Supporting Information

for

Unexpected Preference of the *E. coli* Translation System for Ester-Bond During Incorporation of Backbone-Elongated Substrates

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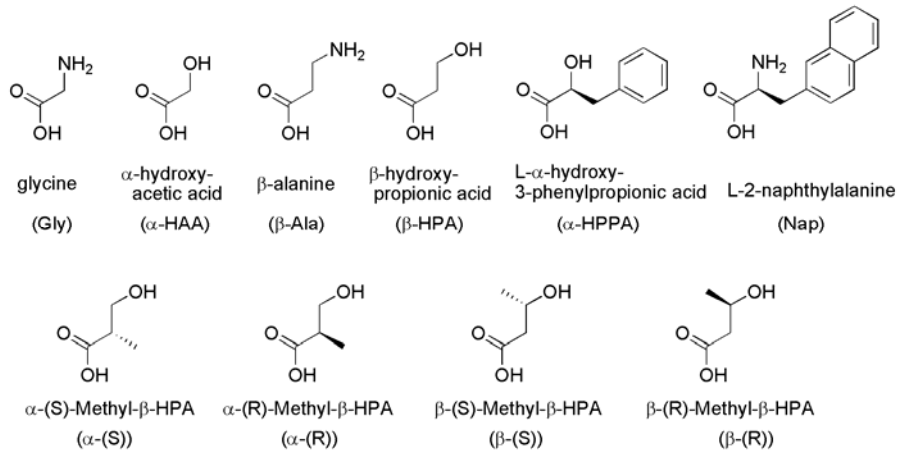
CONTENTS:

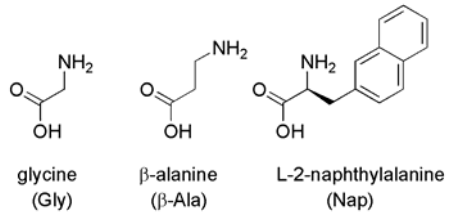
1. General. Reagents and solvents were purchased from standard suppliers and used without further purification. ^{1}H NMR spectra were taken with a Varian Mercury 400 (400 MHz) spectrometer or a JEOL JNM-A500 (500 MHz). FAB mass spectra were recorded on a JEOL JMS DX-300 spectrometer. Dinucleotide pdCpA was prepared on an ABI392 DNA/RNA synthesizer (Perkin-Elmer) using phosphoramidites (Glen Research). DNA oligomers were purchased from Gene Design Inc. (Japan).

2. Synthesis of Misacylated pdCpA.



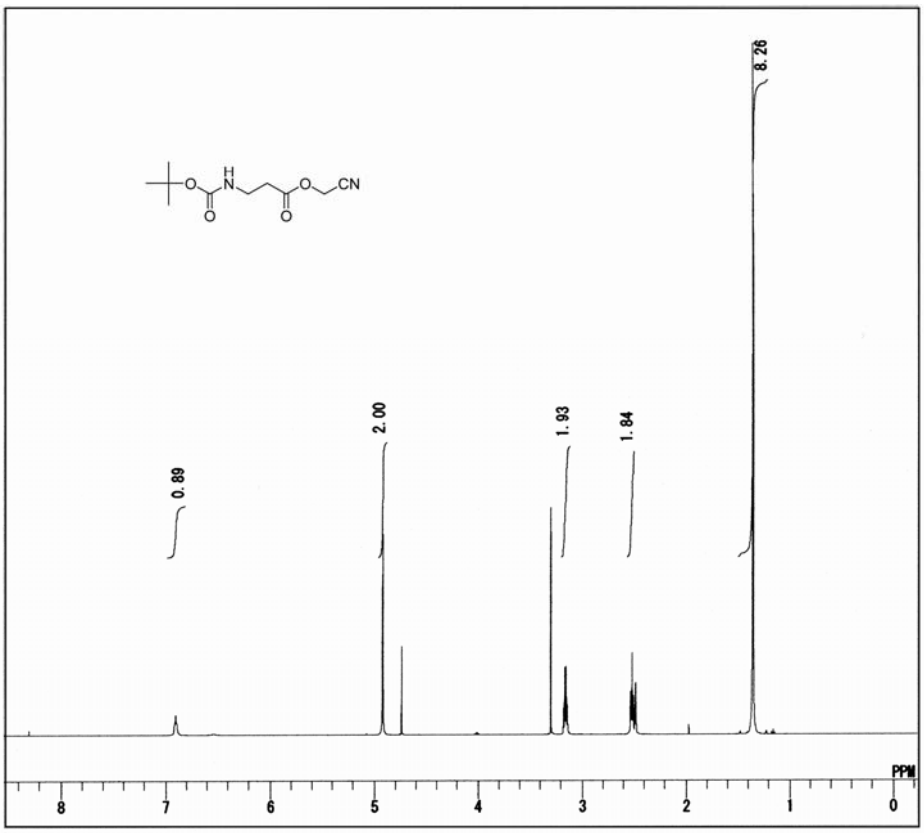
Chemical structures of natural and nonnatural substrates used in this study.

[Gly, β-Ala, and Nap]



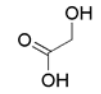
Preparation of *N*-Boc amino acid cyanomethyl ester. All *N*-Boc amino acids were purchased from WATANABE CHEMICAL (Japan). *N*-Boc amino acid was dissolved in 2 equivalents of triethylamine and 3 equivalents of chloroacetonitrile. The reaction solution was stirred at room temperature for 12 h and the resulting mixture was diluted with ethyl acetate, followed by extraction with 1 M aqueous sodium bisulfate. The organic phase was washed with brine, dried over sodium sulfate, and concentrated *in vacuo*. The crude product was used without further purification. *N*-Boc-glycine cyanomethyl ester: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.78 (2H), 3.95 (2H), 1.42 (9H); HRMS (FAB) *m/z* calcd for $C_9H_{15}N_2O_4$ [$(M+H)^+$] 215.1032, found 215.1031. *N*-Boc-β-alanine cyanomethyl ester. ^{1}H NMR (DMSO-d6, 500 MHz) δ 6.90 (1H, -NH), 4.92 (s, 2H), 3.17 (m, 2H), 2.52 (m, 2H), 1.36 (s, 9H); HRMS (FAB) calcd for $C_{10}H_{17}N_2O_4$ [$(M+H)^+$] 229.1188, found 229.1192. L-*N*-Boc naphthylalanine cyanomethyl ester: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.82 (m, 3H), 7.62 (s,

1H), 7.48 (m, 2H), 7.29 (m, 1H), 4.94–4.74 (m, 3H), 3.28 (m, 2H), 1.40 (s, 9H); HRMS (FAB) *m/z* calcd for $C_{20}H_{22}N_2O_4$ [M^+] 354.1580, found 354.1584.



Preparation of aminoacyl pdCpA (Gly, β-Ala, and Nap). The aminoacylation of pdCpA was carried out by adding an *N*-Boc amino acid cyanomethyl ester (10 μmol) to a DMF solution of pdCpA tetra-*n*-butylammonium salt (20 μL, 0.5 μmol) in a microtube. The resulting solution was incubated at room temperature and purified by reverse-phase HPLC on a Wakosil 5C18 column using liner gradient of acetonitrile in 0.1 M TEAA buffer, pH 7.0. Pure *N*-Boc aminoacyl pdCpA obtained was lyophilized to dryness and redissolved in 100 μL of trifluoroacetic acid on ice. The solution was placed on ice for 10 min, and trifluoroacetic acid was removed by blowing N_2 gas. The pellet was washed with 1 mL of ether and the resulting precipitate was collected by centrifugation (13,000 rpm, 4˚C, 30 min). Washing was repeated twice and the precipitate was dried *in vacuo*. Deprotection of Boc-protecting group was checked by HPLC. MALDI-TOF [$(M-H)^-$] data are as follows. α-Glycinyl-pdCpA: calcd 692.45, found 692.13. β-Alanyl-pdCpA: calcd 706.48, found 706.33; HRMS (FAB) *m/z* calcd for $C_{22}H_{32}N_9O_{14}P_2$ [$(M+H)^+$] 708.1544, found 708.1544. L–Naphthylalanyl-pdCpA: calcd 832.19, found 832.47. Each of them was dissolved in DMSO to give a 3 mM solution for subsequent use.

[α-HAA]



α-hydroxy-
acetic acid
(α-HAA)

Preparation of DMTr-oxyacetic acid. To a solution of hydroxyacetic acid (100 mg, 1.31 mmol) in pyridine (5 mL) was added 4,4′-dimethoxytrityl (DMTr) chloride (445 mg, 1.31 mmol). The mixture was stirred at room temperature for 12 h, diluted with ethyl acetate, washed with brine, dried over sodium sulfate, and concentrated *in vacuo*. The mixture was purified by column chromatography on silica gel to give DMTr-oxyacetic acid (438 mg, 88%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.82-7.45 (m, 13H), 3.87 (s, 2H), 3.79 (s, 6H); HRMS (FAB) *m/z* calcd for $C_{23}H_{23}O_5$ [$(M–H)^-$] 377.1389, found 377.1388.

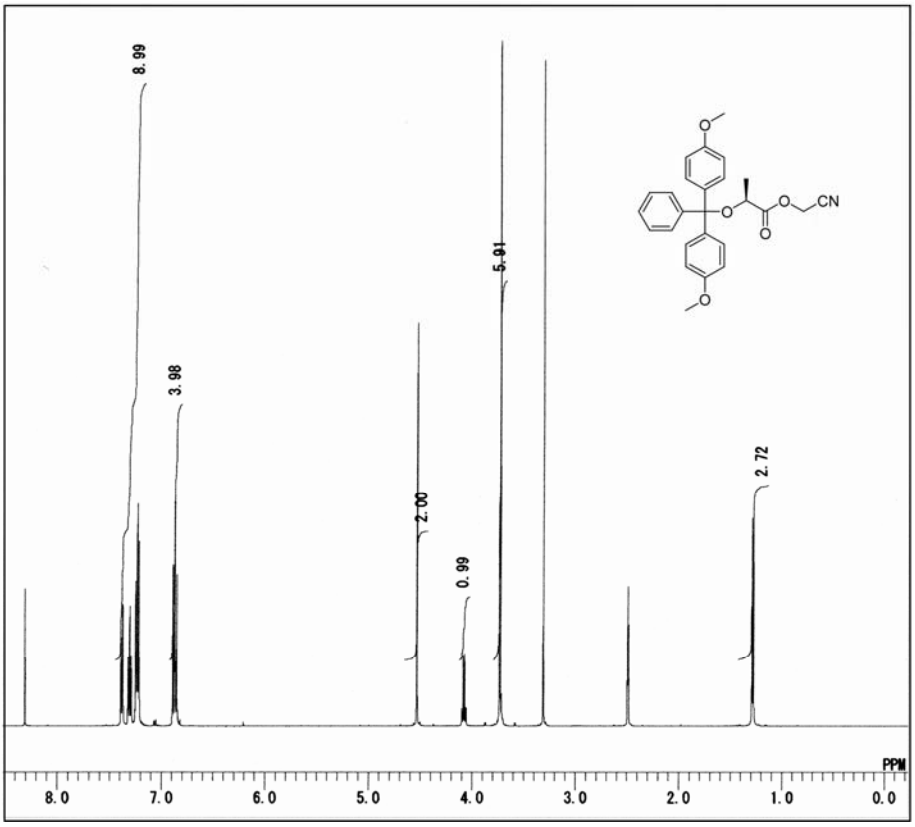
Preparation of DMTr-oxyacetic acid cyanomethyl ester. DMTr-oxyacetic acid cyanomethyl ester was obtained in a similar manner as *N*-Boc amino acid cyanomethyl ester. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.82-7.47 (m, 13H), 4.66 (s, 2H), 3.94 (s, 2H); 3.79 (s, 6H); HRMS (FAB) *m/z* calcd for $C_{25}H_{23}NO_5$ [M^+] 417.1576, found 417.1584.

Preparation of hydroxyacetyl-pdCpA. Hydroxyacetylation of pdCpA was carried out by adding the DMTr-oxyacetic acid cyanomethyl ester (10 μmol) to a DMF solution of pdCpA tetra-*n*-butylammonium salt (20 μL, 0.5 μmol) in a microtube. The resulting solution was incubated at room temperature and purified by reverse-phase HPLC on a Wakosil 5C18 column using liner gradient of acetonitrile (0–75% for 30 min) in 0.1 M TEAA buffer, pH 7.0. Pure DMTr-oxyacetyl-pdCpA was lyophilized to dryness and redissolved in 100 μL of 5% trifluoroacetic acid in CH_2Cl_2/triethylsilane as a Tr^+ scavenger (95/5). The solution was placed on ice for 8 min, and the solvent was removed by blowing N_2 gas. The pellet was washed with 1 mL of ether and the resulting precipitate was collected by centrifugation (13,000 rpm, 4 ˚C, 30 min). Washing was repeated twice and the precipitate was dried *in vacuo*. The product was dissolved in DMSO to give a 3 mM solution for subsequent use. MALDI-TOF *m/z* calcd for [$(M-H)^-$] 693.43, found 692.78.

[L-lactic acid]

L–lactic acid

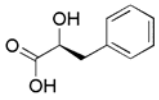
Preparation of 2-DMTr-oxypropionic acid cyanomethyl ester. 2-DMTr-oxypropionic acid cyanomethyl ester was obtained in a similar manner as DMTr-oxyacetic acid cyanomethyl ester. ^{1}H NMR (DMSO-d6, 500 MHz) δ 6.82-7.39 (m, 13H), 4.53 (s, 2H), 4.08 (q, 1H), 3.73 (s, 3H); 3.73 (s, 3H), 1.28 (d, J = 6.5 Hz, 3H); HRMS (FAB) *m/z* calcd for $C_{26}H_{25}NO_5$ [M^+] 431.1733, found 431.1738.



Preparation of 2-hydroxypropionyl-pdCpA

2-Hydroxypropionyl-dpCpA was obtained in a similar manner as hydroxyacetyl-pdCpA. MALDI-TOF *m/z* calcd for [(M-H)$^-$] 707.12, found 707.20.

[α-HPPA]



L–α-hydroxy-
3-phenylpropionic acid
(α-HPPA)

Preparation of L-2-hydroxy-3-phenylpropionic acid cyanomethyl ester.

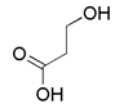
L-2-hydroxy-3-phenylpropionic acid cyanomethyl ester was obtained directly from L-2-hydroxy-3-phenylpropionic acid without DMTr-protection according to the method reported previously.[2] ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.35–7.21 (m, 5H), 4.78 (m, 2H), 4.57 (m, 1H), 3.01-3.19 (m, 2H); HRMS (EI), *m/z* calcd for $C_{11}H_{11}NO_3$ [M^+] 205.0739, found 205.0743.

Preparation of L-2-hydroxy-3-phenylpropionyl-pdCpA.

L-2-Hydroxy-3-phenylpropionyl-pdCpA was obtained in a similar manner as hydroxyacetyl-pdCpA but with the following modifications. After the coupling with pdCpA and purification by HPLC (CH3CN/TEAA), the resulting triethylammonium salt was exchanged three times with 10 mM aqueous acetic acid (100 μL) by repeated lyophilization to provide the free acid of

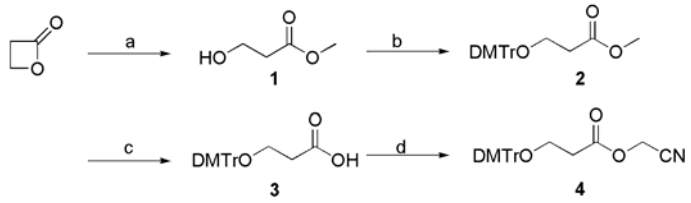
L-2-hydroxy-3-phenylpropionyl-pdCpA as a white solid. The product was dissolved in DMSO to give a 3 mM solution for subsequent use. MALDI-TOF *m/z* calcd for [(M-H)$^-$] 783.55, found 785.28.

[β-HPA]



β-hydroxy-
propionic acid
(β-HPA)

Scheme 1[a]

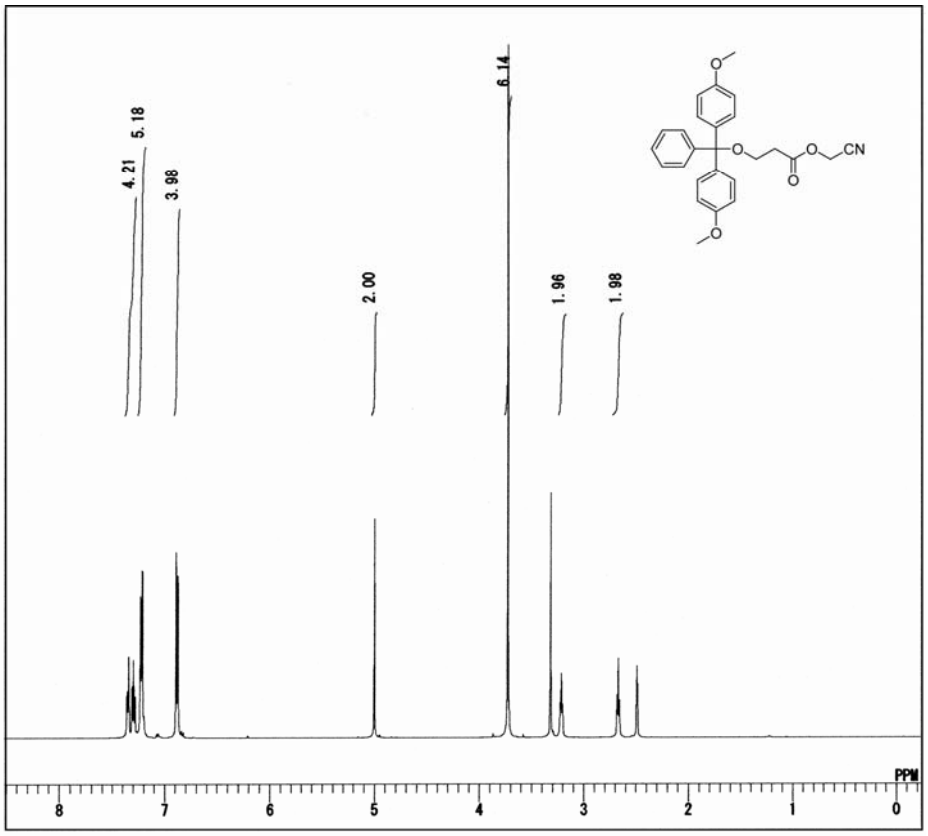


[a]*Reagents*: (a) NaOMe, MeOH, 90%; (b) DMTrCl, pyridine; (c) LiOH, H_2O, THF, 88% in 2 steps; (d) chloroacetnitrile, triethylamine, DMF, 82%

Synthesis of 3-hydroxypropionic acid methyl ester (1). β-Propiolactone (Wako) (1.15 g, 16 mmol) was added to a solution of NaOMe (0.087 mg, 1.6 mmol) in MeOH (5mL) . The mixture was stirred at 50 °C for 90 min, cooled back to room temperature, filtrated, and the residue was washed with ether. Concentration of the filtrate *in vacuo* afforded ester as an oil (1.5 g, 90%); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 3.83 (t, *J* = 5.5 Hz, 2H), 3.69 (s, 3H), 2.54 (t, *J* = 5.5 Hz, 2H).

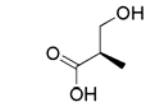
Synthesis of DMTr-oxypropionic acid (3). 3-Hydroxypropionic acid methyl ester (800 mg, 7.7 mmol) was dissolved in dry pyridine. Dimethoxytrityl chloride (4.3 g, 12.7 mmol), dissolved in 10 mL of dry pyridine, was added and the mixture was stirred at room temperature overnight. The reaction mixture was concentrated, dissolved in 10 mL of ethyl acetate, washed with 0.2 M aqueous sodium bisulfite and brine, dried, and concentrated to dryness to give DMTr-oxypropionic acid methyl ester (**2**). The residue was dissolved in 8 mL of THF. To the mixture was added 2.5 M aqueous LiOH (8 mL) and was stirred at 50 °C overnight. The reaction solution was concentrated, dissolved in ethyl acetate and extracted with 0.7 M aqueous LiOH. Aqueous layer was neutralized with saturated aqueous NH_4Cl and re-extracted with ethyl acetate. The organic layer was evaporated to give a mixture of **3**:deprotected **3** (77:23 assigned by NMR) as an yellow oil (total 2.67 g, 68% for **3**). ^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 6.80-7.43 (m, 13H), 3.88 (t, *J* = 5.5 Hz, 2H), 3.78 (s, 6H), 2.63 (t, *J* = 5.5 Hz, 2H); HRMS (FAB) *m/z* calcd for $C_{24}H_{24}O_5$ [M^+] 392.1624, found 392.1623.

Synthesis of DMTr-oxypropionic acid cyanomethyl ester (4). To a solution of DMTr-oxypropionic acid (500 mg, 1.27 mmol) in DMF was added chloroacetonitrile (3 equivalents) followed by triethylamine (7 equivalents). After stirring for 12 h, the mixture was evaporated *in vacuo*. The crude product was purified by silica-gel flash chromatography to give **4** (82%). ^{1}H-NMR (DMSO-d6, 500 MHz) δ = 6.82-7.36 (m, 13H), 5.00 (s, 2H), 3.73 (s, 6H), 3.22 (t, 2H), 2.67 (t, 2H); HRMS (FAB) m/z calcd for $C_{26}H_{25}NO_5$ [M^+] 431.1733, found 431.1732.



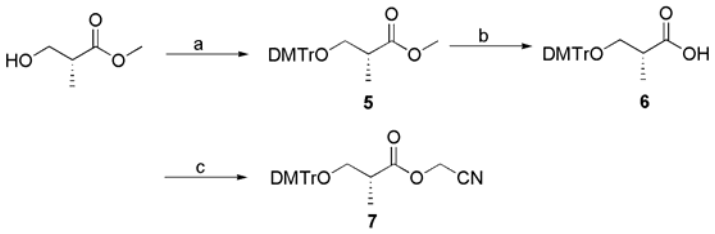
Preparation of 3-hydroxypropionyl-pdCpA. 3-Hydroxypropionyl-pdCpA was prepared by adding the DMTr-oxypropionic acid cyanomethyl ester (10 μmol) to a DMF solution of pdCpA tetra-*n*-butylammonium salt (20 μL, 0.5 μmol) in a microtube. The resulting solution was incubated at room temperature and purified by reverse-phase HPLC on a Wakosil 5C18 column using liner gradient of acetonitrile (0–75% for 40 min) in 0.1 M TEAA buffer, pH 7.0. Pure DMTr-oxypropionyl-pdCpA was lyophilized to dryness and redissolved in 100 μL of trifluoroacetic acid. The solution was placed on ice for 10 min, and the solvent was removed by blowing N_2 gas. The pellet was washed with 1 mL of ether and the resulting precipitate was collected by centrifugation (13,000 rpm, 4 ˚C, 30 min). Washing was repeated twice and the precipitate was dried *in vacuo*. The product was dissolved in DMSO to give a 3 mM solution for subsequent use. MALDI-TOF *m/z* calcd for [$(M-H)^-$] 707.12, found 707.20. HRMS (FAB) *m/z* calcd for $C_{22}H_{31}N_8O_{15}P_2$ [$(M+H)^+$] 709.1384, found 709.1396.

[α-(R)-methyl-β-HPA]



α-(R)-methyl-β-HPA
(α-(R))

Scheme 2[a]



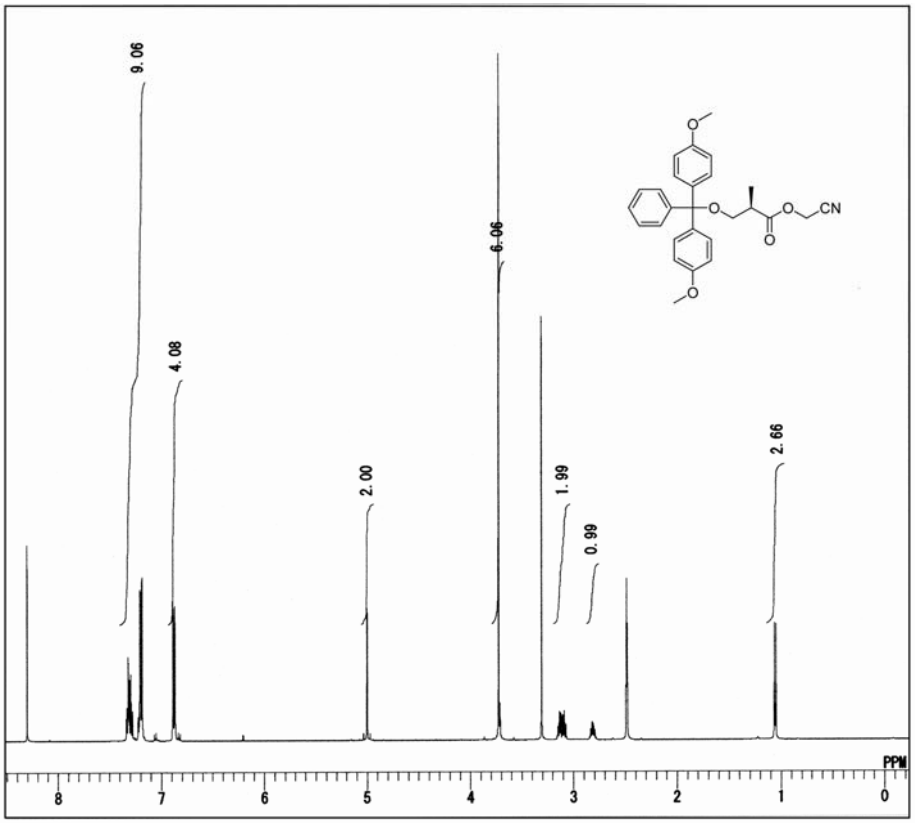
[a]*Reagents*: (a) DMTr-Cl, pyridine; (b) LiOHaq, THF, 21% in 2-steps; (b) chloroacetonitrile, triethylamine, 76%.

Synthesis of (R)-3-DMTr-oxy-2-methylpropionic acid (6).

(R)-(-)-3-hydroxy-2-methylpropionic acid methyl ester (Sigma-Aldrich, 99% ee/GLC) (141 mg, 1.20 mmol) was added to a solution of dimethoxytrityl chrolide (780 mg, 2.3 mmol) in dry pyridine (5 mL). The mixture was stirred at room temperature under nitrogen for 4 hours. The reaction mixture was concentrated, dissolved in 30 mL of ethyl acetate, washed with saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated to dryness to give (R)-3-DMTr-oxy-2-methylpropionic acid methyl ester. The residue was re-dissolved in 1 mL of THF. To the solution was added 2.5 M aqueous LiOH (5mL) and was stirred at 70 °C overnight. The reaction mixture was concentrated and dissolved in 30 mL of ethyl acetate. The solution was washed with brine, saturated aqueous NH_4Cl, dried over $MgSO_4$, filtered, and concentrated. The resulting residue was purified by preparative layer chromatography to give **6** (103 mg, 0.25 mmol, 21% in two steps). ^{1}H-NMR (DMSO-d6, 500 MHz) δ = 6.82-7.36 (m, 13H), 3.72 (6H), 2.99-3.12 (m, 2H), 2.59 (m, 1H), 1.00 (d, *J* = 7 Hz, 3H); HRMS (FAB) *m/z* calcd for $C_{25}H_{26}O_5$ [M^+] 406.1780, found 406.1782.

Syntyhesis of (R)-3-DMTr-oxy-2-methylpropionic acid cyanomethyl ester (7)

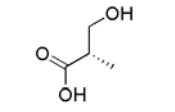
(R)-3-DMTr-oxy-2-methylpropionic acid (294 mg, 0.72 mmol) was dissolved in 0.4 mL of triethylamine and 1 mL of chloroacetonitorile and the solution was stirred at room temperature for 3 h. The reaction mixture was evaporated *in vacuo*, dissolved in ethyl acetate, washed with H_2O, dried over $MgSO_4$, and concentrated to dryness. The resulting residue was purified by silica-gel flash chromatography to give **7** as an oil (247 mg, 0.55 mmol, 76%). ^{1}H-NMR (DMSO-d6, 500 MHz) δ = 6.82-7.34 (m, 13H), 5.00 (2H), 3.73 (s, 6H), 3.08-3.16 (m, 2H), 2.81 (m, 1H), 1.06 (d, *J* = 7 Hz, 3H); HRMS (FAB) *m/z* calcd for $C_{27}H_{27}NO_5$ [M^+] 445.1889, found 445.1892.



Preparation of (R)-3-hydroxy-2-methylpropionyl-pdCpA.

(R)-3-hydroxy-2-methylpropionyl-pdCpA was obtained in a similar manner as 3-hydroxypropionyl-pdCpA. MALDI-TOF m/z calcd for $[(M–H)^-]$ calcd 721.14, found 721.35. HRMS (FAB) *m/z* calcd for $C_{23}H_{33}N_8O_{15}P_2$ $[(M+H)^+]$ 723.1541, found 723.1552.

[α-(S)-methyl-β-HPA]



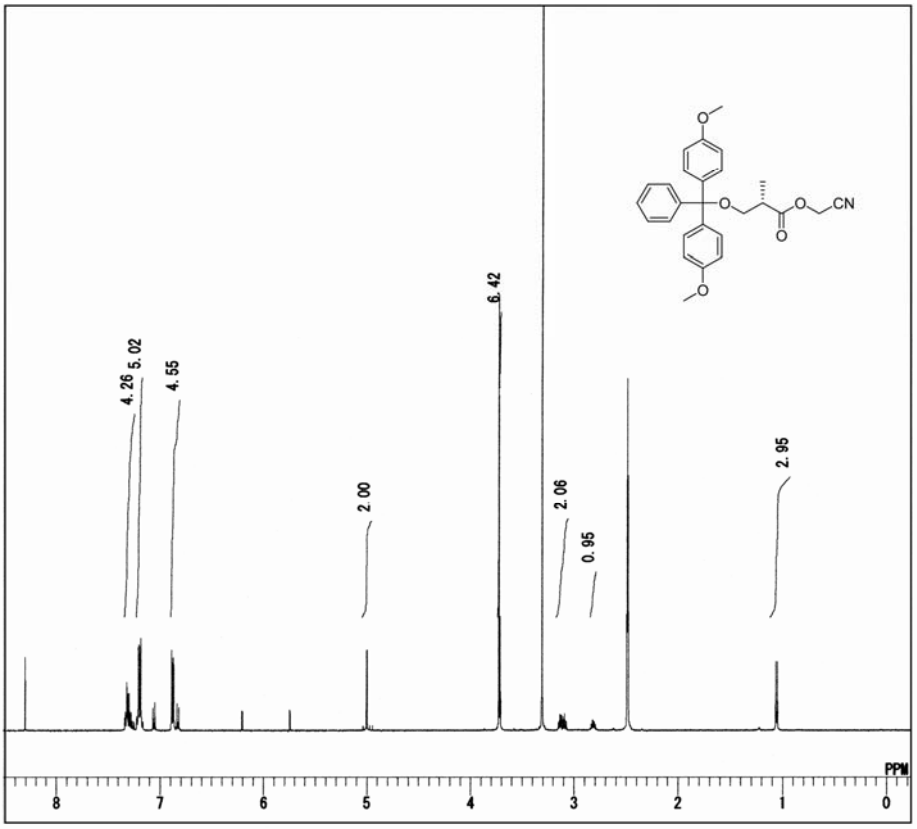
α-(S)-methyl-β-HPA
(α-(S))

Preparation of (S)-3-hydroxy-2-methylpropionyl-pdCpA.

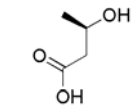
(S)-3-Hydroxy-2-methylpropionyl-pdCpA was prepared in the same manner with (R)-3-hydroxy-2-methylpropionyl-pdCpA using (S)-(+)-3-hydroxy-2-methylpropionic acid methyl ester (Sigma-Aldrich, 99% ee/GLC) as a starting material.

^{1}H-NMR (DMSO-d6, 500 MHz) for (S)-3-DMTr-oxy-2-methylpropionic acid cyanomethyl ester δ = 6.82-7.34 (m, 13H), 5.00 (2H), 3.73 (s, 6H), 3.08-3.15 (m, 2H), 2.82 (m, 1H), 1.06 (d, *J* = 7 Hz, 3H); HRMS (FAB) *m/z* calcd for $C_{27}H_{27}NO_5$ $[M^+]$ 445.1889, found 445.1900.

MALDI-TOF for (S)-3-hydroxy-2-methylpropionyl-pdCpA m/z calcd for [(M–H)$^-$] calcd 721.14, found 721.33. HRMS (FAB) *m/z* calcd for $C_{23}H_{33}N_8O_{15}P_2$ [(M+H)$^+$] 723.1541, found 723.1516.

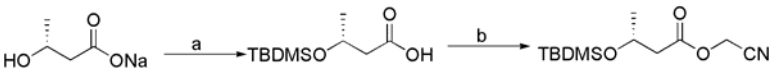


[β-(R)-methyl-β-HPA]



β-(R)-methyl-β-HPA
(β-(R))

Scheme 3[a]



8 **9**

[a]*Reagents*: (a) TBDMS-Cl, imidazole, DMF, 16%; (b) Chloroacetonitrile, triethylamine, DMF, 47%.

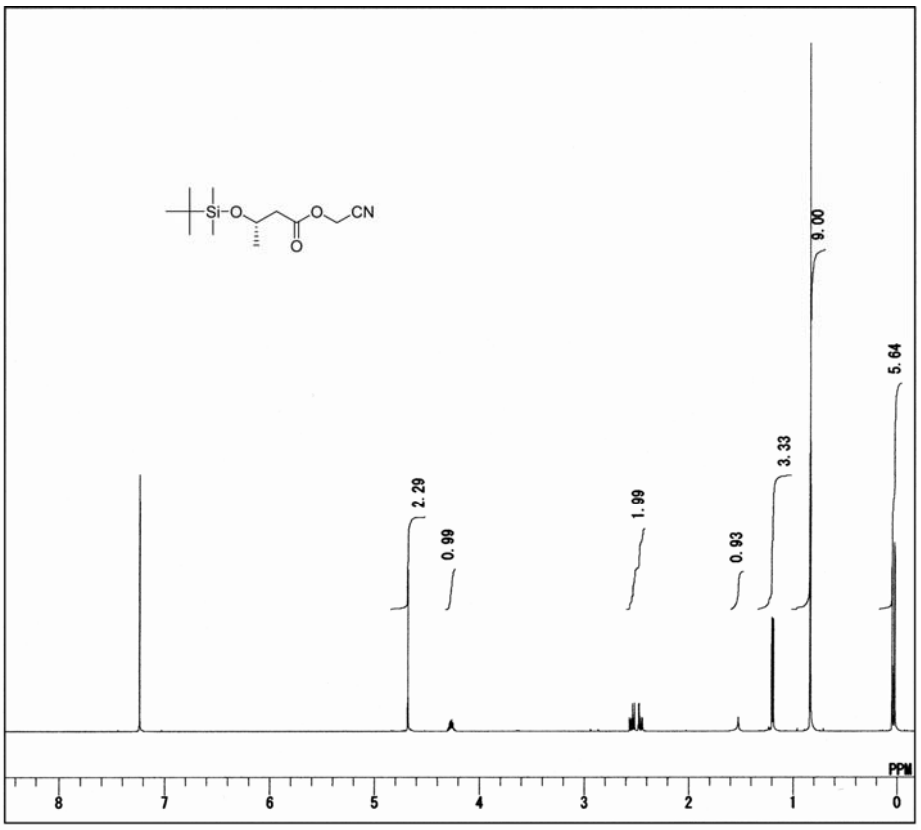
Syntyhesis of (R)-3-TBDMS-oxy-butanoic acid (8).

tert-Butyldimethylsilyl chloride (493 mg, 3.3 mmol) and imidazole (443 mg, 6.5 mmol) was dissoleved in 10 mL of dry DMF. To the solution was added (R)-(-)-3-Hydroxybutyric acid sodium salt (Sigma-Aldrich, 99% ee/GLC) (134 mg, 1.1 mmol) and the mixture was stirred at room temperature under nitrogen for 2 h at 75 °C. The reaction mixture was concentrated, dissolved in ethyl acetate, washed with saturated aqueous NH_4Cl, and extracted with saturated aqueous $NaHCO_3$. The aqueous layer was neutralized with acetic acid and re-extracted with ethyl acetate. The organic

layer was evaporated to give **8** (37 mg, 0.17 mmol, 16%). ^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 4.26 (m, 1H), 2.48 (m, 2H), 1.24 (d, J = 6.5 Hz, 3H), 0.87 (s, 9H), 0.08 (d, 6H); HRMS (FAB) *m/z* calcd for $C_{10}H_{23}O_3Si$ [$(M+H)^+$] 219.1416, found 219.1413.

Syntyhesis of (R)-3-TBDMS-oxy-butyric acid cyanomethyl ester (9).

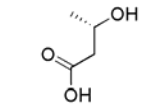
(R)-3-TBDMS-oxy-butyric acid (37 mg, 0.17 mmol) was dissolved in 2.5 mL of dry DMF. To the solution was added 0.5 mL of triethylamine and 0.5 mL of chloroacetonitorile. The resulting solution was stirred at room temperature for 20 h and then at 50 °C for 2h. The reaction mixture was evaporated *in vacuo*, dissolved in ether, washed with H_2O, dried over $MgSO_4$, filtered, and concentrated to give **9** (20 mg, 0.08 mmol, 47%). ^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 4.67 (s, 2H), 4.27 (m, 1H), 2.44-2.57 (m, 2H), 1.20 (d, *J* = 6 Hz, 3H), 0.84 (s, 9H), 0.00 (d, 6H); HRMS (FAB) m/z calcd for $C_{12}H_{24}NO_3S_i$ [$(M+H)^+$] 258.1525, found 258.1524.



Preparation of (R)-3-hydroxy-3-butyril-pdCpA.

(R)-3-hydroxy-3-butyril-pdCpA was prepared in the same manner with 3-hydroxypropionyl-pdCpA. MALDI-TOF m/z calcd for [$(M–H)^–$] calcd 721.14, found 721.32. HRMS (FAB) *m/z* calcd for $C_{23}H_{33}N_8O_{15}P_2$ [$(M+H)^+$] 723.1541, found 723.1517.

[β-(S)-methyl-β-HPA]



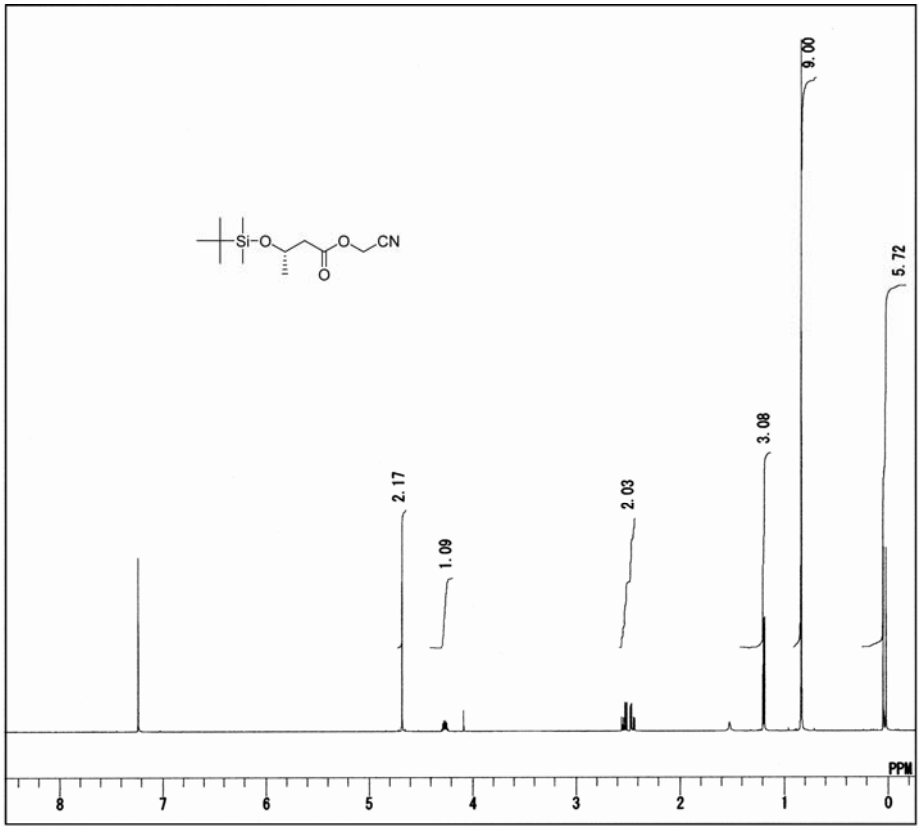
β-(S)-methyl-β-HPA
(β-(S))

Synthesis of (S)-3-TBDMS-oxy-butyric acid.

During the synthesis of (S)-3-TBDMS-oxy-butyric acid, we found that TBDMS-protection condition used in the synthesis of (R)-3-TBDMS-oxy-butanoic acid mainly afford bis-TBDMS product. Therefore, we synthesized (S)-3-TBDMS-oxy-butyric acid from (S)-(+)-3-hydroxybutyric acid sodium salt (WAKO Pure Chemical, >80% ee) in the almost same manner but with slight modifications as follows. Bis-TBDMS product of (S)-(+)-3-hydroxybutyric acid was isolated and was stirred overnight at room temperature in 0.1M K_2CO_3 solution (MeOH/THF/H_2O = 20/10/3) to give (S)-3-TBDMS-oxy-butyric acid (71%).[1] ^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 4.26 (m, 1H), 2.48 (m, 2H), 1.25 (d, *J* = 6.5 Hz, 3H), 0.88 (s, 9H), 0.09 (d, 6H); HRMS (FAB) *m/z* calcd for $C_{10}H_{23}O_3Si$ $[(M+H)^+]$ 219.1416, found 219.1416.

Syntyhesis of (S)-3-TBDMS-oxy-butyric acid cyanomethyl ester.

(S)-3-TBDMS-oxy-butyric acid cyanomethyl ester was prepared in the same manner with (R)-3-TBDMS-oxy-butyric acid cyanomethyl ester. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ = 4.69 (s, 2H), 4.27 (m, 1H), 2.44-2.57 (m, 2H), 1.20 (d, *J* = 6 Hz, 3H), 0.84 (s, 9H), 0.03 (d, 2H); HRMS (FAB) m/z calcd for $C_{12}H_{24}NO_3S_i$ $[(M+H)^+]$ 258.1525, found 258.1525.



Preparation of (S)-3-hydroxy-3-methylpropionyl-pdCpA.

(S)-3-hydroxy-3-butyril-pdCpA was obtained in a similar manner with (R)-3-hydroxy-3-butyril-pdCpA. MALDI-TOF m/z calcd for $[(M–H)^-]$ calcd 721.14, found 721.26. HRMS (FAB) *m/z* calcd for $C_{23}H_{33}N_8O_{15}P_2$ $[(M+H)^+]$ 723.1541, found 723.1509.

3. Preparation of Chemically Misacylated Full-Length tRNA.

Run-off transcription of yeast tRNA$^{Phe}_{CUA}$-CA. The plasmid, encoding the yeast tRNA$^{Phe}_{CUA}$-CA under the control of T7 promoter, was gifted from Dr. M. Endo of Osaka University and Dr. T. Hohsaka of JAIST. DNA templates for transcription were obtained by PCR reaction from the plasmid using the 0.2 μM of forward primer 5'-(GTAAAACGACGGCCAGT)-3' and 0.2 μM of reverse primer 5'-(**GT**GCGAATTCTGTGGATC)-3', in which the two 5'-nucleotides (in bold) were modified with C2'-methoxy (-OCH_3) to inhibit any non-templated nucleotide addition at the 3'-end of the RNA by T7 RNA polymerase.[2] After the PCR reaction, the product was purified by PCR purification kit (Qiagen). The transcription reaction was carried out in 500 μL of a reaction solution containing 1.4 μg of the dsDNA template, 2 μmol each of NTPs (Fermentas), 10 μmol of GMP (Sigma), 200 U of RNase inhibitor (TOYOBO), 2.5 U of inorganic pyrophosphatase (Sigma), 2,000 U of T7 RNA polymerase (NEB), 10 μg of BSA (Invitrogen), 7 μL of 1M $MgCl_2$, and 50 μL of 10× reaction buffer (400 mM Tris-HCl (pH 7.9), 60 mM $MgCl_2$, 100 mM DTT, and 20 mM spermidine). The mixture was incubated at 37 °C for 24 h. Transcripted yeast tRNA$^{Phe}_{CUA}$-CA was purified using QIAGEN RNA/DNA midi kit with modification.[3]

Chemically misacylated full-length tRNA.[4] Ligation reaction was carried out in 20 μL of reaction solution (60 mM Hepes-Na (pH 7.5), 15 mM $MgCl_2$, 3.3 mM DTT, 1 mM ATP) containing 10 μg of tRNA$^{Phe}_{CUA}$-CA, 12 nmol of acylated-pdCpA, and 50 U of T4 RNA ligase (Takara). The mixture was incubated at 4 °C for 2 h and then diluted with 40 μL of 0.45 M aqueous potassium acetate (pH 5.0). The solution was extracted with phenol/chloroform/isoamyl alcohol (25/24/1) and again with chloroform. The ethanol (350 μL)-precipitated tRNAs were collected by centrifugation, washed once with cold 80% (v/v) aqueous ethanol, dried *in vacuo*, and resuspended in 4 μL of 1 mM aqueous sodium acetate (pH 5.0) for subsequent use. Ligation efficiencies were checked by 8% PAGE and stained with 0.05% toluidine blue (pH 7.0, WAKO).

4. Construction of Expression Template.

Expression template for DHFR. The plasmid containing an *E.coli dhfr* gene was gifted from Dr. Y. Shimizu of Post Genome Institute Co., Ltd.. The plasmid encoding $dhfr^{amber}$ gene was obtained by changing codon at position 10, 111, or 140 to UAG amber codon with QuikChange® Site Directed Mutagenesis Kit (Stratagene). DNA templates were obtained by 2-step PCR reaction from these plasmids. The first PCR was carried out in 20 μL of a reaction mixture containing 4 pmol of forward primer with T7-tag and SD sequence 5′-(AAGGAGATATACCAATGGCTAGCATGACTGGTGGACAGCAAATGGGTATCAGTCTGAT TGCG)-3′, 4 pmol of reverse primer 5′-(TATTCATTACCGCCGCTCCAGAATCT)-3′, 5 ng of plasmid encoding *E.coli dhfr* gene, 2.5 U of *Pfu Uitra* HF DNA polymerase (Stratagene), 4 nmol each of dNTPs (TOYOBO), and 2 μL of 10× *Pfu Ultra* HF reaction buffer. After the PCR reaction, the product was purified by agarose gel electrophoresis. The second PCR was carried out in 20 μL of reaction mixture containing 4 pmol of universal primer with T7 promoter, 5'-(GAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTT GTTTAACTTTAAGAAGGAGATATACCA)-3', 4 pmol of reverse primer, 15 ng of the purified first PCR product, 2.5 U of *Pfu Uitra* HF DNA polymerase, 4 nmol each of dNTPs, and 2 μL of 10× *Pfu Ultra* HF reaction buffer. The resulting double-stranded template DNA contains a T7 promoter, an SD sequence, an initiation codon (ATG), a T7-tag sequence at N-terminal, and a stop codon (TAA). The mRNAs were obtained by T7 run-off transcription of these templates using T7 MEGAshortscript kit (Ambion) and were purified with RNeasy MinElute Clean Up kit (Qiagen).

Expression template for oligopeptide. Synthetic DNA template for PCR reaction 5'-(AAGGAGATATACCAATGGACTACAAGGATGACGATGACAAGCAAAAACTGXXXCTGA CGCATAAAGAAACCGCTGCTGCTAAATTCGAACGCCAGCACATGGACAGCTAA)-3' was purchased from GeneDesign (XXX is TAT and TAG for $oligopep^{Y}$ and $oligopep^{amber}$ mRNA templates, respectively). The first PCR was carried out in 20 μL of a reaction mixture containing 4 pmol of forward primer with T7 promoter and SD sequence 5′-(GAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTT GTTTAACTTTAAGAAGGAGATATACCAATGGAC)-3′, 4 pmol of reverse primer 5′-(TATTCATTAGCTGTCCATGTGCTG)-3′, 0.1 μmol of the synthetic DNA template, 2.5 U of *Pfu Uitra* HF DNA polymerase (Stratagene), 4 nmol each of dNTPs (TOYOBO), and 2 μL of 10× *Pfu Ultra* HF reaction buffer. The resulting double-stranded template DNA contains a T7 promoter, an SD sequence, an initiation codon (ATG), FLAG-tag sequence, and S-tag sequence. The mRNAs were obtained by T7 run-off transcription of this template using T7 MEGAshortscript kit (Ambion) and were purified with RNeasy MinElute Clean Up kit (Qiagen).

5. ***Figure S1***

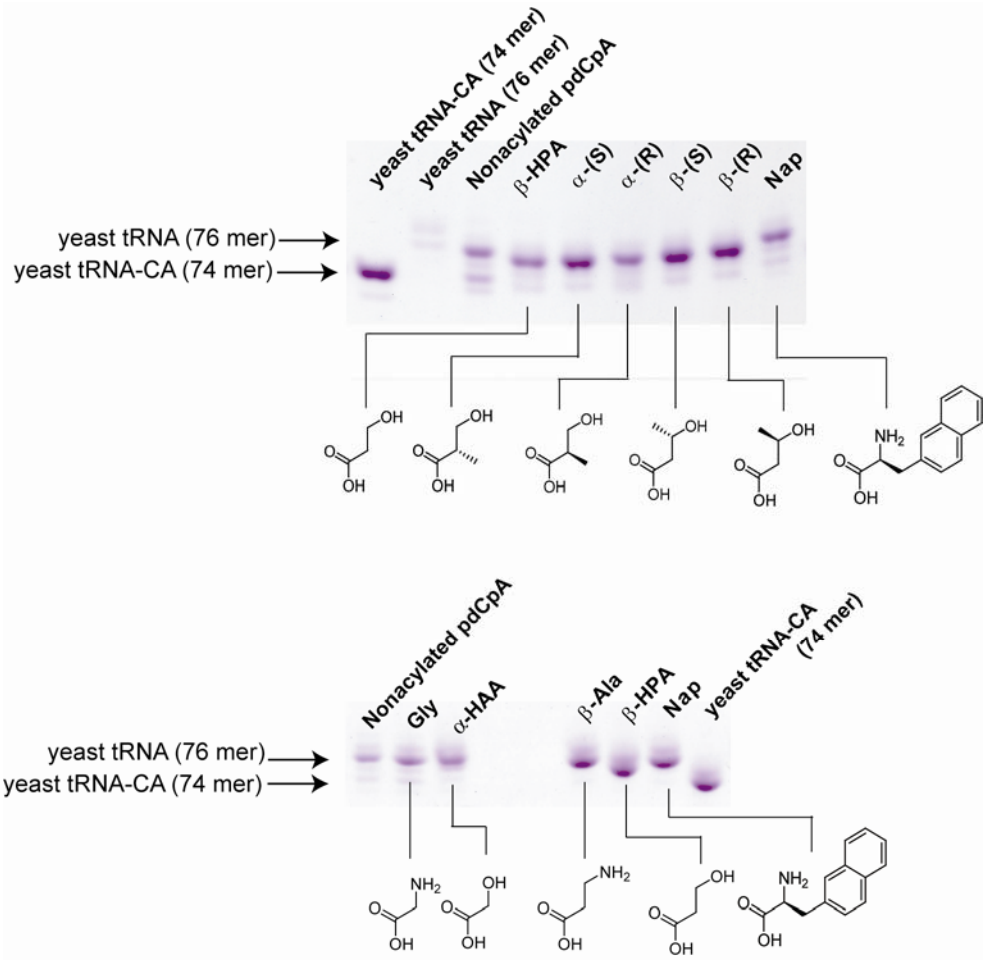


Figure S1. PAGE analysis of the ligation reaction of yeast $tRNA^{Phe}_{CUA}$-CA with pdCpA misacylated with the various substrates indicated in the figure. Run-off transcribed yeast $tRNA^{Phe}_{CUA}$-CA (74-mer) and yeast $tRNA^{Phe}_{CUA}$ (76-mer) were used as size markers on the gel. After the ligation reaction, 0.8 μg of tRNAs were applied to 8% PAGE containing 8 M urea, and the ligation efficiencies were checked by staining with 0.05% toluidine blue (pH 7.0; WAKO).

6. ***Figure S2***

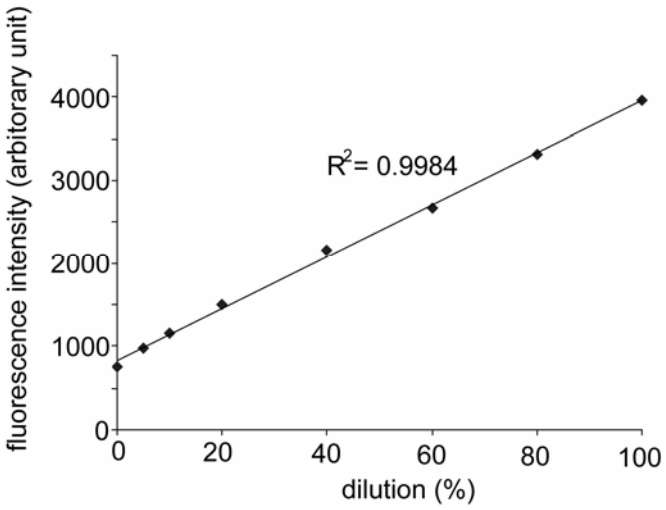
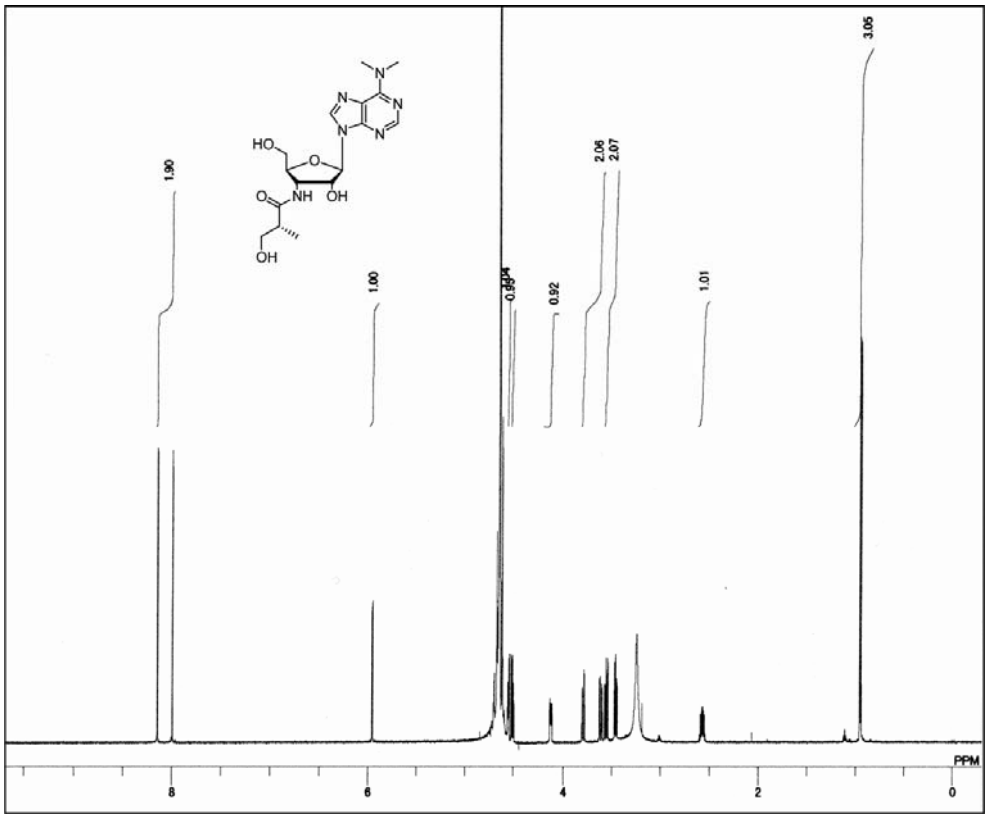


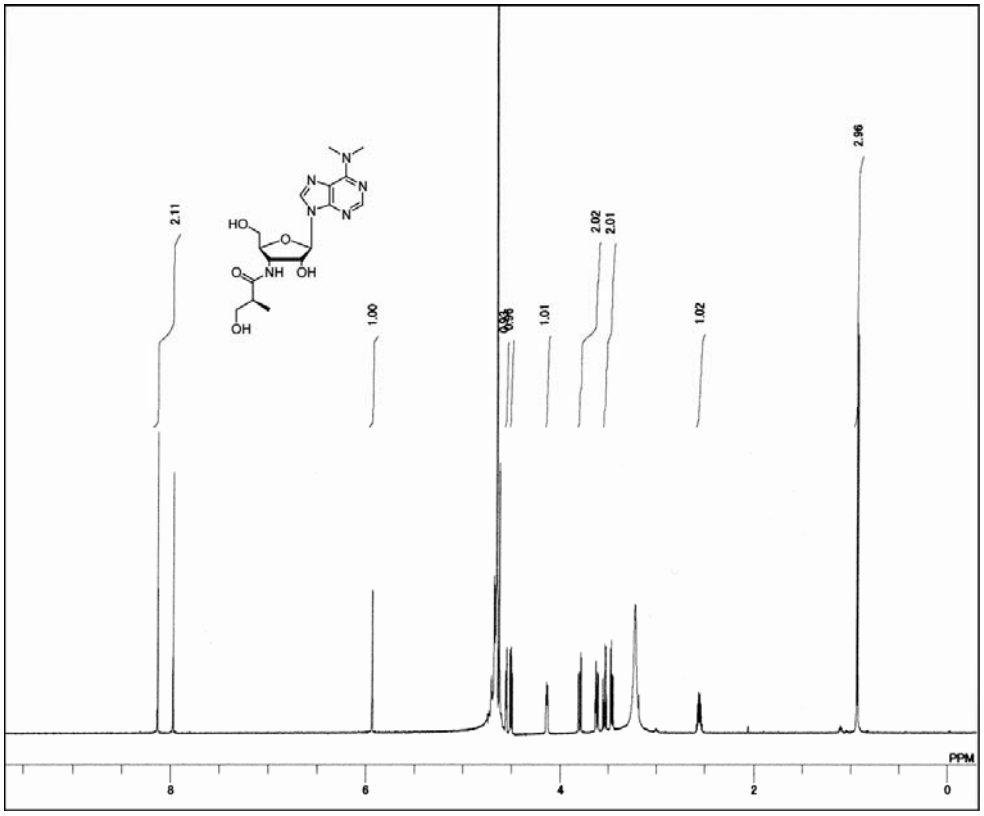
Figure S2. Typical calibration set showing the linearity of fluorescence intensity and amount of full-length oligopep^Y under the S-tag assay conditions used here. A 20 μL aliquot (1/1000 diluted) of serially diluted (0%, 5%, 10%, 20%, 40%, 60%, 80%, or 100%) solutions (2 μg of oligopep^Y mRNA in 10.6 μL of a reconstituted translation solution) was mixed with 180 μL of FRETWorks reaction mix. After 5 min incubation, 20 μL of 10× stop solution was added to the mixture. The fluorescence intensity of the resulting solution was measured with a Wallac 1420 multilabel counter.

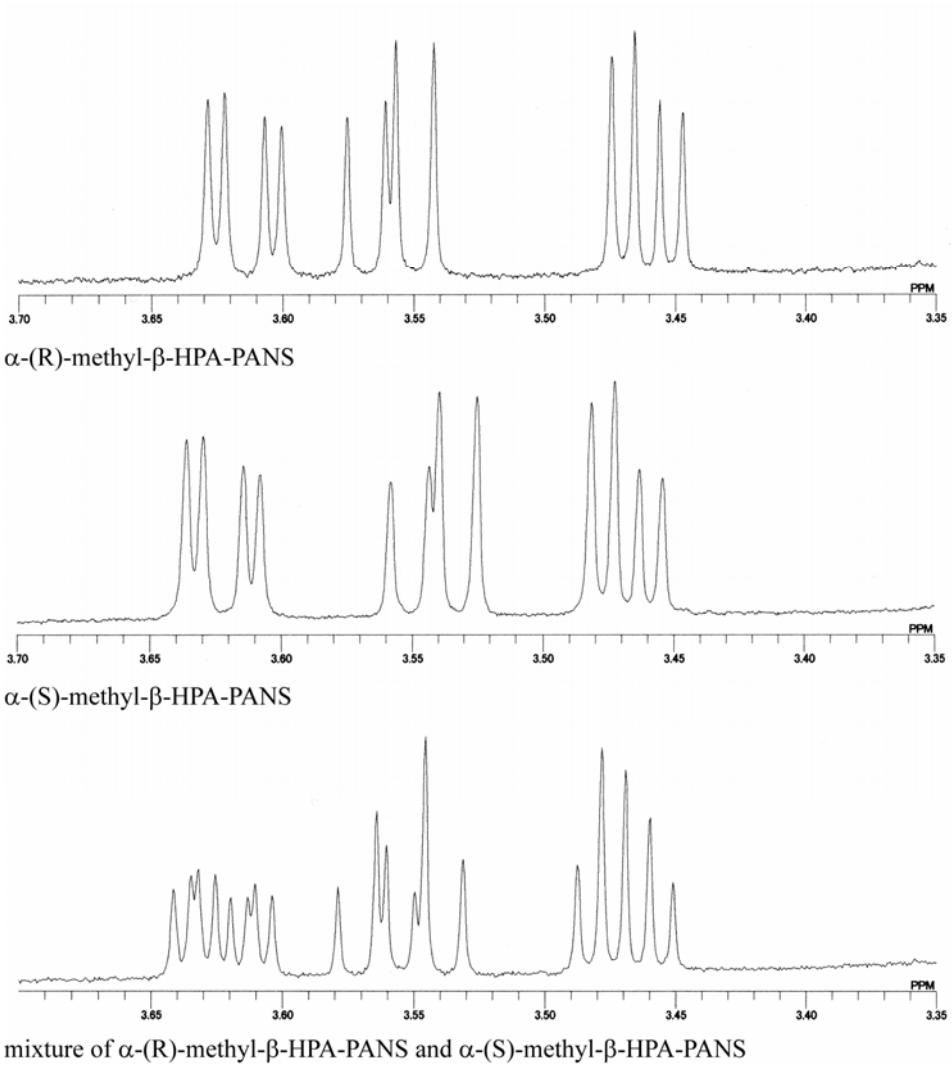
7. *Figure S3*

α-(R)-methyl-β-HPA-PANS



α-(S)-methyl-β-HPA-PANS





Magnified (3.35-3.70 ppm)

Figure S3. NMR analysis of the α-(R)-methyl-β-HPA-PANS and α-(S)-methyl-β-HPA-PANS. The NMR spectra of α-(R)-methyl-β-HPA-PANS and α-(S)-methyl-β-HPA-PANS derived from α-(R)-methyl-β-HPA and α-(S)-methyl-β-HPA used in this study are shown below. They exhibit signals distinguishable from each other, as revealed by the spectrum for a 1:1 mixture thereof. This in turn confirms that the chirality of the methyl-bearing carbon is retained during the synthetic procedure.

8. Reference

1. Jia, Y.; Yuan, W.; Wodzinska, J.; Park, C.; Sinskey, A.J.; Stubbe, J. *Biochemistry* **2001**, *40*, 1011–1019.
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