.1, 36.6, 35.3, 28.6, 27.8, 20.9, 19.7, 10.3 (3 × C). HRMS (ESI): m/z [M-Et_3NH^++2H]$^+$ calcd for $C_{47}H_{48}N_5O_{10}^+$ 842.3401, found 842.3414.

7-[N^6-(2,4-dimethylbenzoyl)-2'-deoxyadenosine-3'-O-yl] N-methyl-N-propargyl-4,7-dioxoheptane-amide (**22**): To a solution of **20** (38 g, 45 mmol) in DCM (400 mL), HOBT (9.1 g, 68 mmol, 1.5 eq.) and EDC (13 g, 68 mmol, 1.5 eq.) were added. After stirring for 2 hours at r.t., *N*-methyl-2-propynylamine (6.2 g, 90 mmol, 2.0 eq.) and Et_3N (9.1 g, 90 mmol, 2.0 eq.) were added. The mixture was stirred for 12 hours at r.t. and washed twice with water. The organic phase was separated, dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified by silica gel flash column chromatography (ethyl acetate/*n*-heptane, 1:1 →1:0, *v/v*) to give **21** as white solid (31 g yield: 96%, HRMS (ESI): *m/z* [M+H]$^+$ calcd for $C_{51}H_{53}N_6O_9^+$ 893.3874, found 893.3864). To a solution of **21** (30 g, 33 mmol) in DCM (300 mL), trifluoroacetic acid (5.7 g, 50 mmol, 1.5 eq.) was added. The mixture was cooled to 0 °C and 1-dodecanethiol (13 g, 66 mmol, 2.0 eq.) was added. The mixture was stirred at 0 °C for 2 h and washed twice with aqueous 5% $NaHCO_3$. The organic phase was separated, dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified by preparative RP-HPLC (MeCN/H_2O, 3:7 → 7:3, *v/v*) to give **22** as white solid (11 g, yield: 56%). ^{1}H NMR (500 MHz, DMSO-*d*6): *δ* 11.03 (s, 1H), 8.71 (s, 2H), 7.48 (d, *J* = 7.8 Hz, 1H), 7.14 (s, 1H), 7.11 (d, *J* = 8.0 Hz, 1H), 6.50 (dd, *J* = 8.4, 6.1 Hz, 1H), 5.40 (d, *J* = 5.8 Hz, 1H), 5.20 (t, *J* = 5.7 Hz, 1H), 4.19 (d, *J* = 2.2 Hz, 0.8H), 4.14 – 4.09 (m, 2.2H), 3.74 – 3.58 (m, 2H), 3.32 (t, *J* = 2.3 Hz, 0.4H), 3.15 (t, *J* = 2.4 Hz, 0.6H), 3.07 – 3.01 (m, 1H), 3.01 (s, 1.8H), 2.87 – 2.79 (m, 3.2H), 2.71 – 2.66 (m, 2H), 2.65 – 2.62 (m, 0.8H), 2.59 – 2.52 (m, 4.2H), 2.43 (s, 3H), 2.34 (s, 3H). ^{13}C{^{1}H} NMR (126 MHz, DMSO-*d*6) (note: rotamers observed): *δ* 208.0 & 208.0, 171.9, 171.1 & 170.9, 167.8, 151.9, 151.7, 150.2, 142.9, 140.2, 136.7, 132.5, 131.5, 128.4, 126.1, 125.3, 85.4, 83.9, 79.7 & 79.3, 75.1 & 74.9, 73.9, 61.6, 38.5, 36.8 & 36.5, 35.6, 33.9, 32.9, 27.9, 26.9 & 26.7, 20.7, 19.8. HRMS (ESI): *m/z* [M+H]$^+$ calcd for $C_{30}H_{35}N_6O_7^+$ 591.2567, found 591.2586.

Tetrapodal soluble support **17**: **22** (4.1 g, 6.9 mmol), tetrakis-*O*-(4-azidomethylphenyl)pentaerythritol[15] (**23**, 0.9 g, 1.4 mmol) and sodium ascorbate (55 mg, 0.28 mmol, 0.2 eq) were dissolved in anhydrous dimethylacetamide (20 ml) and the solution was purged with argon for 60 min. CuI (0.16 g, 0.83 mmol) was added and the mixture was stirred for 48 h at r.t.. The solvent was removed under vacuum and the residue was partitioned between DCM and saturated $NaHCO_3$. The organic phase was washed with 10 % EDTA disodium salt (3 x 20 ml), dried with Na_2SO_4 and evaporated to dryness. The crude material was purified by silica gel chromatography (MeOH/DCM, 5:10 → 10:9, *v/v*) affording **17** (4.0 g, 96 %) as white solid. ^{1}H NMR (500 MHz, $CDCl_3$)(note: rotamers observed approximately in 0.3:0.7 ratio, cf. in Figures S28-S33): *δ* 8.72 (s, 4H), 8.38 (s, 1.2H), 8.37 (s, 2.8H), 7.56 (d, *J* = 7.9 Hz, 4H), 7.53-7.51 (m, 1.2H), 7.41 (s, 2.8H), 7.19-7.15 (m, 8H), 7.09 (s, 4H), 7.06 (d, *J* = 7.9 Hz, 4H), 6.90 – 6.83 (m, 8H), 6.45-6.38 (m, 4H), 5.52 (d, *J* = 5.5 Hz, 4H), 5.41 (s, 2.4H), 5.36 (s, 5.6H), 4.57 (s, 2.4H), 4.52 (s, 5.6H), 4.30 (s, 8H), 4.25 (m, 4H), 3.95 – 3.82 (m, 8H), 3.10-3.02 (m, 12.4H), 2.88 – 2.82 (m, 11.6H), 2.82-2.72 (m, 10H), 2.64 – 2.56 (m, 12H), 2.52 (s, 12H), 2.50-2.43 (m, 4H), 2.35 (s, 12H); ^{13}C{^{1}H} NMR (125 MHz, CDCl3): *δ* 208.1 & 207.9, 172.2 & 172.1, 171.6, 166.9, 159.1 & 159.0, 152.3, 150.7, 150.4, 144.4 & 144.2, 142.9 & 142.7, 142.0, 138.3, 132.7, 131.5, 129.8, 127.8, 127.4 & 127.2, 126.7, 124.1, 122.5 & 121.6, 115.3 & 115.2, 87.6 & 87.5, 87.2 & 87.2, 77.4 & 76.5, 66.6, 63.2, 53.9 & 53.7, 45.5 & 43.0, 44.8, 37.9 & 37.8, 37.3 & 37.3, 37.1, 35.5 & 33.9, 28.3 & 28.2, 27.3 & 27.2, 21.5, 20.5; MS (ESI + TOF): m/z calcd for [(M + 2H)/2]$^{2+}$ 1511.6, found 1511.6.

General procedure for one precipitation / synthesis cycle in LPOS: Coupling: To a solution of soluble support (0.165 mmol) in an anhydrous mixture of DMF (7 ml) and MeCN (4 ml), 5'-O-MIP protected phosphoramidite building block (**1-4**, 1.32 mmol) and 0.45 M tetrazole in MeCN (2.94 ml, 1.32 mmol) were added. The reaction was allowed to proceed for 2 hours at r.t., and quenched by addition of methanol (0.66 mmol). *2. Oxidation:* m-chloroperbenzoic acid (77 m-%, 2.48 mmol, 15 eq) was added and the mixture was shaken for 5 min at r.t. *3. MIP-deprotection:* dichloroacetic acid (DCA, 2.47 ml, 15 % of total volume) was added and the mixture was shaken for 15 minutes at r.t. *4. Precipitation:* The reaction solution was added dropwise to 2-propanol (10 fold volume compared to the reaction mixture). The precipitated material was collected by centrifugation, washed with 2-propanol (100 ml) and dried under vacuum to afford soluble support-oligonucleotide construct (**24**, **25**, **26**, **28**, **30** and **32**) as white powder (cf. precipitation yields in Scheme 3).

General procedure for two precipitations / synthesis cycle in LPOS: 1. Coupling: as above. *2. Oxidation*: 0.2 M I_2 in THF/Py/H_2O (5:4:1 *v/v/v*) was added dropwise to the reaction mixture until the colour of iodine persisted and then the mixture was allowed to stir for further 15 minutes. The excess I_2 was consumed by addition of trimethylphosphite until the colour of iodine cleared. *3. Precipitation:* as

above. *4. MIP-deprotection:* The precipitate was dissolved in a mixture DCA-MeOH-DCM (5:18:27, v/v/v) and the mixture was stirred for 15 min, at r.t. *5. Precipitation:* as above. The soluble support-oligonucleotide constructs (**27**, **29**, **31** or **33**) were obtained as white powders (cf. precipitation yields in Scheme 3).

Aliquots of the precipitates were exposed to concentrated ammonia (overnight at 55°C,) for evaluation of purity, authenticity and yield (cf. Scheme 3) of the released di-, tri-, tetra- and pentanucleotide (Scheme S1).

Solubility measurements. A saturation shake-flask method was adapted for probing the solubilities of the nucleotide linked soluble supports. In a sealed vial, an excess amount of the solid material was equilibrated with each tested solvent (500 µl) on an orbital shaker (400 rpm) at ambient temperature for 24 hours. An upper limit value for the solubility was set at 300 mg/ml and therefore, saturating of samples reaching this value was not attempted. After the equilibration, the clear supernatant solution from each sample was transferred to a centrifuge tube, then briefly centrifuged, and an aliquot of the solution was diluted with dimethyl sulfoxide. The diluted samples were analysed with HPLC, and the mass concentrations of the analytes were determined by the UV peak area at 260 nm. The calculations were performed using a calibration curve fitted to the UV peak areas of five standard solutions of known mass concentrations prepared for each tested compound in dimethyl sulfoxide.

Hydrazine acetate cleavage of protected trinucleotides (**36** and **37**). 5'-O-MIP-protected trinucleotide-soluble supports (**34** and **35**) were assembled on 40 and 90 µmol scale by following the LPOS procedures above. Overall precipitation yields of 96 and 92%, respectively, were obtained. A solution of 0.1 M hydrazine acetate was prepared by adding hydrazine hydrate (25 µl) to a mixture of acetic acid (1 ml) and pyridine (5 ml). Trinucleotide-soluble support construct **34** (190 mg, 29 µmol) was dissolved in the freshly prepared 0.1 M hydrazine acetate (1.4 ml, 140 µmol, 5 eq) and the mixture was stirred for 1 hour. The reaction solution was diluted with DCM and the organic phase was washed with saturated $NaHCO_3$. The organic phase was dried with Na_2SO_4 and concentrated under vacuum. The crude material was purified by a short silica gel column (MeOH/DCM, 5:95 → 7:93, *v/v*, containing 1 % pyridine) affording **36** as white solid (106 mg, 71 %). Trinucleotide-soluble support construct **35** (575 mg, 85 µmol) was treated in a similar manner to afford **36** (292mg, 65 % yield) as white powder. See ^{1}H NMR (500 MHz, CD_3CN), $^{13}C\{^{1}H\}$ NMR (126 MHz, CD_3CN) and ^{31}P NMR (202 MHz, CD_3CN) spectra of **36** and **37** in Figures S34-S43. **36**: MS (ESI-TOF): m/z calcd for $[M + H]^+$ 1287.4, found 1287.4. **37**: MS (ESI-TOF): m/z calcd for $[M + H]^+$ 1318.5, found 1318.5.

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ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

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The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript. / ‡These authors contributed equally. (match statement to author names with a symbol)

Notes

The authors declare no competing final interest.

Supporting Information

NMR data for new compounds (**2**-**6**, **9**, **12**, **15**, **17**, **18**, **20**, **22**, **36** and **37**), RP HPLC profiles, including purity according to peak areas of the HPLC profiles, and MS spectra of di-, tri-, tetra- and pentanucleotides released from oligonucleotide-soluble support constructs **24**-**33**. MS spectra of oligonucleotide-soluble support constructs **32** and **33**. Graphic illustrations of mass content and varying solubility of oligonucleotide-soluble support constructs. HPLC monitoring of hydrazine acetate-mediated orthogonal cleavage of a protected trinucleotide.

The Supporting Information is available free of charge on the ACS Publications website.

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