

# Synthesis of pCpCpA-3'-NH-Phenylalanine as a Ribosomal Substrate

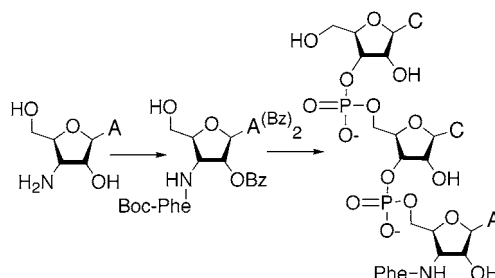
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## ABSTRACT



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*-butoxycarbonyl-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  $^{32}\text{P}$  to yield  $p^*\text{CpCpA-NH-Phe}$ , 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.<sup>1</sup> 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.<sup>2</sup> Recently, X-ray crystal structures of the ribosome suggested that the 23S ribosomal RNA is the peptidyl transferase, but its mechanism is still unclear.<sup>3</sup> 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”<sup>4</sup> or “poly (Phe) synthesis”.<sup>5</sup> 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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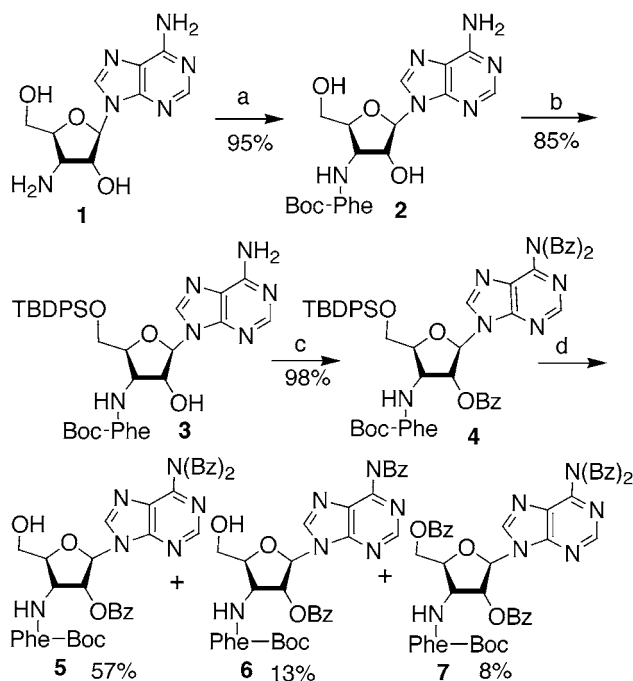
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puromycin serves as an aminoacyl acceptor.<sup>4,6</sup> At the A site, CCA-Phe and CACCA-Phe revealed similar binding activities.<sup>7</sup> Therefore, only nucleotides of the CCA terminus play an important role in the binding of aminoacyl-tRNA to 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.<sup>8a</sup> It was reported that tRNA bearing 3'-amino-3'-deoxyadenosine in the final sequence retained full acceptor activity.<sup>8b</sup> 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 <sup>32</sup>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.<sup>9</sup> Briefly, adenosine was protected with *tert*-butyldiphenylsilyl at the 5'-position and then treated with  $\alpha$ -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*-butoxycarbonyl)-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*-butoxycarbonyl-L-phenylalanine)amido-3'-deoxyadenosine **2** was obtained in 95% yield.<sup>10</sup> 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*-butoxycarbonyl-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<sup>a</sup>



<sup>a</sup> 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,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'-dimethoxytrityl 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*-butoxycarbonyl-L-phenylalanine)amido-3'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-adenosine **10** in 47% yield,<sup>11</sup> unreacted **2** (14%), 3'-(*N*-*tert*-butoxycarbonyl-L-phenylalanine)amido-3'-deoxy-2'-*O*-(4,4'-dimethoxytrityl)-adenosine **8**, and 2'-*O*,5'-*O*-bis-(4,4'-dimethoxytrityl)-3'-(*N*-*tert*-butoxycarbonyl-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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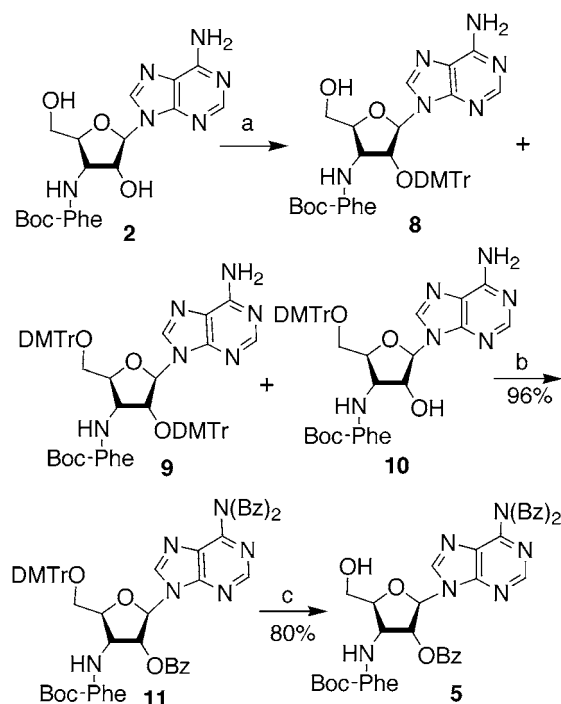
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(10) Compound **2**: TLC (85:15 chloroform/methanol),  $R_f$  = 0.52; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>)  $\delta$  8.39 (s, 1H), 8.14 (s, 1H), 8.02 (d,  $J$  = 7.7 Hz, 1H), 7.32 (s, 2H), 7.28–7.15 (m, 5H), 6.93 (d,  $J$  = 8.4 Hz, 1H), 6.04 (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); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>)  $\delta$  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<sub>24</sub>H<sub>31</sub>N<sub>7</sub>O<sub>6</sub> 513.2, found 536.3 [ $M$  + Na]<sup>+</sup>.

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



<sup>a</sup> 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.<sup>12</sup> Deprotection of the DMTr group with 80% acetic acid at room temperature afforded the desired compound **5** in 80% yield.<sup>13</sup>

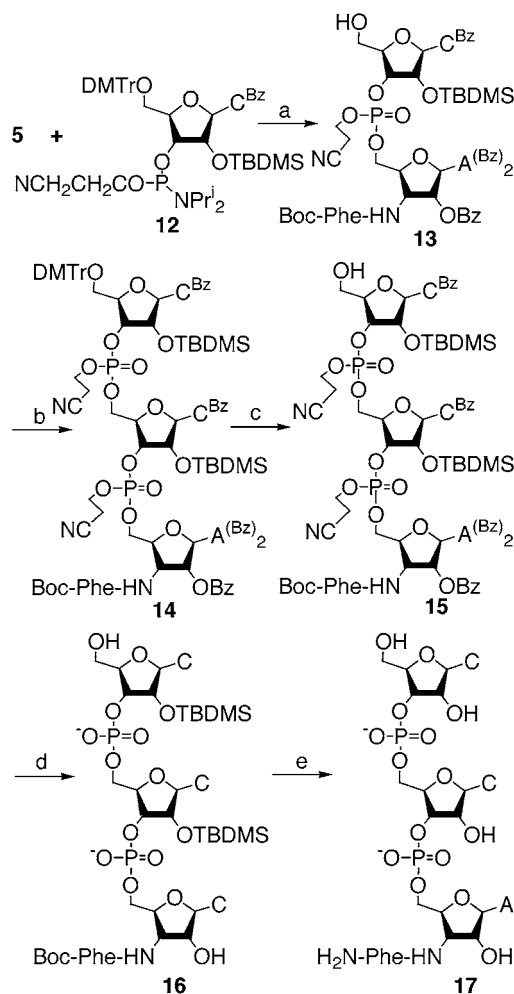
(11) Compound **10**: TLC (9:1 chloroform/methanol),  $R_f = 0.26$ ; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  8.31 (s, 1H), 8.05 (s, 1H), 7.32–6.73 (m, 18H), 6.39 (br, 1H), 5.70 (sh, 1H), 5.65 (br, 2H), 5.15 (br, 1H), 4.84 (br, 1H), 4.47 (br, 1H), 4.32–4.22 (m, 2H), 3.77 (s, 3H), 3.76 (s, 3H), 3.45–3.35 (m, 2H), 3.09–2.92 (m, 2H), 1.42 (s, 9H); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  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<sub>45</sub>H<sub>49</sub>N<sub>7</sub>O<sub>8</sub> 815.4, found 838.3 [M + Na]<sup>+</sup>.

(12) Compound **11**: TLC (9:1 chloroform/methanol),  $R_f = 0.62$ ; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  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<sub>66</sub>H<sub>61</sub>N<sub>7</sub>O<sub>11</sub> 1127.4, found 1150.4 [M + Na]<sup>+</sup>; HRMS ( $m/z$ ) calcd 1127.4429, found 1128.4376 [M + H]<sup>+</sup>.

(13) Compound **5**: TLC (9:1 chloroform/methanol),  $R_f = 0.52$ ; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  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); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  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<sub>45</sub>H<sub>43</sub>N<sub>7</sub>O<sub>9</sub> 825.3, found 848.3 [M + Na]<sup>+</sup>; HRMS ( $m/z$ ) calcd 825.3211, found 826.3189 [M + H]<sup>+</sup>.

The synthesis of 5'-HO-CpCpA-3'-*N*-Phe **17** depicted in Scheme 3 was based upon a similar strategy of phosphor-

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



<sup>a</sup> 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<sub>3</sub> (aq)/ethanol. (e) (i) TFA; (ii) TEA·3HF, 1-methyl-pyrrolidinone, 65 °C.

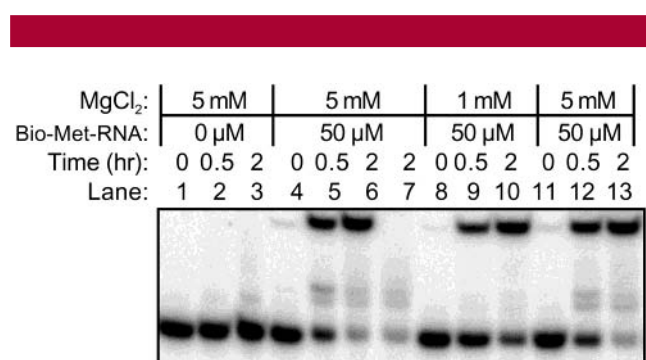
amidite methodology. The 5'-hydroxy group of **5** was coupled with a commercially available cytidine phosphoramidite **12** in the presence of 1*H*-tetrazole in anhydrous acetonitrile, oxidized by *tert*-butyl hydroperoxide, and then treated with 80% acetic acid to yield **13**<sup>14</sup> in 75% yield. Dinucleotide **13** was coupled with **12** again to yield the fully protected trinucleotide **14**.<sup>15</sup> After flash column purification, **14** was treated by 80% acetic acid to produce **15**<sup>16</sup> in 69% yield. Deprotection of benzoyl and cyanoethyl groups with ammonium 3:1 hydroxide/EtOH at 55 °C for 24 h afforded

(14) Compound **13**: <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  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); <sup>31</sup>P NMR (CDCl<sub>3</sub>)  $\delta$  -0.7, -0.9; ESI-MS ( $m/z$ ) calcd for C<sub>70</sub>H<sub>76</sub>N<sub>11</sub>O<sub>17</sub>PSi 1401.5, found 1424.1 (M + Na)<sup>+</sup>.

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-pyrrolidinone (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**<sup>17</sup> 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 [ $\gamma$ -<sup>32</sup>P]ATP. The 5'-end labeled p<sup>32</sup>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'-<sup>32</sup>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 NH<sub>4</sub>Cl, 4 mM MgCl<sub>2</sub>, 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 <sup>32</sup>pCpCpA-*NH*-Phe, and the top bands are <sup>32</sup>pCpCpA-*NH*-Phe-Met-biotin.

*O*-Met-biotin substrate in *E. coli* S30 extract (Promega) with p<sup>32</sup>CpCpA-*NH*-Phe (lanes 1–3, Figure 1). When p<sup>32</sup>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<sup>32</sup>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<sup>8a</sup> 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**: <sup>1</sup>H NMR (CDCl<sub>3</sub>) 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); <sup>13</sup>C NMR (CDCl<sub>3</sub>) 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); <sup>31</sup>P NMR (CDCl<sub>3</sub>) δ 0.7, –1.9 (m); ESI-MS (*m/z*) calcd for C<sub>116</sub>H<sub>127</sub>N<sub>15</sub>O<sub>25</sub>P<sub>2</sub>Si<sub>2</sub> 2279.8, found 2303.4 [M + Na]<sup>+</sup>.

(16) Compound **15**: <sup>1</sup>H NMR (CDCl<sub>3</sub>) 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); <sup>13</sup>C NMR (CD<sub>3</sub>OD) 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); <sup>31</sup>P NMR (CDCl<sub>3</sub>) δ 0.2, –1.3 (m); HRMS (*m/z*) calcd for C<sub>95</sub>H<sub>110</sub>N<sub>15</sub>O<sub>25</sub>P<sub>2</sub>Si<sub>2</sub> 1978.6839 [M + H], found 1978.6713.

(17) Compound **17**: <sup>1</sup>H NMR (D<sub>2</sub>O) 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); <sup>31</sup>P NMR (D<sub>2</sub>O) δ 0.2, 0.0; HRMS (*m/z*) calcd for C<sub>37</sub>H<sub>48</sub>N<sub>13</sub>O<sub>18</sub>P<sub>2</sub> 1024.2716 [M + H], found 1024.2969.