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# Synthesis of puromycin derivatives with backbone-elongated substrates and associated translation inhibitory activities

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ABSTRACT

We have synthesized a series of 5′-phosphorylated and 5′-cytidylyl-(3′–5′)-cytidylyl-(3′–5′)-puromycin derivatives that have backbone-elongated substrates. All the synthesized puromycin derivatives showed good solubility in water and were applied to translation inhibitory assay in a reconstituted *Escherichia coli* translation system.



## 1. Introduction

The ribosome is a highly sophisticated RNA machine that polymerizes amino acids to produce size, shape, and sequence-defined polymers, that is, proteins. Although the ribosomal translation system has evolved for the polymerization of natural α-amino acids, recent efforts have revealed that the translation machinery can use nonnatural substrates beyond the 20 canonical ones. Because of these findings, there has currently been a great deal of interest in using the ribosomal translation system for the synthesis of sequence-programmed peptidomimetics[1–7] or nonnatural biopolymers.[8]

The most intriguing part of this context is the substrate acceptability of the ribosome. Extensive work has revealed a remarkable tolerance of the translation machinery for side-chain modifications of its substrates (amino acids).[9] In contrast, this translation system has strict limitations on substrates with elongated backbones, as demonstrated by the fact that homologous β-amino acid is a less efficient substrate compared to its natural α-counterparts.[1b,4a] Very recently, we found an unexpected loophole in the *Escherichia coli* (*E. coli*) translation system for the incorporation of backbone-elongated substrates.[6b] Interestingly, β-hydroxypropionic acid (β-HPA in Fig. 1) could be incorporated into proteins or oligopeptides with higher efficiency than β-alanine (β-ala in Fig. 1) under our experimental conditions. It is remarkable and even surprising that less-nucleophilic β-HPA is a better substrate than β-ala for the *E. coli* translation system. However, the number of backbone-elongated substrates usable for ribosomal synthesis is still very limited.[10] The next challenge is to find factors affecting the incorporation efficiency of backbone-elongated substrates in order to extend the diversity of the ribosomally decodable products.

Puromycin (Pmn)-based assay is a method used for investigating the substrate adaptability at the A-site of the ribosome.[11–14] Pmn is a simplified mimic of the 3′-end A76 of aminoacyl-tRNA (Fig. 1), which enters the A-site of the ribosome and inhibits translation by attacking the carbonyl group of the nascent peptidyl chain on the P-site. Since the Pmn derivatives enter the A-site of the ribosome by their own ability without any assistance from EF-Tu or the tRNA body, the translation inhibitory activity can be used as an indicator of substrate adaptability at the A-site of the ribosome, supporting our understanding of the permissible modification of substrates. Therefore, Pmn derivatives with backbone-elongated substrates could be useful indicators for investigating the A-site adaptability of substrates. In this report, we show the synthesis of a series of Pmn derivatives with backbone-elongated substrates and the associated translation inhibitory activities in the *E. coli* system.

## 2. Results and discussion

### 2.1. Synthesis of phosphorylated Pmn derivatives

Various Pmn derivatives have so far been synthesized by coupling puromycin aminonucleoside (PANS **1** in Scheme 1) with a substrate. However, such simple Pmn derivatives sometimes suffer from the problem of low solubility, especially when the substrate possesses a large hydrophobic side chain or hydroxyl group as a

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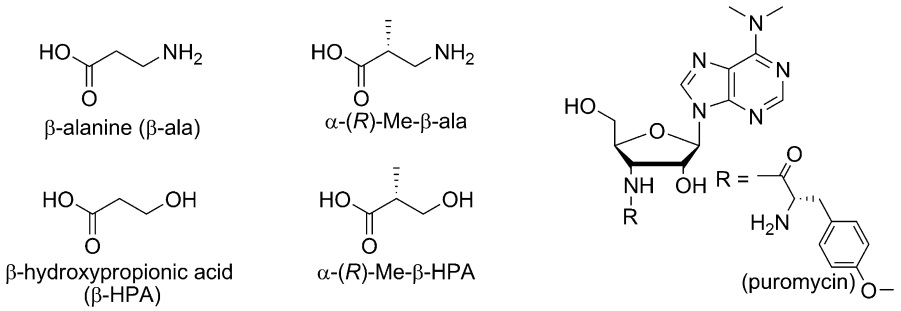
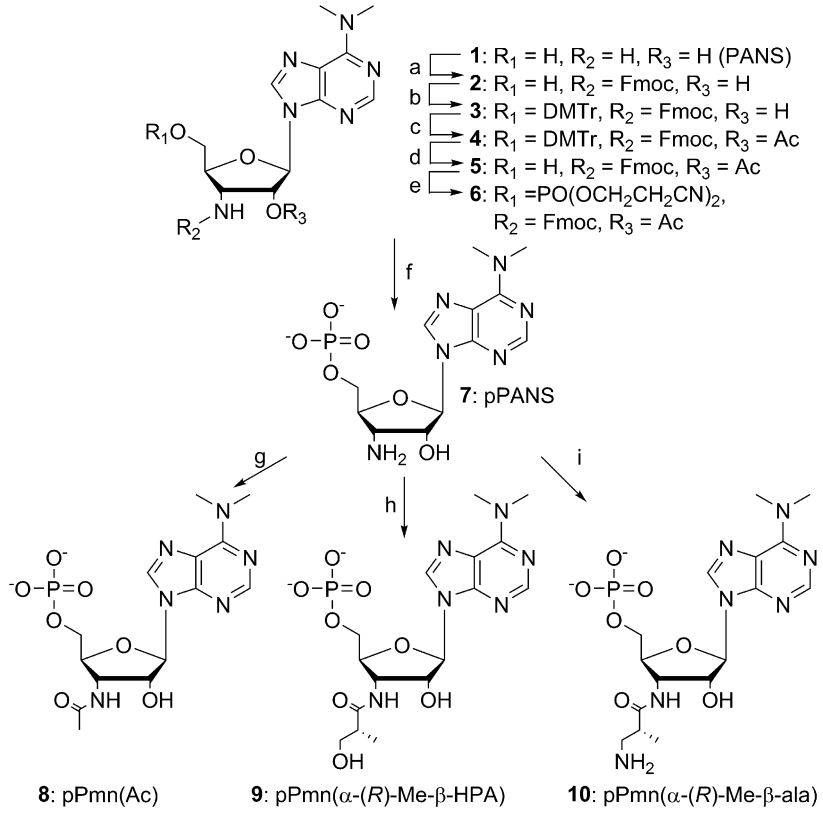



**Figure 1.** Chemical structures of backbone-elongated substrates and puromycin.




**Scheme 1.** Reagents and conditions: (a) FmocOSu, dry DMF, rt, 2 h, 94%; (b) DMTrCl, DMAP, dry pyridine, rt, overnight, quant; (c) $Ac_2O$, dry pyridine, rt, overnight, 61%; (d) 3% TCA in $CH_2Cl_2$, rt, 30 min, 27%; (e) (i) bis(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite, 1*H*-tetrazole, dry $CH_2Cl_2$, rt, 4 h; (ii) 0.02 M $I_2$ in THF/pyridine/$H_2O$, rt, 20 min; (f) 25% ammonium hydroxide/$CH_3OH$ = 15:2, 55 °C, overnight, 5% in three steps; (g) AcOSu in aqueous $NaHCO_3/CH_3CN$, rt, 1 h, 55%; (h) (i) *O*-TBDMS-α-(*R*)-Me-β-HPA-OSu in aqueous $NaHCO_3/CH_3CN$, 42 °C, 2 h; (ii) 50% aqueous TFA, on ice, 30 min, 5% in two steps; (i) (i) *N*-Boc-α-(*R*)-Me-β-ala-OSu in aqueous $NaHCO_3/CH_3CN$, rt, 1 h; (ii) 50% aqueous TFA, on ice, 30 min, 48% in two steps.

nucleophile. This was the case for our substrates. Synthesized Pmn derivative Pmn(β-HPA), wherein the α-amino acid of puromycin was replaced by β-HPA, was highly insoluble in water. In order to overcome this problem of solubility, we used 5′-phosphorylated Pmn (pPmn) as a new scaffold.

pPmn derivatives with backbone-elongated substrates were synthesized according to Scheme 1. 5′-Phosphorylated PANS **7** (pPANS) was prepared from commercially available PANS in six steps. The 3′-amino group of PANS **1** was selectively protected with a Fmoc group to yield 3′-Fmoc protected **2**. Compound **2** was then converted by the selective coupling of 5′- and 2′-hydroxyl groups with *p*,*p*′-dimethoxytrityl chloride (DMTrCl) and anhydrous acetic acid ($Ac_2O$), respectively, to give 5′-*O*-DMTr-3′-*N*-Fmoc-2′-*O*-Ac-protected PANS **4** (57% in three steps). Then, the DMTr group was removed selectively using 3% trichloroacetic acid (TCA) in dichloromethane in a 27% yield. The free 5′-hydroxyl group was again reacted with bis(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite in the presence of 1*H*-tetrazole, and the product was then oxidized using $I_2$ in THF/pyridine/$H_2O$ to give **6**. Compound **6** was fully deprotected and purified via HPLC to yield pPANS **7** (5% yield in three steps, MALDI-TOF [$(M+H)^+$]: calcd 375.30, found 374.97).

The derivatization of the 3′-amino group of pPANS **7** was achieved by coupling with a substrate activated as succinimidyl ester. Acetic acid succinimidyl ester (AcOSu) readily reacted with pPANS **7** to give pPmn derivative **8** in a 55% yield after HPLC purification (MALDI-TOF [$(M+H)^+$]: calcd 417.33, found 416.88). With a facile derivatization method in hand, we then prepared pPmn derivatives that have α-(*R*)-Me-β-HPA and α-(*R*)-Me-β-ala as acylated substrates (Fig. 1). The methyl substituent at the α-(*R*) position on the elongated backbone was revealed to be preferable for *E. coli* translation machinery.[6b] According to the reaction conditions described above, succinimidyl esters of *O*-TBDMS-α-(*R*)-Me-

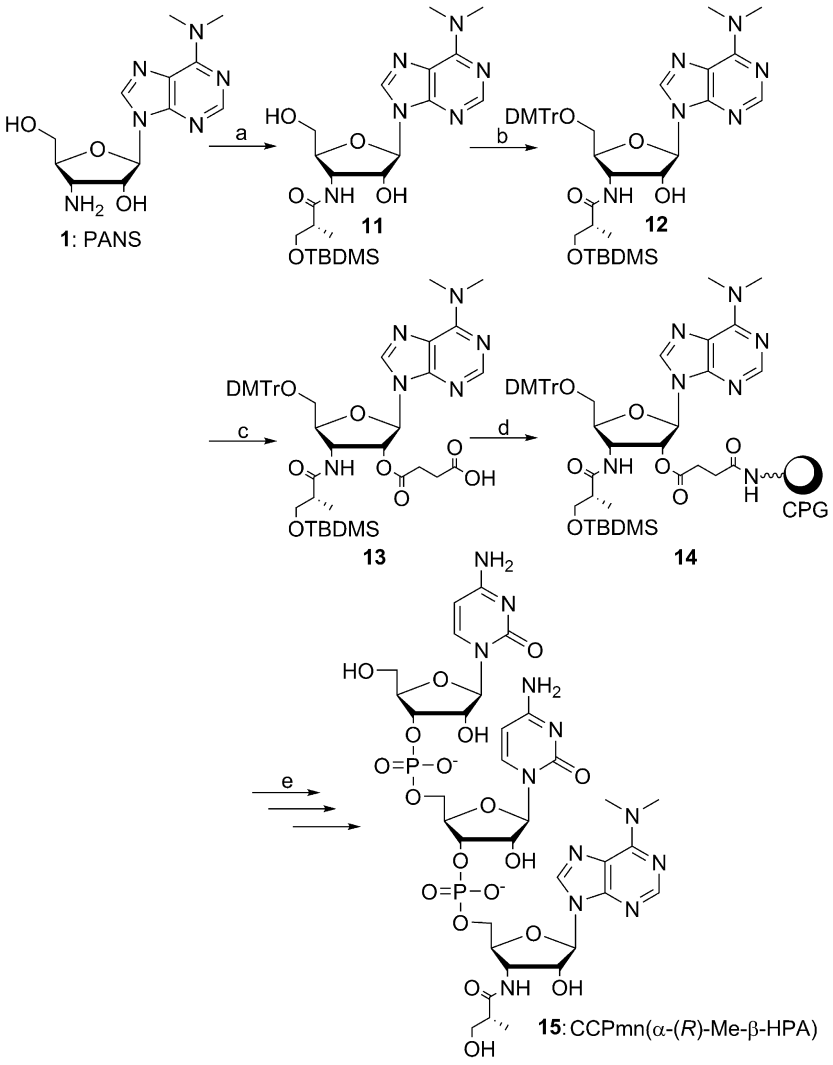
β-HPA and *N*-Boc-α-(*R*)-Me-β-ala were subjected to the coupling reaction with pPANS. The coupling products were isolated by HPLC and then deprotected by acidic treatment to give the final compounds pPmn(α-(*R*)-Me-β-HPA) **9** (MALDI-TOF [(M+H)$^+$]: calcd 461.16, found 460.94) and pPmn(α-(*R*)-Me-β-ala) **10** (MALDI-TOF [(M+H)$^+$]: calcd 460.40, found 459.92).

In contrast to the simple Pmn derivatives, all the synthesized pPmn derivatives are soluble in water at mM range. However, translation inhibition assay indicated that compounds **9** and **10** both showed no apparent inhibitory effects even at high μM range (data not shown). In addition, a recent report has suggested that the C75 and C74 bases of tRNA are indispensable for inducing the conformational change of the ribosome to peptidyl transfer (PT)-reaction active form.[15] Thus, it might be reasonable to assume that simple Pmn or pPmn derivatives are not a true mimic of A-site tRNA, owing to the lack of C75 and C74 sequences. We then focused on cytidylyl-(3′–5′)-cytidylyl-(3′–5′)-puromycin (CCPmn) derivatives as mimics of acylated tRNA at the ribosome A-site.

### 2.2. Synthesis of CCPmn derivatives

The CCPmn derivatives were prepared by solid-phase synthesis.[12c] Fully protected Pmn derivatives were attached onto a solid support and then coupled with the 5′-CC moiety (mimic of C75 and C74 sequences). CCPmn(α-(*R*)-Me-β-HPA) **15** was synthesized as shown in Scheme 2.

The 3′-amino group of PANS **1** was derivatized selectively with *O*-TBDMS-α-(*R*)-Me-β-HPA succinimidyl ester. The 5′ and 2′ hydroxyl groups were reacted with DMTrCl and succinic anhydride, respectively, to give **13** in a 44% yield in three steps. Compound **13** was then attached onto a commercially available LCAA-CPG support and the loading quantities were determined spectroscopically by the DMTr cation method (typically 30 μmol/g loading). The resulting solid support **14** was subjected to solid-phase coupling with N-acetyl-protected cytidine phosphoramidite using normal phosphoramidite coupling chemistry. The resulting trinucleotide was detached from the solid support and was fully deprotected under alkaline conditions. Subsequent purification by reverse-phase HPLC yielded CCPmn(α-(*R*)-Me-β-HPA) **15** (MALDI-TOF [(M−H)$^-$]: calcd 989.26, found 989.53) in a 26% yield from **14**. Other CCPmn derivatives, CCPmn(α-(*R*)-Me-β-ala) **16** (MALDI-TOF [(M−H)$^-$]: calcd 988.27, found 988.61) and CCPmn(Ac) **17** (MALDI-TOF [(M−H)$^-$]: calcd 945.23, found 945.70), were synthesized successfully by the same method using succinimidyl esters of *N*-trifluoroacetyl-α-(*R*)-Me-β-ala and acetic acid, respectively, as a coupling reagent with PANS in the first step (Fig. 2). CCPmn **18** (MALDI-




**Scheme 2.** Reagents and conditions: (a) *O*-TBDMS-α-(*R*)-Me-β-HPA-OSu, dry DMF, rt, overnight, 85%; (b) DMTrCl, DMAP, dry pyridine, rt, 12 h, 89%; (c) succinic anhydride, DMAP, dry pyridine, rt, overnight, 58%; (d) DCC, DMAP, DhbtOH, LCAA-CPG, dry DMF, rt, 24 h, 30 μmol/g; (e) solid-phase DNA synthesis and deprotection.

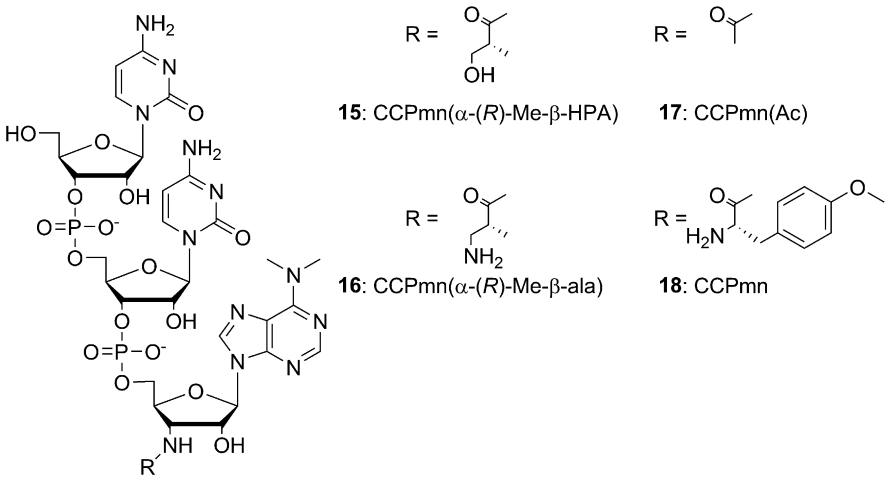



**Figure 2.** Chemical structures of the CCPmn and CCPmn derivatives synthesized and used in this study.

TOF [(M−H)⁻]: calcd 1080.30, found 1080.52) was synthesized using commercially available Puromycin-CPG (Glen Research). All of these products showed good solubility in water up to at least 0.8 mM.

### 2.3. Biological assay

As we now had CCPmn derivatives with backbone-elongated substrates, we then moved on to investigate the translation inhibitory activity of these derivatives. Inhibition experiments were carried out using a reconstituted *E. coli* translation system. An mRNA for firefly luciferase protein was translated in the absence and presence of various concentrations (2.5–30 μM) of CCPmn derivatives **15**, **16**, **17**, and CCPmn **18**. Translation efficiency was determined by comparing the translated luciferase activity with that of a calibration set of control luciferase that was translated in the absence of CCPmns. The results are summarized in Figure 3. Negative control CCPmn(Ac) **17** showed no inhibitory activity in this concentration range ($IC_{50}$ = 66 μM),[16] in marked contrast to the high inhibitory activity of positive control CCPmn **18** ($IC_{50}$ = 5 μM). Interestingly, CCPmn(α-(*R*)-Me-β-HPA) **15** actually inhibited the *E. coli* translation system to give $IC_{50}$ = 12 μM, which is slightly higher than positive control **18**, suggesting that the β-HPAs are substrates that are potentially adaptable to the ribosome A-site. This result is consistent with our previous finding that β-HPA could be incorporated into proteins/peptides through the ribosomal translation system.[6b] Interestingly, CCPmn(α-(*R*)-Me-β-ala) **16** showed an $IC_{50}$ value of 33 μM,[16] lower than that of negative control CCPmn(Ac) **17**. Though careful investigation is indispensable, simply, this suggests that β-amino acids are also adaptable to the ribosomal A-site, implying that β-amino acids are promising substrates for the ribosomal translation system.

## 3. Conclusion

In conclusion, we described the synthesis of a series of pPmn and CCPmn derivatives that have backbone-elongated substrates. The procedure can be easily applied to other Pmn scaffold such as CPmns with backbone-elongated substrates. All of the synthesized derivatives were found to be soluble in an aqueous solution, allowing us to use these probes for translation inhibition assay in an *E. coli* translation system. Our simple inhibition assay suggested that the backbone-elongated substrates can adapt to the ribosomal A-site. However, it should be noted that the present inhibition efficiency is not completely proportional to the chemical compatibility of substrates with ribosomal PT reaction at the A-site. The inhibitory reaction is composed of multiple-step processes including a binding to the A-site and the following PT reaction. Under the pres-

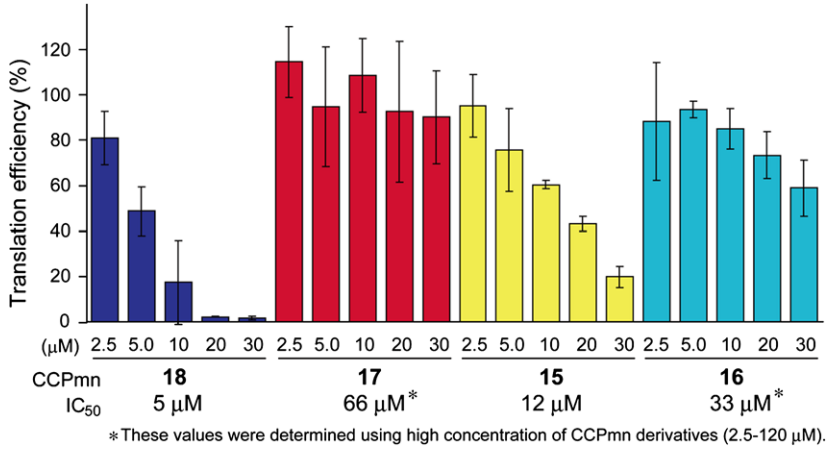



**Figure 3.** Inhibition of luciferase translation in the *E. coli* translation system by the presence of various concentrations of CCPmns (2.5–30 μM). Translation efficiency (%) was determined by comparing the luciferase activity (c.p.s.) with that from the serial dilution of control luciferase translated in the absence of CCPmns. Error bars represent standard deviations of three independent experiments.

ent experimental conditions, there is no guarantee that the PT reaction is a rate limiting step in overall events at the A-site.[17] However, a series of these soluble CCPmns, or CPmns that could be synthesized according to the similar procedure, can also be subjected to further experiments, such as stopped-flow PT reaction assay.[12b,d,13] These experiments could reveal such details, leading to important implications for the rational expansion of ribosomally adaptable substrates. Further work is now in progress along these lines.

## 4. Experiments

### 4.1. Synthesis

#### 4.1.1. General

Reagents and solvents were purchased from standard suppliers and used without further purification. Wakogel C-200 was used for silica gel chromatography. NMR spectra were measured on a JEOL JNM-A500 (500 MHz) NMR. The coupling constants (*J* values) are reported in Hertz. The FAB and MALDI-TOF mass spectra were recorded on a JEOL JMS HX110A spectrometer and an Applied Biosystems Voyager Elite, respectively. HPLC analyses and purifications were performed on a SHIMADZU SCL-10AVP system with Wakosil 5C18 columns.

#### 4.1.2. (9*H*-Fluoren-9-yl)methyl 5-(6-(dimethylamino)-9*H*-purin-9-yl)-4-hydroxy-2-(hydroxymethyl)tetrahydrofuran-3-ylcarbamate (2)

Puromycin aminonucleoside (100 mg, 0.34 mmol) was mixed with 9-fluorenylmethyl succinimidyl carbonate (FmocOSu, 138 mg, 0.41 mmol) in dry DMF (5 ml). The solution was stirred for 2 h at room temperature and the solvent was removed in vacuo. The crude products were purified by preparative layer chromatography to give **2**: Yield = 94%; $^1$H NMR (DMSO-$d_6$, 500 MHz) $\delta$: 8.43 (s, 1H), 8.22 (s, 1H), 7.89–7.33 (m, 9H), 5.97 (m, 1H), 5.94 (br, 1H), 5.23 (br, 1H), 4.46 (br, 1H), 4.28–4.21 (m, 4H), 4.06 (m, 1H), 3.74–3.53 (m, 2H), 3.44 (br, 6H); HRMS (FAB): *m*/*z* calcd for $C_{27}H_{29}O_5N_6$ [(M+H)$^+$] 517.2199, found 517.2218.

#### 4.1.3. (9*H*-Fluoren-9-yl)methyl 2-((bis(4-methoxyphenyl)-(phenyl)methoxy)methyl)-5-(6-(dimethylamino)-9*H*-purin-9-yl)-4-hydroxytetrahydrofuran-3-ylcarbamate (3)

3′-*N*-Fmoc-protected PANS **2** was dried by co-evaporation with pyridine. DMAP (catalytic amount) and DMTrCl (260 mg, 0.77 mmol) were added to **2** (165 mg, 0.32 mmol) in dry pyridine. The mixture was stirred overnight at room temperature. The solvent was removed in vacuo; the resulting residue was redissolved in $CHCl_3$, and washed with aqueous sodium bisulfite (twice). The organic layer was dried with $MgSO_4$ and evaporated to dryness. The crude products were purified by column chromatography (silica gel) to give **3**: Yield = quant; $^1$H NMR (DMSO-$d_6$, 500 MHz) $\delta$: 8.31–7.14 (m, 20H), 6.77 (m, 4H), 6.04 (m, 1H), 6.00 (m, 1H), 4.63 (m, 1H), 4.50 (m, 1H), 4.30–4.18 (m, 4H), 3.66 (s, 6H), 3.45 (br, 6H), 3.29–3.21 (m, 2H); HRMS (FAB): *m*/*z* calcd for $C_{48}H_{47}O_7N_6$ [(M+H)$^+$] 819.3506, found 819.3522.

#### 4.1.4. 4-(((9*H*-Fluoren-9-yl)methoxy)carbonylamino)-5-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-2-(6-(dimethylamino)-9*H*-purin-9-yl)tetrahydrofuran-3-yl acetate (4)

$Ac_2O$ (68.6 μl, 0.73 mmol) was added to 5′-*O*-DMTr-3′-*N*-Fmoc-protected PANS **3** (270 mg, 0.33 mmol) in dry pyridine (5 ml) and the resulting solution was stirred overnight at room temperature. The solvent was removed in vacuo; the resulting residue was redissolved in $CHCl_3$, and washed with aqueous sodium bisulfite (twice) and brine. The organic layer was dried with $MgSO_4$ and evaporated to dryness. The crude products were purified by preparative layer chromatography to give **4**: Yield = 61%; $^1$H NMR (DMSO-$d_6$, 500 MHz) $\delta$: 8.30–7.15 (m, 20H), 6.76 (m, 4H), 6.18 (m, 1H), 5.81 (m, 1H), 4.93 (m, 1H), 4.35 (m, 1H), 4.25–4.18 (m, 3H), 3.66 (s, 6H), 3.44 (br, 6H), 3.28–3.20 (m, 2H), 2.06 (s, 3H); HRMS (FAB): *m*/*z* calcd for $C_{50}H_{49}O_8N_6$ [(M+H)$^+$] 861.3612, found 861.3620.

#### 4.1.5. 4-(((9*H*-Fluoren-9-yl)methoxy)carbonylamino)-2-(6-(dimethylamino)-9*H*-purin-9-yl)-5-(hydroxymethyl)-tetrahydrofuran-3-yl acetate (5)

5′-*O*-DMTr-3′-*N*-Fmoc-2′-*O*-Ac-protected PANS **4** (170 mg, 0.2 mmol) was dissolved in 15 ml of 3% trichloroacetic acid in $CH_2Cl_2$ and the resulting solution was stirred for 30 min at room temperature. The reaction solution was diluted with $CHCl_3$ and washed with $H_2O$. The organic layer was dried with $MgSO_4$ and evaporated to dryness. The crude products were purified by column chromatography (silica gel) to give **5**: Yield = 27%; $^1$H NMR (DMSO-$d_6$, 500 MHz) $\delta$: 8.41 (s, 1H), 8.22 (s, 1H), 7.89 (d, *J* = 7.5 Hz, 2H), 7.83 (m, 1H), 7.69 (d, *J* = 7.5 Hz, 2H), 7.41 (m, 2H), 7.33 (m, 2H), 6.17 (m, 1H), 5.61 (m, 1H), 5.29 (m, 1H), 4.56 (m, 1H), 4.37 (m, 1H), 4.29–4.20 (m, 2H), 4.08 (m, 1H), 3.73–3.52 (m, 2H), 3.47 (br, 6H), 2.00 (s, 3H); HRMS (FAB): *m*/*z* calcd for $C_{29}H_{31}O_6N_6$ [(M+H)$^+$] 559.2305, found 559.2307.

#### 4.1.6. (3-Amino-5-(6-(dimethylamino)-9*H*-purin-9-yl)-4-hydroxytetrahydrofuran-2-yl)methyl phosphate (7)

1*H*-Tetrazole (18 mg, 0.256 mmol) and bis(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (70 mg, 0.258 mmol) were added to the solution of 3′-*N*-Fmoc-2′-*O*-Ac-protected PANS **4** (75 mg, 0.134 mmol) in $CH_2Cl_2$ (3 ml). The resulting mixture was stirred for 2 h at room temperature and additional bis(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (70 mg, 0.258 mmol) was added to the solution. After stirring for 2 h, 0.02 M $I_2$ in THF/pyridine/water (10 ml) was added to the solution and the resulting solution was stirred at room temperature for 20 min. The solvents were evaporated to dryness. The resulting residue was redissolved in $CHCl_3$, and washed with aqueous sodium bisulfite (twice) and brine. The organic layer was dried with $MgSO_4$ and evaporated to dryness. The crude products containing **6** were redissolved in $CH_3OH$ (2 ml). 15 ml of 25% aqueous ammonium solution was added to the solution and the mixture was incubated at 55 °C overnight. The resulting solution was lyophilized and again dissolved in a 100 mM TEAA buffer (3 ml), and purified via HPLC to give pPANS **7**: Yield = 5%; $^1$H NMR ($D_2O$, 500 MHz) $\delta$: 8.27 (s, 1H), 8.03 (s, 1H), 6.02 (m, 1H), 4.77 (m, 1H), 4.42 (m, 1H), 4.02 (m, 1H), 4.00 (m, 2H), 3.26 (br, 6H); MALDI-TOF Mass *m*/*z* calcd for $C_{12}H_{20}O_6N_6P$ [(M+H)$^+$] 375.30, found 374.97.

#### 4.1.7. (3-Acetamido-5-(6-(dimethylamino)-9*H*-purin-9-yl)-4-hydroxytetrahydrofuran-2-yl)methyl phosphate (8)

pPANS **7** (0.89 μmol) in TEAA (100 μl) was added to a solution of acetic acid succinimidyl ester (10 μmol) in acetonitrile (100 μl), and 10% aqueous $NaHCO_3$ (100 μl) was added to the solution. The reaction solution was incubated for 1 h at room temperature and purified by reverse-phase HPLC using a linear gradient of 0–75% acetonitrile in a 100 mM TEAA buffer (pH 7.0). pPmn(Ac) thus obtained was further purified by reverse-phase HPLC using a linear gradient of 0–20% acetonitrile in distillated water: Yield = 55%; MALDI-TOF Mass *m*/*z* calcd for $C_{14}H_{22}O_7N_6P$ [(M+H)$^+$] 417.33, found 416.88.

#### 4.1.8. (5-(6-(Dimethylamino)-9*H*-purin-9-yl)-4-hydroxy-3-((*R*)-3-hydroxy-2-methylpropanamido)tetrahydrofuran-2-yl)methyl phosphate (9)

pPANS **7** (44.3 nmol) in TEAA (5 μl) was added to a solution of (*R*)-3-(*tert*-butyldimethylsilyloxy)-2-methyl-3-hydroxypropionic acid succinimidyl ester (*O*-TBDMS-α-(*R*)-Me-β-HPA-OSu, 5 μmol)

in acetonitrile (5 μl), and 10% aqueous $NaHCO_3$ (5 μl) was added to the solution. The reaction solution was incubated for 2 h at 42 °C, and purified by reverse-phase HPLC using a linear gradient of 0–75% acetonitrile in a 100 mM TEAA buffer (pH 7.0). 50% trifluoroacetic acid (20 μl) was added to pPmn(*O*-TBDMS-α-(*R*)-Me-β-HPA) (3.0 nmol). After incubation for 30 min on ice, the solvent was removed by blowing $N_2$ gas. The product was redissolved in a 100 mM TEAA buffer (100 μl) and purified by HPLC using a linear gradient of 0–40% acetonitrile in a 100 mM TEAA buffer (pH 7.0). The product was further purified by reverse-phase HPLC using a linear gradient of 0–20% acetonitrile in distillated water: Yield = 5% in two steps; MALDI-TOF Mass *m/z* calcd for $C_{16}H_{26}O_8N_6P$ [$(M+H)^+$] 461.16, found 460.94.

#### 4.1.9. (3-((*R*)-3-Amino-2-methylpropanamido)-5-(6-(dimethylamino)-9*H*-purin-9-yl)-4-hydroxytetrahydrofuran-2-yl)methyl phosphate (10)

pPANS **7** (0.74 μmol) in TEAA (100 μl) was added to a solution of *N*-Boc-(*R*)-3-amino-2-methylpropionic acid succinimidyl ester (*N*-Boc-α-(*R*)-Me-β-ala-OSu, 10 μmol) in acetonitrile (100 μl), and 10% aqueous $NaHCO_3$ (100 μl) was added to this solution. The reaction solution was incubated for 1 h at room temperature and purified by reverse-phase HPLC using a linear gradient of 0–75% acetonitrile in a 100 mM TEAA buffer (pH 7.0). 50% trifluoroacetic acid (100 μl) was added to the pPmn(*N*-Boc-α-(*R*)-Me-β-ala) (0.21 μmol). After incubation for 30 min on ice, the solvent was removed by blowing $N_2$ gas. The product was redissolved in a 100 mM TEAA buffer (10 μl) and purified by HPLC using a linear gradient of 0–75% acetonitrile in a 100 mM TEAA buffer (pH 7.0). The product was further purified by reverse-phase HPLC using a linear gradient of 0–20% acetonitrile in distillated water: Yield = 48% in two steps; MALDI-TOF Mass *m/z* calcd for $C_{16}H_{27}O_7N_7P$ [$(M+H)^+$] 460.40, found 459.92.

#### 4.1.10. (2*R*)-3-(*tert*-Butyldimethylsilyloxy)-*N*-(5-(6-(dimethylamino)-9*H*-purin-9-yl)-4-hydroxy-2-(hydroxymethyl)-tetrahydrofuran-3-yl)-2-methylpropanamido (11)

PANS (194 mg, 0.66 mmol) was mixed with (*R*)-3-(*tert*-butyldimethylsilyloxy)-2-methyl-3-hydroxypropionic acid succinimidyl ester (*O*-TBDMS-α-(*R*)-Me-β-HPA-OSu, 215 mg, 0.68 mmol) in dry DMF (6 ml). The solution was stirred overnight at room temperature, and the solvent was removed in vacuo. The crude products were purified by preparative layer chromatography to give **11**. Yield = 85%; $^1$H NMR ($CDCl_3$, 500 MHz) $\delta$: 8.07 (s, 1H), 8.06 (s, 1H), 7.20 (m, 1H), 5.87 (m, 1H), 5.81 (br, 1H), 5.25 (br, 1H), 4.64 (m, 1H), 4.43 (m, 1H), 4.19 (m, 1H), 3.95–3.77 (m, 2H), 3.66 (m, 2H), 3.44 (br, 6H), 2.46 (m, 1H), 1.09 (m, 3H), 0.85 (s, 9H), 0.03 (ds, 6H); HRMS (FAB): *m/z* calcd for $C_{22}H_{38}O_5N_6Si$ [$M^+$] 494.2673, found 494.2666.

#### 4.1.11. (2*R*)-*N*-(2-((Bis(4-methoxyphenyl)(phenyl)methoxy)-methyl)-5-(6-(dimethylamino)-9*H*-purin-9-yl)-4-hydroxytetrahydrofuran-3-yl)-3-(*tert*-butyldimethylsilyloxy)-2-methylpropanamide (12)

Compound **11** (276 mg, 0.56 mmol) was dried by co-evaporation with pyridine. DMAP (26 mg, 0.21 mol) and DMTrCl (392 mg, 1.16 mmol) were added to **11** in dry pyridine (10 ml). The mixture was stirred for 12 h at room temperature. The solvent was removed in vacuo; the resulting residue was redissolved in $CHCl_3$, and washed with aqueous sodium bisulfite (twice). The organic layer was dried with $MgSO_4$ and evaporated to dryness. The crude products were purified by column chromatography (silica gel) to yield **12**. Yield = 89%; $^1$H NMR ($CDCl_3$, 500 MHz) $\delta$: 8.26 (s, 1H), 7.96 (s, 1H), 7.29–7.06 (m, 9H), 6.72 (m, 4H), 6.15 (m, 1H), 5.88 (m, 1H), 4.84 (m, 1H), 4.46 (m, 2H), 3.74 (s, 6H), 3.67–3.65 (m, 2H), 3.51 (br, 6H), 3.45–3.37 (m, 2H), 2.44 (m, 1H), 1.08 (m, 3H), 0.89 (s, 9H), 0.06 (ds, 6H); HRMS (FAB): *m/z* calcd for $C_{43}H_{56}O_7N_6Si$ [$M^+$] 796.3980, found 796.3983.

#### 4.1.12. 4-(5-((Bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-((*R*)-3-(*tert*-butyldimethylsilyloxy)-2-methylpropanamide)-2-(6-(dimethylamino)-9*H*-purin-9-yl) tetrahydrofuran-3-yloxy)-4-oxobutanoic acid (13)

Compound **12** (399 mg, 0.50 mmol) was dried by co-evaporation with pyridine. DMAP (62 mg, 0.51 mmol) and succinic anhydride (493 mg, 4.93 mmol) were added to **12** in dry pyridine (10 ml). The solution was stirred overnight at room temperature. The solvent was removed in vacuo; the resulting residue was redissolved in $CHCl_3$, and subsequently washed with aqueous sodium bisulfite (twice). The organic layer was dried with $MgSO_4$ and evaporated to dryness. The crude products were purified by column chromatography (silica gel) to give **13**: Yield = 58%; $^1$H NMR ($CDCl_3$, 500 MHz) $\delta$: 8.29 (s, 1H), 7.95 (s, 1H), 7.42–7.16 (m, 9H), 6.82–6.76 (m, 4H), 6.48 (m, 1H), 6.18 (m, 1H), 5.66 (m, 1H), 5.17–5.12 (m, 1H), 4.10–4.06 (m, 1H), 3.75 (s, 6H), 3.72–3.52 (m, 2H), 3.51 (br, 6H), 3.43–3.35 (m, 2H), 2.70–2.64 (m, 4H), 2.57 (m, 1H), 0.99 (m, 3H), 0.85 (s, 9H), 0.04 (m, 6H); HRMS (FAB): *m/z* calcd for $C_{47}H_{60}O_{10}N_6Si$ [$M^+$] 896.4140, found 896.4159.

#### 4.1.13. 3′-Amino-3′-deoxy-3′-((*R*)-3-(*tert*-butyldimethylsilyloxy)-2-methylpropionyl)-5′-*O*-(*p*,*p*′-dimethoxytrityl)-$N^6$,$N^6$-dimethyladenosine 2′-*O*-(LCAA-CPG)succinate (14)[18]

*N*,*N*′-Dicyclohexylcarbodiimide (23.7 mg, 0.12 mmol), DMAP (15.6 mg, 0.13 mmol), 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (DhbtOH, 17.6 mg, 0.11 mmol), and LCAA-CPG (201 mg) were added to **13** (62.8 mg, 0.07 mmol) in dry DMF (4 ml). The mixture was stirred for 24 h at room temperature. The CPG was filtered and washed successively with DMF, MeOH, and dry ether. THF/pyridine/$Ac_2O$ (2 ml) and 16% 1-methylimidazole in THF (2 ml) were added to the CPG, and the mixture was stirred for 1 h at room temperature. The support was filtered and washed successively with $CH_3OH$ and $CHCl_3$ to yield the product **14**: Yield = 8%; nucleoside loading = 30 μmol/g.

#### 4.1.14. Cytidylyl-(3′–5′)-cytidylyl-(3′–5′)-3′-amino-3′-deoxy-3′-((*R*)-3-hydroxy-2-methylpropionyl)-$N^6$,$N^6$-dimethyladenosine (CCPmn(α-(*R*)-Me-β-HPA) 15)

CCPmn(α-(*R*)-Me-β-HPA) **15** was prepared by manual oligonucleotide synthesis using standard phosphoramidite coupling chemistry and standard cycles.[12c,17] The solid support **14** (23.0 mg, 0.69 μmol) was loaded into a typical oligonucleotide synthesis column. In each step, the reagents or solvents were loaded via syringe. The 5′-DMTr group of **14** was deprotected with 3% trichloroacetic acid (TCA) in $CH_2Cl_2$ and nucleotide coupling was performed using 5′-dimethoxytrityl-*N*-acetyl-cytidine,2′-*O*-TBDMS-3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (60 mmol) in anhydrous acetonitrile (0.6 ml) and 0.45 M tetrazole in acetonitrile (0.8 ml). After incubation for 15 min, the unreacted 5′-hydroxyl group was capped in THF/pyridine/$Ac_2O$ (1.2 ml) and 16% 1-methylimidazole in THF (1.2 ml) for 1 min. The resulting product was oxidized by treatment with 0.1 M $I_2$ in THF/pyridine/$H_2O$ (1 ml) for 1 min (twice). Additional C was attached according to the same scheme, and the terminal 5′-DMTr group was removed by 3% TCA. The resulting CPG was mixed with 25% ammonium hydroxide (500 μl) and 40% methylamine solution (500 μl). Further it was incubated for 10 min at 65 °C to cleave CCPmns from LCAA-CPG, cooled to room temperature, and the supernatant was collected after centrifugation. The CPG was rinsed twice with $CH_3CH_2OH$/acetonitrile/$H_2O$ (v/v/v = 3/1/1, 1 ml). The combined supernatants were evaporated to dryness under reduced pressure. The resulting products were dissolved in 1.0 M tetrabutylammoniumfluoride in THF (400 μl) and stirred overnight at room temperature to depro-

tect the TBDMS group. The solvent was removed in vacuo and the products were suspended in a 100 mM $NH_4OAc$ buffer (pH 4.5, 1.4 ml). The suspension was filtered and purified by HPLC using 8% acetonitrile in a 100 mM $NH_4OAc$ buffer (pH 4.5), and again further purified using a linear gradient of 0–30% acetonitrile in distillated water to give CCPmn(α-(*R*)-Me-β-HPA) **15**: Yield = 26%; HRMS (FAB): *m*/*z* calcd for $C_{34}H_{49}O_{19}N_{12}P_2$ [(M+H)$^+$] 991.2712, found 991.2700; MALDI-TOF Mass: *m*/*z* calcd for $C_{34}H_{47}O_{19}N_{12}P_2$ [(M−H)$^-$] 989.26, found 989.53.

#### 4.1.15. CCPmn derivatives 16, 17, and 18

CCPmn derivatives **16**, **17**, and **18** were synthesized according to the same method as CCPmn **15** (as described above). For **16**: Yield = 44%; HRMS (FAB): *m*/*z* calcd for $C_{34}H_{50}O_{18}N_{13}P_2$ [(M+H)$^+$] 990.2872, found 990.2885; MALDI-TOF Mass: *m*/*z* calcd for $C_{34}H_{48}O_{18}N_{13}P_2$ [(M−H)$^-$] 988.27, found 988.61. **17**: Yield = 43%; HRMS (FAB): *m*/*z* calcd for $C_{32}H_{45}O_{18}N_{12}P_2$ [(M+H)$^+$] 947.2450, found 947.2454; MALDI-TOF Mass: *m*/*z* calcd for $C_{32}H_{43}O_{18}N_{12}P_2$ [(M−H)$^-$] 945.23, found 945.70. **18**: Yield = 25%; HRMS (FAB): *m*/*z* calcd for $C_{40}H_{54}O_{19}N_{13}P_2$ [(M+H)$^+$] 1082.3134, found 1082.3145; MALDI-TOF Mass: *m*/*z* calcd for $C_{40}H_{52}O_{19}N_{13}P_2$ [(M−H)$^-$] 1080.30, found 1080.52.

### 4.2. Biological assay

#### 4.2.1. Preparation of mRNA for firefly luciferase

The first PCR was carried out in 25 μl of a reaction mixture containing 5 pmol of forward primer 5′-(AAGGAGATATACCA-ATGGAAGACGCCAAAAACATA)-3′, 5 pmol of reverse primer 5′-(TATTCATTACACGGCGATCTTTCCG)-3′, 5 ng of pGL3 vector (Promega), 5 nmol each of dNTPs, 1.25 U of *Pfu* Ultra HF DNA polymerase (Stratagene), and 2.5 μl of 10 × *Pfu* Ultra HF Reaction Buffer. The second PCR was then carried out in 25 μl of a reaction mixture containing 5 pmol of first primer 5′-(GAAATTAATACGACTCACTA TAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTA AGAAGGAGATATACCA)-3′, 5 pmol of reverse primer 5′-(TATTCAT-TACACGGCGATCTTTCCG)-3′, 1.0 μl of the first PCR solution, 5 nmol each of dNTPs, and 2.5 μl of 10 × *Pfu* Ultra HF reaction buffer. The firefly luciferase mRNAs were transcribed from the second PCR product using a T7 MEGAshortscript kit (Ambion) and were purified with an RNeasy MinElute Clean Up kit (Qiagen).

#### 4.2.2. Translation inhibition assay

Translation was carried out in 10 μl of a reaction mixture containing 1 μg of firefly luciferase mRNA, 7 μl of reconstituted *E. coli* translation system (Post Genome Institute, Co., Ltd), and various concentrations of CCPmn derivatives. After 1 h incubation at 37 °C, 2.5 μl aliquots were diluted with 7.5 μl of water. The diluted solution (10 μl) was added to the luciferase assay solution (Promega, 100 μl), and luciferase activity was measured using a Wallac 1420 multilabel counter. Translation efficiency (%) was determined by comparing the luciferase activity (c.p.s.) with that from the serial dilution of control luciferase translated in the absence of CCPmns.

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