Semisynthesis of 3′(2′)-*O*-(Aminoacyl)-tRNA Derivatives as Ribosomal Substrate

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An efficient synthesis of (3′-terminally) 3′(2′)-*O*-aminoacylated pCpA derivatives is described, which could lead to the production of (aminoacyl)-tRNAs following T4 RNA ligase mediated ligation. The tetrahydrofuranyl (thf) group was used as a permanent protective group for the 2′-OH of the cytidine moiety which can be removed during the purification of the 3′(2′)-*O*-aminoacylated-pCpA. This approach allowed for a general synthesis of (3′-terminally) 3′(2′)-*O*-aminoacylated oligonucleotides. The fully protected pCpA **14** was synthesized by phosphoramidite chemistry and treated with NH_3 solution to remove the 2-cyanoethyl and benzoyl groups (→ **15**; *Schemes 1* and *2*). The 2′-*O*-thf-protected-pCpA **15** was coupled with α-amino acid cyanomethyl esters, and the products **20a**–**c** were deprotected and purified with AcOH buffer to afford 3′(2′)-*O*-aminoacylated pCpA **21a**–**c** in high yields. The 3′(2′)-*O*-aminoacylated pCpA were efficiently ligated with tRNA(−CA) to yield (aminoacyl)-tRNA which was an active substrate for the ribosome.

Introduction. – Despite decades of intensive research, the catalytic mechanism of protein synthesis in the ribosome is still largely unknown [1–3]. The peptidyl-transferase reaction in the ribosome has never been well characterized because studies have been hampered by technical problems associated with the complexity of the ribosome and its substrates. The (3′-terminally) 3′(2′)-*O*-aminoacylated pCpA derivatives are the universally conserved terminal sequences of (aminoacyl)-tRNA [4–7]. The enzymatic preparation of (aminoacyl)-tRNA yields only very limited quantities and is not used as a general approach to all natural and unnatural amino acids. Furthermore, the X-ray crystallographic study of the ribosome requires large quantities of (aminoacyl)-tRNAs. The development of a general chemical synthesis of (3′-terminally) 3′(2′)-*O*-aminoacylated oligonucleotides is of considerable importance [8–10]. *Hecht* and coworkers have developed a successful solution to this problem. They coupled tRNA, missing the 3′-terminal pCpA moiety, with 3′(2′)-*O*-aminoacylated pCpA derivatives in the presence of T4 RNA ligase [11–19]. However, the synthesis of aminoacylated pCpA suffered several drawbacks.

Several groups have reported the synthesis of 3′(2′)-*O*-aminoacylated oligonucleotides by either nonchemical methods [20–21] or by various protective-groups/aminoacylating methods [15–19][22–33]. These methods lead to low aminoacylation yields,

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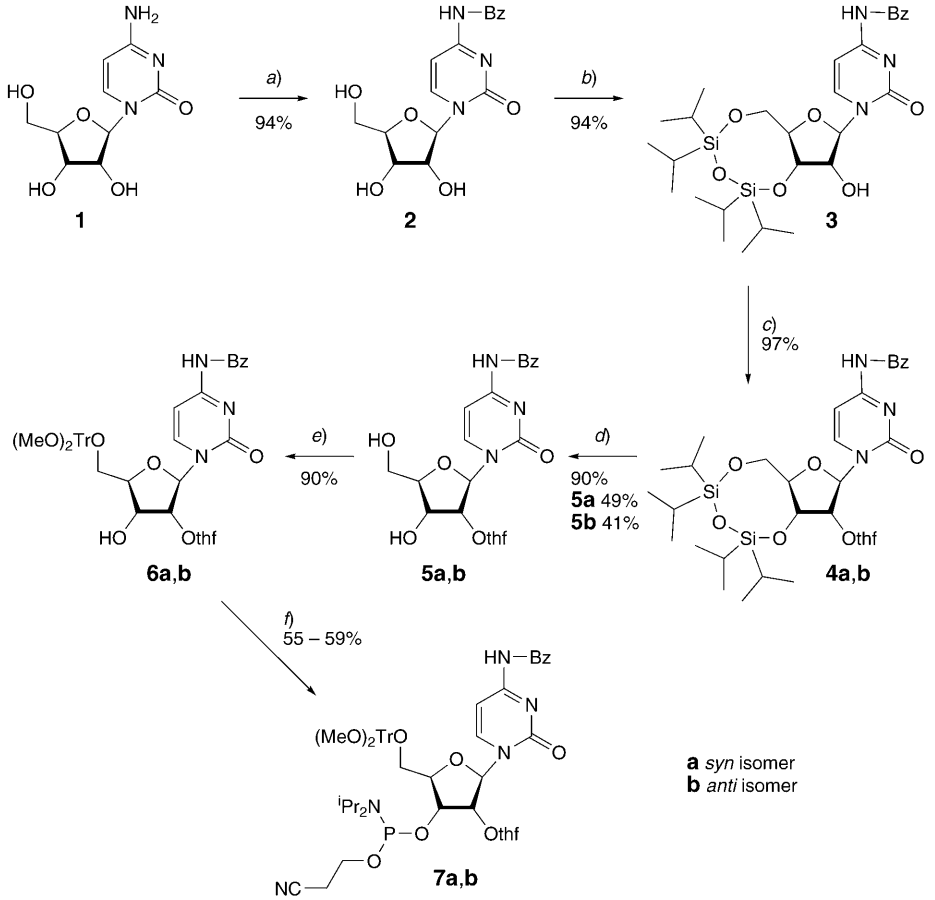
long reaction paths, or racemization of the products. *Schultz* and co-workers reported that the aminoacylation of amino acid cyanomethyl esters with unprotected pdCpA was an efficient method for preparing aminoacylated pdCpA [34]. Although the 2′-deoxycytidine may not be important for the *in vitro* translation system to incorporate a non-natural amino acid into a protein from a suppressor tRNA, it may be considerably critical for studying the peptidyl-transferase reaction in the ribosome. Literature searches revealed that the 2′-OH of the nucleotide was either not protected during aminoacylation [15][27][28] or was protected with the tetrahydro-2*H*-pyran-2-yl (thp) [22], the 4-methoxytetrahydro-2*H*-pyran-2-yl (mthp) [23–26], or a fluoride-labile group [33]. These methods were not satisfactory as they resulted in diacylation of the nucleotide, a missing 5′-terminal phosphate that is required for the ligation with tRNA(–CA) [17][33], extra deprotection steps, or removal of the 2′-OH protecting groups under relatively strong acidic conditions. All these problems led to low overall yields.

Results and Discussion. – The pH range ensuring the best stability of 3′(2′)-aminoacylated oligonucleotides is pH 3–4.5 [35]; thus, the ideal deprotection of the 2′-OH protective group of the cytidine moiety of pCpA should be conducted within this range. It is known that the tetrahydrofuran-2-yl (thf) group can be removed under this condition (acetic acid, pH 4). *Ikehara* and co-workers [36] used thf as a protective group to synthesize oligonucleotides by the phosphotriester method. However, the thf protective group was not widely used in nucleotide synthesis because this group was believed to be incompatible with the deprotection condition for regular 5′-OH protective groups like the 4,4′-dimethoxytrityl $(MeO)_2Tr$, levulinoyl, or 4,4′,4″-tris(4,5-dichlorophthalimido)trityl group under relatively strong acidic conditions. We found that the thf group was stable while $(MeO)_2Tr$ was selectively removed by catalytic hydrogenation in MeOH under 1 atm of H_2. Here, we describe an efficient route for synthesizing 3′(2′)-*O*-aminoacylated pCpA and corresponding (aminoacyl)-tRNAs. The thf group served as a permanent protection group for the 2′-OH of the cytidine moiety while the 5′-OH was protected by the $(MeO)_2Tr$ group.

The cytidine synthon N^4-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(tetrahydrofuran-2-yl)cytidine 3′-(2-cyanoethyl diisopropylphosphoramidite) (**7**) was prepared as shown in *Scheme 1*. Cytidine (**1**) was treated first with chlorotrimethylsilane in pyridine, then with benzoyl chloride, and finally with NH_4OH in a one-pot procedure to produce N^4-benzoylcytidine (**2**) in 94% yield. Compound **2** was treated with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane ($^iPr_2Si(Cl)OSi(Cl)^iPr_2$) in pyridine to give N^4-benzoyl-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)cytidine (**3**) in 94% yield. In the presence of 4-toluenesulfonic acid in THF **3** reacted with 2,3-dihydrofuran to produce N^4-benzoyl-2′-*O*-(tetrahydrofuran-2-yl)-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)cytidine (**4**) in 97% yield. Then, the 3′,5′-tetraisopropyldisiloxanediyl group was removed by treatment with Bu_4NF in THF generating N^4-benzoyl-2′-*O*-(tetrahydrofuran-2-yl)cytidine **5** in 90% yield (49% *anti* isomer and 41% syn isomer). Selective protection of the 5′-OH group with 4,4′-dimethoxytrityl chloride ($(MeO)_2TrCl$) yielded **6**, which was coupled with 2-cyanoethyl diisopropylphosphoramidochloridite in the standard manner to afford the building block **7** in 55–59% yield.

Scheme 2 shows the synthesis of the adenosine synthon and the 2′-*O*-thf-protected pCpA. The N^6-benzoyladenosine was obtained in the same manner as N^4-benzoylcyti-

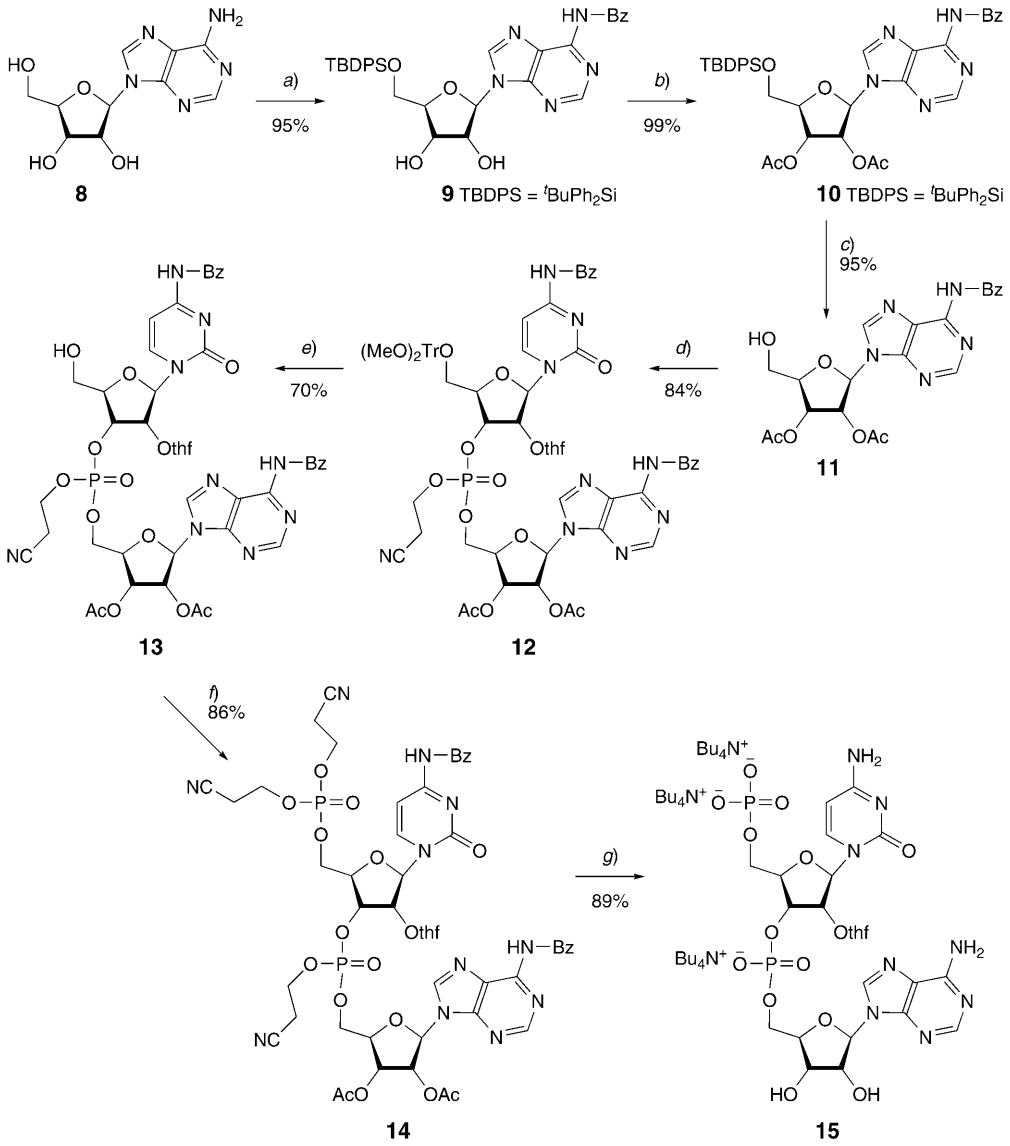
Scheme 1. *Synthesis of* N^4*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(tetrahydrofuran-2-yl)cytidine 3′-(2-Cyanoethyl Diisopropylphosphoramidite)* (**7**)



a) 1. Chlorotrimethylsilane, pyridine; 2. benzoyl chloride, pyridine; 3. NH_4OH. *b*) $^{i}Pr_2Si(Cl)OSi(Cl)^{i}Pr_2$, pyridine. *c*) 2,3-Dihydrofuran, TsOH, THF. *d*) Bu_4NF, THF. *e*) $(MeO)_2TrCl$, pyridine, $^{i}Pr_2EtN$. *f*) 2-Cyanoethyl diisopropylphosphoramidochloridite, $^{i}Pr_2EtN$, CH_2Cl_2.

dine (**2**) and then protected with (*tert*-butyl)chlorodiphenylsilane ($^{t}BuPh_2SiCl$) at 5′-OH to form **9** in 95% yield. The treatment of **9** with Ac_2O in pyridine yielded **10** in quantitative yield. The removal of the (*tert*-butyl)diphenylsilyl group with Bu_4NF produced **11** in 95% yield. The cytidine phosphoramidite **7** was coupled with 2′,3′-di-*O*-acetyl-N^6-benzoyladenosine (**11**) in the presence of 1*H*-tetrazole to yield the fully protected dinucleotide **12** (84% yield). The $(MeO)_2Tr$ group at the 5′-position of dinucleotide **12** was removed by hydrogenation in MeOH over 10% Pd/C. However, the thf group at the 2′-position of cytidine was also partially cleaved on a prolonged reaction time. This might be caused by the accumulation of *in situ* formed protons in the hydrogenation process. Thus, a base (pyridine or Et_3N) was added to the hydrogenation mixture to prevent thf cleavage. But the Pd/C catalyst was poisoned by these amine bases.

Scheme 2. *Synthesis of 2′-O-(Tetrahydrofuran-2-yl)cytidylyl-(3′ → 5′)-adenosine 5′-(Dihydrogen Phosphate) Tris(tetrabutylammonium) Salt* **15**



a) 1. Chlorotrimethylsilane, pyridine; 2. benzoyl chloride, pyridine; 3. NH_4OH; 4. ^{t}BuPh$_2$SiCl, *N*,*N*-dimethylpyridin-4-amine (DMAP). *b*) Ac_2O, pyridine, DMAP. *c*) Bu_4NF, THF. *d*) 1. **7**, 1*H*-tetrazole, MeCN; 2. *t*-BuOOH. *e*) H_2, Pd/C, MeOH, 2 h. *f*) 1. ^{i}Pr$_2$N-P(OCH$_2$CH$_2$CN)$_2$, ^{i}Pr$_2$EtN, 1*H*-tetrazole, MeCN; 2. *t*-BuOOH. *g*) 1. NH_3, EtOH, 55°; 2. ion exchange, $Bu_4N(OH)$.

Replacing the solvent MeOH by EtOH strongly reduced the deprotecting rate. The ^{t}BuPh$_2$Si group was also used to protect the 5′-OH of cytidine while the 2′-OH group

was protected by thf. But the 2-cyanoethyl group at the phosphate moiety was not stable under the cleaving conditions for ^{t}BuPh$_2$Si by Bu$_4$NF. However, a 70% yield of isolated desired product **13** was adieved by treatment of **13** with H_2 over PdC in MeOH for 2 h. Then the 5′-HO-CpA reacted with bis(2-cyanoethyl) diisopropylphosphoramidite [7] in the presence of *1H*-tetrazole in MeCN to yield the fully protected pCpA **14** (86% yield). This was then dissolved in NH_3 solution/EtOH 3 : 1 (*v*/*v*) in a sealed reaction vessel. The mixture was heated at 55° overnight and then exchanged by ion-exchange chromatography (*Amberlite CG-50*, Bu$_4$N$^+$ form) to give 2′-*O*-(tetrahydrofuran-2-yl)cytidylyl-(3′ → 5′)-adenosine 5′-(dihydrogen phoshate) tris(tetrabutylammonium) salt **15** (89%).

The synthesis of 2′(3′)-*O*-[(pent-4-enoyl)amino]acylated pCpAs and {[(pent-4-enoyl)amino]acyl}-tRNAs is shown in *Scheme 3*. The synthesis of [(pent-4-enoyl)-amino] acids was modified from the literature [37][38]. Pent-4-enoic acid (**16**) was treated with *N*-hydroxysuccinimide in the presence of *N*,*N*-dimethylpyridin-4-amine (DMAP) and dicyclohexylcanbodiinide (DCC) to give active ester **17**. The reaction of **17** with three different α-amino acids in DMF yielded the *N*-(pent-4-enoyl)-L-amino acids **18a**–**c**. These were converted to active cyanomethyl esters of *N*-(pent-4-enoyl)-L-amino acids **19a**–**c** in 60–74% yield from **16**. The spectral data were identical with published data. The cyanomethyl esters **19a**–**c** were then treated with (internally) 2′-*O*-thf-protected pCpA dinucleotide **15** in DMF to yield **20a**–**c**. After deprotection and purification, the [(pent-4-enoyl)amino]acylated pCpA **21a**–**c** were obtained in over 80% yield from **15**, as determined by HPLC. The preparation of the {[(pent-4-enoyl)amino]acyl}-tRNAs **22a**–**c** was accomplished by T4-RNA-ligase-mediated ligation with aminoacylated pCpAs **21a**–**c** and *Escherichia coli* tRNA(–CA)^{Phe} transcripts. The pent-4-enoyl protection group can be removed by treatment with 10 mM I_2 for 20 min.

To determine the ligation efficiency, (3′-terminally) 3′(2′)-*O*-{*N*-[(biotinylamino)-caproyl]-L-methionyl}-substituted pCpA and the corresponding {*N*-[(biotinylamino)-caproyl]-L-methionyl}-tRNA were also synthesized in the same manner as described above[2]). The coupling reaction of *N*-[(biotinylamino)caproyl]-L-methionine cyanomethyl ester[2]) with the pCpA tris(tetrabutylammonium) salt **15** in anhydrous DMF yielded 3′(2′)-*O*-{*N*-[(biotinylamino)caproyl]-L-methionyl}-substituded pCpA. This biotinylated aminoacylated pCpA was ligated with an *Escherichia coli* tRNA(–CA)^{Phe} transcript in the presence of T4 RNA ligase. The resulting {*N*-[(biotinylamino)caproyl]-L-methionyl}-tRNA and ligation efficiency were determined by streptavidin-gel-shift analysis (*Fig. 1*). Over 90% of tRNA(–CA) was converted to {*N*-[(biotinylamino)-caproyl]-L-methionyl}-tRNA within 25 min (*Lane 5*). The result demonstrated that the ligation reaction of aminoacylated pCpA with tRNA(–CA) was highly efficient. The determination of the peptidyl-transferase activity of the {*N*-[(biotinylamino)-caproyl]-L-methionyl}-tRNA substrate was performed with 5′-([^{32}P]p)CpCpA-NH-Phe as a peptidyl acceptor in the 50 S ribosome as shown *Fig. 2*. After 30 min., the peptidyl-transferase reaction was complete (*Lane 4*, *Fig. 2*). The results demonstrated that chemically semisynthesized peptidyl-tRNA was an active peptidyl donor for the ribo-

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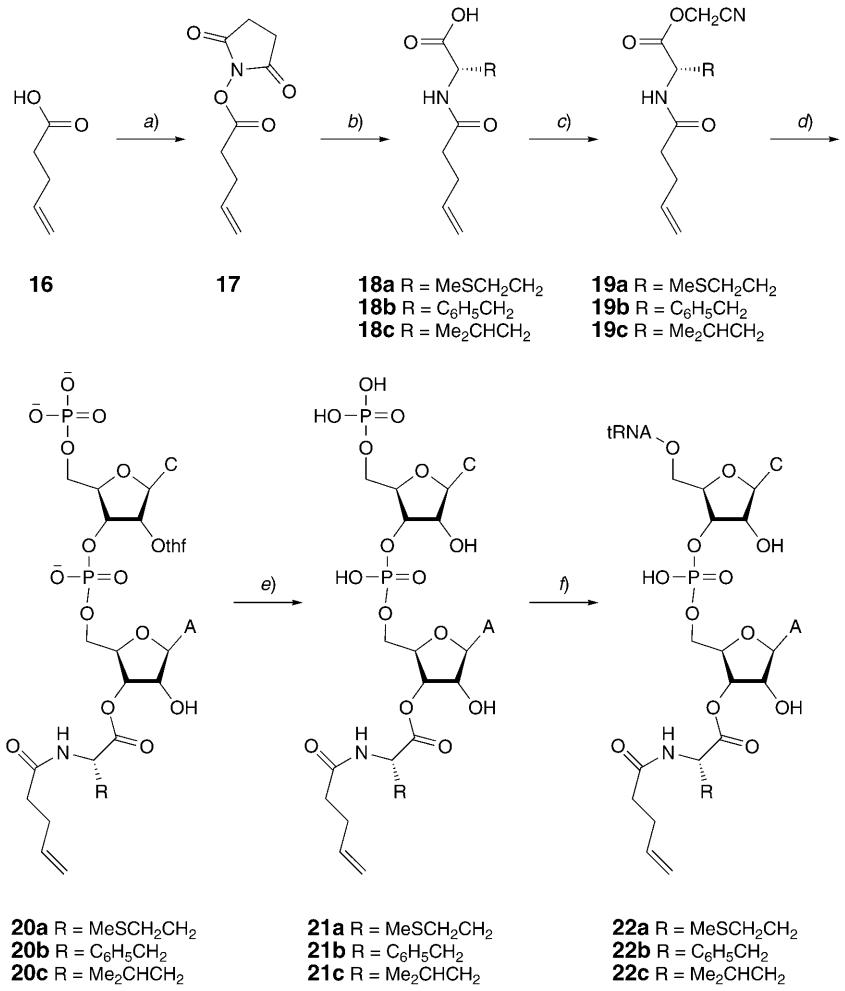
[2]) The systematic name of the acyl group (biotinylamino)caproyl is 6-{{5-[(3a*S*,4*S*,6a*R*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl]-1-oxopentyl}amino}-1-oxohexyl.

Scheme 3. *Synthesis of 2′,(3′)-*O*-Aminoacylated pCpA and tRNA*



a) *N*-Hydroxysuccinimide, DMAP, DCC, THF. *b*) L-Amino acid, $^{i}Pr_2EtN$, DMF. *c*) Chloroacetonitrile, $^{i}Pr_2EtN$, MeCN. *d*) **15**, DMF. *e*) 1. 50 mM NH_4OAc, C_{18} reversed-phase chromatography; 2. 0.87M AcOH, C_{18} reversed-phase chromatography. *f*) tRNA(– CA)^{Phe}, T4 RNA ligase.

some. We also studied the other semisynthesized (aminoacyl)-tRNAs **22a**–**c** as the ribosomal substrate, all of them were active for the peptidyl-transferase reaction in the ribosome (data not shown).

Conclusion. – We have developed a general method for the synthesis of pCpA. The advantage of the synthetic strategy is that the tetrahydrofuran-2-yl protective group is compatible with the $(MeO)_2Tr$ group and can be removed during purification resulting in high aminoacylation yields. Since T4 RNA ligase is a readily available and inexpensive enzyme, a relatively large quantity of (aminoacyl)-tRNA can be prepared by this

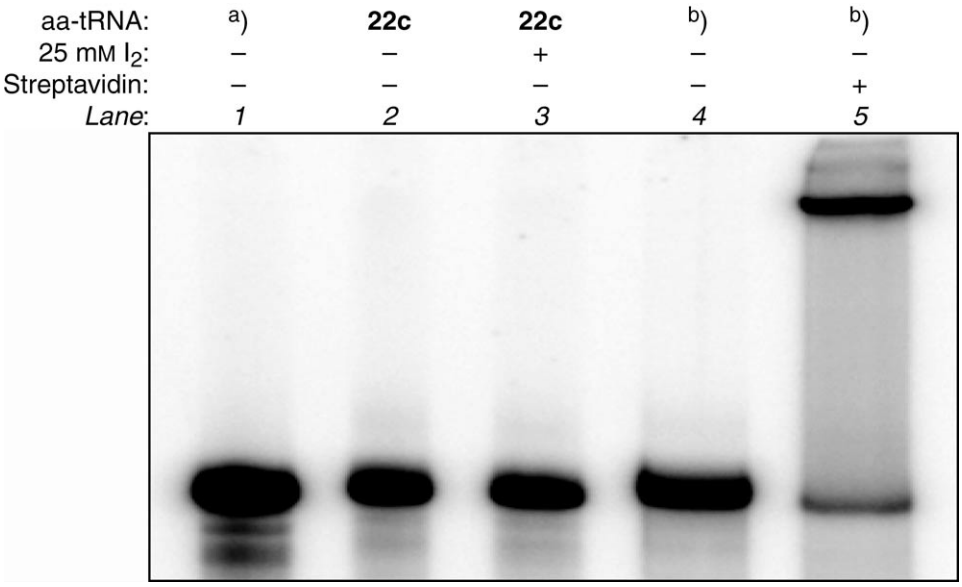


Fig. 1. *Autoradiogram of the streptavidin-gel-shift analysis of ligation products [3′(2′)-*O*-{*N*-[(biotinylamino)caproyl]-*L*-methionyl-pCpA and [5′-^{32}P]-tRNA(−CA)^{Phe}]*. The samples were run on 7.5M urea/10% polyacrylamide gel with 1×TBE buffer at 30 W (TBE = *Tris*/boric acid/Na_4edta). [a]) aa-tRNA = *Escherichia coli* tRNA(−CA)^{Phe}. [b]) aa-tRNA = {*N*-[(biotinylamino)caproyl]-L-methionyl}-tRNA.

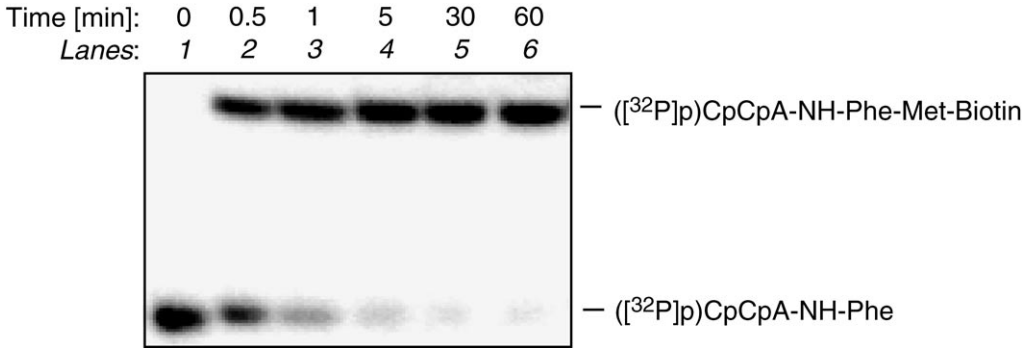


Fig. 2. *Peptidyl-transferase activity of {*N*-[(biotinylamino)caproyl]-*L*-methionyl}-tRNA, determined with ([^{32}P]p)CpCpA-NH-Phe as peptidyl acceptor*

strategy. Semisynthesized peptidyl- or (aminoacyl)-tRNA were active for the peptide synthesis in the ribosome. This synthetic method may provide a potential way to prepare (aminoacyl)-tRNA or unnatural aminoacyl-tRNA for the peptide synthesis in the ribosome.

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Experimental Part

General. All solvents, organic chemicals, and inorganic chemicals were purchased from *Acros* or *Aldrich*. Solvents were dried by using standard methods. CH_2Cl_2, MeCN, and pyridine were refluxed over CaH_2 and freshly distilled before use. Reactions were run under Ar. TLC: precoated silica gel *60 F254* sheets from *EM*. FC: silica gel *60*, 180–240 mesh, from *EM*. ^{1}H-NMR Spectra: *Varian VNMR-400* spectrometer; $CDCl_3$ or (D_6)DMSO as solvents, with the trace-solvent signal as reference; δ in ppm, J in Hz. ESI-MS: *Finnigan LCQ^{DUO}* spectrometer; in m/z.

Ligation Reaction of tRNA(−CA) with 3′(2′)-O-(Aminoacyl)-Substituted pCpA. *E. coli* tRNA-$(-CA)^{Phe}$ was prepared by *in vitro* transcription in the presence of T7 RNA polymerase. Then 40 μg of 3′(2′)-*O*-L-aminoacyl)-substituted pCpA **21a**–**c** or 3′(2′)-*O*-{*N*-[(biotinylamino)caproyl]-L-methionyl}-substituted pCpA was incubated with tRNA$(-CA)^{Phe}$ in the presence of 200 U T4 RNA ligase in the ligation buffer (55 mM HEPES (=4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid); pH 7.5, 250 μM ATP, 15 mM $MgCl_2$, 10% (*v*/*v*) DMSO) at 37° for 1–5 h. The ligated product was purified by 10% polyacrylamide gel electrophoresis.

Labeling of HO-CpCpA-NH-Phe. The kinase reaction was performed with 1 nmol of 5′-HO-CpCpA-NH-Phe, 50 μCi of [γ-^{32}P]-ATP, and 200 units of T4 polynucleokinase (PNK) in the presence of 1×PNK buffer at 37° for 1 h. Excess ATP (100 nmol) was added into the radioactive mix, and the labeling reaction was continued for another hour to convert all 5′-HO-CpCpA-NH-Phe into 5′-([^{32}P]p)CpCpA-NH-Phe which was purified by 20% polyacrylamide gel electrophoresis.

Ribosomal Reactions. Reactions were performed with 1.0 A_{260} unit of 50 S (36 pmol), 200 μM {*N*-[(biotinylamino)caproyl]-L-methionyl} tRNA, and a trace amount of 5′-([^{32}P]p)CpCpA-NH-Phe in the presence of 50 mM *Tris*·HCl buffer (pH 7.4), 35 mM $MgCl_2$, 100 mM NH_4Cl, and 1000 mM KCl at 37°. Samples were loaded onto 24% polyacrylamide/7.5M urea gel.

N^4-*Benzoylcytidine* (**2**). R_f 0.55 (20% MeOH/AcOEt). ^{1}H-NMR ($(D_6)DMSO_6$, 400 MHz): 3.57–3.77 (*m*, 2 H); 3.89–4.01 (*m*, 3 H); 5.06 (*d*, *J*=5.47, 1 H); 5.18 (*t*, *J*=5.08, 1 H); 5.52 (*d*, *J*=5.09, 1 H); 5.79 (*d*, *J*=2.74, 1 H); 7.31 (*m*, 1 H); 7.48–7.63 (*m*, 3 H); 7.97–7.99 (*m*, 2 H); 8.47 (*d*, *J*=7.42, 1 H); 11.16 (br. *s*, 1 H). ^{13}C-NMR ($(D_6)DMSO_6$, 100 MHz): 60.57; 69.32; 75.23; 84.90; 90.88; 96.80; 129.14; 133.42; 133.86; 145.94; 155.22; 163.70; 168.03.

N^4-*Benzoyl-3′,5′*-O-*(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)cytidine* (**3**). R_f 0.57 (9.1% MeOH/$CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): 0.94–1.11 (*m*, 28 H); 2.88 (br. *s*, 1 H); 4.01–4.05 (*dd*, *J*=13.44, 2.69, 1 H) 4.20–4.35 (*m*, 4 H); 5.86 (*s*, 1 H); 7.50–7.63 (*m*, 4 H); 7.87–7.89 (*d*, *J*=6.59, 2 H); 8.22 (*d*, *J*=6.60, 1 H); 8.67 (br. *s*, 1 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 12.70; 13.13; 13.15; 13.60; 17.05; 17.12; 17.21; 17.52; 17.63; 17.68; 60.23; 68.82; 75.46; 82.26; 91.80; 96.24; 127.70; 129.34; 133.49; 144.92; 162.56. ESI-MS: 590.2 ($[M+H]^+$).

N^4-*Benzoyl-2′*-O-*(tetrahydrofuran-2-yl)-3′,5′*-O-*(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)cytidine* (**4**). Compound **3** and TsOH were dried under vacuum (oil pump) for 5 h. To a soln. of **3** (12.89 g, 21.87 mmol) and TsOH (1.46 g, 7.67 mmol) in THF (250 ml), 2,3-dihydrofuran (16.7 ml, 219.2 mmol) was added by syringe at 0°. The mixture was stirred for 3 h at 0° under Ar. Then conc. NH_3 soln. (11.7 ml) was added and the solvent evaporated at low temp. The residue was dissolved in $CHCl_3$ (800 ml) and washed with sat. aq. $NaHCO_3$ soln. (100 ml) and H_2O (100 ml). The aq. layers were extracted with $CHCl_3$ (100 ml). The combined org. phase was dried (Na_2SO_4) and concentrated, and the residue purified by FC (SiO_2, 3.3–75% AcOEt/hexane): **4** (14 g, 97.0%, two diastereoisomers) as a white solid. R_f 0.67 (75% AcOEt in hexane). ^{1}H-NMR (400 MHz, $CDCl_3$): 0.92–1.11 (*m*, 28 H); 1.81–2.00 (*m*, 4 H); 3.82–4.36 (*m*, 7 H); 5.64–5.74 (*m*, 1 H); 5.84 (*d*, *J*=8.99, 1 H); 7.45–7.61 (*m*, 4 H); 7.88 (*d*, *J*=7.42, 2 H); 8.30 (*m*, 1 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 12.76; 12.80; 13.04; 13.23; 13.59; 13.63; 17.06; 17.11; 17.16; 17.18; 17.22; 17.30; 17.46; 17.51; 17.61; 17.68; 22.76; 23.16; 32.23; 32.74; 59.58; 59.64; 66.67; 66.77; 67.21; 68.95; 75.80; 77.80; 81.99; 82.20; 90.71; 90.85; 96.57; 102.61; 103.06; 128.02; 128.99; 133.15; 133.47; 144.34; 154.69; 162.94; 171.34. ESI-MS: 682.1 ($[M+Na]^+$), 660.2 ($[M+H]^+$).

N^4-*Benzoyl-2′*-O-*(tetrahydrofuran-2-yl)cytidine* (**5a**/**5b**). *Data of* **5a**: R_f 0.46 (9.1% MeOH/$CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.84–2.05 (*m*, 4 H); 3.58 (*d*, *J*=5.86, 1 H); 3.80–4.09 (*m*, 6 H); 4.30 (*m*, 1 H); 4.46 (*m*, 1 H); 5.47 (*m*, 1 H); 5.78 (*d*, *J*=2.05, 1 H); 7.44–7.56 (*m*, 4 H); 7.86 (*m*, 2 H); 8.40 (*d*,

$J = 7.32$, 1 H); 9.14 (br. *s*, 1 H). ^{13}C-NMR (100 MHz, (D_6)DMSO): 23.51; 32.47; 60.02; 66.92; 68.21; 79.01; 84.95; 89.40; 96.77; 102.87; 129.12; 129.42; 133.42; 133.77; 145.62; 155.05; 163.82; 168.11. ESI-MS: 416.2 ($[M-H]^-$).

Data of **5b**: R_f 0.39 (9.1% MeOH/$CHCl_3$). ^{1}H-NMR (400 MHz, (D_6)DMSO): 1.02–1.86 (*m*, 4 H); 3.59–3.67 (*m*, 2 H); 3.88 (*m*, 1 H); 4.11–4.18 (*m*, 2 H); 5.14–5.17 (*m*, 2 H); 5.26 (*m*, 1 H); 5.93 (*d*, $J = 4.69$, 1 H); 7.31–7.61 (*m*, 4 H); 7.99 (*d*, $J = 7.42$, 2 H); 8.39 (*d*, $J = 7.04$, 1 H); 11.25 (br. *s*, 1 H). ^{13}C-NMR (100 MHz, (D_6)DMSO): 23.27; 32.54; 61.19; 66.92; 69.20; 78.49; 85.90; 88.78; 96.97; 103.40; 129.12; 129.16; 133.42; 133.84; 146.28; 155.30; 163.70; 168.15. ESI-MS: 416.3 ($[M-H]^-$).

N^4-*Benzoyl-5′*-O-*(4,4′-dimethoxytrityl)-2′*-O-*(tetrahydrofuran-2-yl)-cytidine* (**6b**). R_f 0.36 (4.8% MeOH/$CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.85–2.06 (*m*, 4 H); 2.56 (*d*, $J = 7.03$, 1 H); 3.48–3.58 (*m*, 2 H); 3.82 (*s*, 6 H); 3.87–3.97 (*m*, 2 H); 4.12–4.14 (*m*, 1 H); 4.36–4.42 (*m*, 2 H); 5.54 (*m*, 1 H); 6.27 (*s*, 1 H); 6.86–6.88 (*m*, 4 H); 7.26–7.63 (*m*, 13 H); 7.86 (*m*, 2 H); 8.35 (br. *s*, 1 H); 8.55 (*s*, 1 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 23.46; 32.65; 55.47; 62.24; 68.05; 69.14; 79.43; 83.70; 87.29; 89.47; 97.12; 104.59; 113.56; 127.37; 127.98; 128.28; 128.50; 129.19; 130.30; 130.39; 133.27; 135.59; 135.87; 144.41; 145.39; 155.20; 158.89; 162.61. ESI-MS: 742.3 ($[M+Na]^+$), 720.1 ($[M+H]^+$).

Isomer **6a**: Treatment of **5a** (0.48 g, 1.15 mmol) and 4,4′-dimethoxytrityl chloride (0.779 g, 2.3 mmol) as described for **6b** gave **6a** (0.78 g, 94%). R_f 0.40 (9.1% MeOH/$CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.84–2.08 (*m*, 4 H); 3.55–3.59 (*m*, 3 H); 3.81 (*s*, 6 H); 3.84–4.09 (*m*, 3 H); 4.33–4.43 (*m*, 2 H); 5.56 (*m*, 1 H); 5.94 (*s*, 1 H); 6.86–6.89 (*m*, 4 H); 7.24–7.57 (*m*, 13 H); 7.88–7.91 (*m*, 2 H); 8.54 (*d*, $J = 7.42$, 1 H); 8.95 (br. *s*, 1 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 24.01; 32.78; 55.48; 61.32; 68.04; 68.18; 81.99; 83.40; 87.25; 90.36; 96.57; 105.36; 113.55; 127.39; 127.74; 128.28; 128.51; 129.27; 130.30; 130.39; 133.36; 135.59; 135.90; 144.39; 145.17; 155.08; 158.90; 162.38. ESI-MS: 742.3 ($[M+Na]^+$), 720.1 ($[M+H]^+$).

N^4-*Benzoyl-5′*-O-*(4,4′-dimethoxytrityl)-2′*-O-*(tetrahydrofuran-2-yl)cytidine 3′-(2-Cyanoethyl Diisopropylphosphoramidite)* (**7b**). To a cooled (ice bath) soln. of **6b** (0.8 g, 1.11 mmol; previously dried under vacuum (oil pump) overnight) in CH_2Cl_2 (25 ml), ^{i}Pr$_2$EtN (0.794 ml, 4.56 mmol) and 2-cyanoethyl diisopropylphosphoramidochloridite was added successively under Ar. After 20 min the mixture was stirred at r. t. for 4.5 h. The mixture was diluted with AcOEt (250 ml), the org. layer washed with sat. aq. $NaHCO_3$ soln. (25 ml) and sat. aq. NaCl soln. (25 ml) and dried (Na_2SO_4), the solvent evaporated, and the residue purified by FC (SiO_2, 77% AcOEt/19.2% hexane/3.8% Et_3N): **7b** (0.6 g, 58.5%, two diastereoisomers). Yellow solid. R_f 0.59, 0.70 (4.8% AcOEt/$CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.00–1.15 (*m*, 12 H); 1.76–1.94 (*m*, 4 H); 2.53 (*t*, $J = 6.25$, 1 H); 2.61 (*t*, $J = 6.25$, 1 H); 3.42–3.66 (*m*, 6 H); 3.77–3.76 (*m*, 6 H); 3.83–3.91 (*m*, 2 H); 4.22–4.30 (*m*, 1 H); 4.44–4.53 (*m*, 2 H); 5.44–5.48 (*m*, 1 H); 6.19–6.21 (*m*, 1 H); 6.81–6.85 (*m*, 4 H); 7.02 (br. *s*, 1 H); 7.20–7.54 (*m*, 13 H); 7.88–7.90 (*m*, 2 H); 8.26–8.36 (*m*, 1 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 20.36; 20.43; 20.57; 20.63; 23.17; 23.31; 24.70; 24.77; 24.82; 24.86; 32.58; 32.73; 43.27; 43.40; 43.53; 55.41; 55.44; 58.17; 58.37; 58.71; 58.89; 61.83; 62.23; 67.50; 67.54; 70.47; 70.53; 70.61; 70.68; 82.98; 83.05; 89.34; 89.40; 97.24; 103.65; 103.82; 113.47; 113.50; 117.72; 117.92; 127.37; 127.40; 127.98; 128.21; 128.23; 128.53; 128.64; 129.07; 130.39; 130.51; 133.16; 133.67; 135.37; 135.49; 135.58; 135.70; 144.30; 144.36; 145.15; 158.91; 162.03; 171.29. ^{31}P-NMR (162 MHz, $CDCl_3$): 150.92; 151.38. ESI-MS: 920.1 ($[M+H]^+$).

Treatment of **6a** (0.23 g, 0.32 mmol) as described for **7b** gave **7a** (0.162 g, 55.1%). Light yellow solid. R_f 0.64, 0.81 (4.8% AcOEt/$CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.05–1.15 (*m*, 12 H); 1.80–1.95 (*m*, 4 H); 2.40 (*m*, 2 H); 3.40–3.80 (*m*, 12 H); 3.81–4.10 (*m*, 2 H); 4.24–4.53 (*m*, 3 H); 5.65–5.72 (*m*, 1 H); 6.05–6.11 (*m*, 1 H); 6.85–6.87 (*m*, 4 H); 7.05 (br. *s*, 1 H); 7.26–7.59 (*m*, 13 H); 7.88–7.90 (*m*, 2 H); 8.29–8.55 (*m*, 1 H). ^{31}P-NMR (162 MHz, $CDCl_3$): 150.58; 151.37. ESI-MS: 920.1 ($[M+H]^+$).

N^6-*Benzoyladenosine*. R_f 0.24 (13.3% MeOH/$CHCl_3$). ^{1}H-NMR (400 MHz, (D_6)DMSO): 3.55–3.70 (*m*, 2 H); 3.96–3.99 (*m*, 1 H); 4.17–4.19 (*m*, 1 H); 4.63–4.67 (*m*, 1 H); 5.14 (*t*, $J = 5.50$, 1 H); 5.26 (*d*, $J = 4.77$, 1 H); 5.58 (*d*, $J = 5.87$, 1 H); 6.04 (*d*, $J = 5.51$, 1 H); 7.53–7.64 (*m*, 3 H); 8.03–8.04 (*m*, 2 H); 8.72 (*s*, 1 H); 8.75 (*s*, 1 H); 11.22 (br. *s*, 1 H). ^{13}C-NMR (100 MHz, (D_6)DMSO): 62.00; 71.05; 74.33; 86.40; 88.24; 126.58; 129.18; 133.17; 133.99; 143.86; 151.09; 152.33; 152.91, 166.31.

N^6-*Benzoyl-5′*-O-*[(*tert-*butyl)diphenylsilyl]adenosine* (**9**). Into a soln. of *N*^{6}-benzoyladenosine (1.9 g, 5.12 mmol) and *N,N*-dimethylpyridin-4-amine (0.02 g, 0.164 mmol) in pyridine (100 ml), (*tert*-butyl)-chlorodiphenylsilane (1.6 ml, 6.14 mmol) was injected in one portion. The mixture was stirred at r.t. under Ar for 96 h. The solvent was evaporated, the residue diluted with EtOH (10 ml), and precipitation

induced by adding Et_2O (250 ml). The solid was filtered off and washed with H_2O (2×50 ml): **9** (3.09 g, 99%). White solid. R_f 0.32 (9.1% MeOH/$CHCl_3$). ^{1}H-NMR (400 MHz, (D_6)DMSO): 0.96 (*s*, 1 H); 3.78–4.09 (*m*, 2 H); 4.07 (*m*, 1 H); 4.36 (*m*, 1 H); 4.73 (*t*, *J*=5.14, 1 H); 6.06 (*d*, *J*=5.13, 1 H); 7.32–7.66 (*m*, 13 H); 8.02–8.04 (*m*, 2 H); 8.59 (*s*, 1 H); 8.66 (*s*, 1 H); 11.22 (br. *s*, 1 H). ^{13}C-NMR (100 MHz, (D_6)DMSO): 27.25; 64.62; 70.60; 73.76; 85.25; 88.46; 126.53; 128.20; 128.48; 128.53; 129.16; 129.19; 129.85; 130.59; 132.24; 133.27; 133.44; 134.00; 135.10; 135.69; 135.74; 143.78; 151.09; 152.77.

2′,3′-Di-O-*acetyl*-N^6-*benzoyl-5′*-O-*[(*tert-*butyl)diphenylsilyl]adenosine* (**10**). To a soln. of **9** (10.6 g, 17.4 mmol) and *N*,*N*-dimethylpyridin-4-amine (0.106 g, 0.87 mmol) in dry pyridine (250 ml), Ac_2O (3.6 ml, 38.26 mmol) was added slowly by syringe. The mixture was stirred at r. t. under Ar for 17.5 h, then another portion of Ac_2O (0.164 ml, 0.132 mmol) was added. The mixture was stirred for another 18 h. H_2O (1 ml) was added to quench the reaction. After evaporation of the solvent, the residue was dissolved in CH_2Cl_2 (500 ml) and the soln. washed with sat. aq. NaCl soln. (2×60 ml). The org. phase was dried (Na_2SO_4), the solvent evaporated, and the residue purified by FC (SiO_2, 4.7–40% AcOEt/hexane): **10** (23.2 g, 99%). White solid. R_f 0.72 (13.3% MeOH/$CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.11 (*s*, 9 H); 2.05 (*s*, 3 H); 2.15 (*s*, 3 H); 3.87 (*dd*, *J*=11.72, 3.12, 1 H); 3.99 (*dd*, *J*=11.72, 3.12, 1 H); 4.31 (*m*, 1 H); 5.72 (*dd*, *J*=5.47, 2.74, 1 H); 5.94 (*dd*, *J*=6.64, 5.47, 1 H); 6.40 (*d*, *J*=7.03, 1 H); 7.35–7.45 (*m*, 6 H); 7.51–7.55 (*m*, 2 H); 7.60–7.63 (*m*, 1 H); 7.66–7.68 (*m*, 4 H); 8.00–8.02 (*m*, 2 H); 8.26 (*s*, 1 H); 8.78 (*s*, 1 H); 8.92 (br. *s*, 1 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 20.63; 20.92; 27.16; 63.67; 71.49; 73.72; 84.04; 84.99; 123.40; 128.06; 128.18; 128.20; 129.15; 130.25; 130.33; 132.38; 132.73; 133.08; 133.84; 135.68; 135.89; 141.11; 149.82; 152.25; 153.28; 164.74; 169.58; 169.96. ESI-MS: 694.1 ([*M*+H]$^+$).

2′,3′-Di-O-*acetyl*-N^6-*benzoyladenosine* (**11**). Bu_4NF (25.4 ml, 25.4 mmol) was slowly injected into an ice-cooled soln. of **10** (17.60 g, 25.4 mmol) in THF (300 ml). The mixture was stirred in an ice bath under Ar for 7 h. After the solvent was evaporated, the residue was dissolved in CH_2Cl_2 (800 ml), the soln. washed with H_2O (2×100 ml) and sat. aq. NaCl soln. (100 ml), dried (Na_2SO_4), and concentrated, and the residue purified by FC (SiO_2, 0.5–2.0% MeOH/CH_2Cl_2) **11** (10.95 g, 94.8%). White solid. R_f 0.23 (4.8% MeOH/$CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): 2.01 (*s*, 3 H); 2.16 (*s*, 3 H); 3.83–4.01 (*m*, 2 H); 4.37 (*m*, 1 H); 5.68–5.69 (*m*, 1 H); 5.87–5.90 (*m*, 1 H) 6.00–6.03 (*m*, 1 H); 6.12 (*d*, *J*=7.6, 1 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 20.55; 20.98; 62.78; 72.85; 72.93; 86.54; 88.54; 124.82; 128.15; 129.16; 133.23; 133.53; 142.74; 150.56; 151.12; 152.64; 164.75; 169.24; 169.95. ESI-MS: 454.1 ([*M*−H]$^-$).

N^4-*Benzoyl*-P(O)-*(2-cyanoethyl)-5′*-O-*(4,4′-dimethoxytrityl)-2′*-O-*(tetrahydrofuran-2-yl)cytidylyl-(3′ → 5′)*-N^6-*benzoyl-2′,3′-di*-O-*acetyladenosine* (**12b**). A soln. of **7b** (0.32 g, 0.348 mmol) in MeCN (5 ml) was injected into a soln. of **11** (0.144 g, 0.316 mmol) and 1*H*-tetrazole (0.11 g 1.58 mmol) in MeCN (8 ml). The mixture was stirred at r. t. under Ar for 4 h. *tert*-Butyl hydroperoxide (0.646 ml, 3.16 mmol) was added to the cooled mixture (ice bath), and the mixture was stirred for another 2.5 h. The mixture was diluted with AcOEt (300 ml), the org. phase washed with sat. aq. $NaHCO_3$ soln. (20 ml) and sat. aq. NaCl soln. (20 ml), dried (Na_2SO_4), and concentrated, and the residue purified by FC (SiO_2, 0.1–4% MeOH/CH_2Cl_2): **12b** (0.377 g, 84%). Yellow solid. R_f 0.50 (9.1% MeOH/AcOEt). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.67–1.87 (*m*, 4 H); 2.01–2.11 (*m*, 6 H); 2.58–2.73 (*m*, 2 H); 2.98 (br. *s*, 1 H); 3.42–3.71 (*m*, 4 H); 3.73–3.74 (*m*, 6 H); 4.07–4.67 (*m*, 6 H); 4.62–4.67 (*m*, 1 H); 5.02–5.08 (*m*, 1 H); 5.30–5.38 (*m*, 1 H); 5.67–5.69 (*m*, 1 H); 5.86–5.90 (*m*, 1 H); 6.22–6.30 (*m*, 2 H); 6.79–6.84 (*m*, 4 H); 7.04 (br. *s*, 1 H); 7.19–7.53 (*m*, 16 H); 7.85–7.87 (*m*, 2 H); 7.96–7.98 (*m*, 2 H); 8.05–8.06 (*m*, 1 H); 8.22–8.34 (*m*, 1 H); 8.70–8.71 (*m*, 1 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 19.71; 19.79; 20.60; 20.77; 20.80; 23.26; 23.34; 32.49; 32.62; 55.46; 55.49; 62.55; 62.59; 62.64; 62.84; 62.89; 66.96; 67.00; 67.27; 67.32; 67.81; 67.85; 70.42; 70.58; 73.18; 73.26; 76.31; 76.36; 76.90; 81.09; 81.16; 81.27; 82.56; 82.63; 85.97; 86.27; 87.44; 87.53; 87.68; 87.71; 97.40; 103.86; 103.90; 113.61; 116.71; 116.79; 123.95; 123.96; 127.49; 127.53; 127.98; 128.28; 128.32; 128.49; 128.53; 128.96; 129.11; 130.42; 130.46; 132.93; 133.33; 133.74; 135.09; 141.83; 141.91; 144.07; 145.04; 150.21; 151.90; 152.04; 153.01; 158.96; 158.99; 162.49; 165.24; 169.62; 169.64; 169.87; 169.97. ^{31}P-NMR (162 MHz, $CDCl_3$): −1.77; −1.61. ESI-MS: 1328.4 ([*M*+K]$^+$).

Treatment of **7a** (0.74 g, 0.8 mmol) and **11** (0.333 g, 0.73 mmol) as described for **12b** gave **12a** (0.783 g, 83%). Light yellow solid. R_f 0.23 (4.8% MeOH/$CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.90–1.96 (*m*, 4 H); 2.00–2.11 (*m*, 6 H); 2.55–2.68 (*m*, 2 H); 3.45–3.77 (*m*, 9 H); 3.79–3.96 (*m*, 2 H); 4.15–4.38 (*m*, 6 H); 4.99–5.03 (*m*, 1 H); 5.48–5.54 (*m*, 1 H); 5.65 (*m*, 1 H); 5.82 (*m*, 1 H); 6.04–6.07 (*m*, 1 H);

6.22–6.24 (*m*, 1 H); 6.81–6.85 (*m*, 4 H); 7.00 (br. *s*, 1 H); 7.22–7.54 (*m*, 16 H); 7.85–7.87 (*m*, 2 H); 7.97–7.99 (*m*, 2 H); 8.21–8.30 (*m*, 3 H); 8.71–8.72 (*m*, 1 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 19.66; 19.72; 20.58; 20.75; 23.42; 23.55; 32.70; 55.44; 55.47; 61.07; 61.37; 62.59; 62.64; 62.81; 62.85; 67.15; 67.78; 70.47; 70.56; 73.12; 73.20; 77.34; 77.74; 81.07; 81.14; 86.12; 87.58; 89.10; 89.22; 97.30; 103.72; 103.79; 113.55; 116.81; 116.89; 124.02; 124.08; 127.51; 128.06; 128.31; 128.66; 128.89; 128.97; 130.48; 132.89; 133.18; 133.34; 133.76; 135.09; 135.24; 141.99; 142.11; 143.90; 144.62; 150.22; 151.98; 152.86; 154.92; 158.95; 162.89; 165.43; 169.63; 169.82. ^{31}P-NMR (162 MHz, $CDCl_3$): −1.41; −1.25. ESI-MS: 1328.4 ($[M+K]^+$), 1312.4 ($[M+Na]^+$).

N^4-*Benzoyl*-P(O)-*(2-cyanoethyl)-2′*-O-*(tetrahydrofuran-2-yl)cytidylyl-(3′ → 5′)*-N^6-*benzoyl-2′,3′-di*-O-*acetyladenosine* (**13b**). A suspension of **12b** (0.49 g, 0.38 mmol) and 10% Pd/C (0.10 g) in MeOH (20 ml) was stirred at r. t. under H_2 for 1.5 h. The catalyst was filtered off, the solvent evaporated, and the residue purified by FC (SiO_2, 0.1–5% MeOH/CH_2Cl_2): **13b** (0.263 g, 70.1%). Yellow solid. R_f 0.36 (9.1% MeOH/$CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.54–1.86 (*m*, 4 H); 2.00–2.10 (*m*, 6 H); 2.72–2.76 (*m*, 2 H); 3.12 (br. *s*, 1 H); 3.56–3.79 (*m*, 4 H) 4.19–4.28 (*m*, 3 H); 4.40–4.80 (*m*, 4 H); 5.10–5.21 (*m*, 2 H); 5.66–5.70 (*m*, 1 H); 5.86–5.94 (*m*, 2 H); 6.27–6.30 (*m*, 1 H); 7.37–7.51 (*m*, 7 H); 7.82–8.36 (*m*, 5 H); 8.36 (*m*, 1 H); 8.68 (*s*, 1 H); 9.63 (br., 2 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 19.73; 19.78; 19.81; 19.86; 20.61; 20.80; 23.35; 23.43; 32.40; 32.47; 61.43; 62.85; 62.92; 62.98; 66.87; 67.18; 67.66; 67.72; 70.45; 70.60; 73.21; 73.34; 76.86; 81.19; 81.27; 81.39; 84.65; 84.70; 85.95; 86.21; 90.88; 97.63; 104.34; 104.45; 117.03; 117.05; 123.95; 128.12; 128.37; 128.91; 129.00; 132.98; 133.30; 133.58; 133.61; 141.96; 142.01; 150.20; 152.03; 152.09; 152.93; 163.01; 165.65; 169.72; 169.73; 170.01, 170.03. ^{31}P-NMR (162 MHz, $CDCl_3$): −1.69; −1.67. ESI-MS: 1026.1 ($[M+K]^+$), 1010.2 ($[M+Na]^+$).

Treatment of **12a** (0.3 g, 0.232 mmol) with H_2 over 10% Pd/C (0.1 g) as described for **13b** gave **13a** (0.15 g, 65.2%). Light yellow solid. R_f 0.45 (9.1% MeOH/$CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.79–1.95 (*m*, 4 H); 2.04–2.11 (*m*, 6 H); 2.26 (br. *s*, 1 H); 2.75–2.77 (*m*, 2 H); 3.72–3.96 (*m*, 4 H); 4.24–4.67 (*m*, 7 H); 4.90–4.99 (*m*, 1 H); 5.51 (*m*, 1 H); 5.69–5.72 (*m*, 1 H); 5.85–5.89 (*m*, 2 H); 6.30–6.31 (*m*, 1 H); 7.43–7.58 (*m*, 7 H); 7.86 (*m*, 2 H); 8.01 (*m*, 2 H); 8.34–8.41(*m*, 2 H); 8.75 (*m*, 1 H); 9.32 (br., 2 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 19.80; 19.88; 20.62; 20.81; 23.52; 23.57; 32.66; 60.00; 62.91; 62.96; 67.08; 67.74; 67.83; 70.39; 70.49; 73.26; 73.36; 76.94; 81.22; 81.29; 83.16; 83.22; 85.94; 86.23; 91.09; 97.25; 104.14; 116.76; 116.98; 123.82; 127.96; 128.28; 128.34; 129.05; 129.13; 133.07; 133.35; 133.63; 141.75; 150.09; 152.01; 153.09; 162.83; 165.25; 169.72; 169.99; 170.04. ^{31}P-NMR (162 MHz, $CDCl_3$): −1.11; −0.98. ESI-MS: 1010.2 ($[M+Na]^+$).

N^4-*Benzoyl*-P(O)-*(2-cyanoethyl)-2′*-O-*(tetrahydrofuran-2-yl)-cytidylyl-(3′ → 5′)-N^6-benzoyl-2′,3′-di*-O-*acetyladenosine 5′-[Bis(2-cyanoethyl) Phosphate]* (**14b**). To the soln. of **13b** (0.2 g, 0.203 mmol; dried under vacuum (oil pump) overnight) in dry MeCN (30 ml), bis(2-cyanoethyl) diisopropylphosphoramidite (0.082 g, 0.304 mmol) and 1*H*-tetrazole (0.071 g, 1.01 mmol) were added. The mixture was stirred at r.t. for 3 h, and *tert*-butyl hydroperoxide (0.2 ml, 1.01 mmol) was added. After 2 h, the solvent was evaporated, the residue dissolved in AcOEt (200 ml), the soln. washed with sat. aq. $NaHCO_3$ soln. (20 ml) and sat. aq. NaCl soln. (20 ml), dried (Na_2SO_4), and concentrated, and the residue purified by FC (silica gel, 0.1–5% MeOH/CH_2Cl_2): **14b** (0.204 g, 86.1%). White solid. R_f 0.44 (9.1% MeOH/$CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.67–1.92 (*m*, 4 H); 2.03–2.13 (*m*, 6 H); 2.75–2.78 (*m*, 6 H); 3.65–3.73 (*m*, 2 H); 4.26–4.67 (*m*, 13 H); 5.09–5.12 (*m*, 1 H); 5.22–5.27 (*m*, 1 H); 5.68–5.73 (*m*, 1 H); 5.83–5.91 (*m*, 2 H); 6.30–6.32 (*m*, 1 H); 7.43–7.57 (*m*, 7 H); 7.78–7.89 (*m*, 3 H); 7.99–8.02 (*m*, 2 H); 8.36–8.38 (*m*, 1 H); 8.74–8.75 (*m*, 1 H); 9.43 (br., 2 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 19.84; 19.86; 19.91; 20.61; 20.63; 20.83; 23.50; 23.56; 32.51; 32.59; 62.92; 62.97; 62.99; 66.62; 67.25; 67.86; 67.91; 70.52; 70.56; 73.18; 73.29; 74.60; 74.75; 76.45; 76.56; 81.05; 81.12; 81.24; 81.31; 85.95; 86.08; 92.40; 97.44; 105.05; 117.03; 117.14; 124.07; 124.12; 128.06; 128.33; 129.00; 129.12; 133.02; 133.42; 133.71; 141.96; 142.06; 146.60; 150.21; 152.14; 154.75; 163.04; 165.38; 169.66; 169.71, 169.98. ^{31}P-NMR (162 MHz, $CDCl_3$): −1.59; −1.43; −1.41. ESI-MS: 1212.1 ($[M+K]^+$), 1196.1 ($[M+Na]^+$).

Treatment of **13a** (0.096 g, 0.0972 mmol) with bis(2-cyanoethyl) diisopropylphosphoramidite (0.039 g, 0.144 mmol) as described for **14b** gave **14a** (0.1g, 87.7%). White solid. R_f 0.44 (9.1% MeOH/$CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.77–1.99 (*m*, 4 H); 2.02–2.14 (*m*, 6 H); 2.74–2.83 (*m*, 6 H); 3.75–3.84 (*m*, 2 H); 4.27–4.60 (*m*, 13 H); 4.85–4.90 (*m*, 1 H); 5.54 (*m*, 1 H); 5.68–5.73 (*m*, 1 H); 5.80–5.88 (*m*, 2 H); 6.29–6.33 (*m*, 1 H); 7.44–7.58 (*m*, 7 H); 7.88–7.92 (*m*, 2 H); 8.00–8.05 (*m*, 3 H); 8.38–8.41 (*m*, 1 H);

8.72–8.76 (*m*, 1 H); 9.37 (br., 2 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 19.82; 19.91; 19.98; 20.57; 20.63; 20.85; 23.52; 23.57; 32.14; 32.62; 63.03; 63.06; 63.08; 63.14; 65.74; 67.38; 67.43; 67.82; 67.88; 70.48; 70.58; 73.31; 76.47; 76.51; 80.17; 80.23; 81.22; 81.29; 85.81; 86.06; 90.81; 97.28; 103.99; 104.10; 116.99; 117.02; 117.06; 117.17; 123.94; 128.01; 128.31; 128.35; 129.03; 129.15; 133.04; 133.42; 133.71; 141.80; 142.12; 144.78; 150.16; 150.22; 152.09; 153.01; 154.93; 162.95; 165.26; 169.73; 169.75; 170.00; 170.02. ^{31}P-NMR (162 MHz, $CDCl_3$): −1.34; −1.31; −1.19; −0.98. ESI-MS: 1196.3 ($[M+Na]^+$).

*2′-*O*-(Tetrahydrofuran-2-yl)cytidylyl-(3′ → 5′)-adenosine 5′-(Dihydrogen Phosphate) Tris(tetrabutylammonium) Salt* (**15a**). A soln. of **14a** (0.098 g, 0.0835 mmol) in conc. NH_3 soln. (9 ml) and EtOH (3 ml) in a sealed pressure flask was stirred at 55° for 16 h. The reaction was shown to be complete by ^{31}P-NMR. After the solvent was evaporated, the residue in H_2O (60 ml) was first extracted by $CHCl_3$ (2 × 15 ml) and then lyophilized. The solid was exchanged to the tetrabutylammonium salt by ion exchange (*Amberlite-CG-50* resin (100–200 mesh), Bu_4N^+ form). The eluate was lyophilized overnight: **15a** (0.1 g, 83.3%). White solid. R_f 0.32 (^{i}PrOH/NH_3 soln./H_2O 7:1:2). ^{1}H-NMR (400 MHz, D_2O): 0.75 (*m*, 36 H); 1.17 (*m*, 24 H); 1.44 (*m*, 24 H); 1.65–1.81 (*m*, 4 H); 2.98 (*m*, 24 H); 3.62–3.66 (*m*, 1 H); 3.77–4.00 (*m*, 5 H); 4.19 (*m*, 2 H); 4.26 (*m*, 1 H); 4.36 (*m*, 1 H); 4.46–4.52 (*m*, 2 H); 5.28 (*m*, 1 H); 5.75 (*d*, *J* = 7.42, 1 H); 5.84 (*d*, *J* = 4.29, 1 H); 5.90 (*d*, *J* = 6.25, 1 H); 7.95 (*d*, *J* = 7.43, 1 H); 8.04 (*s*, 1 H); 8.38 (*s*, 1 H). ^{13}C-NMR (100 MHz, D_2O): 12.98; 19.27; 22.56; 23.22; 31.83; 58.19; 62.39; 65.05; 67.99; 70.76; 73.01; 75.29; 77.62; 84.32; 84.40; 86.89; 87.50; 96.87; 103.78; 118.71; 139.70; 141.43; 149.11; 153.00; 155.68; 157.39; 165.87. ^{31}P-NMR (162 MHz, D_2O): 3.75; −1.03. ESI-MS: 721.3 ($[M-H]^-$).

Treatment of **14b** (0.1 g, 0.0852 mmol) with NH_3 soln. (15 ml) and EtOH (5 ml) as described for **15a** gave **15b** (0.109 g, 88.6%). White solid. R_f 0.30 (^{i}PrOH/NH_3 soln./H_2O 7:1:2). ^{1}H-NMR (400 MHz, D_2O): 0.73 (*m*, 36 H); 1.15 (*m*, 24 H); 1.42 (*m*, 24 H); 1.55–1.72 (*m*, 4 H); 2.96 (*m*, 24 H); 3.41–3.50 (*m*, 2 H); 3.78 (*m*, 2 H); 3.94 (*m*, 2 H); 4.18 (*m*, 3 H); 4.34 (*m*, 1 H); 4.59 (*m*, 2 H); 5.03 (*m*, 1 H); 5.87–5.90 (*m*, 2 H); 5.93 (*d*, *J* = 6.97, 1 H); 7.86 (*d*, *J* = 7.70, 1 H); 8.03 (*s*, 1 H); 8.37 (*s*, 1 H). ^{13}C-NMR (100 MHz, D_2O): 12.98; 19.25; 22.65; 23.20; 31.65; 58.16; 63.39; 65.24; 67.58; 70.93; 73.80; 74.64; 77.85; 83.99; 84.35; 86.46; 86.77; 97.03; 104.12; 118.65; 139.84; 141.93; 149.25; 153.00; 155.68; 157.72; 165.99. ^{31}P-NMR (162 MHz, D_2O): −0.46; −4.89. ESI-MS: 721.3 ($[M-H]^-$).

*2′-*O*-(Tetrahydrofuran-2-yl)cytidylyl-(3′ → 5′)-3′(2′)-*O*-[*N*-(1-oxopent-4-enyl)-*L*-methionyl]adenosine 5′-(Dihydrogen Phosphate)* (**20a**·3 H^+). To a soln. of **15** (0.05 g, 0.0296 mmol) in DMF (0.61 ml), a stock soln. of **19a** [37][38] (0.0182 g, 0.0673 mmol) in DMF (0.25 ml) was slowly added in drops in several portions within 4 h under N_2. A mixture of 50 mM NH_4Ac (2 ml, pH 4.5) and MeCN (1 ml) was added to quench the reaction, and the solvent was evaporated. The residue was redissolved in 50 mM NH_4Ac (pH 4.5) and MeCN and applied to reversed-phase chromatography (1–50% of MeCN/50 mM NH_4Ac). After lyophilization, the residue was applied to reversed phase chromatography (1–30% MeCN/0.87M AcOH): **20a**·3 H^+ (0.01 g, 39%). R_f 0.45 (BuOH/AcOH/H_2O 5:2:3). ^{1}H-NMR (400 MHz, D_2O; 2:1 mixture of monoacylated diastereoisomers): 1.95 (*s*, 3 H); 2.06–2.54 (*m*, 8 H); 3.90 (*m*, 2 H); 4.04 (*m*, 2 H); 4.18 (*t*, *J* = 5.13, 1 H); 4.46 (*m*, 1 H); 4.39–4.49 (*m*, 3 H); 4.83–4.96 (*m*, 3 H); 5.38–5.74 (*m*, 3 H); 6.00–6.19 (*m*, 2 H); 7.92 (*m*, 1 H); 8.20 (*s*, 1 H); 8.37 (*s*, 1 H). ^{31}P-NMR (162 MHz, D_2O): 0.03; −0.71. ESI-MS: 864.1 ($[M-H]^-$).

*2′-*O*-(Tetrahydrofuran-2-yl)cytidylyl-(3′ → 5′)-3′(2′)-*O*-[*N*-(1-oxopent-4-enyl)-*L*-phenylalanyl]adenosine 5′-(Dihydrogen Phosphate)* (**20b**·3 H^+). Treatment of **15** (0.050 g, 0.0296 mmol) and **19b** [37][38] as described for **20a**·3 H^+ gave **20b**·3 H^+ (0.01 g, 38.5%). White solid. R_f 0.37 (BuOH/AcOH/H_2O 5:2:3). ^{1}H-NMR (400 MHz, D_2O, 2:1 mixture of monoacylated diastereoisomers): 1.98–2.21 (*m*, 4 H); 3.05 (*m*, 2 H); 3.91 (*m*, 2 H); 3.97 (*m*, 2 H); 4.17–4.23 (*m*, 2 H); 4.69–4.85 (*m*, 3 H); 5.27–5.58 (*m*, 2 H); 5.69–5.74 (*m*, 1 H); 5.84–6.11 (*m*, 2 H); 7.00–7.26 (*m*, 5 H); 7.90–7.92 (*m*, 1 H); 8.14 (*s*, 1 H); 8.33 (*s*, 1 H). ^{31}P-NMR (162 MHz, D_2O): 0.05; −0.76. ESI-MS: 904.1 ($[M+Na]^+$).

*2′-*O*-(Tetrahydrofuran-2-yl)cytidylyl-(3′ → 5′)-3′(2′)-*O*-[*N*-(1-oxopent-4-enyl)-*L*-leucinyl]adenosine 5′-(Dihydrogen Phosphate)* (**20c**·3 H^+). Treatment of **15** (0.050 g, 0.0296 mmol) and **19c** as described for **20a**·3 H^+ gave **20c**·3 H^+ (0.012 g, 48%). White solid. R_f 0.50 (BuOH/AcOH/H_2O 5:2:3). ^{1}H-NMR (400 MHz, D_2O, 2:1 mixture of monoacylated diastereoisomers): 0.75 (*m*, 6 H); 1.57 (*m*, 3 H); 2.11–2.27 (*m*, 4 H); 3.90 (*m*, 2 H); 4.04 (*m*, 2 H); 4.18 (*t*, *J* = 5.14, 1 H); 4.23 (*m*, 1 H); 4.36–4.49 (*m*, 3 H); 5.36–5.74 (*m*, 3 H); 6.00–6.17 (*m*, 2 H); 7.91–7.94 (*m*, 1 H); 8.19 (*s*, 1 H); 8.36 (*s*, 1 H). ^{31}P-NMR (162 MHz, D_2O): 0.03; −0.73; ESI-MS: 870.1 ($[M+Na]^+$).

N-*[(Biotinylamino)caproyl]*-L-*methionine Cyanomethyl Ester (*=N-*{6-{[5-[(3a*S*,4*S*,6a*R*)-Hexahydro-2-oxo-1*H-*thieno[3,4-*d*]imidazol-4-yl]-1-oxopentyl}amino}-1-oxoheyl}*-L-*methionine Cyanomethyl Ester)*. To a soln. of *N*-[(biotinylamino)caproyl]-L-methione (0.958 g, 1.96 mmol) in DMF (20 ml), $^{i}Pr_2$-EtN (2.4 ml, 13.72 mmol) and chloroacetonitrile (0.24 ml, 3.92 mmol) were added at 0° under N_2. The mixture was stirred at r. t. overnight. The solvent was evaporated and the residue washed with acetone (2×6 ml), dried under vacuum overnight, and then suspended in CH_2Cl_2 (15 ml) for 1 h. The solid was filtered off washed with CH_2Cl_2 (2×10 ml) and Et_2O (2×10 ml), and then dried *in vacuo* overnight: *N*-[(biotinylamino)caproyl]-L-methionine cyanomethyl ester (0.75 g, 72.8%). White solid. ^{1}H-NMR (400 MHz, (D_6)DMSO): 1.52–1.64 (*m*, 12 H); 1.87–2.10 (*m*, 9 H); 2.41–2.6 (*m*, 3 H); 2.81 (*dd*, *J*=12.48, 5.14, 1 H); 2.99 (*m*, 2 H); 3.08 (*m*, 1 H); 4.11 (*m*, 1 H); 4.28 (*m*, 1 H); 4.40 (*m*, 1 H); 4.99 (*s*, 2 H); 6.37 (*s*, 1 H); 6.43 (*s*, 1 H); 7.74 (*t*, *J*=5.13, 1 H); 8.36 (*d*, *J*=7.33, 1 H). ^{31}C-NMR (400 MHz, (D_6)DMSO): 15.18; 25.57; 26.00; 26.66; 28.71; 28.91; 29.63; 30.03; 30.72; 35.47; 35.88; 38.96; 40.53; 50.11; 51.30; 56.12; 59.85; 61.71; 116.50; 163.38; 171.84; 172.46; 173.37.

Cytidylyl-(3′ → 5′)-3′(2′)-O-*{*N-*[(biotinylamino)caproyl]*-L-*methionyl}adenosine 5′-(Dihydrogen Phosphate)*=*Cytidylyl-(3′ → 5′)-3′(2′)*-O-*{*N-*{6-{[5-[(3a*S*,4*S*,6a*R*)-hexahydro-2-oxo-1*H-*thieno[3,4-*d*]-imidazol-4-yl]-1-oxopentyl}amino}-1-oxohexyl}*-L-*methionyl}adenosine 5′-(Dihydrogen Phosphate)*. To a soln. of **15** (0.035 g, 0.0242 mmol) in DMF (0.34 ml), *N*-[(biotinylamino)caproyl]-L-methionine cyanomethyl ester was added under N_2. The mixture was stirred for 2 h. A mixture of 50 mM NH_4Ac (2 ml, pH 4.5) and MeCN (1 ml) was added to quench the reaction, and the solvent was evaporated. The residue was subjected to reversed-phase chromatography (1–50% MeCN/50 mM NH_4Ac). After lyophilization, the dry residue was applied to reversed-phase chromatography (1–50% of MeCN/0.87M AcOH): 3′(2′)-*O*-{*N*-[(biotinylamino)caproyl]-L-methionyl}-substituted pCpA (5 mg, 21.4% for two steps). White solid after lyophilization. R_f 0.32 (BuOH/AcOH/H_2O 5:2:3). ^{1}H-NMR (400 MHz, D_2O, 2:1 mixture of monoacylated diastereoisomers): 1.12–1.48 (*m*, 12 H); 1.91–2.16 (*m*, 9 H); 2.32–2.55 (*m*, 3 H); 2.72–2.77 (*m*, 1 H); 2.93–2.98 (*m*, 2 H); 3.04–3.09 (*m*, 1 H); 3.85–4.86 (*m*, 12 H); 5.35–5.53 (*m*, 1 H); 5.67–5.72 (*m*, 1 H); 5.96–6.15 (*m*, 2 H); 7.90–7.93 (*m*, 1 H); 8.19–9.21 (*m*, 1 H); 8.35–8.42 (*m*, 1 H). ESI-MS: 1121.3 ([*M*−H]$^-$).

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