Synthesis of aspartic acid derivatives useful for the preparation of misacylated transfer RNAs

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Abstract: Several derivatives of aspartic acid were protected on N^{α} as their NVOC derivatives, and on the side chain carboxylates as nitroveratryl esters. Following activation as the cyanomethyl esters, these fully protected aspartate derivatives were converted to the respective pdCpA esters. The protected aspartyl-pdCpA esters were then utilized as substrates for T4 RNA ligase in the presence of in vitro transcripts of tRNA lacking the pCpA dinucleotide normally found at the 3′-end. In this fashion, several misacylated tRNAs were prepared; following photolytic deprotection, these were employed successfully for incorporation into proteins at predetermined positions.

Key words: aminoacylated nucleotides, amino acid protection, protein synthesis, tRNA activation.

Résumé : On a préparé plusieurs dérivés de l'acide aspartique dans lesquels le N^{α} est protégé par un groupe "NVOC" et les groupes carboxyles de la chaîne latérale sont protégés par des esters nitrovératryles. Ces dérivés de l'acide aspartique totalement protégés, activés sous la forme d'esters cyanométhyles, ont été transformés en esters "pdCpA." Les esters aspartyl-pdCpA protégés ont alors été utilisés comme substrats pour la ligase d'ARN T4, en présence de produits de transcriptions in vitro de *t*-ARN ne portant pas le dinucléotide pCpA que l'on retrouve normalement à l'extrémité 3′. De cette façon, on a pu préparer plusieurs tARN anormalement aminoacétylés; après déprotection photolytique, ces produits ont été incorporés avec succès dans des protéines à des positions prédéterminées.

Mots clés : nucléotides aminoacétylés, protection d'acides aminés, synthèse de protéines, activation de la tARN.

[Traduit par la Rédaction]

Introduction

In 1962, Chapeville et al. (1) demonstrated that a cysteine-specific transfer RNA (tRNA) misactivated with alanine could be used to direct the incorporation of alanine into protein at sites intended for cysteine. While the lack of a general method for elaborating misacylated tRNAs initially limited the strategy to misacylated tRNAs accessible by chemical modification of enzymatically activated tRNAs (2), the Hecht laboratory discovered that virtually any misacylated tRNA could be prepared via the T4 RNA ligase-mediated condensation of a chemically synthesized aminoacylated pCpA derivative with a tRNA from which the last two nucleotides had been removed (3). Technical modifications, including the use of aminoacylated pdCpA derivatives (4) and in vitro RNA transcripts lacking the terminal CpA moiety (5) have facilitated the preparation of misacylated tRNAs by the use of this strategy, as have the development of methods for reversible *N*-protection of the aminoacyl moiety, resulting in aminoacylated dinucleotides and tRNAs of enhanced stability (3, 6).

The availability of misacylated tRNAs of diverse structure has permitted the elaboration of modified peptides (3, 7, 8) and proteins (9). That the elaborated species can include peptide and protein analogues of altered connectivity (8, 10) underscores the potential of this strategy for creating novel biomaterials, and for defining the chemistry that the ribosome is actually capable of mediating in the presence of suitable aminoacyl-tRNA analogues.

While the synthesis of aminoacylated dinucleotides for the preparation of misacylated tRNAs has been described (3, 6–9), modifications of the chemistry are required when the amino acid of interest has side-chain functionality. Presently, we describe the chemistry employed for the synthesis of pdCpA derivatives esterified with aspartate analogues, and the use of the derived dinucleotides for the elaboration of misacylated aspartyl-tRNAs. We have previously described the use of such species for the preparation of structurally modified analogues of dihydrofolate reductase (11) and HIV-1 protease (12).

Results and discussion

The aspartic acid derivatives shown in Fig. 1 were commercially available with the exception of the carboxyproline

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This manuscript is dedicated to Professor Stephen Hanessian on the occasion of his 65th birthday, in appreciation of his many contributions to organic chemistry and to the chemical community.

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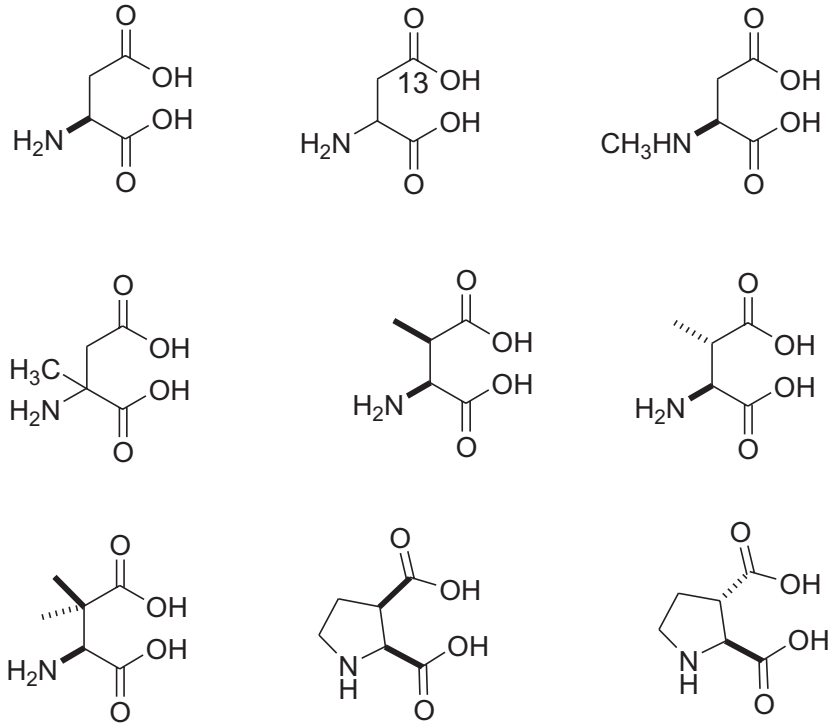
Fig. 1. Aspartic acid derivatives used for the preparation of misacylated tRNAs.



derivatives and β,β-dimethylaspartic acid. *Threo*- and *erythro*-carboxyproline were prepared as described (13).

With the exception of β,β-dimethylaspartic acid, all of the aspartic acid derivatives shown in Fig. 1 were converted to suitably protected cyanomethyl esters as exemplified in Scheme 1 for [α-^{13}C]aspartic acid. In all cases, N^{α} was protected with the NVOC group, while the side-chain carboxylate was protected as the nitroveratryl ester. As shown in Scheme 1, treatment of [α-^{13}C]aspartic acid (**1**) with 6-nitroveratryl chloroformate (14), followed by treatment with 6-nitroveratryl bromide (15) provided fully protected **3** in good yield. Selective hydrolysis of the substituted benzyl ester with 1 equiv. of LiOH (16) gave β-monoester **4** in nearly quantitative yield. This intermediate was converted to cyanomethyl ester **5** (17) in 70% yield by treatment with chloroacetonitrile and triethylamine. It may be noted that this method has been utilized previously for the synthesis of **5** lacking ^{13}C enrichment (4). The other aspartic derivatives shown in Fig. 1 were converted to their fully protected cyanomethyl esters analogously, and in reasonable yields.

The strategy used for the synthesis and protection of β,β-dimethylaspartic acid is shown in Scheme 2. Known compound **6** (18) was treated with potassium bis(trimethylsilyl)amide and methyl iodide to provide the respective dimethylated derivative **7**. Removal of the 9-phenylfluorenyl group by hydrogenation, followed by treatment with 6-nitroveratryl chloroformate gave NVOC protected **8** in 98% yield. Hydrolysis of the methyl esters and treatment with 6-nitroveratryl bromide then provided bis-nitroveratryl ester **9**. Finally, in analogy with the transformations described for compound **3**, compound **9** was converted to cyanomethyl ester **11** by selective hydrolysis and treatment with chloroacetonitrile.

Table 1. Yields of incorporation of aspartic acid analogues into position 25 of HIV-1 protease.

Aspartic acid derivative	Yield (%)
Aspartic acid	22
erythro-β-Methylaspartic acid	33
threo-β-Methylaspartic acid	17
β,β-Dimethylaspartic acid	17

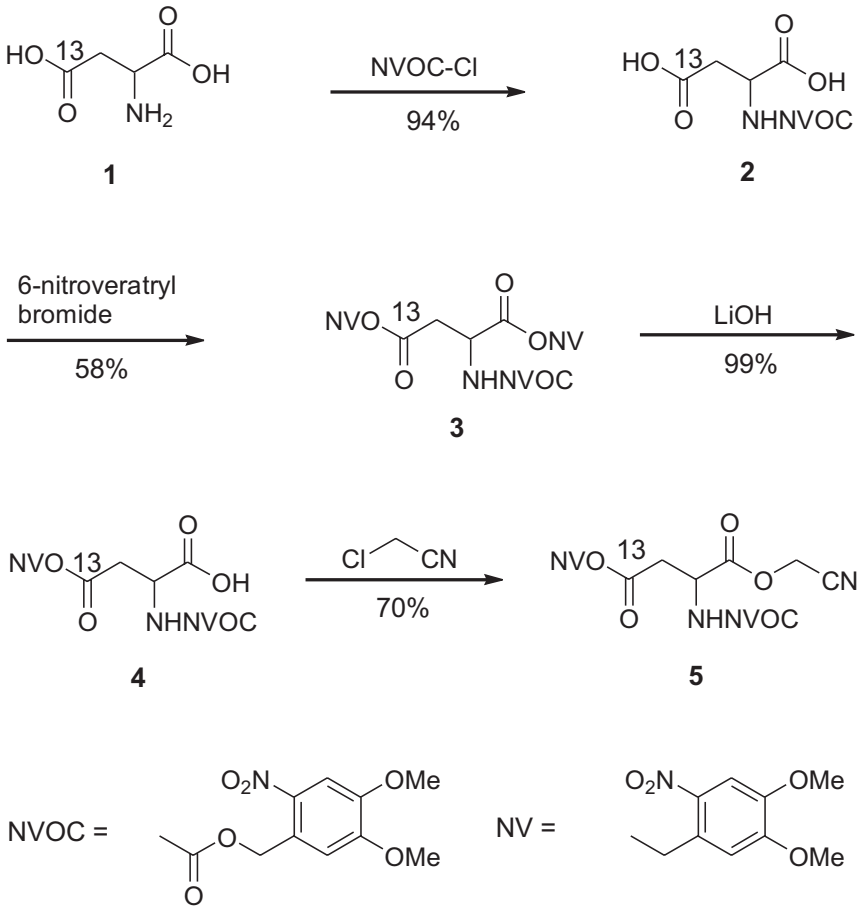
Scheme 3 shows an example of the attachment of an aspartic acid analogue to the dinucleotide pdCpA (4) and the subsequent ligation of the aminoacylated pdCpA derivative to a tRNA transcript lacking the 3′-terminal pCpA moiety. The cyanomethyl ester of (*2S, 3R*)-carboxyproline (**12**) was treated with the tetrabutylammonium salt of pdCpA as described (4). The protected aminoacylated dinucleotide **13** was then ligated to a truncated tRNA (tRNA-C_{OH}) via the agency of T4 ligase (3, 8–11). Removal of the protecting groups was accomplished by irradiation with a 500 W mercury–xenon lamp for 5 min. The deblocked misacylated tRNAs (e.g., **II**) were used promptly in an in vitro protein biosynthesizing system, yielding proteins containing the unnatural aspartic acid derivatives at predetermined sites (11, 12). The incorporation yields for several aspartic derivatives are illustrated in Table 1 using an optimized bacterial protein synthesizing system (19) programmed to elaborate analogues of HIV-1 protease.

Experimental

General information

Melting points were taken on a capillary melting point apparatus and are uncorrected. Moisture sensitive reactions were conducted under argon in oven-dried glassware.

Scheme 1.



Acetonitrile and dichloromethane were distilled from CaH_2, and DMF was distilled from CaH_2 under diminished pressure. THF was distilled from $LiAlH_4$. Triethylamine was distilled from P_2O_5. All other chemicals were purchased from Aldrich Chemicals or Sigma Chemicals and were used without further purification. Analytical thin layer chromatography was performed on 60 F_{254} (E. Merck) plates. Flash chromatography was performed using 230–400 mesh silica gel. High resolution mass spectra were recorded at the Michigan State University-NIH Mass Spectrometry Facility in East Lansing, MI. T4 RNA ligase was purchased from Amersham Pharmacia Biotech.

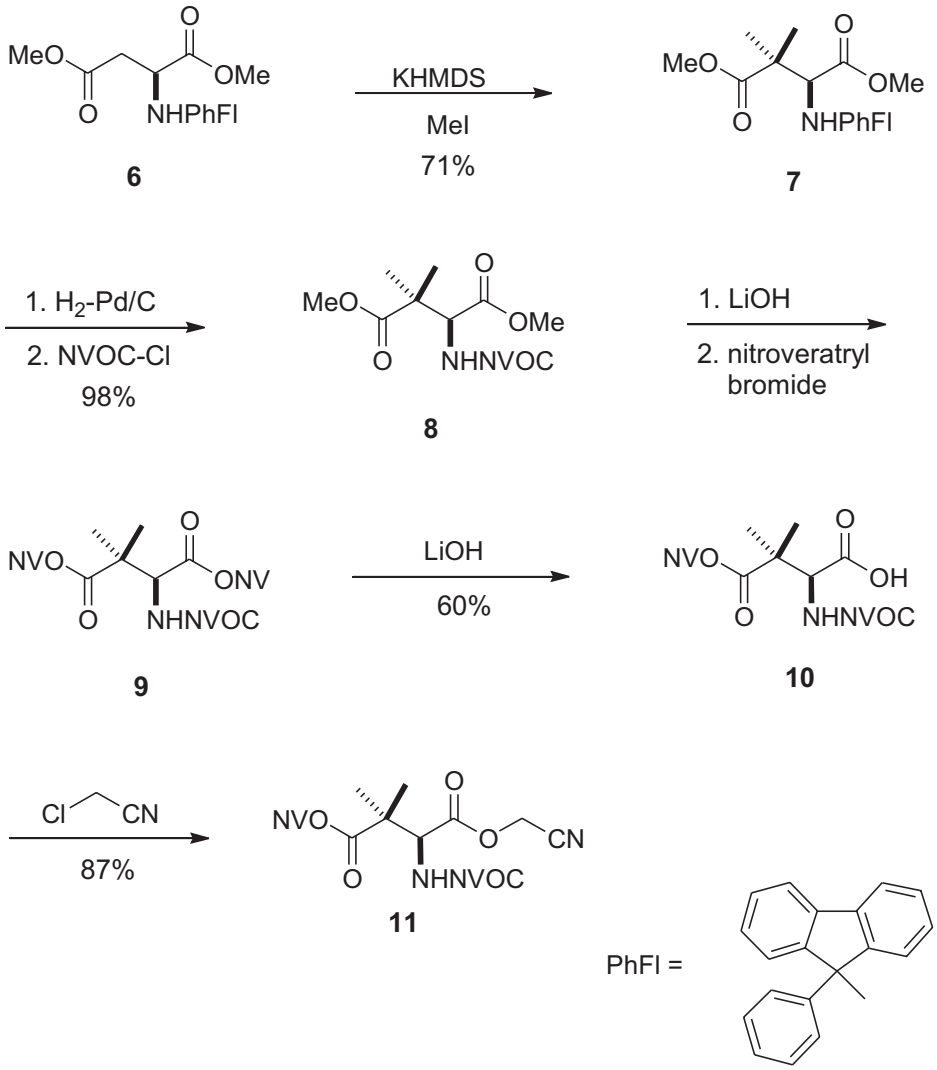
N-*(6-Nitroveratryloxycarbonyl)-4-*^{13}*C-(*RS*)-aspartate (**2**)*

To a solution containing 50 mg (0.37 mmol) of 4-^{13}C-(*RS*)-aspartic acid (**1**) and 157 mg (1.87 mmol) of $NaHCO_3$ in 5 mL of dioxane and 2 mL of H_2O was added a solution of 184 mg (0.67 mmol) of 6-nitroveratryl chloroformate. After stirring at room temperature for 5 h the reaction mixture was diluted with 10 mL of 1 N $NaHSO_4$ and extracted with four 6-mL portions of ethyl acetate. The combined extract was dried (Na_2SO_4) and concentrated under diminished pressure. The crude product was applied to a silica gel column (25 × 2 cm); elution with 5:1:4 ethyl acetate – acetic acid – hexanes provided *N*-(6-nitroveratryloxycarbonyl)-4-^{13}C-(*RS*)-aspartate (**2**) as an orange solid: yield 131 mg (94%), mp 168–172°C. Silica gel TLC R_f: 0.31 (5:1:4 ethyl acetate – acetic acid – hexanes). ^{1}H NMR ($CDCl_3$) δ: 2.73–2.83 (m, 1H), 2.90–2.98 (m, 1H), 3.87 (s, 3H), 3.92 (s, 3H), 4.47–4.53 (m, 1H), 5.43–5.53 (m, 3H), 6.99 (s, 1H), 7.64 (s, 1H). ^{13}C NMR ($CDCl_3$) δ: 30.0, 56.6, 64.1, 108.5, 109.8, 139.6, 148.3, 154.4, 156.5, 173.6. Mass spectrum (chemical ionization) *m/z*: 374.090 $(M+H)^+$ ($C_{13}{}^{13}CH_{17}N_2O_{10}$ requires 374.0917).

Dinitroveratryl N-*(6-nitroveratryloxycarbonyl)-4-*^{13}*C-(*RS*)-aspartate (**3**)*

To a solution containing 131 mg (0.35 mmol) of *N*-(6-nitroveratryloxycarbonyl)-4-^{13}C-(*RS*)-aspartate (**2**) and 292 mg (1.06 mmol) of 6-nitroveratryl bromide in 5 mL of DMF was added 161 mg (1.06 mmol) of CsF. After stirring at room temperature for 24 h the reaction mixture was diluted with 10 mL of ethyl acetate and washed with 10 mL of saturated aqueous $NaHCO_3$ and with 10 mL of brine. The organic layer was dried (Na_2SO_4) and concentrated under diminished pressure. The crude product was applied to a silica gel column (27 × 2 cm); elution with 10–50% ethyl acetate in hexanes provided dinitroveratryl *N*-(6-nitroveratryloxycarbonyl)-4-^{13}C-(*RS*)-aspartate (**3**) as a yellow solid: yield 160 mg (58%), mp 126–134°C. Silica gel TLC R_f: 0.17 (1:1 ethyl acetate–hexanes). ^{1}H NMR ($CDCl_3$) δ: 2.90–3.15 (m, 2H), 3.88 (m, 18H), 4.71 (m, 1H), 5.37–5.45 (m, 6H), 5.50 (br, 1H), 6.89 (s, 1H), 6.98 (s, 2H), 7.62 (s, 3H). ^{13}C NMR ($CDCl_3$) δ: 30.1, 36.3, 37.9, 50.9, 56.7, 64.4, 64.5, 108.6,

Scheme 2.



111.0, 111.2, 126.2, 128.2, 140.1, 148.9, 154.1, 156.7, 170.7. Mass spectrum (electron impact) *m*/*z*: 763.192 ($C_{31}{}^{13}CH_{34}N_4O_{18}$ requires 763.1902).

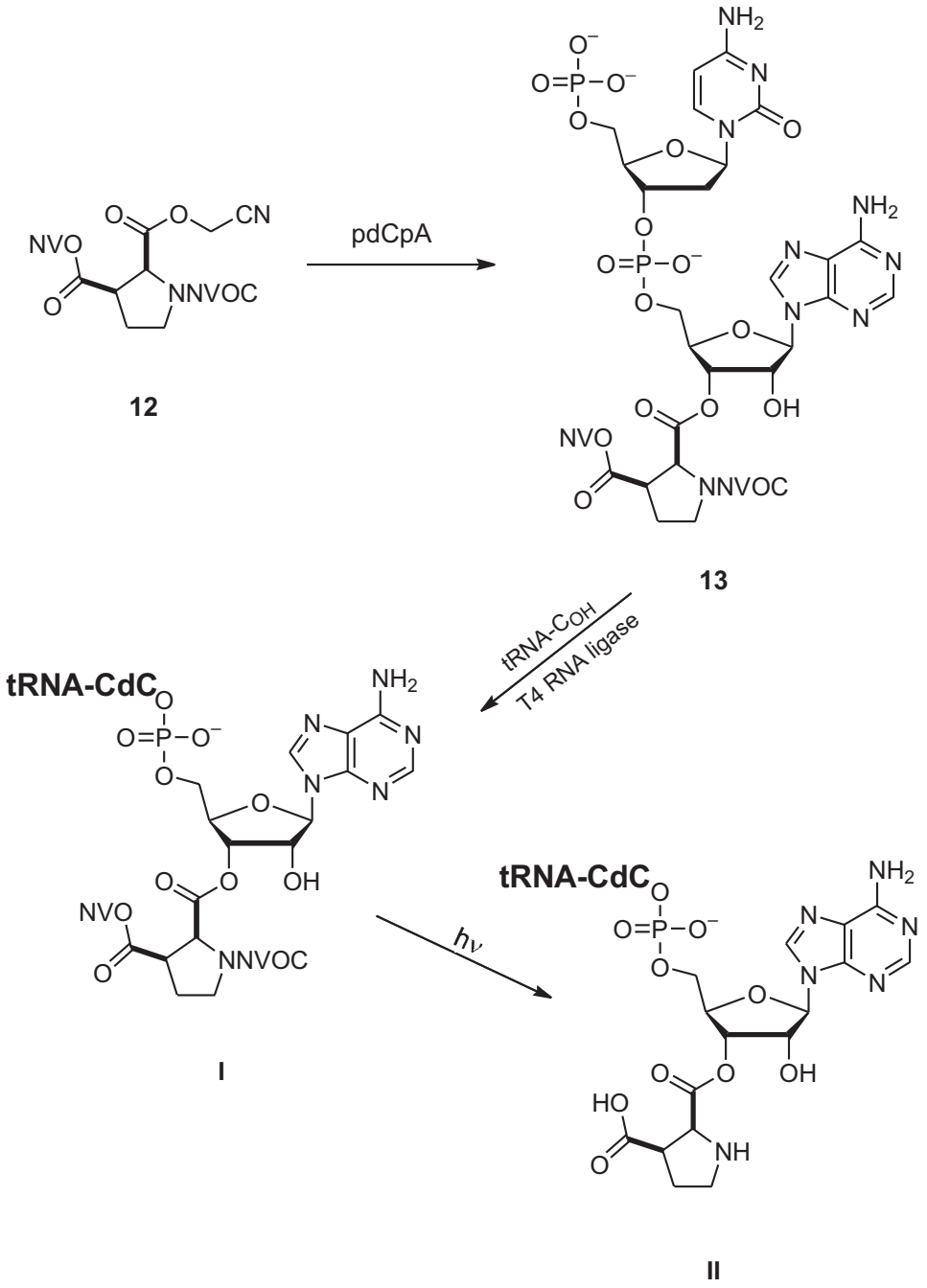
β-*Nitroveratryl* N-*(6-nitroveratryloxycarbonyl)-4-*^{13}*C-(*RS*)-aspartate (4)*

To a cooled (0–5°C) solution containing 89 mg (0.12 mmol) of dinitroveratryl *N*-(6-nitroveratryloxycarbonyl)-4-^{13}C-(*RS*)-aspartate (**3**) in 13 mL of acetone and 2 mL of H_2O was added 12 mL of a 0.1 N solution of LiOH in H_2O. After stirring at room temperature for 16 h, the reaction mixture was diluted with 20 mL of 1 N $NaHSO_4$ and extracted with five 10-mL portions of CH_2Cl_2. The combined organic extract was washed with 10 mL of brine, dried ($MgSO_4$), and concentrated under diminished pressure. The crude product was applied to a silica gel column (26 × 2 cm); elution with 10:1:1:8 ethyl acetate – acetic acid – MeOH – hexanes provided β-nitroveratryl *N*-(6-nitroveratryloxycarbonyl)-4-^{13}C-(*RS*)-aspartate (**4**) as a yellow solid: yield 65 mg (99%). Silica gel TLC R_f: 0.18 (10:1:1:8 ethyl acetate – acetic acid – MeOH – hexanes). ^{1}H NMR ($CDCl_3$) δ: 2.95–3.04 (m, 1H), 3.16–3.24 (m, 1H), 3.93–3.97 (m, 12H), 4.78 (m, 1H), 5.48–5.56 (m, 4H), 5.97 (br, 1H), 6.94 (m, 2H), 7.02 (s, 1H), 7.68–7.70 (m, 2H). Mass spectrum (electron impact) *m*/*z*: 569.142 $(M+H)^+$ ($C_{22}{}^{13}CH_{26}N_3O_{14}$ requires 569.1448).

Cyanomethyl β-*nitroveratryl* N-*(6-nitroveratryloxycarbonyl)-4-*^{13}*C-(*RS*)-aspartate (5)*

To a solution containing 66 mg (0.12 mmol) of β-nitroveratryl *N*-(6-nitroveratryloxycarbonyl)-4-^{13}C-(*RS*)-aspartate (**4**) in 2 mL of acetonitrile was added 100 μL (0.72 mmol) of Et_3N, followed by 0.5 mL (7.90 mmol) of chloroacetonitrile. After stirring at room temperature for 24 h, the reaction mixture was diluted with 10 mL of 1 N HCl and extracted with five 4-mL portions of CH_2Cl_2. The combined organic extract was washed with 5 mL of brine, dried (Na_2SO_4), and concentrated under diminished pressure. The crude product was applied to a silica gel column (12 × 2 cm); elution with 10–50% ethyl acetate in hexanes provided cyanomethyl β-nitroveratryl *N*-(6-nitroveratryloxycarbonyl)-4-^{13}C-(*RS*)-aspartate (**5**) as a yellow solid: yield 50 mg (70%), mp 129–134°C. Silica gel TLC R_f: 0.51 (1:4 ethyl acetate–CH_2Cl_2). ^{1}H

Scheme 3.



NMR ($CDCl_3$) δ: 3.00 (m, 1H), 3.21 (m, 1H), 3.96–4.02 (m, 12H), 4.78 (m, 3H), 5.43–5.59 (m, 4H), 5.80 (br, 1H), 6.95 (s, 1H), 7.05 (s, 1H), 7.73 (s, 2H). ^{13}C NMR (acetone-d_6) δ: 35.0, 35.8, 48.5, 50.2, 55.2, 55.4, 63.24, 63.39, 107.6, 109.3, 110.2, 124.7, 125.8, 127.4, 127.6, 128.4, 139.0, 147.8, 148.1, 153.5, 155.1, 169.0, 169.2. Mass spectrum (chemical ionization) *m/z*: 608.152 $(M+H)^+$ ($C_{24}{}^{13}CH_{27}N_4O_{14}$ requires 608.1557).

Dimethyl N-*(9-phenylfluorenyl)*-β,β-*dimethyl-(*S*)-aspartate (7)*

To a cooled (–78°C) solution containing 5.17 mL of a 0.5 M solution of potassium bis(trimethylsilyl)amide in toluene (2.58 mmol) in 30 mL of THF was added dropwise a solution of 683 mg (1.70 mmol) of dimethyl *N*-(9-phenylfluorenyl)-(*S*)-aspartate (**6**) in 20 mL of THF. After stirring for 1 h, a solution containing 0.45 mL (7.18 mmol) of methyl iodide in 1 mL of THF was added. After stirring for an additional 1 h, another 5.20 mL portion of the 0.5 M potassium bis(trimethylsilyl)amide solution was added. After 2 h the reaction mixture was quenched with 2 mL of MeOH, diluted with 30 mL of 1 N H_3PO_4, and extracted with three 15-mL portions of ethyl acetate. The organic extract was washed with 10 mL of brine, dried ($MgSO_4$), and concentrated under diminished pressure. The crude product was applied to a silica gel column (20 × 2 cm); elution with 4:1 hexanes–ethyl acetate provided dimethyl *N*-(9-phenylfluorenyl)-β,β-dimethyl-(*S*)-aspartate (**7**) as a pale yellow oil: yield 522 mg

(71%). Silica gel TLC R_f: 0.24 (1:1 ethyl acetate–hexanes). ^{1}H NMR ($CDCl_3$) δ: 1.12 (s, 3H), 1.28 (s, 3H), 2.93 (d, 1H, J = 11 Hz), 3.19 (s, 3H), 3.55 (s, 3H), 5.20 (s, 1H), 7.20–7.75 (m, 13H). ^{13}C NMR ($CDCl_3$) δ: 21.4, 22.7, 46.9, 51.7, 52.4, 54.0, 61.5, 73.1, 120.4, 127.3, 127.7, 127.8, 128.1, 148.4, 148.7, 174.6, 176.4. Mass spectrum (electron impact) *m*/*z*: 429.194 ($C_{27}H_{27}NO_4$ requires 429.1940).

Dimethyl N-*(6-nitroveratryloxycarbonyl)*-β,β-*dimethyl-(*S*)-aspartate (**8**)*

To a solution containing 250 mg (0.58 mmol) of dimethyl *N*-(9-phenylfluorenyl)-β,β-dimethyl-(*S*)-aspartate (**7**) in MeOH was added 125 mg of 10% Pd/C. The reaction mixture was shaken under H_2 at 50 psi for 24 h. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was dissolved in 5 mL of H_2O and washed with three 5-mL portions of CH_2Cl_2. The aqueous layer was diluted with 6 mL of dioxane and 122 mg (1.45 mmol) of $NaHCO_3$ was added, followed by a solution containing 289 mg (1.05 mmol) of 6-nitroveratryl chloroformate in 4 mL of dioxane. After stirring at room temperature for 16 h, the reaction mixture was diluted with 20 mL of 1 N $NaHSO_4$ and extracted with four 25-mL portions of ethyl acetate. The combined extract was washed with 15 mL of brine, dried ($MgSO_4$), and concentrated under diminished pressure. The crude product was applied to a silica gel column (20 × 2 cm); elution with 10–50% ethyl acetate in hexanes gave dimethyl *N*-(6-nitroveratryloxycarbonyl)-β,β-dimethyl-(*S*)-aspartate (**8**) as a yellow solid: yield 244 mg (98%). Silica gel TLC R_f: 0.24 (2:3 ethyl acetate–hexanes). ^{1}H NMR ($CDCl_3$) δ: 1.14 (s, 3H), 1.24 (s, 3H), 3.64 (s, 3H), 3.65 (s, 3H) 3.87 (s, 3H), 3.91 (s, 3H), 4.55 (d, 1H, J = 10 Hz), 4.88 (s, 2H), 5.44 (br, 1H), 6.92 (s, 1H), 7.61 (s, 1H). ^{13}C NMR ($CDCl_3$) δ: 22.3, 23.3, 52.9, 56.7, 56.8, 60.5, 62.8, 64.4, 108.4, 108.5, 110.1, 110.9, 133.1, 139.8, 148.1, 154.3, 156.4, 171.2, 176.2. Mass spectrum (electron impact) *m*/*z*: 428.142 ($C_{18}H_{24}N_2O_{10}$ requires 428.1431).

Dinitroveratryl N-*(6-nitroveratryloxycarbonyl)*-β,β-*dimethyl-(*S*)-aspartate (**9**)*

To a solution containing 249 mg (0.58 mmol) of dimethyl *N*-(6-nitroveratryloxycarbonyl)-β,β-dimethyl-(*S*)-aspartate (**8**) in 12 mL of acetone and 6 mL of H_2O was added 14.6 mL of a 0.1 N solution of LiOH in H_2O. After stirring at 40°C for 2 days the reaction mixture was diluted with 10 mL of ethyl acetate and the layers were separated. The aqueous layer was acidified with 1 N $NaHSO_4$ and extracted with four 10-mL portions of ethyl acetate. The combined extract was washed with 10 mL of brine, dried ($MgSO_4$), and concentrated under diminished pressure. The crude diacid was dissolved in 2 mL of DMF and 155 mg (0.56 mmol) of 6-nitroveratryl bromide was added, followed by 85 mg (0.56 mmol) of CsF. After stirring at room temperature for 2 days, the reaction mixture was diluted with 10 mL of ethyl acetate and washed with 10 mL of saturated aqueous $NaHCO_3$ and 10 mL of brine. The organic layer was dried (Na_2SO_4) and concentrated under diminished pressure. The crude product was applied to a silica gel column (18 × 2 cm); elution with 10–50% ethyl acetate in hexanes gave dinitroveratryl *N*-(6-nitroveratryloxycarbonyl)-β,β-dimethyl-(*S*)-aspartate (**9**) as a yellow syrup: yield 124 mg (27%). Silica gel TLC R_f: 0.50 (3:2 ethyl acetate–hexanes). ^{1}H NMR ($CDCl_3$) δ: 1.29 (s, 3H), 1.41 (s, 3H), 3.90–3.93 (m, 18H), 4.71 (d, 1H, J = 10 Hz), 5.39–5.50 (m, 6H), 6.02 (br, 1H), 6.91 (s, 1H), 6.97 (s, 2H), 7.62–7.66 (m, 3H). ^{13}C NMR ($CDCl_3$) δ: 22.8, 23.8, 45.8, 60.7, 64.5, 64.9, 108.5, 110.0, 110.9, 111.3, 126.2, 126.6, 128.2, 140.0, 140.3, 148.6, 148.8, 154.0, 154.2, 156.4, 170.3, 175.3. Mass spectrum (FAB) *m*/*z*: 791.226 $(M+H)^+$ ($C_{34}H_{39}N_4O_{18}$ requires 791.2259).

β-*Nitroveratryl* N-*(6-nitroveratryloxycarbonyl)*-β,β-*dimethyl-(*S*)-aspartate (**10**)*

To a cooled (0–5°C) solution containing 119 mg (0.15 mmol) of dinitroveratryl *N*-(6-nitroveratryloxycarbonyl)-β,β-dimethyl-(*S*)-aspartate (**9**) in 5 mL of acetone and 2 mL of H_2O was added 1.6 mL (0.16 mmol) of a 0.1 N solution of LiOH in H_2O. After stirring at room temperature for 24 h, the reaction mixture was concentrated under diminished pressure. The residue was dissolved in 4 mL of saturated aqueous $NaHCO_3$ and washed with three 5-mL portions of ethyl acetate. The aqueous layer was acidified with 1 N $NaHSO_4$ and extracted with three 6-mL portions of ethyl acetate. The combined extract was washed with 6 mL of brine, dried ($MgSO_4$), and concentrated under diminished pressure. The crude product was applied to a silica gel column (13 × 2 cm); elution with 10:1:1:8 ethyl acetate – MeOH – acetic acid – hexanes gave β-nitroveratryl *N*-(6-nitroveratryloxycarbonyl)-β,β-dimethyl-(*S*)-aspartate (**10**) as a pale yellow syrup: yield 54 mg (60%). Silica gel TLC R_f: 0.48 (10:1:9 ethyl acetate – MeOH – hexanes). ^{1}H NMR ($CDCl_3$) δ: 1.23 (s, 3H), 1.41 (s, 3H), 3.94–3.97 (m, 12H), 4.65 (d, 1H, J = 10 Hz), 5.51 (m, 4H), 6.02 (br, 1H), 7.02 (s, 2H), 7.67 (s, 2H), 11.0 (br, 1H). Mass spectrum (FAB) *m*/*z*: 596.170 $(M + H)^+$ ($C_{25}H_{30}N_3O_{14}$ requires 596.1728).

Cyanomethyl β-*nitroveratryl* N-*(6-nitroveratryloxycarbonyl)*-β,β-*dimethyl-(*S*)-aspartate (**11**)*

To a solution containing 52 mg (0.087 mmol) of β-nitroveratryl *N*-(6-nitroveratryloxycarbonyl)-β,β-dimethyl-(*S*)-aspartate (**10**) in 2 mL of acetonitrile was added 61 μL (0.44 mmol) of Et_3N, followed by 100 μL (1.58 mmol) of chloroacetonitrile. After stirring at room temperature for 24 h the reaction mixture was diluted with 5 mL of CH_2Cl_2 and washed successively with 4 mL of 1 N HCl, two 4-mL portions of saturated aqueous $NaHCO_3$, and 5 mL of brine. The organic layer was dried (Na_2SO_4) and concentrated under diminished pressure. The crude product was applied to a silica gel column (15 × 2 cm); elution with 10–50% ethyl acetate in hexanes provided cyanomethyl β-nitroveratryl *N*-(6-nitroveratryloxycarbonyl)-β,β-dimethyl-(*S*)-aspartate (**11**) as a pale yellow solid: yield 48 mg (87%), mp 69–72°C. Silica gel TLC R_f: 0.24 (1:1 ethyl acetate–hexanes). ^{1}H NMR ($CDCl_3$) δ: 1.33 (s, 3H), 1.44 (s, 3H), 3.96 (s, 6H), 4.00 (s, 6H), 4.65 (m, 1H), 4.71 (m, 2H), 5.51 (m, 2H), 5.56 (m, 2H), 5.92, (br, 1H), 7.03 (s, 2H), 7.72 (s, 2H). Mass spectrum (FAB) *m*/*z*: 634.172 ($C_{27}H_{30}N_4O_{14}$ requires 634.1759).

β-*Nitroveratryl* N-*(6-nitroveratryloxycarbonyl)-(2*S*,3*R*)-carboxyproline pdCpA ester (**13**)*

A solution of 4 mg (2.94 μmol) of the tris(tetrabutylammonium) salt of pdCpA and 9.8 mg (13.7 μmol) of β-nitroveratryl *N*-(6-nitroveratryloxycarbonyl)-(*2S,3R*)-carboxy-

proline cyanomethyl ester (**12**) in 50 μL of DMF was stirred at room temperature for 4 h. A 10-μL aliquot of the mixture was diluted with 90 μL of 1:1 CH_3CN – 50 mM NH_4OAc, pH 4.5. Ten μL of the diluted aliquot was analyzed by HPLC on a C_{18} reversed phase column (250 × 10 mm). The column was washed with 1→63% CH_3CN in 50 mM NH_4OAc, pH 4.5, over a period of 45 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). The reaction mixture was diluted to a total volume of 400 μL of 1:1 CH_3CN – 50 mM NH_4OAc, pH 4.5, and purified using the same semipreparative C_{18} reversed phase column, affording β-nitroveratryl *N*-(6-nitroveratryloxycarbonyl)-(*2S*,*3R*)-carboxyproline pdCpA ester (**13**) as a pale yellow foam: yield 0.5 mg (14%). Mass spectrum (FAB) *m/z*: 1212.257 $(M+H)^+$ ($C_{44}H_{52}N_{11}O_{26}P_2$ requires 1212.2560).

Synthesis of misacylated tRNAs

Ligation reactions were carried out in 50 μL of 50 mM Na Hepes buffer, pH 7.5, containing 5 μL (~0.2 nmol) of $tRNA_{(CUA)}C_{OH}$, 0.5 A_{260} unit (~20 nmol) of an aminoacyl-pdCpA, 0.5 mM ATP, 15 mM $MgCl_2$, 10% dimethylsulfoxide, and 100 units of T4 RNA ligase. Reaction mixtures were incubated at 37°C for 25 min and then quenched by the addition of 5 μL of 3 M Na acetate, pH 4.5. The aminoacylated tRNA (**I**) was precipitated with 2.5 volumes of ethanol, collected by centrifugation, washed with 70% ethanol, and dried. The product was redissolved in 1 mM KOAc to a final concentration of 1 μg/μL and then irradiated with a 500 W mercury–xenon lamp using Pyrex and water filters. The protected aminoacyl tRNA (**I**) was cooled in an ice bath during irradiation, which was typically carried out for 5 min for amino acid derivatives containing an NVOC group and a nitroveratryl ester. The deprotected aminoacyl-tRNAs (**II**) were used in in vitro suppression experiments soon after deprotection.

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