

## 5',5'-Phosphodiester and esterase labile triesters of 2'-C-methylribonucleosides

Anna Leisvuori,<sup>a</sup> Zafar Ahmed,<sup>a</sup> Mikko Ora,<sup>a</sup> Lawrence Blatt,<sup>b</sup> Leonid Beigelman<sup>b</sup> and Harri Lönnberg<sup>\*a</sup>

<sup>a</sup>Department of Chemistry, University of Turku, FIN-20014 Turku, Finland

<sup>b</sup>AliosBiopharma, 260 E. Grand Ave, 2nd Floor, South San Francisco, CA 94080, USA

E-mail: [harlon@utu.fi](mailto:harlon@utu.fi)

Dedicated to Prof Ferenc Fülöp on the occasion of his 60<sup>th</sup> birthday

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### Abstract

Bis(2'-C-methyladenosin-5'-yl) (**11**), bis(2'-C-methylguanosin-5'-yl) (**13**), bis(2'-C-methyluridin-5'-yl) (**15**) and 2'-C-methylguanosin-5'-yl 2'-C-methyluridin-5'-yl (**16**) phosphodiester have been prepared as pro-drug candidates for the respective 2'-C-methylribonucleoside 5'-monophosphates, expectedly exhibiting antiviral activity against Hepatitis C virus. Additionally, the bis(2'-C-methyladenosine) diester has been converted to 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl (**19**) or pivaloyloxymethyl (**20**) triester. The underlying idea is that the 5',5'-phosphodiester is first released by intracellular carboxyesterases and subsequently cleaved to nucleoside and nucleoside 5'-monophosphate by phosphodiesterases.

**Keywords:** Pro-drug, antiviral, nucleotide, phosphoester, synthesis

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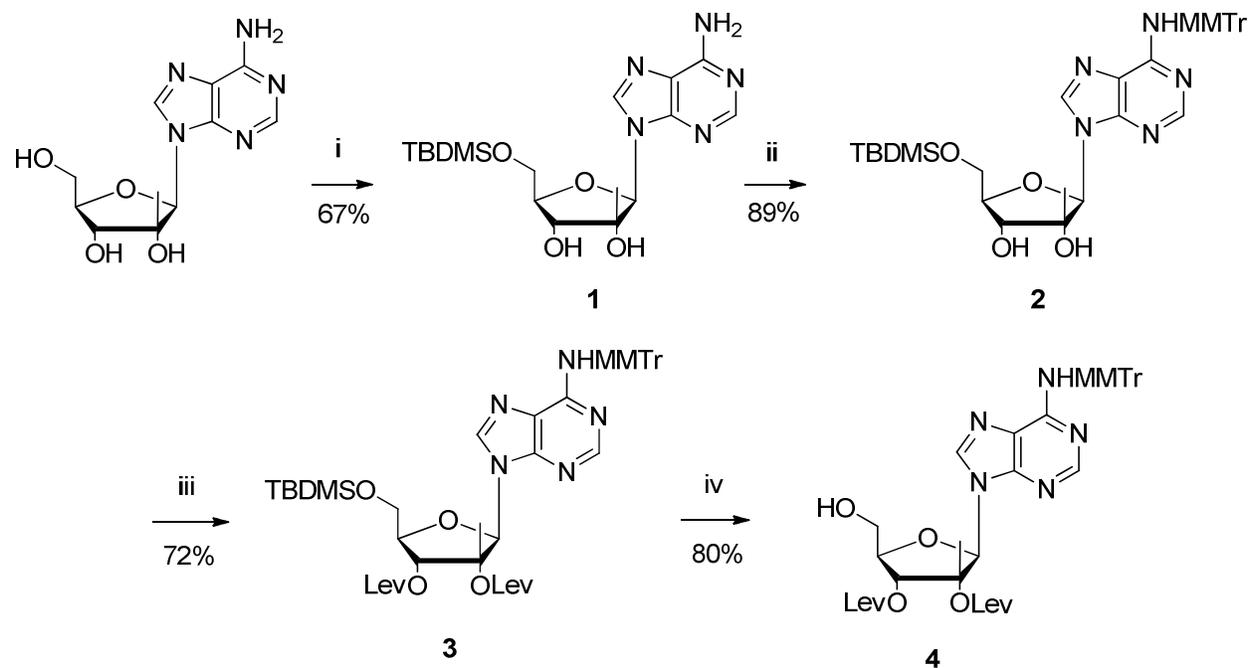
### Introduction

Hepatitis C virus (HCV) infections constitute a worldwide health threat with an estimated 170 million infected individuals.<sup>1,2</sup> Besides interferon based therapies,<sup>3</sup> inhibitors of the viral NS3-4A serine protease and the NS5B RNA polymerase have emerged as potential drug candidates.<sup>4,5</sup> Among the RNA polymerase inhibitors, 2'-C-methylribonucleosides have received particular attention. 2'-C-methyl-3'-O-(L-valinyl)cytidine (Valopicitabine)<sup>6,7</sup> and 4'-azido-3',5'-di-O-(2-methylpropanoyl)cytidine<sup>8</sup> showed antiviral activity in phase II and phase I clinical trials, respectively, and 2'-C-methyladenosine and 2'-C-methylguanosine inhibited HCV RNA replication *in vitro*.<sup>9</sup> To terminate the synthesis of viral RNA, antiviral nucleoside analogues need to be converted to their 5'-triphosphates. Since conversion to the monophosphate often is

the rate-limiting step, low antiviral activity may result from lack of this transformation by nucleoside kinases, and biodegradably protected nucleotides might, hence, be more potent drugs.<sup>10</sup> For example, 2'-*C*-methylguanosine has been reported to suffer from inefficient uptake and phosphorylation.<sup>11</sup> We now report on synthesis of novel type of *pro*-2'-*C*-methylribonucleotides. Two 2'-*C*-methylribonucleosides are linked to each other via a 5',5'-phosphodiester bond that additionally bears an esterase labile protecting group, either a pivaloyloxymethyl or 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl group. The underlying idea is that intracellular carboxyesterases first releases the 5',5'-phosphodiester, which is then cleaved by phosphodiesterases to nucleoside and nucleoside 5'-monophosphate.

## Results and Discussion

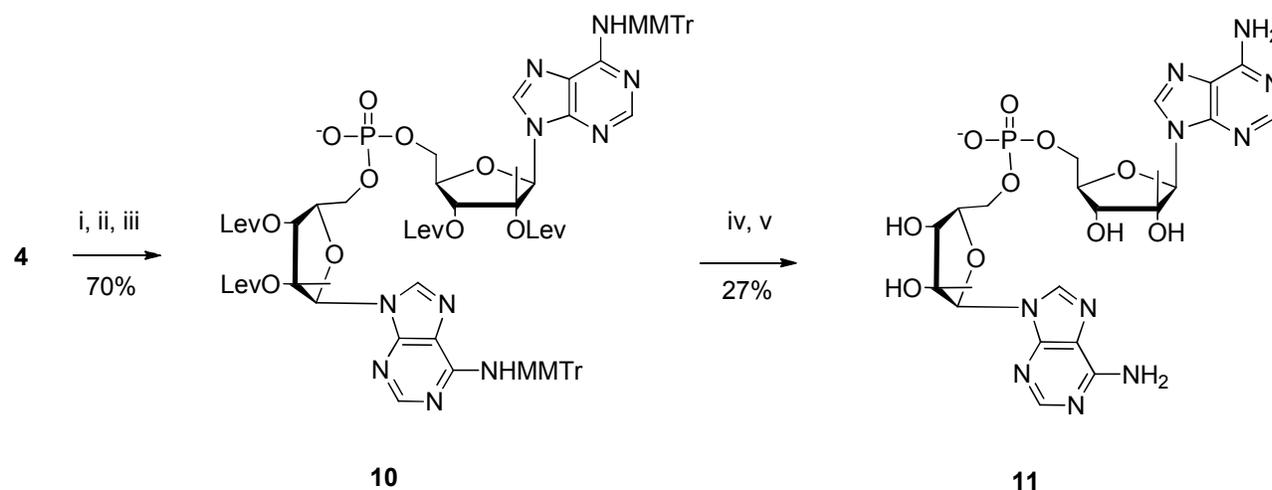
Conversion of 2'-*C*-methyladenosine, 2'-*C*-methylguanosine and 2'-*C*-methyluridine to a protected form allowing their 5'-phosphitylation and, hence, the assembly of their 5',5'-phosphodiester is outlined in Schemes 1-3, respectively. Accordingly, the 5'-OH of 2'-*O*-methyladenosine was first protected with a *tert*-butyldimethylsilyl group to give **1** and the 6-amino function then with a 4-methoxytrityl group (**2**; Scheme 1). Finally, the 2'- and 3'-OH groups were esterified with levulinic anhydride, prepared from levulinic acid *in situ* (**3**), and the 5'-silyl protection was removed (**4**). These two reactions deserve special attention.



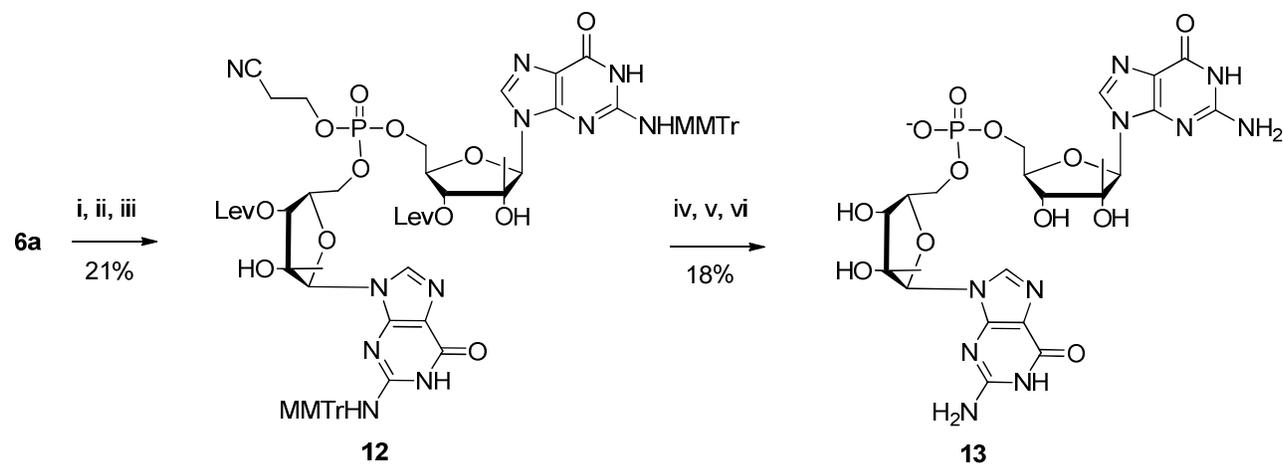
**Scheme 1.** Reagents and conditions: (i) TBDMSCl, Py; (ii) MMTrCl, Py,  $\Delta$ ; (iii) 1. Lev<sub>2</sub>O, Py, 1,4-dioxane, 1 day 2. Lev<sub>2</sub>O, DCM, 4 days ; (iv) TBAF, THF, AcOH.



and the phosphite triester was oxidized to phosphate ester (**12**; Scheme 5). The levulinoyl and 2-cyanoethyl groups were removed with methanolic ammonia and the 4-methoxytrityl with aqueous acetic acid (**13**). Finally, the ammonium counter ion was exchanged to sodium ion with the aid of a strong cation exchange resin.



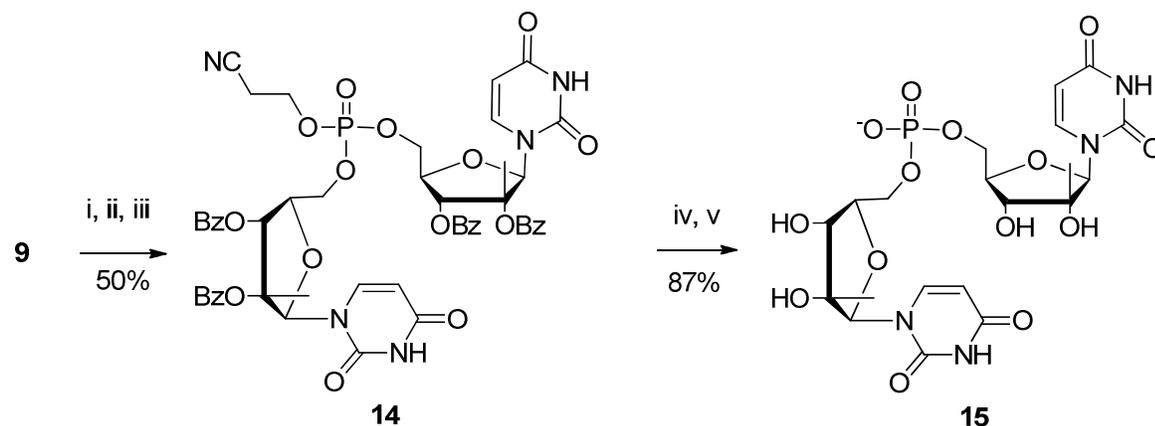
**Scheme 4.** Reagents and conditions: (i)  $i\text{Pr}_2\text{NPCl}_2$ ,  $\text{Et}_3\text{N}$ , DCM; (ii)  $\text{H}_2\text{O}$ , TetH, MeCN; (iii)  $\text{I}_2$ , 2,6-lutidine,  $\text{H}_2\text{O}$ , THF; (iv)  $\text{H}_2\text{NNH}_3\text{OAc}$ , MeOH; (v) 80% AcOH



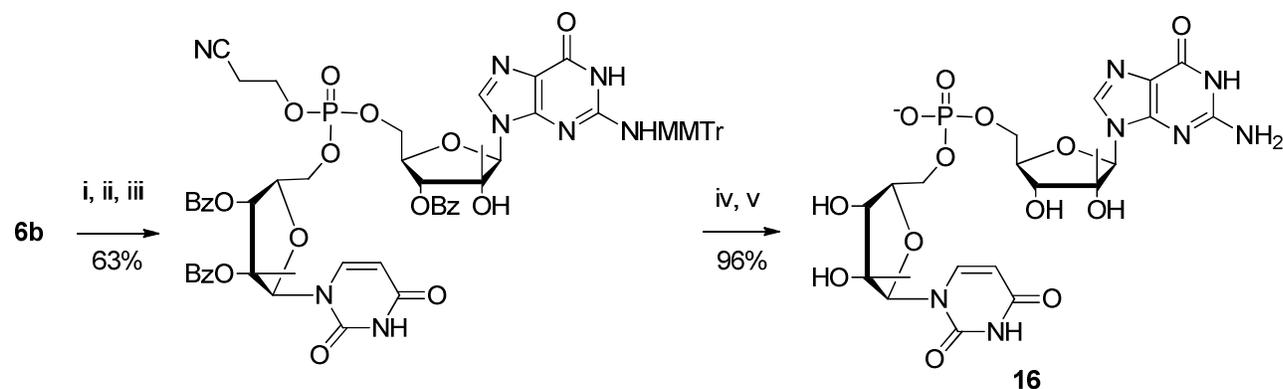
**Scheme 5.** Reagents and conditions: (i) 1-chloro-1-(2-cyanoethoxy)- $N,N$ -diisopropylphosphinamine,  $\text{Et}_3\text{N}$ , DCM; (ii) **6a**, TetH, MeCN; (iii)  $\text{I}_2$ , 2,6-lutidine,  $\text{H}_2\text{O}$ , THF; (iv)  $\text{NH}_3$ , MeOH; (v) 80% AcOH; (vi) Dowex 50WX8  $\text{Na}^+$  form,  $\text{H}_2\text{O}$ .

The same method was applied to preparation of bis(2'- $C$ -methyluridin-5'-yl) phosphate (**15**; Scheme 6) from 2',3'-di- $O$ -benzoyl-2'- $C$ -methyluridine (**9**) and preparation of 2'- $C$ -methylguanosin-5'-yl 2'- $C$ -methyluridin-5'-yl phosphate (**16**; Scheme 7) from **9** and 3'- $O$ -

benzoyl-*N*<sup>2</sup>-(4-methoxytrityl)-2'-*C*-methylguanosine (**6b**). The low yields of compounds **11**, **12** and **13** resulted from difficulties in chromatographic purification. The migration of the derivatives of 2'-*C*-methylguanosine, in particular, was accompanied by marked broadening of the bands



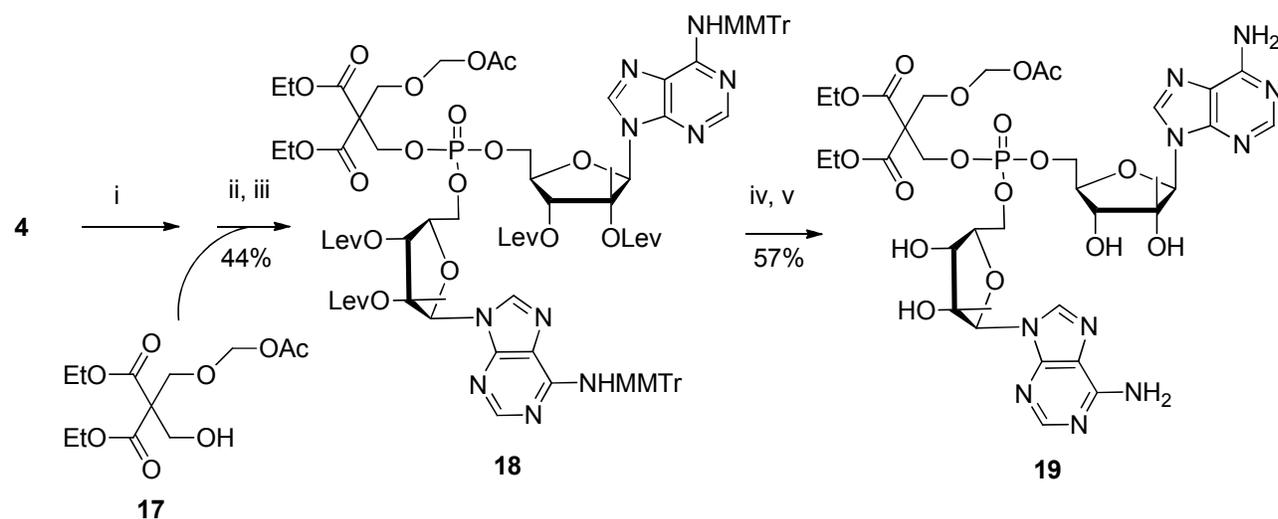
**Scheme 6.** Reagents and conditions: (i) 1-chloro-1-(2-cyanoethoxy)-*N,N*-diisopropylphosphinamine, Et<sub>3</sub>N, DCM; (ii) **9**, TetH, MeCN; (iii) I<sub>2</sub>, 2,6-lutidine, H<sub>2</sub>O, THF; (iv) NH<sub>3</sub>, MeOH; (v) Dowex 50WX8 Na<sup>+</sup> form, H<sub>2</sub>O



**Scheme 7.** Reagents and conditions: (i) 1-chloro-1-(2-cyanoethoxy)-*N,N*-diisopropylphosphinamine, Et<sub>3</sub>N, DCM; (ii) **9**, TetH, MeCN; (iii) I<sub>2</sub>, 2,6-lutidine, H<sub>2</sub>O, THF; (iv) NH<sub>3</sub>, MeOH; (v) 80% AcOH

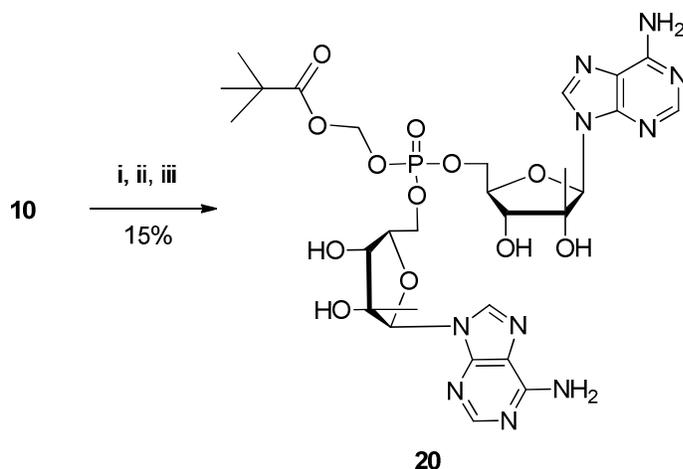
Two different esterase labile groups were used to protect the internucleosidic 5',5'-phosphodiester linkage. 3-Acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl group was introduced by phosphitylating the corresponding alcohol (**17**)<sup>12</sup> with bis[2',3'-di-*O*-levulinoyl-*N*<sup>6</sup>-(4-methoxytrityl)-2'-*C*-methyladenosin-5'-yl-*N,N*-diisopropylphosphoramidite and oxidizing the phosphite triester obtained to a phosphate triester (**18**; Scheme 8). The levulinoyl protections were removed by hydrazinium acetate in methanol. The rather labile acetyloxymethoxy function

of the phosphate protection group withstood this treatment. The deprotected triester (**19**) was obtained by removal of the 4-methoxytrityl group with 80% aq acetic acid.



**Scheme 8.** Reagents and conditions: (i)  $i\text{Pr}_2\text{NPCl}_2$ ,  $\text{Et}_3\text{N}$ , DCM; (ii) **17**, TetH, MeCN; (iii)  $\text{I}_2$ , 2,6-lutidine,  $\text{H}_2\text{O}$ , THF; (iv)  $\text{H}_2\text{NNH}_3\text{OAc}$ , MeOH; (v) 80% AcOH.

Introduction of the commonly used pivaloyloxymethyl group turned out to be difficult. Reaction of bis[2',3'-di-*O*-levulinoyl-*N*<sup>6</sup>-(4-methoxytrityl)-2'-*C*-methyladenosin-5'-yl] phosphate with chloromethyl pivalate was attempted in many solvents. The best result was obtained in *N*-methylpyrrolidone. The overall yield after removal of the levulinoyl and 4-methoxytrityl protections was, however, only 15% (**20**; Scheme 9).



**Scheme 9.** Reagents and conditions: (i)  $\text{PivOCH}_2\text{Cl}$ , NMP,  $\text{Et}_3\text{N}$ , 60 °C, 4 days ; (ii)  $\text{H}_2\text{NNH}_3\text{OAc}$ , DCM, MeOH; (iii) 80% AcOH.

The antiviral activity of the compounds will be tested by AliosBiopharma.

## Experimental Section

**General.** Chemicals were purchased from Sigma-Aldrich, Fluka, Merck and Ramidus. MeCN, DCM, THF and pyridine were dried over 4Å molecular sieves and 1,4-dioxane over 3Å molecular sieves. Et<sub>3</sub>N was dried by refluxing over CaH<sub>2</sub>, distilled before use and stored over CaH<sub>2</sub> lumps. Reactions were monitored with TLC (*Merck* silica gel 60 F<sub>254</sub> aluminium sheets) or with <sup>31</sup>P-NMR. Column chromatography was performed on silica gel (Fluka silica gel 60 230-400 mesh). <sup>1</sup>H-, <sup>13</sup>C-, <sup>31</sup>P- and 2D-NMR spectra were recorded on Bruker Avance 400 or 500 spectrometer at 25°C. The chemical shifts are given in ppm with reference to internal TMS, the coupling constants *J* are given in Hz. High resolution mass spectra were recorded on Bruker Daltonics microTOF-Q using electrospray ionization.

**5'-O-(*tert*-Butyldimethylsilyl)-2'-C-methyladenosine (1).** 2'-C-Methyladenosine **1** (5.26 mmol; 1.48 g) was coevaporated twice from dry pyridine and dissolved in the same solvent (10 mL). *tert*-Butyldimethylsilyl chloride (5.21 mmol; 0.81 g) was added and the reaction mixture was stirred for 18 h at r.t. Volatiles were removed and the residue was dissolved in DCM, washed with saturated aqueous NaHCO<sub>3</sub> and brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and evaporated to dryness. The crude product was purified on a silica gel column eluting with 5% MeOH in DCM. Compound **1** was obtained as white solid in 67% yield (1.40 g). <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>) δ 8.41 (s, 1H, H2), 8.35 (s, 1H, H8), 6.83 (br. s, 2H, NH<sub>2</sub>), 6.29 (s, 1H, H1'), 6.22 (br. s, 1H, OH), 4.62 (br. s, 1H, OH), 4.12-4.20 (m, 3H, H3', H4', H5'), 3.97 (d, *J* = 10.2 Hz, 1H, H5'), 1.05 (s, 3H, 2'-Me), 0.94 (s, 9H, Me<sub>3</sub>CSi), 0.15 (s, 3H, MeSi), 0.14 (s, 3H, MeSi). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>) δ 155.82 (C5), 152.66 (C8), 148.98 (C4), 138.80 (C2), 119.50 (C6), 91.38 (C1'), 83.67 (C4'), 79.21 (C2'), 72.98 (C3'), 61.57 (C5'), 26.06 (Me<sub>3</sub>CSi), 20.25 (Me<sub>3</sub>CSi), 18.63 (2'-Me), -5.24 (MeSi), -5.39 (MeSi). HR-ESI-MS: [M+H]<sup>+</sup> obsd. 396.2041, calcd. 396.2062.

**5'-O-(*tert*-Butyldimethylsilyl)-N<sup>6</sup>-(4-methoxytrityl)-2'-C-methyladenosine (2).** Compound **1** (3.54 mmol; 1.40 g) was dried over P<sub>2</sub>O<sub>5</sub> for one day and dissolved in dry pyridine (10 mL). 4-Methoxytrityl chloride (3.92 mmol; 1.21 g) was added and the reaction mixture was stirred on an oil bath at 51 °C for 17 hours. The reaction was quenched with MeOH and the mixture was evaporated to dryness. The residue was purified on a silica gel column eluting with 50% EtOAc in petroleum ether. Compound **2** was obtained as white solid in 89% yield (2.11 g). <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>) δ 8.22 (s, 1H, H2), 7.98 (s, 1H, H8), 7.22-7.34 (m, 12H, MMTr), 7.05 (s, 1H, NH), 6.79 (d, *J* = 9.0 Hz, 2H, MMTr), 6.10 (s, 1H, H1'), 4.05-4.11 (m, 3H, H3', H4', H5'), 3.93 (dd, *J* = 8.7 and *J* = 3.6 Hz, 1H, H5'), 3.77 (s, 3H, OMe), 1.01 (s, 3H, Me2'), 0.95 (s, 9H, Me<sub>3</sub>CSi), 0.14 (s, 3H, MeSi), 0.14 (s, 3H, MeSi). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>) δ 158.34 (MMTr), 154.29 (C5), 151.75 (C8), 147.83 (C4), 145.04 (MMTr), 138.11 (C2), 137.07 (MMTr),

130.25, 128.92, 127.89, 126.91 (MMTr), 120.98 (C6), 113.15 (MMTr), 91.42 (C1'), 84.32 (C4'), 78.75 (C2'), 73.64 (C3'), 71.18 (MMTr), 61.90 (C5'), 55.23 (MeO of MMTr), 26.07, 26.01, 25.94 (*Me*<sub>3</sub>CSi), 20.33 (Me2'), 18.61 (*Me*<sub>3</sub>CSi), -5.26, -5.36 (MeSi). HR-ESI-MS: [M+H]<sup>+</sup> obsd. 668.3268, calcd. 668.3263, [M+Na]<sup>+</sup> obsd. 690.3084, calcd. 690.3082.

**5'-O-(*tert*-Butyldimethylsilyl)-2',3'-di-O-levulinoyl-N<sup>6</sup>-(4-methoxytrityl)-2'-C-methyladenosine (3).** Levulinic acid (16.36 mmol; 1.90 g) was dissolved in dry 1,4-dioxane (20 mL) and cooled on an ice bath. Dicyclohexylcarbodiimide (DCC; 8.58 mmol; 1.77 g) was added portion wise within an hour. The reaction mixture was filtered and the precipitate was washed with dry 1,4-dioxane (10 mL). Compound **2** (3.16 mmol; 2.11 g) was coevaporated with dry pyridine and dissolved in the same solvent (10 mL) and the levulinic anhydride in 1,4-dioxane was added together with a catalytic amount of 4-dimethylaminopyridine (DMAP). The reaction mixture was stirred at r.t. overnight, heated on an oil bath at 54 °C for 5 hours and stirred again over night at r.t. The reaction mixture was evaporated to dryness, the residue was dissolved in DCM and washed with H<sub>2</sub>O, saturated aqueous NaHCO<sub>3</sub> and brine. The crude product, dried over Na<sub>2</sub>SO<sub>4</sub>, was purified twice by silica gel chromatography, first eluting with 2% MeOH in DCM and then with 1% MeOH in DCM. According to the NMR spectrum, a mixture of products containing one or two levulinoyl groups was obtained, the singly acylated product predominating. The mixture of products was subjected to another levulinoylation. Levulinic acid (11.19 mmol; 1.30 g) was dissolved in dry DCM (20 mL) and DCC (5.48 mmol; 1.13 g) was added in one portion. The reaction mixture was stirred at r.t. overnight and filtered. The precipitate was washed with dry DCM (5 mL) and the DCM phases were combined. The filtrate and a catalytic amount of DMAP were added to the mixture (2.11 g) in dry DCM (5 mL). Owing to sluggish progress of the reaction, dry Et<sub>3</sub>N (0.50 mL) and some DMAP were after one day added to the reaction mixture. The reaction was completed in 4 days. The reaction mixture was washed with H<sub>2</sub>O, saturated aqueous NaHCO<sub>3</sub> and brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and evaporated to dryness. The crude product was purified on a silica gel column eluting with 1% MeOH in DCM. Compound **3** was obtained as yellowish solid in 72% yield (1.98 g). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 8.09 (s, 1H, H2), 8.07 (s, 1H, H8), 7.22-7.36 (m, 12H, MMTr), 6.93 (s, 1H, NH), 6.80 (d, *J* = 11.2 Hz, 2H, MMTr), 6.38 (s, 1H, H1'), 5.59 (d, *J* = 6.8 Hz, 1H, H3'), 4.14 (m, 1H, H4'), 4.01 (dd, *J* = 11.7 and 2.4 Hz, 1H, H5'), 3.96 (dd, *J* = 11.6 and 3.2 Hz, 1H, H5'), 3.78 (s, 3H, Me of MMTr), 2.59-2.79 (m, 8H, CH<sub>2</sub> of Lev), 2.19 (s, 3H, Me of Lev), 2.08 (s, 3H, Me of Lev), 1.41 (s, 3H, Me2'), 0.93 (s, 9H, *Me*<sub>3</sub>CSi), 0.12 (s, 6H, MeSi). <sup>13</sup>C-NMR (101 MHz, CDCl<sub>3</sub>) δ 206.34, 206.23 (CH<sub>2</sub>COCH<sub>3</sub> Lev), 171.73, 171.10 (OCOCH<sub>2</sub> Lev), 158.32 (MMTr), 154.14 (C6), 152.52 (C2), 148.68 (C4), 145.20 (MMTr), 138.54 (C8), 137.23 (MMTr), 130.23, 128.91, 127.87, 126.85 (MMTr), 120.85 (C5), 113.15 (MMTr), 87.87 (C1'), 84.69 (C2'), 82.89 (C4'), 74.26 (C3'), 71.02 (MMTr), 62.16 (C5'), 55.22 (MeO of MMTr), 37.86, 37.74 (MeCOCH<sub>2</sub> Lev), 29.76, 29.65 (Me of Lev), 28.54, 27.72 (CH<sub>2</sub>COO Lev), 25.99 (*Me*<sub>3</sub>CSi), 18.52 (*Me*<sub>3</sub>CSi), 17.04 (Me2'), -5.43, -5.45 (MeSi). HR-ESI-MS: [M+H]<sup>+</sup> obsd. 864.4001, calcd. 864,3998.

**2',3'-Di-O-levulinoyl-N<sup>6</sup>-(4-methoxytrityl)-2'-C-methyladenosine (4).** Compound **3** (2.29 mmol; 1.98 g) was dissolved in a mixture of THF (32 mL) and AcOH (6 mL) and

tetrabutylammonium fluoride hydrate (4.63 mmol; 1.21 g) was added. The reaction mixture was stirred at r.t for 42 hours and evaporated to dryness. The residue was dissolved in EtOAc, washed with H<sub>2</sub>O, saturated aqueous NaHCO<sub>3</sub> and brine. The crude product dried over Na<sub>2</sub>SO<sub>4</sub> was purified on a silica gel column eluting with 3% MeOH in DCM. Compound **4** was obtained as white solid in 80% yield (1.37 g). <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>) δ 8.00 (s, 1H, H2), 7.90 (s, 1H, H8), 7.21-7.33 (m, 12H, MMTr), 7.01 (s, 1H, NH), 6.80 (d, *J* = 10.1 Hz, 2H, MMTr), 6.44 (s, 1H, H1'), 5.83 (d, *J* = 6.8 Hz, 1H, H3'), 5.62 (m, 1H, OH5'), 4.30 (m, 1H, H4'), 4.09 (dd, *J* = 12.7 and 1.3 Hz, 1H, H5'), 3.78 (s, 3H, Me of MMTr), 3.76 (m, 1H, H5'), 2.62-2.84 (m, 8H, CH<sub>2</sub> of Lev), 2.21 (s, 3H, Me of Lev), 2.19 (s, 3H, Me of Lev), 1.31 (s, 3H, Me2'). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>) δ 206.49, 206.28 (CH<sub>2</sub>COMe Lev), 172.21, 171.78 (OCOCH<sub>2</sub> Lev), 158.37 (MMTr), 154.51 (C6), 152.18 (C2), 147.69 (C4), 144.99, 144.97 (MMTr), 139.66 (C8), 136.95 (MMTr), 130.20, 128.86, 127.92, 126.94 (MMTr), 121.695 (C5), 113.18 (MMTr), 91.50 (C1'), 86.78 (C2'), 81.36 (C4'), 73.69 (C3'), 71.16 (MMTr), 60.28 (C5'), 55.22 (MeO of MMTr), 37.89, 37.85 (MeCOCH<sub>2</sub> Lev), 29.81, 29.78 (Me of Lev), 28.71, 27.67 (CH<sub>2</sub>COO Lev), 18.47 (Me2'). HR-ESI-MS: [M+H]<sup>+</sup> obsd. 750.3109, calcd. 750.3134.

**5'-O-(tert-Butyldimethylsilyl)-N<sup>2</sup>-(4-methoxytrityl)-2'-C-methylguanosine (5).** 2'-C-Methylguanosine (12.28 mmol; 3.65 g) was dried over P<sub>2</sub>O<sub>5</sub> for two days, dissolved in dry pyridine (30 mL) and *tert*-butyldimethylsilyl chloride (12.27 mmol; 1.85 g) was added. The reaction mixture was stirred at r.t. for 24 hours and evaporated to dryness. The residue was coevaporated three times with dry pyridine and dissolved in the same solvent (15 mL). 4-Methoxytrityl chloride (12.27 mmol; 3.79 g) was added and the reaction mixture was heated on an oil bath at 57 °C for 20 hours and evaporated to dryness. The residue was dissolved in DCM, washed with saturated aqueous NaHCO<sub>3</sub> and brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and evaporated to dryness. The crude product was purified three times on a silica gel column: first with 8% MeOH in DCM, then with 4% MeOH in DCM and finally by using gradient elution from 1% to 4% MeOH in DCM. Compound **5** was obtained as white solid in 82% yield (6.88 g). <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>) δ 7.74 (s, 1H, H8), 7.11-7.36 (m, 13H, MMTr, NH), 6.79 (d, *J* = 8.7 Hz, 2H, MMTr), 5.37 (s, 1H, H1'), 3.85-4.02 (m, 5H, H3', H4', H5', H5'', OH), 3.70 (s, 3H, OMe), 2.89 (br. s, 1H, OH), 0.92 (s, 9H, Me<sub>3</sub>CSi), 0.54 (s, 3H, Me2'), 0.11 (s, 6H, MeSi). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>) δ 158.40 (MMTr), 158.26 (C6), 151.38 (C4), 149.89 (C2), 144.73, 144.61 (MMTr), 136.33 (MMTr), 135.44 (C8), 130.29, 128.82, 128.71, 127.87, 126.83 (MMTr), 117.25 (C5), 113.17 (MMTr), 90.64 (C1'), 83.36 (C4'), 78.69 (C2'), 72.86 (C3'), 70.52 (MMTr), 62.15 (C5'), 55.15 (MeO of MMTr), 26.07 (Me<sub>3</sub>CSi), 19.77 (Me2'), 18.55 (Me<sub>3</sub>CSi), -5.30, -5.38 (MeSi). HR-ESI-MS: [M+H]<sup>+</sup> obsd. 684.3213, calcd. 684.3212, [M+Na]<sup>+</sup> obsd. 706.3054, calcd. 706.3031.

**N<sup>2</sup>-(4-Methoxytrityl)-3'-O-levulinoyl-2'-C-methylguanosine (6a).** Levulinic acid (4.82 mmol; 0.56 g) was dissolved in dry DCM (20 mL), DCC (2.90 mmol; 0.60 g) was added and the reaction mixture was stirred at r.t. for 4 hours. Precipitated dicyclohexylurea was removed by filtration and compound **5** (1.94 mmol; 1.33 g) in DCM (2 mL) and a catalytic amount of DMAP and Et<sub>3</sub>N (0.80 ml) were added. The reaction mixture was stirred at r.t. over night. The mixture was washed with H<sub>2</sub>O and brine. The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to

dryness. The crude product was purified on a silica gel column eluting with 1 to 3% MeOH in DCM. According to NMR spectroscopy, the main product bore only one levulinoyl group. The product was used as such for the next step. Accordingly, the levulinoylated nucleoside was dissolved in a mixture of dry THF (20 mL) and acetic acid (4 mL). Tetrabutylammonium fluoride hydrate (2.95 mmol, 0.77 g) was coevaporated from dry THF and added to the reaction mixture in the same solvent (1 mL). The mixture was evaporated to dryness after 21 hours stirring at r.t. The residue was equilibrated between EtOAc and saturated aqueous NaHCO<sub>3</sub>. The organic layer was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and evaporated to dryness. The crude product was purified on a silica gel column eluting with 5% MeOH in DCM. The product was obtained as white solid in 50% yield (0.64 g). <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>) δ 7.76 (s, 1H, H8), 7.17-7.34 (m, 13H, MMTr, NH), 6.78 (d, *J* = 8.8 Hz, 2H, MMTr), 5.52 (s, 1H, H1'), 5.22 (m, 1H, H3'), 4.16 (m, 1H, H4'), 4.02 (m, 1H, H5'), 3.74 (s, 3H, OMe), 3.70 (m, 1H, H5''), 2.80-2.83 (m, 2H, MeCOCH<sub>2</sub>CH<sub>2</sub> Lev), 2.51-2.65 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>COO Lev), 2.20 (s, 3H, Me of Lev), 0.73 (s, 3H, Me2'). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>) δ 207.16 (CH<sub>2</sub>COCH<sub>3</sub> Lev), 172.35 (OCOCH<sub>2</sub> Lev), 158.15 (MMTr), 151.56 (C6), 149.79 (C4), 143.38 (MMTr), 135.86 (MMTr), 135.71 (C8), 130.13, 128.65, 128.22, 127.11 (MMTr), 117.67 (C5), 113.47 (MMTr), 80.60 (C4'), 80.13 (C2'), 73.62 (C3'), 70.54 (MMTr), 60.44 (C5'), 55.23 (MeO of MMTr), 38.23 (MeCOCH<sub>2</sub> Lev), 29.77 (Me of Lev), 27.97 (CH<sub>2</sub>COO Lev), 20.80 (Me2'). HR-ESI-MS: [M+H]<sup>+</sup> obsd. 668.2713, calcd. 668.2715, [M+Na]<sup>+</sup> obsd. 690.2553, calcd. 690.2534.

***N*<sup>2</sup>-(4-Methoxytrityl)-3'-*O*-benzoyl-2'-*C*-methylguanosine (6b).** Compound **5** (1.74 mmol; 1.19 g) was coevaporated three times with dry pyridine and the residue was dissolved in the same solvent (5.0 mL). The solution was cooled on an ice bath and benzoyl chloride (4.39 mmol; 0.22 mL) was added. The solution was stirred at r.t. overnight and evaporated to dryness. The residue was dissolved in DCM (5.0 mL) and washed with brine. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The crude product was isolated by Silica gel chromatography eluting with DCM containing MeOH from 5% to 8% MeOH. HR-ESI-MS: [M+H]<sup>+</sup> obsd. 788.3393, calcd. 788.3474. According to <sup>1</sup>H- and <sup>13</sup>C-NMR spectroscopic characterization, the desired product, 5'-*O*-(*tert*-butyldimethylsilyl)-*N*<sup>2</sup>-(4-methoxytrityl)-3'-*O*-benzoyl-2'-*C*-methylguanosine, was contaminated with some impurities. In spite of this, the crude product was subjected as such to desilylation that was carried out as described above for compound **6a**. The crude product was purified by silica gel chromatography eluting with DCM that contained 5% MeOH. Compound **6b** was obtained as white solid in 53% yield (0.52 g). <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>) δ: 8.05 (dd, 2H, *J* = 8.45 and 1.35 Hz, Bz), 7.84 (br. s, 1H, H8), 7.60 (m, 1H, Bz), 7.48 (t, 2H, *J* = 7.90 Hz, Bz), 7.36-7.11 (12H, MMTr), 6.72 (d, *J* = 8.75 Hz, 2H, MMTr), 5.44 (s, 1H, H1'), 5.28 (m, 1H, H3'), 4.24 (m, 1H, H4'), 4.05 (m, 1H, H5''), 3.76-3.72 (m, 2H, H5', OH), 3.68 (s, 3H, OMe), 2.25 (br s, 1H, N<sup>2</sup>H), 0.73 (s, 3H, 2'-Me). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>) δ: 165.92 (C=O Bz), 158.21 (C6), 151.45 (C4), 149.89 (C2), 144.82 (C8), 158.1, 144.9, 133.74, 130.28, 129.89, 129.00, 128.87 128.77, 128.60, 127.86, 127.81 and 126.75 (MMTr and Bz), 117.26 (C5), 113.17 (MMTr), 90.81 (C1'), 80.98 (C4'), 79.29 (MMTr), 74.00

(C3'), 70.76 (C2'), 60.59 (C5'), 55.19 (MeO of MMTr), 20.55 (2'-CH<sub>3</sub>). HR-ESI-MS: [M+H]<sup>+</sup> obsd. 674.2603, calcd. 674.2609.

**5'-O-(4-Methoxytrityl)-2'-C-methyluridine (7).** 2'-C-Methyluridine (0.79 g; 3.05 mmol) was dissolved in dry pyridine (20 mL) under argon. 4-Methoxytrityl chloride (1.03 g; 3.36 mmol) was added and the mixture was stirred at 50 °C for 20 hours. The reaction was quenched with saturated aq NaHCO<sub>3</sub> (50 mL) and subjected to DCM (3 × 100 mL) workup. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The residue was purified by Silica gel chromatography using DCM containing 5% MeOH as eluent. Compound **7** was obtained as solid in 92% yield (1.50 g). <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>) δ: 8.16 (d, 1H, *J* = 8.0 Hz, H6), 7.41 (m, 4H, MMTr), 7.26 (m, 6H, MMTr), 7.19 (m, 2H, MMTr), 6.86 (d, 2H, *J* = 9.0 Hz, MMTr), 6.09 (s, 1H, H1'), 5.23 (d, 1H, *J* = 8.0 Hz, H5), 4.05-4.17 (m, 2H, H3'&H4'), 3.81-3.84 (m, 4H, MeO-MMTr & H5'), 3.61 (br s, 2H, 2'-&3'-OH); 3.23 (d, 1H, *J* = 12.5 Hz, H5'), 1.36 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C-NMR (100 MHz, CD<sub>3</sub>OD) δ: 164.64 (C4), 158.76 (MMTr), 151.02 (C2), 144.52 (MMTr), 141.12 (C6), 135.35 (MMTr), 130.18, 128.10, 127.34, 126.46 and 112.64 (MMTr), 102.94 (C5), 91.67 (C1'), 87.66 (MMTr), 82.44 (C2'), 78.66 (C4'), 71.94 (C3'), 59.05 (C5'), 54.32 (OMe), 18.80 (Me). HR-ESI-MS: [M+Na]<sup>+</sup> obsd. 553.1921, calcd. 553.1945.

**2',3'-di-O-Benzoyl-5'-O-(4-methoxytrityl)-2'-C-methyluridine (8).** 5'-O-(4-Methoxytrityl)-2'-C-methyluridine (1.50 g; 2.87 mmol) (**7**) was dissolved in dry pyridine (60 mL). Benzoyl chloride (0.83 mL, 7.17 mmol) was added and the mixture was stirred overnight at room temperature. The reaction was quenched with cold water and the mixture was evaporated to dryness. The residue was dissolved in DCM and washed with water (3 × 100 mL). The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The residue was purified by Silica gel chromatography using DCM containing 10% EtOAc as eluent. Compound **8** was obtained as solid in 77% yield (1.63 g). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ: 8.29 (d, 1H, *J* = 8.4 Hz, H6), 8.07 (d, 2H, *J* = 7.6 Hz, Bz), 7.90 (d, 2H, *J* = 7.6 Hz, Bz), 7.64 (t, 2H, *J* = 7.2 Hz, Bz), 7.51-7.42 (m, 8H, Bz and MMTr), 7.33-7.17 (m, 8H, Bz and MMTr), 6.80 (d, 2H, *J* = 8.8 Hz, MMTr), 6.07 (s, 1H, H1'), 5.77 (d, 1H, *J* = 8.4 Hz, H5), 5.45 (d, 1H, *J* = 9.2 Hz, H3'), 4.45 (d, 1H, *J* = 8.8 Hz, H4'), 3.74 (s, 3H, OCH<sub>3</sub>), 3.63 (d, 1H, *J* = 11.2 Hz, H5'), 3.46 (d, 1H, *J* = 9.6 Hz, H5'), 2.88 (br s, 1H, NH), 1.36 (s, 3H, CH<sub>3</sub>). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>) δ: 168.30 (C=O Bz), 165.43 (C4), 161.78 (C=O Bz), 158.80 (MMTr), 149.75 (C2), 143.71, 143.49 (MMTr), 139.79 (C6), 135.15, 134.35, 134.17, 133.88, 131.39, 130.52, 130.39, 129.94, 129.20, 128.72, 128.69, 128.44, 128.35, 128.08, 127.88, 127.30, 127.25, 127.20, 113.34, 113.22 (Bz and MMTr), 102.52 (C5), 91.66 (C1'), 87.55 (spiro C, MMTr), 79.64 (C2'), 79.41 (C4'), 73.39 (C3'), 60.45 (C5'), 55.20 (MeO of MMTr), 21.00 (Me). HR-ESI-MS: [M+Na]<sup>+</sup> obsd. 761.2450, calcd. 761.2470.

**2',3'-Di-O-benzoyl-2'-C-methyluridine (9).** 2',3'-Di-O-benzoyl-5'-O-(4-methoxytrityl)-2'-C-methyluridine (1.88g; 2.54 mmol) (**8**) was dissolved in 80% (v/v) aq AcOH (36 mL). After stirring overnight at room temperature, the mixture was evaporated to dryness. The residue was dissolved in dichloromethane (100 mL) and washed with water (2 × 100 mL). Organic phase was dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The residue was purified by Silica gel chromatography eluting with a 7:3 mixture of DCM and EtOAc. Compound **9** was obtained as

white foam in 78% yield (0.90 g).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$ : 8.21 (d, 1H,  $J = 8.2$  Hz, H6), 8.07 (dd, 2H,  $J = 8.0$  and 1.2 Hz, Bz), 7.91 (dd, 2H,  $J = 8.0$  and 1.3 Hz, Bz), 7.66 (m, 2H, Bz), 7.50 (t, 4H,  $J = 7.2$  Hz, Bz), 6.09 (s, 1H, H1'), 5.91 (d, 1H,  $J = 8.2$  Hz, H5), 5.22 (d, 1H,  $J = 8.7$  Hz, H3'), 4.32 (d, 1H,  $J = 8.5$  Hz, H4'), 4.12 (dd, 1H,  $J = 13.2$  Hz and 1.3 Hz, H5''), 3.85 (dd, 1H,  $J = 13.2$  Hz and 2.2 Hz, H5'), 1.38 (s, 3H,  $\text{CH}_3$ ).  $^{13}\text{C-NMR}$  (100 MHz,  $\text{CDCl}_3$ )  $\delta$ : 168.29 (C=O Bz), 166.45 (C4), 161.76 (C=O Bz), 149.78 (C2), 139.49 (C6), 135.18, 134.24, 131.40, 130.40, 129.96, 129.21, 128.80, 128.34 (Bz), 102.66 (C5), 91.55 (C1'), 80.74 (C4'), 79.71 (C2'), 73.50 (C3'), 60.36 (C5'), 20.82 ( $\text{CH}_3$ ). HR-ESI-MS:  $[\text{M}+\text{Na}]^+$  obsd. 489.1241, calcd. 489.1268.

**Bis[2',3'-di-*O*-levulinoyl-*N*<sup>6</sup>-(4-methoxytrityl)-2'-*C*-methyladenosin-5'-yl] phosphate (10).**

Compound **4** (1.82 mmol; 1.37 g) was dried on  $\text{P}_2\text{O}_5$  over two nights and dissolved in dry DCM (6 mL) under nitrogen. Dry  $\text{Et}_3\text{N}$  (9.28 mmol; 1.29 mL) and 1,1-dichloro-*N,N*-diisopropylphosphinamine (0.97 mmol; 180  $\mu\text{L}$ ) were added and the reaction mixture was stirred at r.t. for 1.5 hours. To complete the reaction, another portion of 1,1-dichloro-*N,N*-diisopropylphosphinamine (0.27 mmol; 50  $\mu\text{L}$ ) was added and the mixture was stirred for 30 minutes. The reaction mixture was then passed through a short silica gel column eluting with EtOAc/hexane (8:2, v/v) containing 0.5%  $\text{Et}_3\text{N}$ . The solvents were removed under reduced pressure and the residue was co-evaporated two times from dry MeCN.  $^{31}\text{P-NMR}$  signal at 149.66 ppm verified the formation of the desired dinucleoside phosphoramidite. The product (0.44 mmol; 0.72 g) was dissolved in MeCN (1 mL), and 1-*H*-tetrazole (5.22 mmol; 11.60 mL of 0.45 mol  $\text{L}^{-1}$  solution in MeCN) and  $\text{H}_2\text{O}$  (8.00 mL) were added. The progress of the reaction was followed by  $^{31}\text{P-NMR}$  spectroscopy. After 75 min, 7 mL of 0.15 mol  $\text{L}^{-1}$  solution of  $\text{I}_2$  (1.05 mmol) in a 4:2:1 mixture of THF,  $\text{H}_2\text{O}$  and 1,2-lutidine was added. The oxidation was completed in 2.5 hours. The product was isolated by conventional 5% aq  $\text{NaHSO}_3/\text{DCM}$  workup. The crude product was purified on a silica gel column with gradient elution from 5% to 10% MeOH in DCM. Compound **10** was obtained as yellowish solid in 70% yield (0.39 g).  $^1\text{H-NMR}$  (500 MHz,  $\text{CDCl}_3$ )  $\delta$  8.32 (s, 2H, H8), 8.03 (s, 2H, H2), 7.19-7.35 (m, 24H, MMTr), 6.78 (d,  $J = 9.0$  Hz, 4H, MMTr), 6.36 (s, 2H, H1'), 5.59 (d,  $J = 5.0$  Hz, 2H, H3'), 4.15-4.23 (m, 6H, H4', H5' & H5''), 3.75 (s, 6H, OMe of MMTr), 2.51-2.79 (m, 16H,  $\text{CH}_2$  of Lev), 2.17, 2.05 (2s, 12H, Me of Lev), 1.37 (s, 6H, Me2').  $^{13}\text{C-NMR}$  (126 MHz,  $\text{CDCl}_3$ )  $\delta$  207.74, 207.05 ( $\text{CH}_2\text{COCH}_3$  Lev), 171.99, 171.08 ( $\text{OCOCH}_2$  Lev), 158.11 (MMTr), 153.73 (C6), 152.23 (C2), 148.44 (C4), 144.79 (MMTr), 139.03 (C8), 136.86 (MMTr), 130.06, 128.70, 127.64, 126.70 (MMTr), 119.88 (C5), 112.90 (MMTr), 87.67 (C1'), 84.20 (C2'), 80.94 (C4'), 74.13 (C3'), 70.85 (MMTr), 63.97, 63.95 (C5'), 54.97 (MeO of MMTr), 37.62, 37.45 ( $\text{MeCOCH}_2$  Lev), 29.34, 29.29 (Me of Lev), 28.19, 27.60 ( $\text{CH}_2\text{COO}$  Lev), 16.77 (Me2').  $^{31}\text{P-NMR}$  (202 MHz,  $\text{CDCl}_3$ )  $\delta$  0.10. HR-ESI-MS:  $[\text{M-H}]^-$  obsd. 1559.5598, calcd. 1559.5606.

**Bis(2'-*C*-methyladenosin-5'-yl) phosphate (11).** Compound **10** (0.04 mmol; 0.06 g) was dissolved in DCM (0.50 mL) and hydrazinium acetate (0.32 mmol; 0.03 g) in MeOH (0.2 mL) was added. After 20 hours stirring at r.t., the reaction mixture was evaporated to dryness and the residue was dissolved in 80% aqueous AcOH (1 mL) and stirred at r.t. for 4 hours. The volatiles were removed under reduced pressure and the residue was equilibrated between  $\text{H}_2\text{O}$  and ethyl

acetate. The aqueous layer was separated and evaporated to dryness. The crude product was purified by HPLC on a Sun Fire™ Prep C18 column (250×10 mm, 5 μm, flow rate 3.0 mL min<sup>-1</sup>), using isocratic elution with 9% buffer A in buffer B (Buffer A: 20 mmol L<sup>-1</sup> aq NH<sub>4</sub>OAc in 50% MeCN, Buffer B: 20 mmol L<sup>-1</sup> aq NH<sub>4</sub>OAc). Compound **11** was obtained as white solid in 27% yield (6 mg as an ammonium salt). <sup>1</sup>H-NMR (500 MHz, D<sub>2</sub>O) δ 7.99 (s, 2H, H<sub>2</sub>), 7.96 (s, 2H, H<sub>8</sub>), 5.87 (s, 2H, H<sub>1</sub>'), 4.36-4.40 (m, 2H, H<sub>5</sub>'), 4.22-4.27 (m, 4H, H<sub>4</sub>', H<sub>5</sub>''), 4.01 (m, 2H, H<sub>3</sub>'), 0.71 (s, 6H, Me<sub>2</sub>'). <sup>13</sup>C NMR (126 MHz, D<sub>2</sub>O) δ 154.59 (C<sub>6</sub>), 152.13 (C<sub>2</sub>), 147.29 (C<sub>4</sub>), 139.27 (C<sub>8</sub>), 117.60 (C<sub>5</sub>), 90.76 (C<sub>1</sub>'), 80.62, 80.55 (C<sub>4</sub>'), 79.18 (C<sub>2</sub>'), 72.21 (C<sub>3</sub>'), 65.60, 65.56 (C<sub>5</sub>'), 18.30 (Me<sub>2</sub>'). <sup>31</sup>P-NMR (202 MHz, CDCl<sub>3</sub>) δ 0.28. HR-ESI-MS: [M-H]<sup>-</sup> obsd. 623.1735, calcd. 623.1733.

**2-Cyanoethyl bis[3'-O-levulinoyl-N<sup>6</sup>-(4-methoxytrityl)-2'-C-methylguanosin-5'-yl] phosphate (12).** Compound **6a** (0.48 mmol; 0.32 g) dried over P<sub>2</sub>O<sub>5</sub> over night was dissolved in dry DCM (3 mL) under N<sub>2</sub>. Dry Et<sub>3</sub>N (2.41 mmol; 335 μL) and 1-chloro-1-(2-cyanoethoxy)-*N,N*-diisopropylphosphinamine (0.53 mmol; 118 μL) were added. After 1 hour stirring at r.t., the mixture was passed through a short silica gel column eluting with EtOAc that contained 0.5% Et<sub>3</sub>N. The solvents were removed under reduced pressure and the residue was co-evaporated from dry MeCN. The residue was dissolved in dry MeCN (2 mL) and compound **6** (0.48 mmol; 0.32 g) and 1-*H*-tetrazole (0.96 mmol; 2.13 mL of 0.45 mol L<sup>-1</sup> solution in MeCN) were added under N<sub>2</sub>, and the mixture was stirred for 1.5 hours. The phosphite ester obtained was oxidized as described above for compound **10**. The phosphate triester **12** was isolated by conventional 5% aq NaHSO<sub>3</sub>/DCM workup. The crude product was purified on a silica gel column eluting with 5 to 10% MeOH in DCM. Compound **12** was obtained as white solid in 21% yield (0.15 g). <sup>1</sup>H-NMR (500 MHz, MeOD) δ 7.82 (s, 1H, H<sub>8</sub>), 7.81 (s, 1H, H<sub>8</sub>), 7.19-7.38 (m, 24H, MMTr), 6.86 (m, 4H, MMTr), 5.35 (s, 2H, H<sub>1</sub>'), 5.15 (m, 2H, H<sub>4</sub>'), 4.25-4.42 (m, 8H, H<sub>3</sub>', H<sub>5</sub>', H<sub>5</sub>'', CH<sub>2</sub>), 3.77 (s, 6H, OMe), 2.86-2.89 (m, 2H, CH<sub>2</sub>CN), 2.77-2.84 (m, 4H, MeCOCH<sub>2</sub>CH<sub>2</sub> Lev), 2.61-2.64 (m, 4H, CH<sub>2</sub>CH<sub>2</sub>COO Lev), 2.15 (s, 3H, Me of Lev), 2.13 (s, 3H, Me of Lev), 0.63 (s, 3H, Me<sub>2</sub>'), 0.63 (s, 3H, Me<sub>2</sub>'). <sup>13</sup>C-NMR (126 MHz, MeOD) δ 208.33 (CH<sub>2</sub>COCH<sub>3</sub> Lev), 158.48 (MMTr), 157.00 (C<sub>6</sub>), 151.21 (C<sub>4</sub>), 149.25 (C<sub>2</sub>), 144.78, 144.80, 144.81, 144.83 (MMTr), 136.50 (MMTr), 135.25 (C<sub>8</sub>), 130.05, 128.64, 127.48, 126.50 (MMTr), 120.75 (CN), 112.76 (MMTr), 78.55 (C<sub>4</sub>'), 77.89 (C<sub>2</sub>'), 73.13 (C<sub>3</sub>'), 70.42, 70.45 (MMTr), 66.17 (C<sub>5</sub>'), 63.00 (OCH<sub>2</sub>CH<sub>2</sub>CN), 54.37 (MeO of MMTr), 37.30 (MeCOCH<sub>2</sub> Lev), 28.32 (Me of Lev), 27.36 (CH<sub>2</sub>COO Lev), 18.96 (Me<sub>2</sub>') 18.62, 18.57 (OCH<sub>2</sub>CH<sub>2</sub>CN). <sup>31</sup>P-NMR (202 MHz, MeOD) δ -2.24.

**Bis(2'-C-methylguanosin-5'-yl) phosphate (13).** Compound **12** (0.10 mmol; 0.15 g) was dissolved in 30% methanolic ammonia (25 mL) and the mixture was stirred at r.t. overnight, and evaporated to dryness under reduced pressure. The residue was dissolved in 80% aq AcOH (10 mL) and the mixture was stirred at r.t. for 3 days. The crude product obtained by evaporation to dryness was equilibrated between H<sub>2</sub>O and hexane. The aqueous phase was evaporated to dryness and the product was purified by HPLC as described above for compound **11**. The product was dissolved in water and passed through an ion-exchange column (Dowex Na<sup>+</sup>-form,

50Wx8, 100-200 mesh) and evaporated to dryness. Compound **13** was obtained as white solid in 18% yield (12 mg).  $^1\text{H-NMR}$  (500 MHz,  $\text{D}_2\text{O}$ )  $\delta$  7.55 (s, 2H, H8), 5.51 (s, 2H, H1'), 4.18 (m, 2H, H5'), 4.02-4.11 (m, 4H, H4', H5''), 3.88 (m, 2H, H5'), 0.65 (s, 6H, 2'-Me).  $^{13}\text{C-NMR}$  (126 MHz, MeOD)  $\delta$  163.26 (C6), 90.72, 90.70 (C1'), 80.60, 80.59, 80.52, 80.50 (C4'), 79.25 (C2'), 72.55, 72.53 (C3'), 65.70, 65.66 (C5'), 18.52, 18.50 (2'-Me).  $^{31}\text{P-NMR}$  (202 MHz,  $\text{D}_2\text{O}$ )  $\delta$  0.20. HR-ESI-MS:  $[\text{M-H}]^-$  obsd. 655.1624, calcd. 655.1631.

**2-Cyanoethyl bis(2',3'-di-*O*-benzoyl-2'-*C*-methyluridin-5'-yl) phosphate (14).** 2',3'-Di-*O*-benzoyl-2'-*C*-methyluridine (0.225 g; 0.48 mmol) (**9**) dried over  $\text{P}_2\text{O}_5$  overnight was dissolved in dry DCM (5 mL) under argon. Anhydrous triethylamine (0.336 mL; 2.41 mmol) and 1-chloro-1-(2-cyanoethoxy)-*N,N*-diisopropylphosphinamine (0.118 mL; 0.53 mmol) were added. The mixture was stirred at room temperature for 1 hour. The product was isolated by passing the mixture through a short silica gel column with dry EtOAc containing 0.5%  $\text{Et}_3\text{N}$ . The solvent was removed under reduced pressure and the product was coevaporated twice with dry acetonitrile (2  $\times$  20 mL) to remove the traces of  $\text{Et}_3\text{N}$ . The phosphitylated nucleoside was dissolved in dry MeCN (1 mL) under argon. Nucleoside **9** (0.225 g; 0.48 mmol, dried over  $\text{P}_2\text{O}_5$  overnight) in dry MeCN (2 mL) and tetrazole (1.92 mmol, 4.2 mL of 0.45 mol  $\text{L}^{-1}$  solution in MeCN) were added. The mixture was stirred for 2 hours. The phosphite ester formed was oxidized with  $\text{I}_2$  as described above from compound **10** and subjected to 5% aq  $\text{NaHSO}_3/\text{DCM}$  workup. The product was purified by Silica gel chromatography, eluting with DCM containing 5% MeOH. Compound **14** was obtained as solid in 52% yield (0.26 g).  $^1\text{H-NMR}$  (500 MHz,  $\text{CDCl}_3$ )  $\delta$ : 8.01-7.99 (m, 4H, Bz), 7.87-7.85 (m, 4H, Bz), 7.73 (t,  $J = 8.0$  Hz, 2H, H6), 7.61-7.54 (m, 4H, Bz), 7.44-7.38 (m, 8H, Bz), 5.99 (s, 2H, 2  $\times$  H1'), 5.91 and 5.90 (d,  $J = 8.0$ , 2H, 2  $\times$  H5), 5.23-5.29 (m, 2H, 2  $\times$  H3'), 4.51-4.36 (m, 6H, 2  $\times$  H4', H5', 5''), 4.29 (m, 2H,  $\text{OCH}_2$ ), 2.70 (m, 2H,  $\text{CH}_2\text{CN}$ ), 1.28 and 1.27 (s, 6H, 2  $\times$  Me).  $^{13}\text{C-NMR}$  (100 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$ : 168.38 (C=O Bz), 165.61 and 165.60 (C4), 161.72 (C=O Bz), 149.70 and 149.66 (C2), 139.25 (C6), 133.54, 131.95, 129.88, 128.62, 128.24, 127.57, 127.46, 126.78 (Bz), 116.67 (CN), 102.73 (C5), 91.71 (C1'), 78.94 (C2'), 78.71 (C4'), 74.25 and 74.15 (C3'), 66.43 and 66.40 (C5'), 62.67 and 62.63 ( $\text{OCH}_2$ ), 20.88 (Me), 19.66 and 19.60 ( $\text{CH}_2\text{CN}$ ).  $^{31}\text{P-NMR}$  (202 MHz,  $\text{CDCl}_3$ )  $\delta$ : -1.85. HR-ESI-MS:  $[\text{M+H}]^+$  obsd. 1048.2608, calcd. 1048.2648,  $[\text{M+Na}]^+$  obsd. 1070.2413, calcd. 1070.2468.

**Bis(2'-*C*-methyluridin-5'-yl) phosphate (15).** Compound **14** (130mg; 0.124 mmol) was dissolved in methanolic ammonia (2.0 mL; 7 mol  $\text{L}^{-1}$ ) on an ice bath. After stirring overnight (20 h) at room temperature, the mixture was evaporated to dryness. The product was purified by RP-HPLC on a Lichrochart<sup>®</sup> Prep C18 column (250  $\times$  10 mm, 5  $\mu\text{m}$ , flow rate 3.0 mL  $\text{min}^{-1}$ ) eluting with water that contained 3% MeCN. Compound **15** was obtained as an ammonium salt in 87% yield (62 mg). Finally, the product was converted to sodium salt as described above for compound **13**.  $^1\text{H-NMR}$  (500 MHz,  $\text{D}_2\text{O}$ )  $\delta$ : 7.85 (d,  $J = 8.1$  Hz, 2H, H6), 5.97 (s, 2H, H1'), 5.89 (d,  $J = 8.1$  Hz, 2H, H5), 4.30-4.33 (m, 2H, 2  $\times$  H5''), 4.13-4.17 (m, 4H, 2  $\times$  H5' and 2  $\times$  H4'), 3.97 (d,  $J = 9.3$  Hz, 2H, H3'), 1.18 (s, 6H,  $\text{CH}_3$ ).  $^{13}\text{C-NMR}$  (126 MHz,  $\text{D}_2\text{O}$ )  $\delta$ : 165.80 (C4), 151.41 (C2), 141.36 (C6), 102.35 (C5), 91.56 (C1'), 80.28 and 80.22 (C4'), 78.96 (C2'), 71.97

(C3'), 63.58 and 63.54 (C5'), 18.80 (Me). <sup>31</sup>P-NMR (202 MHz, D<sub>2</sub>O) δ: 0.01. HR-ESI-MS: [M-H]<sup>-</sup> obsd. 577.1186, calcd. 577.1189.

**2'-C-Methylguanosin-5'-yl 2'-C-methyluridin-5'-yl phosphate (16).** Compound **6b** (0.260 g; 0.38 mmol) dried over P<sub>2</sub>O<sub>5</sub> overnight was phosphitylated with 1-chloro-1-(2-cyanoethoxy)-*N,N*-diisopropylphosphinamine, as described above for its 3'-*O*-levulinoyl analog **6a**. The phosphitylated nucleoside was dissolved in dry MeCN (1 mL) under argon and 2',3'-di-*O*-benzoyl-2'-*C*-methyluridine (**9**, 0.18 g, 0.38 mmol, dried over P<sub>2</sub>O<sub>5</sub> overnight) in dry MeCN (2 mL) and tetrazole (1.92 mmol, 4.2 mL of 0.45 mol L<sup>-1</sup> solution in MeCN) were added. The mixture was stirred for 2 hours and the phosphite ester formed was then oxidized as described above for compound **10**. The product was purified by silica gel chromatography, eluting with 10% MeOH in DCM that contained 0.5% Et<sub>3</sub>N. The 2-cyanoethyl group was cleaved during the chromatography and, hence, 2',3'-di-*O*-benzoyl-2'-*C*-methyluridin-5'-yl 3'-*O*-benzoyl-*N*<sup>2</sup>-(4-methoxytrityl)-2'-*C*-methylguanosin-5'-yl phosphate was obtained as solid in 63% yield (0.29 g). HR-ESI-MS: [M-H]<sup>-</sup> obsd. 1200.3393, calcd. 1200.3398. The benzoyl groups were then removed with ammoniacal methanol, as described above for compound **15**. Silica gel chromatography gave the product in 96% yield (71 mg). ESI-MS: [M-H]<sup>-</sup> obsd. 888.6, calcd. 888.8. The *N*<sup>2</sup>-(4-methoxytrityl) group was finally removed with aq 80% acetic acid (1.5 mL) at room temperature. The product was purified by HPLC and converted to Na<sup>+</sup> salt, as described above for compound **13**. The yield was 56% (27 mg). <sup>1</sup>H-NMR (400 MHz, D<sub>2</sub>O) δ: 7.93 (s, 1H, H8 Guo), 7.67 (d, *J* = 8.4 Hz, 1H, H6 Urd), 5.91 (s, 1H, H1ouG'), 5.85 (s, 1H, H1'Urd), 5.70 (d, *J* = 8.4 Hz, 1H, H5 Urd), 4.10-4.38 (m, 7H, 2 × H5'', 2 × H5', 2 × H4' and H3' Guo), 3.87 (d, *J* = 9.6 Hz, 1H, H3' Urd), 1.13 (s, 3H, Me), 1.01 (s, 3H, Me). <sup>13</sup>C-NMR (100 MHz, D<sub>2</sub>O) δ: 165.54 (C4 Urd), 158.69 (C6 Guo), 153.72 (C2 Guo), 151.06 and 150.95 (C4 Guo and C2 Urd), 140.88 (C6 Urd), 137.61 (C8 Guo), 116.09 (C5 Guo), 101.84 (C5 Urd), 91.56 and 91.15 (C1' Urd and Guo), 80.92, 80.84, 79.28 and 79.02 (C4' Urd and Guo), 79.28 and 79.02 (C2' Urd and Guo), 72.85 and 71.95 (C3' Urd and Guo), 65.23, 65.19 64.24, 64.21 (C5' Urd and Guo), 18.70 (2Me). <sup>31</sup>P-NMR (162 MHz, D<sub>2</sub>O) δ: 0.24. HR-ESI-MS: [M-H]<sup>-</sup> obsd. 616.1418, calcd. 616.1410.

**3-Acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl bis[2',3'-di-*O*-levulinoyl-*N*<sup>6</sup>-(4-methoxytrityl)-2'-*C*-methyladenosin-5'-yl] phosphate (18).** Compound **4** (1.82 mmol; 1.37 g) was dried over P<sub>2</sub>O<sub>5</sub> for 2 days and dissolved in dry DCM (6 mL) under N<sub>2</sub>. Dry Et<sub>3</sub>N (9.28 mmol; 1.29 ml) and 1,1-dichloro-*N,N*-diisopropylphosphinamine (0.97 mmol; 180 μl) were added and the reaction mixture was stirred at r.t. for 1,5 hours. Since according to TLC some unreacted nucleoside was still present, another portion of 1,1-dichloro-*N,N*-diisopropylphosphinamine (0.27 mmol; 50 μl) was added and the mixture was stirred for 30 minutes. The mixture was then passed through a short silica gel column eluting with EtOAc/hexane (8:2, v/v) that contained 0.5% Et<sub>3</sub>N. The solvents were removed under reduced pressure and the residue was co-evaporated two times from dry MeCN. <sup>31</sup>P-NMR spectroscopy exhibited a signal at 149.66 ppm (202 MHz, CD<sub>3</sub>CN), consistent with formation of the desired dinucleoside phosphoramidite. The compound (0.65 mmol; 0.77 g) was dissolved in dry MeCN (1 mL) and diethyl 2-acetyloxymethyl-2-hydroxymethylmalonate<sup>1</sup> (1.16 mmol; 0.34 g) in dry MeCN (2

mL) and 1-*H*-tetrazole (1.89 mmol; 4.20 mL of 0.45 mol L<sup>-1</sup> solution in MeCN) were added under N<sub>2</sub> and the reaction mixture was stirred at r.t. for 1 hour. Formation of the phosphite ester was accompanied by appearance of a <sup>31</sup>P-NMR signal at 140.12. Oxidation to phosphate ester was carried out as described above for compound **10**. Conventional 5% aq NaHSO<sub>3</sub>/DCM workup gave the crude product that was purified twice on a silica gel column; first elution with 3% MeOH in DCM and the second with 3% MeOH in EtOAc. Compound **18** was obtained as white solid in 44% yield (0.38 g). <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>) δ 8.09 (s, 1H, H8), 8.06 (s, H, H2), 8.05 (s, H, H2), 8.02 (s, 1H, H8), 7.19-7.36 (m, 24H, MMTr), 6.98 (s, 1H, NH), 6.95 (s, 1H, NH), 6.77-6.80 (m, 4H, MMTr), 6.41 (s, 1H, H1'), 6.39 (s, 1H, H1'), 5.62-5.68 (m, 2H, H4'), 5.19 (s, 2H, OCH<sub>2</sub>OAc), 4.56 (d, *J* = 5.4 Hz, 2H, POCH<sub>2</sub>C), 4.39-4.47 (m, 4H, H5' & H5''), 4.30 (m, 2H, H4'), 4.13-4.18 (m, 4H, MeCH<sub>2</sub>CO), 4.11 (s, 2H, CH<sub>2</sub>OCH<sub>2</sub>OAc), 3.77 (s, 3H, OMe of MMTr), 3.76 (s, 3H, OMe of MMTr), 2.69-2.80 (m, 8H, MeCOCH<sub>2</sub>CH<sub>2</sub> Lev), 2.53-2.65 (m, 8H, CH<sub>2</sub>CH<sub>2</sub>COO Lev), 2.17, 2.16 (2s, 6H, Me of Lev), 2.07, 2.06 (2s, 6H, Me of Lev), 2.02 (s, 3H, AcO), 1.37, 1.34 (2s, 6H, Me2'), 1.15-1.21 (m, 6H, MeCH<sub>2</sub>O). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>) δ 171.91, 171.88 (CH<sub>2</sub>COCH<sub>3</sub> Lev), 171.37, 171.32 (OCOCH<sub>2</sub> Lev), 170.28 (CO of Ac), 166.64 (COOEt), 158.30 (MMTr), 154.15 (C6), 152.42 (C2), 148.48, 148.37 (C4), 145.19, 145.15 (MMTr), 139.33, 139.25 (C8), 137.20 (MMTr), 130.23, 128.90, 127.87, 126.85 (MMTr), 121.14, 121.05 (C5), 113.14 (MMTr), 88.78 (OCH<sub>2</sub>O), 88.06, 87.96 (C1'), 84.40, 84.08 (MMTr), 80.31, 80.25, 80.16, 80.06 (C4'), 74.62 (C3'), 70.98 (C2'), 67.22, 67.09 (C5'), 65.23, 65.20 (CCH<sub>2</sub>OP), 62.12 (MeCH<sub>2</sub>O), 58.98, 58.91 (CCH<sub>2</sub>OCH<sub>2</sub>OAc), 55.21 (MeO of MMTr), 45.23 (-C-), 37.86, 37.76 (MeCOCH<sub>2</sub> Lev), 29.73, 29.62 (Me of Lev), 27.68 (CH<sub>2</sub>COO Lev), 20.91 (Me of Ac), 17.22 (Me2'), 13.90 (MeCH<sub>2</sub>O). <sup>31</sup>P-NMR (202 MHz, CDCl<sub>3</sub>) δ -1.54. HR-ESI-MS: [M+H]<sup>+</sup> obsd. 1835.6900, calcd. 1835.6805, [M+Na]<sup>+</sup> obsd. 1857.6726, calcd. 1857.6624.

**3-Acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl bis(2'-C-methyladenosin-5'-yl) phosphate (19)**. Compound **18** (80.23 mmol; 0.34 g) was deprotected and purified as described above for compound **10** to obtain **11**. Compound **19** was obtained as white solid in 57% yield (0.12 g). <sup>1</sup>H-NMR (500 MHz, MeOD) δ 8.22 (s, 1H, H2), 8.21 (s, 1H, H2), 8.20 (s, H, H8), 8.19 (s, H, H8), 6.10 (s, 1H, H1'), 6.10 (s, 1H, H1'), 5.22 (d, *J* = 6.4 Hz, 1H, OCH<sub>2</sub>O), 5.19 (d, *J* = 6.4 Hz, 1H, OCH<sub>2</sub>O), 4.53-4.58 (m, 6H, POCH<sub>2</sub>C, H5', H5''), 4.13-4.29 (m, 10H, H3', H4', MeCH<sub>2</sub>COO, CH<sub>2</sub>OCH<sub>2</sub>OAc), 2.05 (s, 3H, AcO), 1.20 (t, *J* = 7.1 Hz, 3H, MeCH<sub>2</sub>COO), 0.94 (s, 6H, 2×Me). <sup>13</sup>C-NMR (126 MHz, MeOD) δ 170.66 (CO of Ac), 166.56, 166.55 (COOEt), 155.94 (C6), 152.57 (C2), 148.85 (C4), 139.21 (C8), 118.94 (C5), 91.97 (C1'), 88.35 (OCH<sub>2</sub>O), 80.57, 80.55, 80.51, 80.50 (C4'), 78.54, 78.53 (C3'), 73.10, 73.05 (C2'), 67.56, 67.52, 67.48 (C5'), 66.72 (CCH<sub>2</sub>OCH<sub>2</sub>OAc), 65.23, 65.19 (CCH<sub>2</sub>OP), 61.90, 61.89 (MeCH<sub>2</sub>O), 58.84, 58.77 (-C-), 19.50 (Me of Ac), 18.71 (2'-Me), 12.85 (MeCH<sub>2</sub>O). <sup>31</sup>P-NMR (202 MHz, MeOD) δ -1.61. HR-ESI-MS: [M+H]<sup>+</sup> obsd. 899.2970, calcd. 899.2931.

**Pivaloyloxymethyl bis(2'-C-methyladenosin-5'-yl) phosphate (20)**. Compound **10** (0.14 g; 0.09 mmol) was dissolved in *N*-methylpyrrolidone (1 mL), triethylamine (25 μL; 0.18 mmol) was added and the reaction mixture was incubated at 60 °C for 40 minutes. Chloromethyl

pivalate (POMCl, 25  $\mu$ L, 0.18 mmol) was added and the reaction was allowed to proceed at 60 °C for 3 days. One equivalent of Et<sub>3</sub>N (13  $\mu$ L, 0.09 mmol) and POMCl (13  $\mu$ L, 0.09 mmol) was added and the reaction mixture was stirred for one more day. The mixture was equilibrated between water and EtOAc. The organic layer was washed with brine and evaporated to dryness. The residue was filtered through a short silica gel column eluting with 5% MeOH in DCM. The crude product was dissolved in DCM (600  $\mu$ L) and hydrazinium acetate (0.044 g, 0.48 mmol) in MeOH (200  $\mu$ L) was added. The reaction mixture was stirred at r.t. for 18 hours. The unreacted hydrazinium acetate was destroyed with acetone and the reaction mixture was evaporated to dryness. The residue was dissolved in 80% aqueous acetic acid (1 mL) and the mixture was stirred at r.t. for 24 hours. The crude product was purified by HPLC as described above for compound **11**. Compound **20** was obtained as white solid in 15% yield (10 mg). <sup>1</sup>H-NMR (500 MHz, MeOD)  $\delta$  8.20 (s, 1H, H2), 8.20 (s, 1H, H2), 8.19 (s, 2H, H8), 9.08 (s, 1H, H1'), 6.08 (s, 1H, H1'), 5.73 (d,  $J$  = 1.1 Hz, 1H, OCH<sub>2</sub>O), 5.70 (d,  $J$  = 1.1 Hz, 1H, OCH<sub>2</sub>O), 4.56-4.60 (m, 4H, H5', H5''), 4.22-4.28 (m, 4H, H3', H4''), 1.19 (CMe<sub>3</sub>), 0.93, 0.92 (s, 6H, 2'-Me). <sup>13</sup>C-NMR (126 MHz, MeOD)  $\delta$  176.59 (CO), 155.90 (C6), 152.53 (C2), 148.82 (C4), 139.20, 139.15 (C8), 117.51 (C5), 91.94, 91.90 (C1'), 83.05, 83.01 (OCH<sub>2</sub>O), 80.49, 80.46, 80.42 (C4'), 78.54 (C2'), 73.05, 72.86 (C3'), 67.53, 67.49, 67.39, 67.34 (C5'), 38.31 (CMe<sub>3</sub>), 25.78 (CMe<sub>3</sub>), 18.30 (2'-Me). <sup>31</sup>P-NMR (202 MHz, CDCl<sub>3</sub>)  $\delta$  -2.50. HR-ESI-MS: [M+H]<sup>+</sup> obsd. 739.2533, calcd. 739.2567, [M+Na]<sup>+</sup> obsd. 761.2348, calcd. 761.2379.

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