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Synthesis of pCpCpA-3′-*NH*-Phenylalanine as a Ribosomal Substrate

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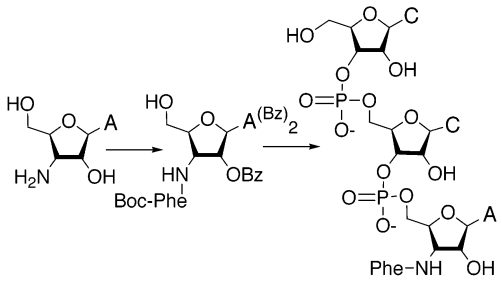
ABSTRACT



The trinucleotide cytidylyl(3′→5′phosphoryl)cytidylyl(3′→5′phosphoryl)-3′-deoxy-3′-(L-phenylalanyl) amido adenosine (CpCpA-NH-Phe) was synthesized by phosphoramidite chemistry from 3′-amino-3′-deoxyadenosine as the ribosomal substrate. The 3′-amino-3′-deoxyadenosine was first converted to 3′-(*N-tert*-butyloxycarbonyl-L-phenylalanine)amido-3′-deoxy-6-*N*,6-*N*,2′-*O*-tribenzoyl-adenosine and then coupled with cytidine phosphoramidite to produce the fully protected CpCpA-*NH*-Phe-Boc. The title product was obtained after removing all protection groups and then radiolabeled with ^{32}P to yield p*CpCpA-*NH*-Phe, which demonstrated high activity for the peptidyl transferase reaction in the ribosome.

The 2′(3′)-*O*-aminoacyl-pCpCpA derivatives are the universally conserved terminal sequences of aminoacyl-tRNA and potential substrates for ribosomal peptidyl transferase.[1] Evidence from cross-linking and chemical footprinting experiments has suggested specific tRNA−rRNA interactions, in which the universally conserved CCA of the 3′-end of tRNA and its attached aminoacyl moiety are involved in the interactions between tRNA and 23S rRNA.[2] Recently, X-ray crystal structures of the ribosome suggested that the 23S ribosomal RNA is the peptidyl transferase, but its mechanism is still unclear.[3] Biochemical studies are important in understanding the mechanism of the peptidyl transferase reactions, which might require 2′(3′)-*O*-aminoacylated oligonucleotides or tRNAs. Most assays of rRNA function involve in vitro reconstitution of 50S subunits. The peptidyl transferase activity is measured by either "fragment reaction"[4] or "poly (Phe) synthesis".[5] In these assays, the reaction products are analyzed by high-voltage paper electrophoresis or by quantitating the radioactivity in the ethyl acetate-extracted fraction through scintillation counting. In fragment reactions of the ribosome, CAACCA-(fMet), CCA-(fMet), or even CA-fMet can replace the P-site tRNA, while

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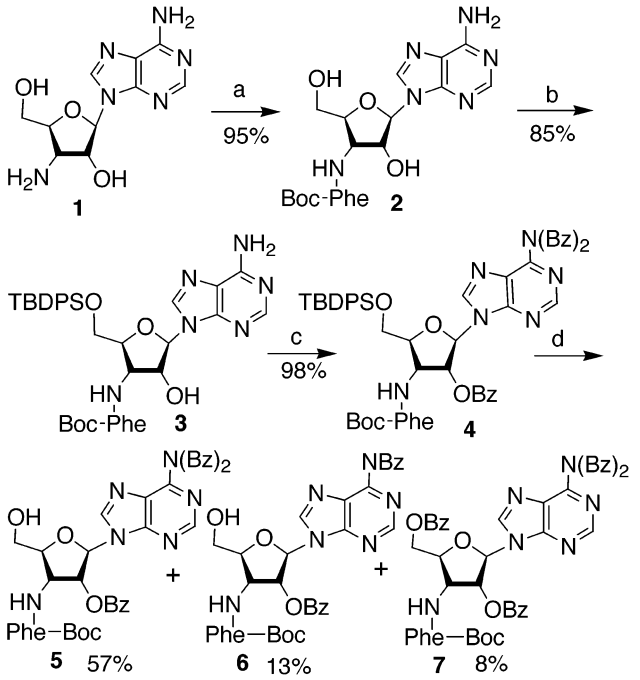
puromycin serves as an aminoacyl acceptor.[4,6] At the A site, CCA-Phe and CACCA-Phe revealed similar binding activities.[7] Therefore, only nucleotides of the CCA terminus play an important role in the binding of aminoacyl-tRNA to the A site. In most fragment reactions, puromycin was used as a peptidyl acceptor and did not exhibit full acceptor activity but required addition of alcohol. Recently, Starck and Roberts reported that puromycin-oligonucleotides revealed steric restrictions for ribosome entry and multiple modes of translation inhibition.[8a] It was reported that tRNA bearing 3′-amino-3′-deoxyadenosine in the final sequence retained full acceptor activity.[8b] Thus, the synthesized pCpCpA-*NH*-Phe might likely exhibit full activity for the peptidyl transferase reaction in the ribosome. Here, we report the first synthesis of the new compound of CpCpA-3′-deoxy-3′-*N*-phenylalanine and the enzymatic activity of ^{32}pCpCpA-*NH*-Phe.

The target compound was synthesized from 3′-amino-3′-deoxy-adenosine **1** by phosphoramidite chemistry. We have modified Robins' nine-step route for the synthesis of 3′-amino-3′-deoxyadenosine **1** into a seven-step process with 55% overall yield.[9] Briefly, adenosine was protected with *tert*-butyldiphenylsilyl at the 5′-position and then treated with α-acetoxyisobutyryl bromide to yield 2′-*O*-acetyl-3′-bromo-3′-deoxy-5′-*O*-*tert*-butyldiphenylsilyl-adenosine. This was treated with 0.5 N ammonia in methanol and then reacted with benzylisocyanate to yield 3′-(benzylamino)-5′-*O*-(*tert*-butyl)diphenylsilyl-3′-*N*,2′-*O*-carbonyl-3′-deoxyadenosine. This product was reacted with sodium hydride and then with 1.0 N NaOH and finally deprotected by hydrogenation with Pd−C (10%) to yield 3′-deoxy-3′-amino-3′-deoxyadenosine **1**. Since compound **1** contains multiple functional groups, finding a suitable protection group for each functional group is critical toward the synthesis of CpCpA-*NH*-Phe.

Boc-L-phenylalanine was first introduced into the 3′-position of 3′-amino-3′-deoxyadenosine **1**, which also acted as a protection group of the 3′-amino group. The reaction in DMF was not successful because of the poor solubility of **2** in DMF and a high racemization of L-phenylalanine. When *N*-(*tert*-butyloxycarbonyl)-L-phenylalanine *N*-hydroxy succinimide ester was stirred with 3′-amino-3′-deoxyadenosine **1** in anhydrous dimethyl sulfoxide (DMSO) at room temperature for 4 h, an optically pure 3′-(*N*-*tert*-butyloxycarbonyl-L-phenylalanine)amido-3′-deoxyadenosine **2** was obtained in 95% yield.[10] The *tert*-butyl-diphenylsilyl (TBDPS) group was first used to protect the 5′-hydroxyl group of **2** to yield 5′-*O*-(*tert*-butyl-diphenylsilyl)-3′-(*N*-*tert*-butyloxycarbonyl-L-phenylalanine) amido-3′-deoxy-adenosine **3** in 85% yield (Scheme 1). This reaction was highly regioselective

Scheme 1. Synthesis of 2′-*O*-Benzoyl-*N*,*N*-dibenzoyl-3′-deoxy-3′-*N*-(Boc-phenylalanyl) Adenosine with 5′-TBDPS Protection[a]



[a] Reaction conditions: (a) Boc-Phe-NHS ester, DMSO, rt; (b) TBDPS−Cl, pyridine, 2 days; (c) benzoyl chloride, pyridine, 0−25 °C; (d) TBAF, THF, 0−25 °C.

to form the 5′-protected compound. After benzoylation, the fully protected 3′-amino-3′-deoxyadenosine (98%) was treated with *tert*-butylammonium fluoride (TBAF) in anhydrous THF to remove the 5′-*O*-TBDPS group. Unfortunately, the deprotection of TBDPS produced the desired compound **5** (57%) as well as 6-*N*-monobenzoyl compound **6** (13%) and 6-*N*,2′-*O*,5′-*O*-tetrabenzoyl compound **7** (8%), which was formed via benzoyl migration.

We have also investigated the DMTr protection of the 5′-hydroxy of **2**. Reaction of **2** with 4,4′-dimethoxyltrityl chloride in dry pyridine yielded a mixture of components (Scheme 2). The mixture was separated by a flash column of silica gel to give the desired product 3′-(*N*-*tert*-butyloxycarbonyl-L-phenylalanine)amido-3′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-adenosine **10** in 47% yield,[11] unreacted **2** (14%), 3′-(*N*-*tert*-butyloxycarbonyl-L-phenylalanine)amido-3′-deoxy-2′-*O*-(4,4′-dimethoxytrityl)-adenosine **8**, and 2′-*O*,5′-*O*-bis-(4,4′-dimethoxytrityl)-3′-(*N*-*tert*-butyloxycarbonyl-L-phenylalanine)amido-3′-deoxyadenosine **9**. Compounds **8** and **9** were treated with 80% acetic acid at room temperature to regenerate the starting material **2** without the cleavage of

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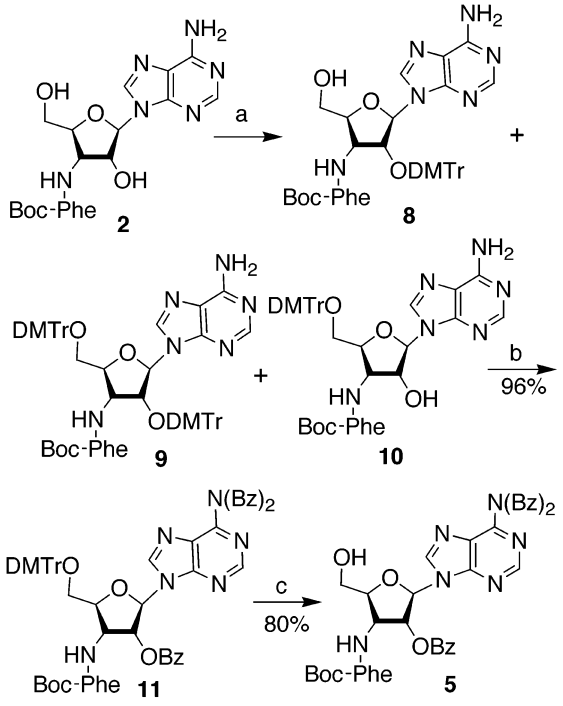
(10) Compound **2**: TLC (85:15 chloroform/methanol), R_f = 0.52; ^{1}H NMR (DMSO-d_6) δ 8.39 (s, 1H), 8.14 (s, 1H), 8.02 (d, J = 7.7 Hz, 1H), 7.32 (s, 2H), 7.28−7.15 (m, 5H), 6.93 (d, J = 8.4 Hz, 1H), 6.04 (d, J = 3.3 Hz, 1H), 5.94 (d, J = 2.9 Hz, 1H), 5.17 (t, J = 5.5 Hz, 1H), 4.51 (br, 1H), 4.46 (m, 1H), 4.25 (m, 1H), 3.90 (m, 1H), 3.66−3.43 (dm, 2H), 2.98−2.71 (dm, 2H), 1.28 (s, 9H); ^{13}C NMR (DMSO-d_6) δ 168.1, 152.2, 151.3, 148.7, 145.0, 135.3, 134.2, 125.4, 124.1, 122.3, 115.2, 85.4, 79.7, 74.2, 69.1, 57.2, 51.9, 46.5, 33.9, 24.2, 21.2. ESI-MS (m/z) calcd for $C_{24}H_{31}N_7O_6$ 513.2, found 536.3 $[M + Na]^+$.

Scheme 2. Synthesis of 2′-*O*-Benzoyl-*N*,*N*-dibenzoyl-3′-deoxy-3′-*N*-(Boc-phenylalanyl) Adenosine with 5′-DMTr Protection



[a] Reaction conditions: (a) DMTr-Cl, pyridine; (b) benzoyl chloride, pyridine; (c) 80% acetic acid.

N-Boc group of phenylalanine (30% yield). On the basis of the recovery of the starting material, the calculated yield of **10** was 84%.

Benzoylation of **10** with a large excess (6 equiv) of benzoyl chloride produced 3′-(*N*-*tert*-butyloxycarbonyl-L-phenylalanine)amido-3′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-6-*N*,6-*N*,2′-*O*-tribenzoyl-adenosine **11** in 96% yield.[12] Deprotection of the DMTr group with 80% acetic acid at room temperature afforded the desired compound **5** in 80% yield.[13]

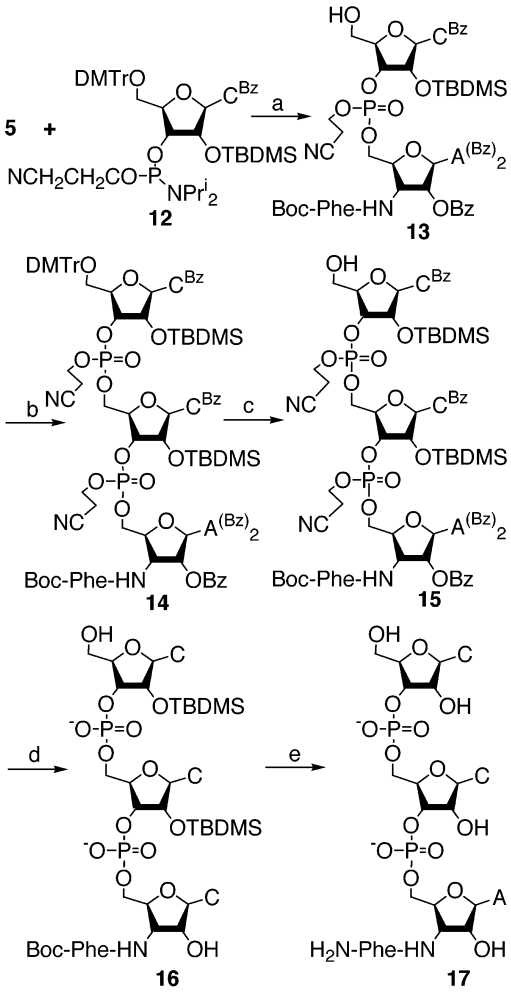
(11) Compound **10**: TLC (9:1 chloroform/methanol), $R_f = 0.26$; ^{1}H NMR ($CDCl_3$) δ 8.31 (s, 1H), 8.05 (s, 1H), 7.32−6.73 (m, 18H), 6.39 (br, 1H), 5.70 (sh, 1H), 5.65 (br, 2H), 5.15 (br, 1H), 4.84 (br, 1H), 4.47 (br, 1H), 4.32−4.22 (m, 2H), 3.77 (s, 3H), 3.76 (s, 3H), 3.45−3.35 (m, 2H), 3.09−2.92 (m, 2H), 1.42 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 172.5, 158.7, 156.0, 152.8, 148.8, 144.6, 138.8, 136.9, 135.9, 135.8, 130.4, 129.5, 128.9, 128.5, 128.1, 127.2, 127.1, 120.1, 113.4, 91.2, 86.7, 82.8, 80.4, 74.6, 63.4, 56.2, 55.4, 53.7, 51.5, 39.5, 28.5; ESI-MS (*m*/*z*) calcd for $C_{45}H_{49}N_7O_8$ 815.4, found 838.3 $[M + Na]^+$.

(12) Compound **11**: TLC (9:1 chloroform/methanol), $R_f = 0.62$; ^{1}H NMR ($CDCl_3$) δ 8.61 (s, 1H), 8.26 (s, 1H), 7.92−6.78 (m, 28H), 6.20 (d, $J = 2.0$ Hz, 1H), 5.92 (d, $J = 8.6$ Hz, 1H), 5.84 (dd, $J = 6.2$, 2.0 Hz, 1H), 5.31 (dt, $J = 8.6$, 6.0 Hz, 1H), 5.04 (d, $J = 7.4$ Hz, 1H), 4.21 (m, 1H), 3.95 (br, 1H), 3.75 (s, 6H), 3.48 (m, 2H), 3.04 (m, 1H), 2.73 (m, 1H), 1.32 (s, 9H); ESI-MS (*m*/*z*) calcd for $C_{66}H_{61}N_7O_{11}$ 1127.4, found 1150.4 $[M + Na]^+$; HRMS (*m*/*z*) calcd 1127.4429, found 1128.4376 $[M + H]^+$.

(13) Compound **5**: TLC (9:1 chloroform/methanol), $R_f = 0.52$; ^{1}H NMR ($CDCl_3$) δ 8.68 (s, 1H), 8.43 (s, 1H), 7.97−7.18 (m, 20H), 6.48 (d, $J = 3.3$ Hz, 1H), 6.22 (d, $J = 2.7$ Hz, 1H), 5.75 (dd, $J = 6.1$, 2.9 Hz, 1H), 5.10 (dt, $J = 7.0$, 6.6 Hz, 1H), 4.95 (br, 1H), 4.33 (dt, $J = 7.4$, 7.0 Hz, 1H), 4.23 (br, 1H), 4.11 (m, 1H), 4.02 (m, 1H), 3.76 (m, 1H), 3.05 (m, 2H), 1.32 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 172.6, 172.5, 165.3, 152.6, 152.5, 152.3, 144.2, 136.7, 134.5, 134.2, 133.4, 130.2, 129.8, 129.4, 129.2, 129.1, 129.0, 128.5, 128.4, 127.5, 89.4, 84.8, 81.0, 76.7, 61.6, 56.3, 49.7, 38.2, 28.4; ESI-MS (*m*/*z*) calcd for $C_{45}H_{43}N_7O_9$ 825.3, found 848.3 $[M + Na]^+$; HRMS (*m*/*z*) calcd 825.3211, found 826.3189 $[M + H]^+$.

The synthesis of 5′-HO-CpCpA-3′-*N*-Phe **17** depicted in Scheme 3 was based upon a similar strategy of phosphoramidite methodology. The 5′-hydroxy group of **5** was coupled with a commercially available cytidine phosphoramidite **12** in the presence of 1*H*-tetrazole in anhydrous acetonitrile, oxidized by *tert*-butyl hydroperoxide, and then treated with 80% acetic acid to yield **13**[14] in 75% yield. Dinucleotide **13** was coupled with **12** again to yield the fully protected trinucleotide **14**.[15] After flash column purification, **14** was treated by 80% acetic acid to produce **15**[16] in 69% yield. Deprotection of benzoyl and cyanoethyl groups with ammonium 3:1 hydroxide/EtOH at 55 °C for 24 h afforded

Scheme 3. Synthesis of Cytidylyl(3′→5′Phosphoryl) Cytidylyl(3′→5′Phosphoryl)-3′-deoxy-3′-(L-phenylalanyl) Amido Adenosine



[a] Reaction conditions: (a) (i) 1*H*-tetrazole, MeCN; (ii) *t*-BuOOH; (iii) 80% AcOH, rt. (b) (i) **12**, 1*H*-tetrazole, MeCN; (ii) *t*-BuOOH. (c) 80% AcOH, rt. (d) 3:1 NH_3 (aq)/ethanol. (e) (i) TFA; (ii) TEA·3HF, 1-methyl-pyrollidinone, 65 °C.

(14) Compound **13**: ^{1}H NMR ($CDCl_3$) δ 8.68 (s, 1H), 8.33 (s, 1H), 8.27−7.14 (m, 25H), 6.24 (br, 1H), 5.86 (br 1H), 5.60 (s, 1H), 5.15−3.70 (m, 13H), 3.02 (m, 2H), 2.70 (br, 2H), 1.20 (s, 9H), 0.88 (s, 9H), 0.12 (s, 3H), 0.10 (s, 3H); ^{31}P NMR ($CDCl_3$) δ −0.7, −0.9; ESI-MS (*m*/*z*) calcd for $C_{70}H_{76}N_{11}O_{17}PSi$ 1401.5, found 1424.1 $(M + Na)^+$.

trinucleotide **16** in 89% yield. The *N*-Boc group of **16** was removed by stirring with trifluoroacetic acid at 0 °C for 30 min.

After removal of trifluoroacetic acid, the solid compound was heated with a mixture of triethylamine/triethylamine trifluoric acid/1-methyl-pyrollidinone (v/v/v = 3:4:6) at 65 °C for 1.5 h. The final product was purified by a C18 reversed-phase column eluted with a gradient of water and methanol to give a white solid **17**[17] in 61% yield from **15**. The overall yield from **5** was 28%.

Labeling the 5′-end of trinucleotide **17** was accomplished through phosphorylation using T4 polynucleotide kinase (PNK) and [γ-^{32}P]ATP. The 5′-end labeled p^{32}CpCpA-*NH*-Phe was used as a peptidyl acceptor for the peptidyl transferase reactions in the ribosome. These reactions were monitored by polyacrylamide gel electrophoresis (PAGE) (Figure 1). No product was formed in the absence of tRNA-

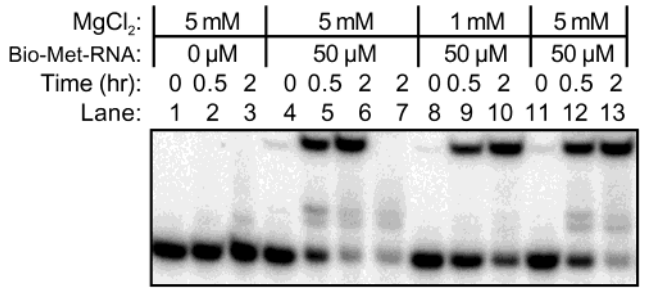


Figure 1. Autoradiogram of the peptidyl transferase reaction of 2′(3′)-biotin-methionyl-tRNA with 5′-^{32}pCpCpA-*NH*-Phe in the presence of *E. coli* 70S ribosome or S30 extract in the reaction buffer [20 mM Tris·HCl (pH 7.4), 6 μM Spermidine, 400 mM NH4Cl, 4 mM MgCl2, 330 mM KCl]. Samples were run on 7.5 M urea/20% polyacrylamide gel with 1 × TBE buffer at 30 W. The bottom bands are ^{32}pCpCpA-*NH*-Phe, and the top bands are ^{32}pCpCpA-*NH*-Phe-Met-biotin.

O-Met-biotin substrate in *E. coli* S30 extract (Promega) with p*CpCpA-*NH*-Phe (lanes 1−3, Figure 1). When p*CpCpA-*NH*-Phe was incubated with 50 μM tRNA-*O*-Met-biotin in the presence of 1.0 U of *E. coli* S30 extract (lanes 4−7, Figure 1) under the reaction buffer, a new band was formed. When p*CpCpA-*NH*-Phe was incubated with 50 μM tRNA-*O*-Met-biotin in the presence of 1.0 U of *E. coli* 70S ribosome under the same reaction buffer, a similar product band was formed at an approximate 90% yield at the reaction's final extent (lanes 8−13, Figure 1). After treatment of streptavidin, the product band migrated much slower (the band not shown in Figure 1, lane 7), indicating that a biotin moiety was transferred to *pCpCpA-*NH*-Phe. This suggested that a peptide bond was formed between pCpCpA-*NH*-Phe and tRNA-*O*-Met-biotin. These results demonstrated that pCpCpA-*NH*-Phe is fully active for the peptidyl transferase reaction in the ribosome. It has been well-known that puromycin is a universal protein synthesis inhibitor by covalent attachment to the nascent chain of peptide. Recently, a report[8a] suggested that puromycin was shown to be more complicated than thought previously and, in some cases, to not form peptide bonds. Therefore, pCpCpA-*NH*-Phe is a new compound and should be a useful peptidyl acceptor for studying the peptide bond formation in the ribosome.

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Supporting Information Available: Experimental details and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) Compound **14**: ^{1}H NMR ($CDCl_3$) δ 9.95−9.94 (br, 1H), 8.96−6.88 (m, 47H), 6.39−3.54 (m, 24H), 3.81 (s, 6H), 3.76−3.54 (m, 4H), 3.05−2.84 (m, 2H), 2.74−2.53 (m, 4H), 1.11−0.83 (m, 27H), 0.20−0.02 (m, 12H); ^{13}C NMR ($CDCl_3$) 173.2, 172.9, 172.5, 172.4, 167.1, 165.7, 163.2, 159.0, 156.1, 155.7, 155.4, 152.5, 152.1, 144.5, 144.1, 137.3, 135.1, 135.0, 134.1, 133.2 (m), 130.4 (m), 129.6, 128.9, 128.4 (m), 127.6, 126.9, 116.8, 116.7, 113.6, 113.5, 97.7, 97.3, 90.1−86.8 (m), 82.2, 81.5, 80.4−79.8 (m), 75.9−72.9 (m), 68.3, 67.5, 66.4, 62.7 (m), 59.7, 55.4, 50.9, 40.4, 29.8, 28.0, 25.8, 19.7, 18.2, −4.6 (m); ^{31}P NMR ($CDCl_3$) δ 0.7, −1.9 (m); ESI-MS (*m*/*z*) calcd for $C_{116}H_{127}N_{15}O_{25}P_2Si_2$ 2279.8, found 2303.4 [M + Na]$^+$.

(16) Compound **15**: ^{1}H NMR ($CDCl_3$) δ 9.96−9.94 (br, 1H), 8.68−7.12 (m, 40H), 6.39−3.85 (m, 23H), 3.04−2.88 (m, 2H), 2.74−2.48 (m, 4H), 1.10 (m, 9H), 0.90−0.80 (m, 18H), 0.10−0.01 (m, 12H); ^{13}C NMR (CD_3OD) δ 172.8, 172.5, 172.4, 167.2, 165.6, 163.5, 163.0, 156.3, 155.7, 152.6, 152.1, 146.1, 144.6, 137.1, 134.1, 133.9, 133.3, 130.2−127.8 (m), 127.0, 116.9, 116.8, 97.4, 91.8, 91.1, 89.1, 88.7, 83.1, 80.5, 78.9, 76.0, 75.1−73.2 (m), 68.4−65.5 (m), 65.9, 62.9, 60.6, 60.1, 59.4, 55.6, 50.4, 39.5, 28.1, 25.8, 19.7, 18.2, −4.6, −4.9 (m); ^{31}P NMR ($CDCl_3$) δ 0.2, −1.3 (m); HRMS (*m*/*z*) calcd for $C_{95}H_{110}N_{15}O_{25}P_2Si_2$ 1978.6839 [M + H], found 1978.6713.

(17) Compound **17**: ^{1}H NMR (D_2O) δ 8.43 (s, 1H), 8.23 (s, 1H), 7.93 (d, J = 8.0 Hz, 1H), 7.86 (d, J = 8.0 Hz, 1H), 7.46−7.28 (m, 5H), 5.98 (d, J = 3.2 Hz, 1H), 5.94 (d, J = 8.0 Hz, 1H), 5.85 (d, J = 8.0 Hz, 1H), 5.74 (m, 2H), 4.61−3.80 (m, 16H), 3.31−3.15 (m, 2H); ^{31}P NMR (D_2O) δ 0.2, 0.0; HRMS (*m*/*z*) calcd for $C_{37}H_{48}N_{13}O_{18}P_2$ 1024.2716 [M + H], found 1024.2969.