Pergamon

Tetrahedron 56 (2000) 9421–9429

TETRAHEDRON

N-Substituted 2-Amino-4-pentenoic Acids for Amino Acid Protection and Resolution

Michiel Lodder, Bixun Wang and Sidney M. Hecht*

Departments of Chemistry and Biology, University of Virginia, Charlottesville, VA 22901, USA

Received 24 February 2000; accepted 2 August 2000

Abstract—*N*-Substituted 2-amino-4-pentenoic acid derivatives employed for the protection of racemic amino acids were shown to be capable of permitting the chromatographic separation of *R* and *S* isomers. When N^{α} was disubstituted with benzyl and phenylfluorenyl groups, the racemic amino acids could be separated with facility on open silica gel columns. Further, the optically pure *N*-protected amino acids so derived could be used for the preparation of misacylated suppressor transfer RNAs.

Introduction

During the past two decades our laboratory has developed a method for the chemical preparation of transfer RNAs bearing a noncognate amino acid.[1–10] The general strategy of T4 RNA ligase-mediated ligation of a synthetic aminoacylated pdCpA derivative with a truncated tRNA lacking the 3′-terminal cytidine and adenosine moieties has proven to be successful for a wide variety of unnatural amino acids.[1–19] The resulting misacylated transfer RNAs have been used for probing the peptidyltransferase reaction[4,6] and for the in vitro preparation of proteins containing unnatural amino acids at single, predetermined sites.[11–19]

An interesting aspect of the utilization of misacylated transfer RNAs involves the chirality of the synthetic amino acids attached to the tRNA. Based on the composition of ribosomally synthesized proteins, it has been assumed that nature incorporates only the *S*-isomers of the proteinogenic amino acids. Studies using misacylated transfer RNAs bearing the *R*-isomer of a few different amino acids showed that the unnatural isomers do not participate in peptide bond formation to any reasonable extent.[8,10,14] Although not studied with precision, the use of racemic amino acids apparently results in the formation of peptide bonds to almost the same extent as when the optically pure *S*-isomer is employed. However, it has also been shown that tRNAs bearing the *R*-isomer of an amino acid can bind to the ribosomes in a fashion similar to the *S*-isomer.[8] This implies that although the *R*-isomer is not involved in peptide bond formation, it could sometimes hinder peptide bond formation by tRNAs bearing the respective *S*-isomer. Therefore, when possible it seems advisable to utilize optically pure amino acids for the preparation of misacylated transfer RNAs that are employed for in vitro protein synthesis.

Many different methods have been developed for the synthesis of optically enriched amino acids.[20] Few of these methods, however, produce amino acids consisting exclusively of one isomer and many require an additional separation step to obtain compounds that can be considered optically pure. Another approach to enantiomerically pure compounds involves the separation of racemic mixtures of unnatural amino acids. Over the years many methods have been used. They have included preferential crystallization, crystallization from optically active solvents, fractional crystallization of diastereomeric salts and selective destruction of one enantiomer.[21] Advances in chromatographic techniques, especially HPLC, have also made it possible to use chiral stationary phases for the separation of enantiomers.[22] Another possibility is the use of chiral derivatizing reagents to convert the enantiomers into diastereomers, which in turn can be separated based on their physical differences.[23,24] The choice of the chiral reagent has a significant effect upon the degree of separation of the derived diastereomers, as well as the detectability and the recovery of the individual enantiomers. With many of the currently used reagents, recovery of the optically pure amino acids can be a problem. In most cases, a stable amide bond is formed between the amino acids and the chiral reagent. Recovery of the optically pure isomers is, therefore, only possible by treatment with reagents such as strong acid. Accordingly, the development of a resolving reagent for the separation of racemic amino acids that can be removed under mild conditions would be very useful.

Keywords: amino acids and derivatives; protecting groups; resolution; optical properties.

* Corresponding author. Tel.: +804-924-3906; fax: +804-924-7856; e-mail: sidhecht@virginia.edu

0040–4020/00/$ - see front matter
PII: S0040-4020(00)00826-7

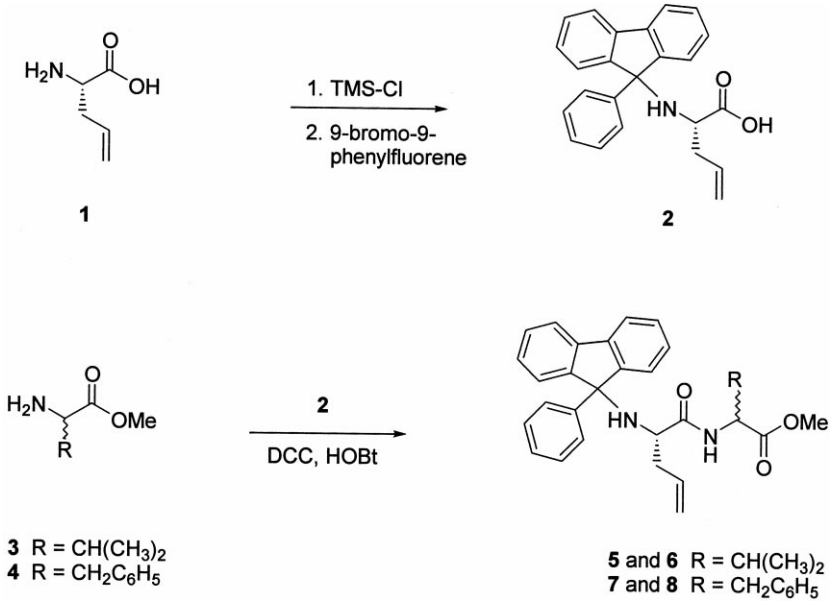
Described herein is the development of a reagent that combines the function of separating racemic mixtures with the function of a protecting group. Combining these functions into one group limits the number of steps necessary for the preparation and utilization of optically pure amino acids, and also ensures that the derivatizing reagent can be removed after synthesis of the final compound is completed. The utility of this reagent is illustrated by the resolution and activation of an amino acid for ribosomally mediated protein synthesis.

Results and Discussion

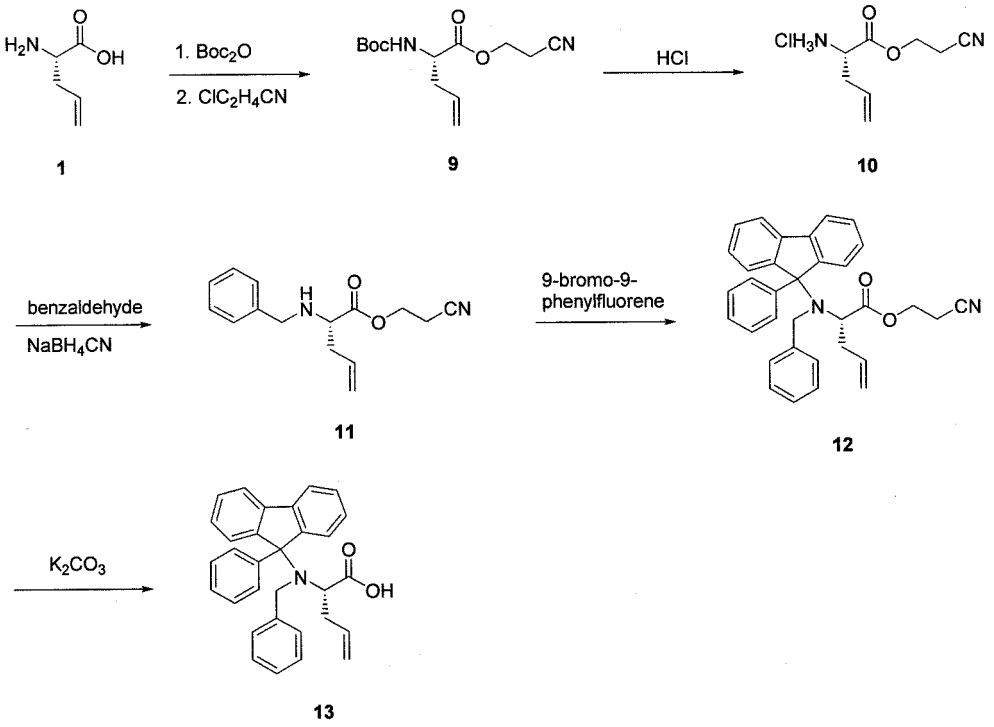
Recently we investigated substituted 4-pentenoyl groups for their ability to serve as amine protecting groups during the synthesis of misacylated transfer RNAs.[25,26] During this investigation 2-amino-4-pentenoic acid (allylglycine) was identified as a possible core structure for the preparation of a chiral derivatizing reagent useful for the separation of racemic amino acids. The amino group provides a suitable functional group for introducing bulky substituents potentially capable of differentiating the optical antipodes of conjugated amino acids. Further, based on an earlier study,[26] it seemed likely that N^{α} substituents would not seriously affect the iodolactonization reaction (vide infra) necessary for the removal of the amino acid protecting group.

In a first attempt to develop a novel derivatizing reagent, a 9-phenylfluorenyl[27–29] group was attached to the amine. This bulky substituent has been reported to be 6000 times more stable to acid than the trityl group.[30] Further, the rigid planar ring system may improve the separation properties of the substituted pentenoyl derivative. The 9-phenylfluorenyl group was introduced as described (Scheme 1).[29] In situ protection of the carboxylate moiety with trimethylsilyl chloride, followed by alkylation of the amine with 9-bromo-9-phenylfluorene afforded, after acidic workup, *N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoic acid (**2**) in 84% yield. Compound **2** was conjugated to racemic valine methyl ester (**3**) by a DCC-mediated coupling. The resulting diastereomers **5** and **6** were obtained in a 1:1 ratio in 92% yield. A similar reaction of **2** with racemic phenylalanine methyl ester (**4**) gave a 1:1 mixture of diastereomers **7** and **8** (Scheme 1). HPLC analysis, using a normal phase silica gel column, showed reasonable separation of the diastereomers in both mixtures.[31] The degree of separation did not seem to depend on the side chain moiety of the amino acids involved. Treatment of *S*-valine benzyl ester conjugated to *N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoic acid with iodine in aqueous THF led to the recovery of the free amine in 71% yield.[31] These results showed that a substituted 2-amino-4-pentenoyl derivative could function as an effective derivatizing reagent for the separation of racemic amino acids and could be removed under the same conditions as the unsubstituted 4-pentenoyl group. While compound **2** may be useful for analytical purposes, the necessity of using an HPLC column for the separation of the derived diastereomers of a racemic amino acid diminishes its attractiveness for the preparative synthesis of optically pure derivatives.

In an effort to improve the facility of separation of the derived diastereomers, a substituted 2-amino-4-pentenoic acid derivative was prepared that contained an additional *N*-benzyl group (Scheme 2). Reversible protection of the carboxylate moiety proved to be more complicated than originally envisioned. A methyl ester was used at first, but after introduction of the bulky substituents on the amine, it was not possible to remove the methyl group. Many different methods for the removal of methyl esters were used,[32] but none afforded the desired free acid. Apparently, the steric bulk of the *N*-substituent shielded the carboxylate, thereby hindering the hydrolysis of the methyl ester.[33] It was decided to use a cyanoethyl protecting group instead, which is not removed by direct hydrolysis, but rather by base induced β-elimination.[34,35] Thus, commercially available



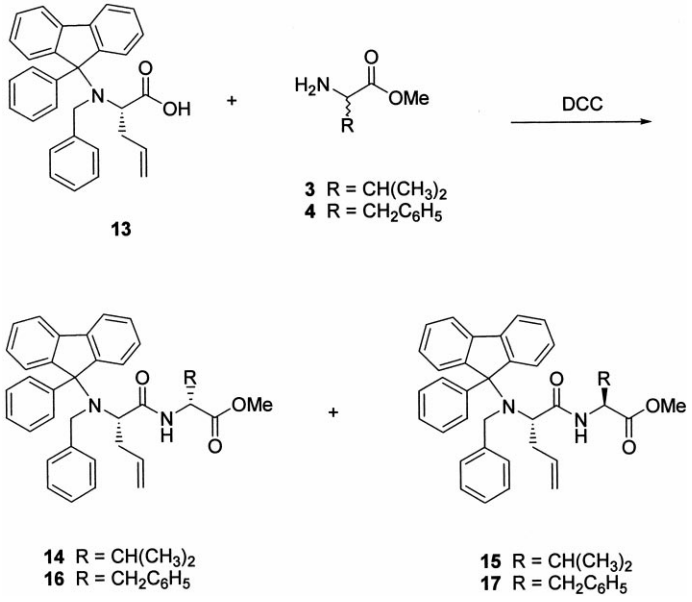
Scheme 1.



Scheme 2.

(*S*)-2-amino-4-pentenoic acid was treated with Boc anhydride, followed by a DCC-mediated coupling of 3-hydroxypropionitrile[34] to afford protected amino acid **9** in 67% yield. The Boc group was removed by treatment with HCl in dioxane, affording hydrochloride salt **10**. The benzyl group was introduced by reductive benzylation of compound **10** with benzaldehyde and sodium cyanoborohydride.[36] The use of the hydrochloride salt instead of the free amine proved very effective for providing the correct pH for the reductive benzylation. Compound **11** was treated with 9-bromo-9-phenylfluorene resulting in bulky compound **12** in 83% yield. Removal of the cyanoethyl group by treatment with K_2CO_3 afforded desired free acid **13** in good yield.

Substituted pentenoyl derivative **13** was conjugated to racemic valine methyl ester (**3**) via DCC-mediated coupling (Scheme 3). Interestingly, the addition of HOBt to the reaction mixture gave an unreactive benzotriazole ester of **13**. Therefore, the coupling was conducted in a mixture of CH_2Cl_2 and pyridine without HOBt. The resulting diastereomers **14** and **15** could be separated easily by the use



Scheme 3.

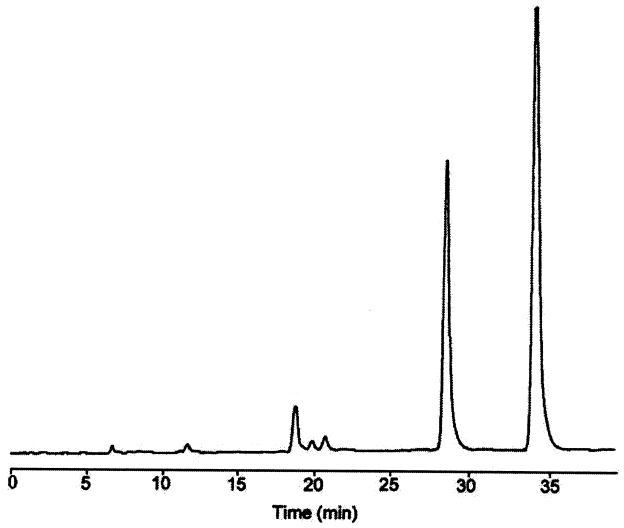
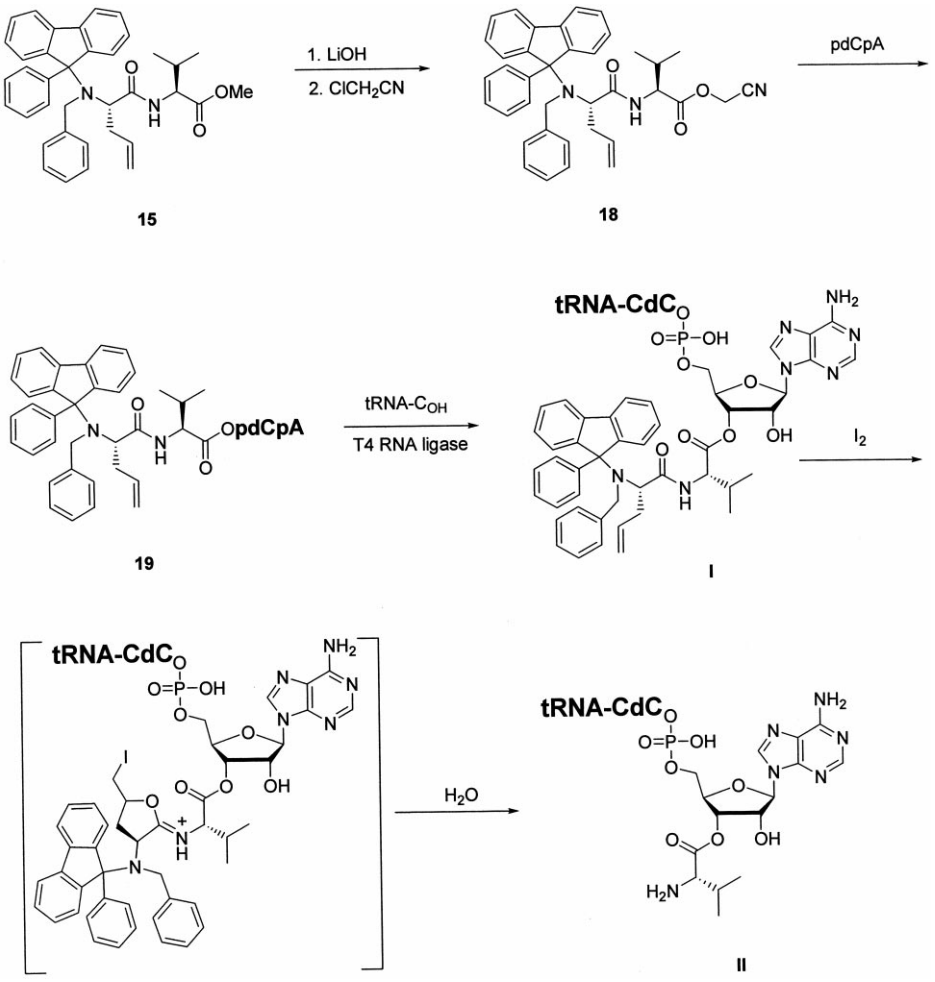


Figure 1. Separation of diastereomic **16** (28.6 min) and **17** (34.5 min) by normal phase HPLC.

of an open silica gel column. Similar results were obtained when compound **13** was conjugated to racemic phenylalanine methyl ester (**4**). HPLC analysis of diastereomers **16** and **17** (Fig. 1) showed that the separation of the diastereomers was much improved as compared to the separation obtained by using compound **2** as a derivatizing reagent. Comparison of the products with diastereomers that were derived from the optically pure isomers of the respective amino acids showed that the diastereomers obtained from the *R*-isomers eluted before those obtained from the *S*-isomers. Interestingly, in case of both valine and phenylalanine the ratio of diastereomers formed was approximately 1:2 in favor of the (*S*,*S*) derivatives over the (*S*,*R*) derivatives. Based on the racemic starting materials a 1:1 ratio would have been expected, but apparently bulky **13** reacts preferentially with the *S*-isomers of the amino acids.

To show that substituted pentenoyl derivative **13** could also be used as a protecting group for subsequent transformations, (*S*)-valine derivative **15** was used for the preparation of the corresponding misacylated transfer RNA (Scheme 4). Treatment of optically pure **15** with LiOH, followed by reaction with chloroacetonitrile afforded cyanomethyl ester **18** in good yield. Attachment to the dinucleotide as described[37] gave pdCpA derivative **19**. Enzymatic ligation of the aminoacyl-pdCpA derivative to an abbreviated suppressor tRNA, i.e. a tRNA lacking the 3′-terminal CA moiety, provided full length protected aminoacyl-tRNA **I**. Subsequent treatment with iodine should result in the



Scheme 4.

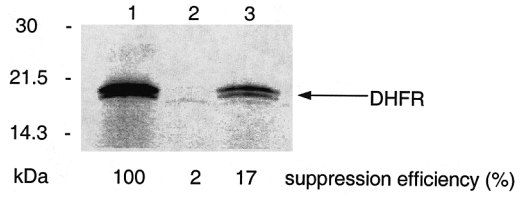


Figure 2. Elaboration of DHFR by suppression of an UAG codon at position 10 using suppressor tRNA **I**. Protein synthesis was carried out in the presence of ^{35}S-methionine using a rabbit reticulocyte lysate, mRNA containing UAG at codon position 10 and deblocked valyl-tRNA$_{CUA}$ derived from **I**. Following incubation at 30°C for 1.5 h, the reaction mixture was analyzed on a SDS-polyacrylamide gel; lane 1, wild type, no suppressor tRNA; lane 2, suppressor tRNA **I** before iodine treatment; lane 3, suppressor tRNA **I** after iodine treatment. The minor band migrating ahead of the full length protein was due to initiation of DHFR synthesis at an internal methionine codon.

removal of the pentenoyl derivative (presumably via the imidolactone intermediate shown in the Scheme), affording unprotected valyl-tRNA **II**. To show that the bulky pentenoyl group could indeed be removed from the aminoacyl-tRNA by treatment with iodine, protected valyl-tRNA **I** and an iodine-treated sample were used in an in vitro protein synthesizing system. The protein synthesizing system employed rabbit reticulocyte lysate and an mRNA for dihydrofolate reductase (DHFR) containing a nonsense (UAG) codon at position 10.[38] The results are shown in Fig. 2. Before treatment with iodine, use of aminoacyl-tRNA **I** did not result in the synthesis of full length DHFR (lane 2). As shown in lane 3, after treatment with iodine a product was obtained with the same molecular weight as wild type DHFR. This indicated that the bulky pentenoyl derivative had been removed from the aminoacyl-tRNA and that valine was incorporated into the protein. Comparison with an incubation mixture containing valyl-tRNA derived from *N*-(4-pentenoyl)-(*S*)-valyl-tRNA showed that the same product was obtained in both.

Conclusion

Substituted 2-amino-4-pentenoic acid derivatives **2** and **13** are useful tools for the protection and resolution of racemic amino acids. Although the examples shown here involved naturally occurring amino acids, the method should be readily applicable to unnatural amino acids, since the side chain moiety appears to have little effect on the separation of the derived diastereomers. The ability to remove the derivatizing reagent under mild conditions after the separation makes these compounds especially attractive for the large scale synthesis of optically pure unnatural amino acids and the synthesis of misacylated transfer RNAs activated with optically pure amino acids.

Experimental

General methods

Melting points were taken on a Thomas Hoover melting point apparatus and are not corrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a General Electric QE-300 spectrometer. Chemical shifts are expressed relative to $CHCl_3$ (7.26 ppm). Moisture sensitive reactions were conducted under argon in oven-dried glassware. All chemical reagents were purchased from Aldrich Chemicals or Sigma Chemicals and used without further purification. Acetonitrile and dichloromethane were distilled from CaH_2; DMF was distilled from CaH_2 under diminished pressure. Analytical thin layer chromatography was performed on 60 F_{254} (E. Merck) plates and visualized using iodine. Flash chromatography was performed using 230–400 mesh silica gel. Elemental analyses were carried out by Atlantic Microlab, Inc., Norcross, GA. High resolution mass spectra were recorded at the Nebraska Center for Mass Spectrometry.

[^{35}S] Methionine (1000 Ci/mmol, 10 μCi/μL) was purchased from Amersham Corporation. Nuclease-treated rabbit reticulocyte lysate system was obtained from Promega Corporation. Restriction endonucleases and T4 RNA ligase were obtained from New England Biolabs. Kits for plasmid isolation were purchased from PGC Scientific. AmpliScribe transcription kits were purchased from Epicentre Technologies. Acrylamide, *N*,*N*-methylenebisacrylamide, urea and Tris base were obtained from Sigma Chemicals.

Plasmid DNAs were isolated using a JetStar plasmid midi kit (PGC Scientific) according to the protocol provided. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out using the standard Laemmli procedure.[39] Gels were visualized and quantified utilizing a Molecular Dynamics 400E PhosphorImager equipped with ImageQuant version 5.0 software. UV spectral measurements were made using a Perkin–Elmer Lambda 20 UV/VIS spectrometer. Run-off transcription of abbreviated suppressor tRNA$_{CUA}$ (-CA) and DHFR mRNAs was carried out as described.[38] All procedures involving water employed distilled, deionized water from a Milli-Q system.

***N*-Phenylfluorenyl-(*S*)-2-amino-4-pentenoic acid (2).** To a suspension of 300 mg (2.61 mmol) of (*S*)-2-amino-4-pentenoic acid (**1**) in 5 mL of CH_2Cl_2 was added 364 μL (2.87 mmol) of trimethylsilyl chloride. After stirring at room temperature for 2 h, 0.8 mL (5.74 mmol) of Et_3N was added. The reaction mixture was stirred for 15 min and 563 mg (1.70 mmol) of $Pb(NO_3)_2$ was added, followed by 1.05 g (3.26 mmol) of 9-bromo-9-phenylfluorene. After stirring at room temperature for 2 days, the reaction mixture was filtered through Celite and concentrated under diminished pressure. The residue was dissolved in 40 mL of CH_2Cl_2 and washed with two 40-mL portions of 5% citric acid and with 40 mL of brine. The organic phase was dried ($MgSO_4$) and concentrated. The crude product was applied to a silica gel column (25×2 cm); elution with 2% MeOH in CH_2Cl_2 provided *N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoic acid (**2**) as a colorless solid: yield 775 mg (84%); mp 63–64°C; silica gel TLC R_f 0.30 (3% MeOH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 1.94–2.03 (m, 1H), 2.40–2.49 (m, 1H), 2.70 (t, 1H, J=5.4 Hz), 5.12–5.23 (m, 2H), 5.43–5.54 (m, 1H) and 7.19–7.76 (m, 13H); ^{13}C NMR ($CDCl_3$) δ 38.5, 55.6, 73.3, 120.2, 120.8, 125.5, 126.2, 126.4, 128.2, 128.6, 128.7,129.1, 129.5, 129.6, 133.6,

141.0, 141.4, 143.9, 147.6, 148.9 and 177.1; mass spectrum (CI, methane) *m/z* 356 $(M+H)^+$. Anal. Calcd for $C_{24}H_{21}NO_2$: C, 81.10; H, 5.96. Found: C, 80.84; H, 6.35.

***N*-(*N*-Phenylfluorenyl-(*S*)-2-amino-4-pentenoyl)-(*R*,*S*)-valine methyl ester (5 and 6).** To a cooled (0–5°C) solution containing 100 mg (0.28 mmol) of *N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoic acid (**2**), 47 mg (0.28 mmol) of (*R*,*S*)-valine methyl ester hydrochloride (**3**) and 62 μL (0.56 mmol) of *N*-methylmorpholine in 5 mL of CH_2Cl_2 was added 42 mg (0.31 mmol) of hydroxybenzotriazole, followed by 64 mg (0.31 mmol) of DCC. After stirring at room temperature for 16 h, the reaction mixture was filtered, diluted with 25 mL of CH_2Cl_2 and washed successively with two 25-mL portions of 0.5N HCl, two 25-mL portions of saturated aqueous $NaHCO_3$ and 25 mL of brine. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The crude product was applied to a silica gel column (25×2 cm); elution with 2% MeOH in CH_2Cl_2 gave *N*-(*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoyl)-(*R*,*S*)-valine methyl ester as a colorless oil (1:1 mixture of diastereomers **5** and **6**): yield 121 mg (92%); ^{1}H NMR δ 0.92–0.98 (m, 6H), 1.78–1.95 (m, 1H), 2.11–2.20 (m, 1H), 2.57–2.65 (m, 2H), 3.77 (s, 1.5H), 3.81 (s, 1.5H), 4.32–4.41 (m, 1H), 5.06–5.14 (m, 2H), 5.38–5.54 (m, 1H) and 7.03–7.73 (m, 13H); mass spectrum (FAB) *m/z* 469.2472 $(M+H)^+$ ($C_{30}H_{33}N_2O_3$ requires 469.2491).

***N*-(*N*-Phenylfluorenyl-(*S*)-2-amino-4-pentenoyl)-(*R*,*S*)-phenylalanine methyl ester (7 and 8).** In the same fashion as described for compounds **5** and **6**, compounds **7** and **8** were prepared as a 1:1 mixture (obtained as a colorless oil): yield 139 mg (96%); ^{1}H NMR δ 1.82–1.89 (m, 1H), 2.34–2.60 (m, 2H), 3.00–3.18 (m, 2H), 3.73 (s, 1.5H), 3.77 (s, 1.5H), 4.51–4.56 (m, 0.5 H), 4.71–4.77 (m, 0.5H), 5.01–5.14 (m, 2H), 5.23–5.31 (m, 0.5H), 5.49–5.55 (m, 0.5H) and 6.90–7.73 (m, 18H); mass spectrum (FAB) *m/z* 517.2502 $(M+H)^+$ ($C_{34}H_{33}N_2O_3$ requires 517.2491).

HPLC analysis of diastereomers 5 and 6 and diastereomers 7 and 8

The mixture of diastereomers **5** and **6** was dissolved in 4 mL of 4:1 hexanes–ethyl acetate and analyzed by HPLC. A 10-μL aliquot was diluted with 70 μL of 4:1 hexanes–ethyl acetate and injected on a Partisil silica column (250×10 mm). Elution was performed with a gradient of 10–50% ethyl acetate in hexanes over a period of 45 min at a flow rate of 3.5 mL/min. Detection was at 275 nm. Two major peaks were found at 19.2 and 20.3 min in approximately 1:1 ratio. Analysis of the mixture of diastereomers **7** and **8** under the same conditions resulted in two major peaks at 27.1 and 28.0 min in a 1:1 ratio.

2-Cyanoethyl *N*-butyloxycarbonyl-(*S*)-2-amino-4-pentenoate (9). To a cooled (0–5°C) solution containing 1.0 g (8.67 mmol) of (*S*)-2-amino-4-pentenoic acid (**1**) in 30 mL of dioxane and 30 mL of 1 M $NaHCO_3$ was added 2.10 g (9.62 mmol) of di-*t*-butyl pyrocarbonate. The reaction mixture was stirred for 24 h at room temperature. The mixture was diluted with 75 mL of H_2O, washed with two 60-mL portions of ether, acidified to pH 1 with 1N H_2SO_4 and extracted with four 30-mL portions of CH_2Cl_2. The combined organic extract was dried ($MgSO_4$) and concentrated. The residue was dissolved in 30 mL of CH_2Cl_2 and 0.75 mL (10.8 mmol) of 3-hydroxypropionitrile and 122 mg (1.0 mmol) of 4,4-(dimethylamino)pyridine (DMAP) were added. The mixture was cooled in an ice bath and 1.97 g (9.54 mmol) of DCC was added. The reaction was stirred for 20 h and filtered. The filtrate was diluted with 25 mL of CH_2Cl_2 and washed successively with two 50-mL portions of 0.5N HCl, two 50-mL portions of saturated aqueous $NaHCO_3$ and 50 mL of brine. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was applied to a silica gel column (15×3 cm): elution with 2% MeOH in CH_2Cl_2 afforded 2-cyanoethyl *N*-butyloxycarbonyl-(*S*)-2-amino-4-pentenoate (**9**) as a colorless oil that solidified upon standing: yield 1.55 mg (67%); mp 58–59°C; ^{1}H NMR ($CDCl_3$) δ 1.45 (s, 9H), 2.48–2.57 (m, 2H), 2.72 (t, 2H, *J*=6 Hz), 4.23–4.38 (m, 3H), 4.95 (br, 1H), 5.15–5.21 (m, 2H) and 5.65–5.76 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 18.4, 28.7, 29.3, 53.4, 59.9, 80.5, 117.1, 119.9, 132.5, 155.6 and 172.1; mass spectrum (CI, methane) *m/z* 269 $(M+H)^+$. Anal. Calcd for $C_{13}H_{20}N_2O_4$: C, 58.19; H, 7.51. Found: C, 58.29; H, 7.55.

2-Cyanoethyl (*S*)-2-amino-4-pentenoate (10). To a flask containing 1.47 g (5.48 mmol) of 2-cyanoethyl *N*-butyloxycarbonyl-(*S*)-2-amino-4-pentenoate (**9**) was added 14 mL of a solution of 4N HCl in dioxane. After stirring at room temperature for 2 h the solvent was removed under diminished pressure. The residue was crystallized from ethanol–ether to afford 2-cyanoethyl (*S*)-2-amino-4-pentenoate (**10**) as the hydrochloride: yield 947 mg (84%); ^{1}H NMR ($CDCl_3$–MeOD) δ 2.63–2.72 (m, 2H), 2.77 (t, 2H, *J*=6 Hz), 4.06 (t, 1H, *J*=6 Hz), 4.31–4.43 (m, 2H), 5.21–5.28 (m, 2H) and 5.63–5.76 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 17.5, 34.7, 52.6, 61.3, 117.7, 121.0, 130.6 and 168.8. Anal. Calcd for $C_8H_{13}N_2O_2Cl$: C, 46.95; H, 6.40. Found: C, 47.05; H, 6.35.

2-Cyanoethyl *N*-benzyl-(*S*)-2-amino-4-pentenoate (11). To a solution containing 780 mg (3.81 mmol) of 2-cyanoethyl (*S*)-2-amino-4-pentenoate hydrochloride (**10**) in 20 mL of freshly distilled MeOH was added 360 mg (5.72 mmol) of $NaBH_3CN$, followed by 0.39 mL (3.81 mmol) of benzaldehyde. After stirring at room temperature for 19 h the mixture was cooled in an ice bath, acidified with conc. HCl and stirred for an additional hour. The solvent was removed under diminished pressure. The residue was dissolved in 20 mL of H_2O and the pH adjusted to 10 with saturated Na_2CO_3. The aqueous solution was extracted with four 25-mL portions of CH_2Cl_2 and the combined extract was dried ($MgSO_4$) and concentrated. The residue was applied to a silica gel column (35×2 cm): elution with 3% MeOH in CH_2Cl_2 gave 2-cyanoethyl *N*-benzyl-(*S*)-2-amino-4-pentenoate (**11**) as a colorless oil: yield 715 mg (73%); ^{1}H NMR ($CDCl_3$) δ 2.48 (t, 2H, *J*=7 Hz), 2.70 (t, 2H, *J*=7 Hz), 3.44 (t, 1H, *J*=7 Hz), 3.72 (d, 1H, *J*=13 Hz), 3.86 (d, 1H, *J*=13 Hz), 4.31 (t, 2H, *J*=6 Hz), 5.10–5.17 (m, 2H), 5.70–5.84 (m, 1H) and 7.24–7.38 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 18.6, 38.2, 52.5, 59.3, 60.6, 117.3, 118.9, 127.7, 128.8, 129.0, 129.3, 129.4, 133.8, 140.0 and 174.6; mass spectrum (CI, methane) *m/z* 259 $(M+H)^+$. Anal. Calcd for $C_{15}H_{18}N_2O_2$: C, 69.74; H, 7.02. Found: C, 69.69; H, 7.00.

2-Cyanoethyl *N*-benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoate (12). To a solution containing 715 mg (2.77 mmol) of 2-cyanoethyl *N*-benzyl-(*S*)-2-amino-4-pentenoate (**11**) in 15 mL of anhydrous CH_3CN was added 1.11 g (3.46 mmol) of 9-bromo-9-phenylfluorene, followed by 647 mg (3.05 mmol) of K_3PO_4 and 734 mg (2.22 mmol) of $Pb(NO_3)_2$. The reaction mixture was stirred for 17 h at room temperature, filtered through Celite and concentrated under diminished pressure. The residue was dissolved in 30 mL of CH_2Cl_2 and washed with two 30-mL portions of 5% citric acid and with 30 mL of brine. The organic phase was dried ($MgSO_4$) and concentrated. The crude product was applied to a silica gel column (20×2 cm): elution with 4:1 hexanes–EtOAc gave 2-cyanoethyl *N*-benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoate (**12**) as a colorless foam: yield 1.15 g (83%); ^{1}H NMR ($CDCl_3$) δ 1.61–1.73 (m, 1H), 2.21–2.37 (m, 1H), 2.43 (t, 2H, J=6 Hz), 3.26–3.30 (m, 1H), 3.52–3.60 (m, 1H), 3.73–3.80 (m, 1H), 4.05 (d, 1H, J=14 Hz), 4.40 (d, 1H, J=14 Hz), 4.48 (d, 1H, J=17 Hz), 4.66 (d, 1H, J=12 Hz), 5.18–5.32 (m, 1H) and 7.18–7.83 (m, 18H); ^{13}C NMR ($CDCl_3$) δ 18.2, 34.0, 51.6, 59.0, 61.0, 80.1, 117.2, 117.7, 120.4, 121.1, 127.1, 127.6, 127.8, 128.0, 128.2, 128.2, 128.5, 129.0, 129.3, 130.0, 135.9, 140.0, 140.7, 141.3, 143.9, 147.3, 148.0 and 172.7; mass spectrum (CI, methane) m/z 499 $(M+H)^+$. Anal. Calcd for $C_{34}H_{30}N_2O_2$: C, 81.90; H, 6.06. Found: C, 81.67; H, 6.25.

***N*-Benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoic acid (13).** To a solution of 1.1 g (2.21 mmol) of 2-cyanoethyl *N*-benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoate (**12**) in 10 mL of THF and 5 mL of MeOH was added a solution of 3.65 g (22.1 mmol) of $K_2CO_3{\cdot}1.5H_2O$ in 15 mL of H_2O. After stirring at room temperature for 14 h the solvent was removed under diminished pressure. The residue was dissolved in 25 mL of CH_2Cl_2 and washed with two 25-mL portions of 5% citric acid and with 25 mL of brine. The organic phase was dried ($MgSO_4$) and concentrated to afford *N*-benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoic acid (**13**) as a colorless foam: yield 0.91 g (92%); ^{1}H NMR ($CDCl_3$) δ 1.63–1.69 (m, 1H), 2.37–2.47 (m, 1H), 3.08–3.12 (m, 1H), 4.00 (d, 1H, J=14 Hz), 4.38 (d, 1H, J=13 Hz), 4.40 (d, 1H, J=19 Hz), 4.63 (d, 1H, J=10 Hz), 5.29–5.40 (m, 1H) and 7.19–7.82 (m, 18H); ^{13}C NMR ($CDCl_3$) δ 33.4, 51.8, 61.3, 80.1, 116.8, 120.6, 121.2, 127.0, 127.6, 127.7, 128.0, 128.1, 128.4, 128.5, 128.7, 129.2, 129.4, 129.5, 130.0, 136.4, 138.9, 140.6, 141.6, 143.6, 146.6, 147.8 and 177.6; mass spectrum (FAB) m/z 446.2118 $(M+H)^+$ ($C_{31}H_{28}NO_2$ requires 446.2120).

***N*-(*N*-Benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoyl)-(*R*)-valine methyl ester and *N*-(*N*-benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoyl)-(*S*)-valine methyl ester (14 and 15).** To a solution containing 200 mg (0.45 mmol) of *N*-benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoic acid (**13**) in 3 mL of CH_2Cl_2 was added a solution of 92 mg (0.56 mmol) of (*R*,*S*)-valine methyl ester hydrochloride (**3**) in 1 mL of pyridine, followed by 116 mg (0.56 mmol) of DCC. After stirring at room temperature for 18 h the solvent was removed under diminished pressure. The residue was dissolved in 25 mL of CH_2Cl_2, filtered, and washed with two 25-mL portions of 0.5N HCl, two 25-mL portions of saturated aqueous $NaHCO_3$ and with 25 mL of brine. The organic phase was dried ($MgSO_4$) and concentrated. The mixture of diastereomers **14** and **15** was separated by silica gel column chromatography (30×2 cm), elution with 15–20% ethyl acetate in hexanes. *N*-(*N*-benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoyl)-(*R*)-valine methyl ester (**14**) was isolated as an oil: yield 31 mg (12%); ^{1}H NMR ($CDCl_3$) δ 0.67 (d, 3H, J=7 Hz), 0.78 (d, 3H, J=7 Hz), 1.37–1.50 (m, 1H), 1.89–1.98 (m, 1H), 2.44–2.58 (m, 1H), 2.89 (d, 1H, J=10 Hz), 3.66–3.70 (m, 1H), 3.93 (d, 1H, J=14 Hz), 3.95 (s, 3H), 4.27–4.34 (m, 2H), 4.56 (d, 1H, J=10 Hz), 5.28–5.36 (m, 1H) and 7.11–7.82 (m, 18H); ^{13}C NMR ($CDCl_3$) δ 18.5, 18.7, 31.6, 32.2, 51.4, 52.5, 57.6, 61.2, 80.0, 115.6, 120.6, 121.4, 125.7, 127.6, 127.8, 127.8, 127.9, 128.1, 128.3, 128.6, 129.3, 130.8, 138.0, 138.3, 140.4, 141.6, 143.5, 146.6, 148.9, 172.9 and 173.1; mass spectrum (FAB) m/z 559.2945 $(M+H)^+$ ($C_{37}H_{39}N_2O_3$ requires 559.2961).

N-(*N*-benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoyl)-(*S*)-valine methyl ester (**15**) was isolated as an oil: yield 68 mg (27%); ^{1}H NMR ($CDCl_3$) δ 0.68 (d, 3H, J=7 Hz), 0.73 (d, 3H, J=7 Hz), 1.51–1.60 (m, 1H), 1.84–1.93 (m, 1H), 2.42–2.52 (m, 1H), 3.06 (dd, 1H, J=9 Hz, J=4 Hz), 3.75 (s, 3H), 4.00–4.04 (m, 1H), 4.06 (d, 1H, J=15 Hz), 4.28 (d, 1H, J=14 Hz), 4.49 (d, 1H, J=17 Hz), 4.63 (d, 1H, J=10 Hz), 5.27–5.38 (m, 1H) and 7.13–7.78 (m, 18H); ^{13}C NMR ($CDCl_3$) δ 19.1, 19.5, 32.1, 33.8, 51.5, 52.3, 58.0, 62.6, 80.7, 115.8, 120.6, 121.2, 126.4, 127.7, 127.8, 127.9, 128.3, 128.4, 129.0, 129.2, 129.4, 130.1, 137.8, 140.0, 140.4, 141.7, 143.8, 146.6, 148.1, 173.0 and 173.7; mass spectrum (FAB) m/z 559.2949 $(M+H)^+$ ($C_{37}H_{39}N_2O_3$ requires 559.2961).

***N*-(*N*-Benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoyl)-(*R*)-phenylalanine methyl ester and *N*-(*N*-benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoyl)-(*S*)-phenylalanine methyl ester (16 and 17).** Compounds **16** and **17** were prepared in the same fashion as described for compounds **14** and **15**. *N*-(*N*-benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoyl)-(*R*)-phenylalanine methyl ester (**16**): yield 51 mg (19%); ^{1}H NMR ($CDCl_3$) δ 1.41–1.48 (m, 1H), 2.45–2.60 (m, 1H), 2.82–3.00 (m, 3H), 3.75–3.93 (m, 3H), 3.86 (s, 3H), 4.26–4.36 (m, 2H), 4.59 (d, 1H, J=8 Hz), 5.31–5.41 (m, 1H) and 6.83–7.82 (m, 23H); ^{13}C NMR ($CDCl_3$) δ 31.5, 38.2, 51.5, 52.7, 54.2, 61.5, 80.0, 115.6, 120.6, 121.4, 125.5, 127.1, 127.6, 127.7, 127.9, 128.1, 128.2, 129.0, 129.2, 129.4, 129.9, 130.9, 136.9, 138.0, 138.3, 140.2, 141.7, 143.6, 146.6, 148.6, 172.7 and 173.2; mass spectrum (FAB) m/z 607.2962 $(M+H)^+$ ($C_{41}H_{38}N_2O_3$ requires 607.2961).

N-(*N*-benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoyl)-(*S*)-phenylalanine methyl ester (**17**): yield 78 mg (29%); ^{1}H NMR ($CDCl_3$) δ 1.66–173 (m, 1H), 2.41–2.65 (m, 3H), 2.97 (dd, 1H, J=9Hz, 2 Hz), 3.57 (s, 3H), 4.00 (d, 1H, J=13 Hz), 4.09–4.40 (m, 3H), 4.59 (d, 1H, J=10 Hz), 5.26–5.37 (m, 1H) and 6.98–7.78 (m, 23H); ^{13}C NMR ($CDCl_3$) δ 32.9, 38.6, 51.3, 52.4, 53.9, 61.8, 80.4, 115.7, 120.6, 121.3, 125.9, 127.6, 127.9, 128.0, 128.3, 128.6, 128.9, 129.1, 129.2, 129.4, 130.4, 137.1, 137.8, 139.5, 140.3, 141.6, 143.6, 146.7, 148.4, 173.0 and 173.3; mass spectrum (FAB) m/z 607.2933 $(M+H)^+$ ($C_{41}H_{38}N_2O_3$ requires 607.2961).

HPLC analysis of diastereomers 14 and 15 and diastereomers 16 and 17

A small portion of the crude reaction mixture of diastereomers **14** and **15** was analyzed by HPLC. A 10-μL aliquot was diluted with 70 μL of 4:1 hexanes–ethyl acetate and injected on a Partisil silica column (250×10 mm). Elution was performed with a gradient of 5–25% ethyl acetate in hexane over a period of 30 min at a flow rate of 3.5 mL/min. Detection was at 275 nm. Two product peaks were found at 22.3 and 28.0 min in approximately 1:2.5 ratio. Analysis of the mixture of diastereomers **16** and **17** under the same conditions resulted in two major peaks at 28.6 and 34.5 min in a 1:2 ratio.

***N*-(*N*-Benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoyl)-(*S*)-valine cyanomethyl ester (18).** To a solution containing 48 mg (0.086 mmol) of *N*-(*N*-benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoyl)-(*S*)-valine methyl ester (**15**) in 2 mL of THF was added a solution of 18 mg (0.43 mmol) LiOH monohydrate in 1 mL of H_2O. After stirring at room temperature for 20 h, the reaction mixture was diluted with 20 mL of ethyl acetate and washed with two 20-mL portions of 1N $NaHSO_4$. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was applied to a silica gel column (20×1 cm); elution with 4% MeOH and 1% AcOH in CH_2Cl_2 gave the free acid as a colorless oil. The residue was dissolved in 1 mL of CH_3CN and 60 μL (0.43 mmol) of Et_3N was added, followed by 27 μL (0.43 mmol) of chloroacetonitrile. After stirring at room temperature for 17 h the reaction mixture was diluted with 10 mL of ethyl acetate and washed with two 10-mL portions of 1N $NaHSO_4$ and then with 10 mL of brine. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was applied to a silica gel column (20×1 cm); elution with 2:3 ethyl acetate–hexanes gave *N*-(*N*-benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoyl)-(*S*)-valine cyanomethyl ester (**18**) as a colorless solid: yield 38 mg (76%); mp 77–78 °C; ^{1}H NMR ($CDCl_3$) δ 0.82 (d, 3H, *J*=6.9 Hz), 0.87 (d, 3H, *J*=6.9 Hz), 1.58–1.72 (m, 1H), 1.75–1.84 (m, 1H), 2.42–2.56 (m, 1H), 3.06 (dd, 1H, *J*=9.2 Hz, *J*=2.5 Hz), 3.86–3.90 (m, 1H), 4.04 (d, 1H, *J*=13.5 Hz), 4.29 (d, 1H, *J*=13.5 Hz), 4.44 (d, 1H, *J*=17.3 Hz), 4.62 (d, 1H, *J*=15.8 Hz), 4.63 (d, 1H, *J*=13.0 Hz), 4.76 (d, 1H, *J*=15.4 Hz), 5.26–5.34 (m, 1H), 6.90 (d, 1H, *J*=8.8 Hz) and 7.19–7.79 (m, 18H). Anal. Calcd for $C_{38}H_{37}N_3O_3$: C, 78.19; H, 6.39. Found: C,77.83; H, 6.65.

***N*-(*N*-Benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoyl)-(*S*)-valine pdCpA ester (19).** To a conical vial containing 5 mg (15.7 μmol) of *N*-(*N*-benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoyl)-(*S*)-valine cyanomethyl ester (**18**) was added a solution of 5 mg (3.7 μmol) of the tris(tetrabutylammonium) salt of pdCpA[40] in 50 μL of DMF. The reaction mixture was stirred at room temperature for 2 h. A 5-μL aliquot of the mixture was diluted with 45 μL of 1:2 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). After 3 h 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 semi-preparative C_{18} reversed phase column. After lyophilization of the appropriate fractions *N*-(*N*-benzyl-*N*-phenylfluorenyl-(*S*)-2-amino-4-pentenoyl)-(*S*)-valine pdCpA ester (**19**) was obtained as a colorless solid: yield 2.2 mg (51%); mass spectrum (FAB) *m*/*z* 1163.3760 $(M+H)^+$ ($C_{55}H_{61}N_{10}O_{15}P_2$ requires 1163.3793).

Synthesis and deprotection of aminoacyl-tRNA I

Transfer RNA activation reactions were carried out in 100 μL (total volume) of 50 mM Na Hepes buffer, pH 7.5, containing 0.5 mM ATP, 15 mM $MgCl_2$, 50 μg (2 nmol) of suppressor $tRNA_{CUA}$ transcript lacking the 3′-terminal pCpA moiety, 1.0 A_{260} unit (40 nmol) of pdCpA derivative **19**, 20% dimethyl sulfoxide (v/v), and 200 units of T4 RNA ligase. After incubation at 37°C for 45 min, the reaction was quenched by the addition of 0.1 vol. of 3 M sodium acetate, pH 5.2, and the tRNA was precipitated with 2.5 vol. of ethanol, collected by centrifugation, washed with 70% ethanol, and dried. The product was redissolved in 1 mM KOAc to a final concentration of 3 μg/μL. The efficiency of ligation was estimated by gel electrophoresis (pH 5.0).[41]

Deprotection was accomplished by admixture of 0.25 vol. of 40 mM I_2 in 4:1 THF–H_2O. The combined solution was maintained at 25°C for 60 min, and aminoacyl-tRNA **II** was recovered by centrifugation following successive additions of 0.1 vol. of 3 M NaOAc, pH 5.2, and 2.5 vol. of cold ethanol. The tRNA pellet was washed with 70% ethanol, dried, and then dissolved in RNase-free water to a final concentration of 3 μg/μL and used in in vitro suppression in a protein biosynthesis experiment immediately following deprotection.

In vitro protein biosynthesis

In vitro protein synthesis reactions contained per 100 μL: 70 μL of methionine-depleted, nuclease-treated rabbit reticulocyte lysate, 80 μCi of [^{35}S]-*S*-methionine (1000 Ci/mmol), 2 μL of a solution 1 mM in the 19 amino acids used in ribosomal protein synthesis (but lacking methionine), 10 μg of the DHFR mRNA, and 25 μg (~1.0 nmol) of protected misacylated $tRNA_{CUA}$ **I** or deprotected misacylated $tRNA_{CUA}$ **II**. The reaction mixture was incubated at 30°C for 1.5 h and quenched by cooling to 0°C. Aliquots (typically 1 μL) were utilized for analysis by 15% SDS–PAGE. After electrophoresis, the gel was fixed in 40% methanol-10% acetic acid and dried. Autoradiography of the gels was carried out to determine the location of ^{35}S-labeled protein; quantification of the bands was carried out using a phosphorimager.

Acknowledgements

This work was supported by NIH Research Grant CA77359 awarded by the National Cancer Institute.

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