Misacylated Transfer RNAs Having a Chemically Removable Protecting Group

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The 4-pentenoyl group and a number of derivatives have been studied as protecting groups for N^{α} of the aminoacyl moiety in misacylated tRNAs. The unsubstituted 4-pentenoyl group itself was found to function as efficiently as any of the derivatives studied. Four different *N*-(4-pentenoyl)-aminoacyl-$tRNA_{CUA}$s were prepared and shown to undergo deprotection readily upon admixture of aqueous iodine; the derived misacylated tRNAs all functioned well as suppressors of a nonsense codon in an in vitro protein biosynthesizing system. Also prepared were four N^{α}-(4-pentenoyl)-aspartyl-$tRNA_{CUA}$s that were protected on the side chain carboxylate as the nitroveratryl ester. Following treatment with aqueous iodine, the misacylated suppressor tRNAs incorporated the aspartate derivatives into position 27 of dihydrofolate reductase by suppression of a UAG codon in the mRNA. The suppression yields were significantly better than those obtained when side chain protection was absent. The resulting "caged proteins" were inactive, but full catalytic potential was restored by irradiation under conditions sufficient to effect deprotection of the side chain carboxylate moiety.

Since the pioneering work of the Lipmann laboratory which demonstrated that a tRNA bearing a noncognate amino acid could be employed for the elaboration of a modified protein,[1] there has been great interest in the elaboration of misacylated tRNAs. Initial efforts focused on the chemical modification of tRNAs bearing a cognate amino acid,[1,2] but the Hecht laboratory described a potentially general strategy in which tRNAs lacking the 3′-terminal dinucleotide, which is always pCpA, were converted to misacylated tRNAs by incubation with an aminoacylated pCpA derivative in the presence of T4 RNA ligase.[3]

Several improvements in the methodology have greatly facilitated the preparation of misacylated tRNAs, including the use of aminoacylated pdCpA derivatives[4] and in vitro RNA transcripts elaborated without the normal 3′-terminal CA moiety.[5] The greatest challenge, however, has involved the aminoacyl moiety itself, which is not completely stable under the conditions of the ligation reaction; to the extent that hydrolysis of this group occurs, the product of the ligation reaction is a full length, deacylated tRNA.

Strategies for obtaining ligated tRNAs bearing the aminoacyl moiety of interest have included the use of unprotected aminoacyl-pCpA derivatives in a ligation reaction of limited duration[6] and the use of protecting groups for N^{α} of the amino acid. The latter afford increased stability of the aminoacyl moiety toward hydrolysis, but their subsequent removal without concomitant deacylation of the amino acid from the misacylated tRNA has proved problematic. Until recently, the most successful protecting groups had been the pyroglutamyl[3i] and nitroveratryloxycarbonyl (NVOC)[7] groups; these can be removed from the derived misacylated tRNAs via the agency of an enzyme (pyroglutamate aminopeptidase) and light, respectively. Chemical deprotection strategies have not been used extensively.[8]

On the basis of the work of Fraser-Reid and coworkers[9] who studied the protection of simple amines, we have recently described the use of the *N*-pentenoyl group as a suitable aminoacyl protecting group for the preparation of misacylated tRNAs.[10] This group can be removed under very mild chemical conditions by treatment with aqueous iodine, presumably via an iodolactonization reaction (Scheme 1). That this reagent is

(1) Chapeville, F.; Lipmann, F.; von Ehrenstein, G.; Weisblum, B.; Roy, W. J.; Benzer, S. *Proc. Natl. Acad. Sci. U.S.A.* **1962**, *48*, 1086.

(2) (a) Fahnestock, S.; Rich, A. *Nature, New Biol.* **1971**, *229*, 8. (b) Fahnestock, S.; Rich, A. *Science* **1971**, *173*, 340. (c) Johnson, A. E.; Woodward, W. R.; Herbert, E.; Menninger, J. R. *Biochemistry* **1976**, *15*, 569.

(3) (a) Hecht, S. M.; Alford, B. L.; Kuroda, Y.; Kitano, S. *J. Biol. Chem.* **1978**, *253*, 4517. (b) Pezzuto, J. M.; Hecht, S. M. *J. Biol. Chem.* **1980**, *255*, 865. (c) Heckler, T. G.; Zama, Y.; Naka, T.; Hecht, S. M. *J. Biol. Chem.* **1983**, *258*, 4492. (d) Heckler, T. G.; Chang, L. H.; Zama, Y.; Naka, T.; Hecht, S. M. *Tetrahedron* **1984**, *40*, 87. (e) Heckler, T. G.; Chang, L. H.; Zama, Y.; Naka, T.; Chorghade, M. S.; Hecht, S. M. *Biochemistry* **1984**, *23*, 1468. (f) Roesser, J. R.; Chorghade, M. S.; Hecht, S. M. *Biochemistry* **1986**, *25*, 6361. (g) Payne, R. C.; Nichols, B. P.; Hecht, S. M. *Biochemistry* **1987**, *26*, 3197. (h) Heckler, T. G.; Roesser, J. R.; Cheng, X.; Chang, P.-I.; Hecht, S. M. *Biochemistry* **1988**, *27*, 7254. (i) Roesser, J. R.; Xu, C.; Payne, R. C.; Surratt, C. K.; Hecht, S. M. *Biochemistry* **1989**, *28*, 5185. (j) Hecht, S. M. *Acc. Chem. Res.* **1992**, *25*, 545.

(4) Robertson, S. A.; Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Nucleic Acids Res.* **1989**, *17*, 9649.

(5) Noren, C. J.; Anthony-Cahill, S. J.; Suich, D. J.; Noren, K. A.; Griffith, M. C.; Schultz, P. G. *Nucleic Acids Res.* **1990**, *18*, 83.

(6) Baldini, G.; Martoglio, B.; Schachenmann, A.; Zugliani, C.; Brunner, J. *Biochemistry* **1988**, *27*, 7951.

(7) Robertson, S. A.; Ellman, J. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 2722.

(8) See, however, ref 3a and (a) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182. (b) Mendel, D.; Ellman, J. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 2758.

(9) (a) Debenham, J. S.; Madsen, R.; Roberts, C.; Fraser-Reid, B. *J. Am. Chem. Soc.* **1995**, *117*, 3302. (b) Madsen, R.; Roberts, C.; Fraser-Reid, B. *J. Org. Chem.* **1995**, *60*, 7920.

(10) Preliminary results on the preparation of *N*-(4-pentenoyl)valyl-tRNA have been published: Lodder, M.; Golovine, S.; Hecht, S. M. *J. Org. Chem.* **1997**, *62*, 778.

(11) Letsinger, R. L.; Lunsford, W. B. *J. Am. Chem. Soc.* **1976**, *98*, 3655.

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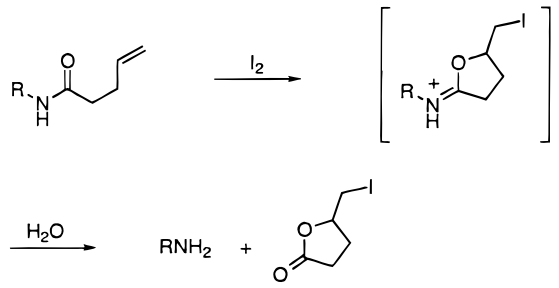
Scheme 1. Putative Mechanism of Deblocking of Pentenoyl Derivatives via an Iodolactonization Reaction



employed in the chemical synthesis of nucleic acids[11] suggested that its use would be compatible with the integrity of the derived misacylated tRNAs.

Presently, we describe the investigation of nine different *N*-pentenoyl derivatives as potential protecting groups for misacylated tRNAs, and provide a complete description of the preparation and assay of four misacylated susppressor tRNAs. Also described is the elaboration of four suppressor tRNAs misacylated with aspartic acid (analogues) bearing a protecting group on the side chain carboxylate whose removal obtains under conditions orthogonal to those employed for removal of the pentenoyl group. Side chain protection was demonstrated to substantially increase the suppression of nonsense codons during translation and provided access to caged analogues[12] of dihydrofolate reductase (DHFR); subsequent deblocking of the caged proteins afforded catalytically active analogues of DHFR.

Results

Synthesis and Deprotection of *N*-Protected Valine Benzyl Esters. (*S*)-Valine benzyl ester was used as a model compound for investigation of protecting groups that could be removed via an iodolactionization reaction. The modified pentenoic acids were attached to (*S*)-valine benzyl ester *p*-toluenesulfonate[13] by *N*,*N*′-dicyclohexylcarbodiimide (DCC) or *N*,*N*-bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP-Cl)[14]-mediated coupling (Scheme 2). The yield of *N*-(4-pentenoyl)-(*S*)-valine benzyl ester (**2**) (87%) was comparable to the yield achieved previously for the introduction of the pentenoyl group using pentenoic anhydride.[9]

Removal of the protecting groups by treatment with iodine was then studied. Thus compounds **2**−**10** were dissolved in aqueous THF and treated with 3 equiv of iodine. After 5 min the reactions were quenched with solid $Na_2S_2O_3$ and concentrated under diminished pressure. After purification the formed (*S*)-valine benzyl ester was converted to the *p*-toluenesulfonate salt and the yield of isolated amino acid was calculated (Table 1). As shown in the table, two of the pentenoyl derivatives (**3** and **10**) were converted to valine benzyl ester to a significant extent within 5 min upon admixture of I_2, but neither was converted more efficiently than the parent *N*-pentenoyl derivative on this time scale. Most of the derivatives were deblocked very inefficiently and analogues (**4**−**6**) could not be deblocked at all under these conditions. Interestingly, treatment of **10** with I_2 produced iodohydrin **10a** in 29% yield in addition to valine benzyl ester. The iodohydrin presumably arises from solvolytic ring opening of the iminolactone intermediate resulting from iodolactonization (vide supra).

Scheme 2. Synthesis of *N*-Blocked Valine Benzyl Esters

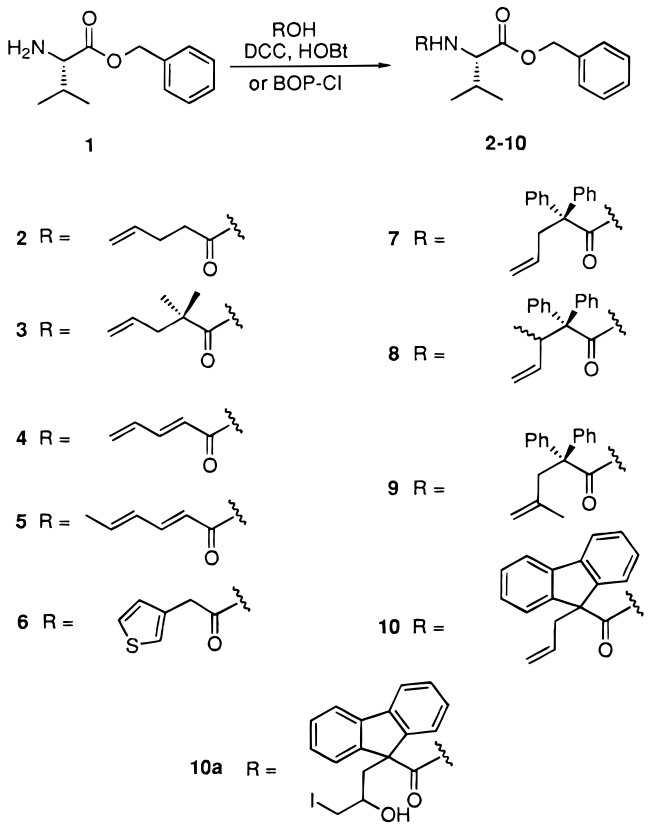


Table 1. Deprotection of Pentenoyl Derivatives 2−10

compound	deprotection after 5 min (%)[a]	compound	deprotection after 5 min (%)[a]
2	92	**7**	<3
3	70	**8**	<3
4	0	**9**	<3
5	0	**10**	18[b]
6	0		

[a] Percentage of deprotection is based on the amount of isolated valine benzyl ester after purification. [b] Also isolated in 29% yield was iodohydrin derivative **10a**.

Synthesis of *N*-(4-Pentenoyl)aminoacyl-pdCpA Esters. Aminoacyl-pdCpA esters **14a**−**d** were prepared as shown in Scheme 3. Methyl esters **11a**−**d** were N^{α}-protected by *N*,*N*′-dicyclohexylcarbodiimide-mediated coupling with 4-pentenoic acid, affording amides **12a**−**d**. Subsequent hydrolysis of the esters with LiOH in aqueous THF and reaction with chloroacetonitrile gave the respective cyanomethyl esters **13a**−**d**.[15] Admixture of the active esters with the tris(tetrabutylammonium) salt of pdCpA[4] in DMF provided *N*-(4-pentenoyl)aminoacyl-pdCpA esters **14a**−**d** in good yield. For comparative purposes in the deblocking experiments, *N*-(6-nitrovera-

(12) For a review on caged compounds, see: Adams, S. R.; Tsien, R. Y. *Annu. Rev. Physiol.* **1993**, *55*, 755.

(13) Zervas, L.; Winitz, M.; Greenstein, J. P. *J. Org. Chem.* **1957**, *22*, 1515.

(14) Tung, R. D.; Rich, D. H. *J. Am. Chem. Soc.* **1985**, *107*, 4342.

(15) Bodanszky, M.; Bodanszky, A. In *The Practice of Peptide Synthesis*, 2nd ed.; Springer-Verlag: Berlin, Heidelberg, New York, 1994; p 96.

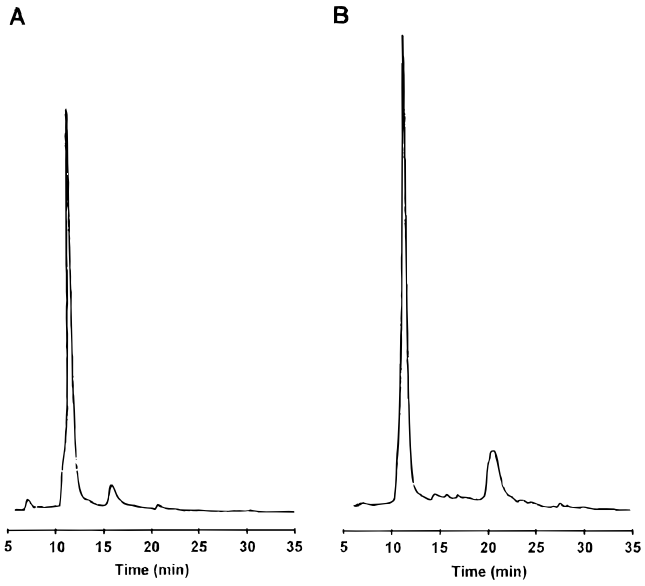
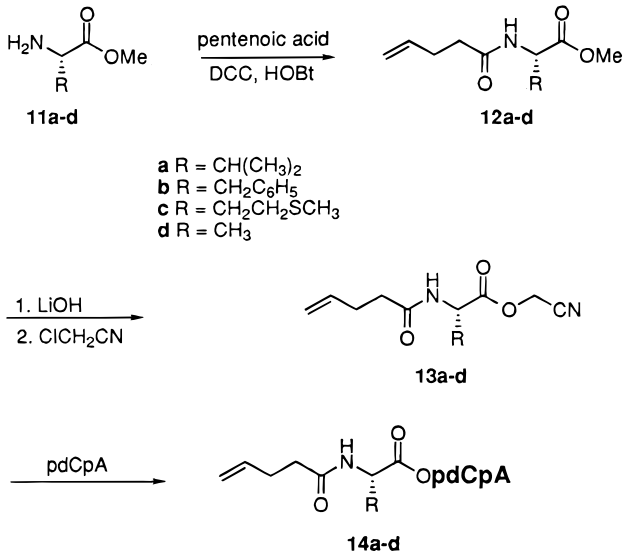


Figure 1. HPLC analysis of deprotection of valyl-pdCpAs. (A) Deprotection of *N*-(4-pentenoyl)valyl-pdCpA by treatment with aqueous iodine for 5 min at 25 °C. (B) Deprotection of *N*-(6-nitroveratryloxycarbonyl)valyl-pdCpA by irradiation with a 500 W mercury−xenon lamp for 5 min at 2 °C. The deblocked valyl-pdCpA eluted at 11.2 min. Co-injection of the reaction mixtures confirmed comigration of the products obtained from both deprotection reactions.

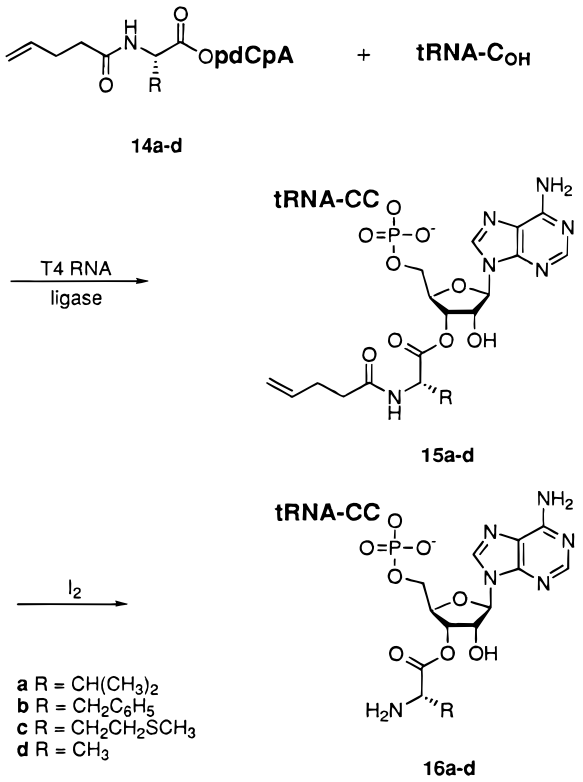
Scheme 3. Synthesis of *N*-(4-Pentenoyl)Aminoacyl-pdCpAs



tryloxycarbonyl)valyl-pdCpA and *N*-(6-nitroveratryloxycarbonyl)methionyl-pdCpA were prepared by a known method.[7]

Valine derivative **14a** was deprotected by treatment with iodine in aqueous THF. HPLC analysis (Figure 1) of the reaction mixture showed complete deprotection within 5 min. To ensure the identity of the products, the *N*-(6-nitroveratryloxycarbonyl)valyl-pdCpA, which is known[7] to be converted to valyl-pdCpA upon irradiation with a 500 W mercury−xenon lamp, was deblocked and the product compared with that obtained from **14a**. As shown in the Figure, the major products from both protected valyl-pdCpA derivatives had essentially the same retention times; co-injection confirmed that they were indistinguishable by reverse phase HPLC analysis.

Scheme 4. Synthesis and Deprotection of *N*-(4-Pentenoyl)Aminoacyl-tRNAs



The purity of the product obtained from **14a** also compared favorably with that accessible from (NVOC)valyl-pdCpA.

N-(4-Pentenoyl)methionyl-pdCpA (**14c**) was deblocked in the same fashion with iodine and the product was compared with methionyl-pdCpA accessible by deblocking of (NVOC)methionyl-pdCpA. Again, the deblocked methionyl-pdCpAs comigrated when analyzed by reverse phase HPLC (not shown).

Misacylated tRNA Preparation and Use in *in Vitro* Protein Synthesis. Preparation of misacylated tRNAs was accomplished by T4 RNA ligase-mediated coupling of 2′(3′)-*O*-[*N*-(4-pentenoyl)]aminoacyl-pdCpAs **14a**−**d** with yeast suppressor $tRNA_{CUA}^{Phe}$ transcripts lacking the 3′-terminal cytidine and adenosine moieties (Scheme 4).[3−5,7] The resulting *N*-(4-pentenoyl)aminoacyl-tRNAs (**15a**−**d**) were deprotected in aqueous THF containing 5 mM iodine, affording misacylated suppressor tRNAs **16a**−**d**.

The misacylated tRNAs so prepared were then used in an in vitro protein biosynthesizing system that employed rabbit reticulocyte lysate and an mRNA for dihydrofolate reductase (DHFR) containing a nonsense (UAG) codon at position 10.[16] As shown in Table 2, the synthesis of DHFR, which involved readthrough of the UAG codon by a misacylated suppressor tRNA, proceeded in reasonably good yield for each misacylated $tRNA_{CUA}$ studied. No synthesis of DHFR was observed if iodine treatment was omitted. Direct comparison with valyl-tRNA prepared by photodeprotection of *N*-(6-nitroveratryloxycarbonyl)valyl-tRNA showed that the reaction proceeded to the same extent for both deblocked valyl-tRNAs.

(16) Karginov, V. A.; Mamaev, S. V.; An, H.; Van Cleve, M. D.; Hecht, S. M.; Komatsoulis, G. A.; Abelson, J. N. *J. Am. Chem. Soc.* **1997**, *119*, 8166−8176.

Table 2. Efficiency of Suppression by Aminoacyl-tRNA$_{CUA}$ at Position 10 of Dihydrofolate Reductase

amino acid	suppression efficiency (%)[a]
valine[b]	30
valine[c]	25
methionine	17
alanine	20
phenylalanine	30

[a] Defined as the percentage of DHFR produced via nonsense codon suppression relative to production of DHFR from wild-type mRNA. [b] In the absence of I_2 treatment of *N*-(4-pentenoyl)valyl-tRNA$_{CUA}$, the suppression efficiency was <1%. [c] From *N*-(nitroveratryloxycarbonyl)-(*S*)-valyl-tRNA.

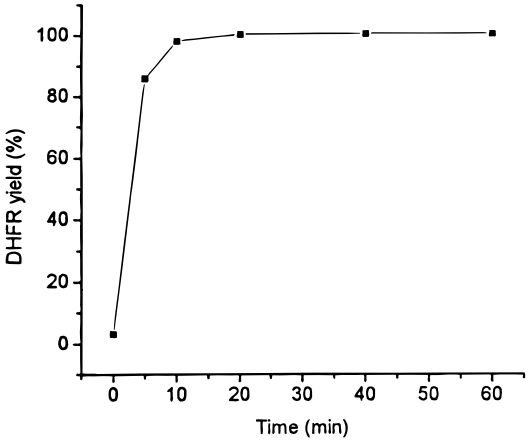
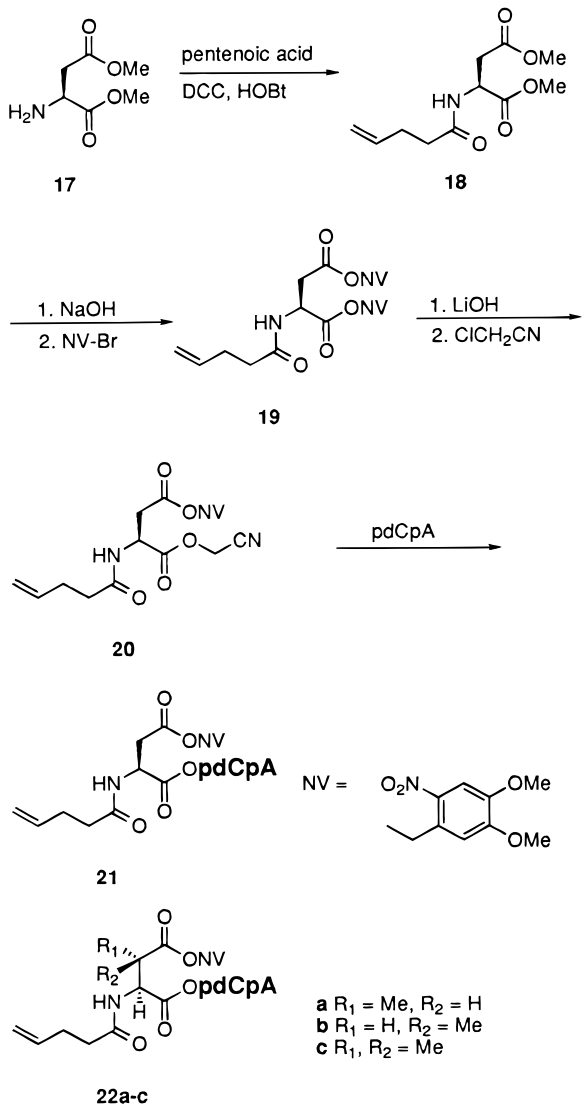


Figure 2. Effect of time of deprotection of *N*-(4-pentenoyl)-valyl-tRNA on the production of DHFR. Aliquots of *N*-(4-pentenoyl)valyl-tRNA taken at predetermined times after treatment with iodine were added to in vitro protein synthesis reactions programmed with an mRNA containing a nonsense codon (UAG) at position 10. More than 90% of the modified protein was obtained in the first 10 min of the reaction, suggesting that deprotection was almost complete after 10 min.

As shown in Figure 2, the use of aliquots of *N*-(4-pentenoyl)valyl-tRNA treated with iodine for varying lengths of time prior to addition to the protein biosynthesizing system afforded maximal yields of full length protein after 5−10 min of iodine treatment. The yields of DHFR did not diminish if longer I_2 treatment was employed. Because a substantial percentage of all of the activated valine moieties are incorporated into protein, this suggested that deprotection was largely complete within 5−10 min. In addition, *N*-(4-pentenoyl)-(*S*)-valyl-tRNA proved to be stable in cacodylate buffer, pH 7.4, at 37 °C for at least 24 h (data not shown).

Synthesis of Side-Chain-Protected *N*-(4-Pentenoyl)aspartyl-pdCpAs. Dimethyl (*S*)-aspartate (**17**) was N^{α} protected with the 4-pentenoyl group (Scheme 5). The resulting amide **18** was treated with NaOH in aqueous THF and then with 6-nitroveratryl bromide[17] and CsF[18] to afford nitroveratryl diester **19**. Selective hydrolysis of the α-ester with LiOH and treatment with chloroacetonitrile gave cyanomethyl ester **20**. Reaction of **20** with the tris(tetrabutylammonium) salt of pdCpA in DMF afforded γ-nitroveratryl *N*-(4-pentenoyl)aspartyl-pdCpA (**21**) as a pale yellow foam.

Scheme 5. Synthesis of γ-Nitroveratryl *N*-(4-Pentenoyl)Aspartyl-pdCpAs



In the same fashion as described for dimethyl (*S*)-aspartate (**17**), dimethyl *threo*-β-methyl-(*S*)-aspartate,[19] dimethyl *erythro*-β-methyl-(*S*)-aspartate,[19] and dimethyl β,β-dimethyl-(*S*)-aspartate[19] were converted to their respective γ-nitroveratryl *N*-(4-pentenoyl)aspartyl-pdCpA derivatives (**22a**−**c**).

Side-Chain-Protected Aspartyl-tRNAs. Preparation of Aspartyl-tRNAs and in Vitro Protein Synthesis. Aspartyl-pdCpAs **21** and **22a**−**c** were ligated onto an abbreviated *Escherichia coli* tRNA$_{CUA}^{Ala}$ transcript as described for pdCpA derivatives **14a**−**d**. The resulting aspartyl-tRNAs were treated with iodine and employed for the in vitro synthesis of DHFR (Figure 3). A DHFR mRNA containing a nonsense (UAG) codon at position 27 was used as described previously.[16] Analogues of DHFR containing side-chain-protected aspartyl derivatives at position 27 were obtained in good yields (Table 3). A 2- to 3-fold increase in suppression efficiency was observed as compared to aspartyl-tRNA$_{CUA}$ derivatives lacking side chain protection.[16]

(17) Wilcox, M.; Viola, R. W.; Johnson, K. W.; Billington, A. P.; Carpenter, B. K.; McCray, J. A.; Guzikowski, A. P.; Hess, G. P. *J. Org. Chem.* **1990**, *55*, 1585.

(18) Dijkstra, G.; Kruizinga, W. H.; Kellogg, R. M. *J. Org. Chem.* **1987**, *52*, 4230.

(19) Crasto, C. F.; Laikhter, A. L.; An, H.; Lodder, M.; Hecht, S. M. Manuscript in preparation.

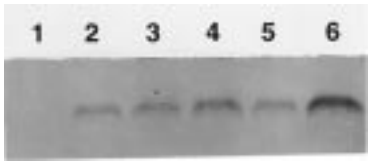


Figure 3. Elaboration of dihydrofolate reductase analogues containing derivatives of aspartic acid with 6-nitroveratryl protected side chain carboxylates at position 27. Protein synthesis was carried out in the presence of ^{35}S-methionine using a rabbit reticulocyte lysate, mRNA containing a nonsense codon at position 27, and no suppressor tRNA (lane 1) or a suppressor tRNA activated with one of several amino acids (lanes 2−5). Following incubation at 30 °C for 1 h, the reactions were analyzed on a 20% SDS−polyacrylamide gel: lane 1, no $tRNA_{CUA}$; lane 2, aspartic acid; lane 3, *β*,*β*-dimethylaspartic acid; lane 4, *erythro*-*β*-methylaspartic acid; lane 5, *threo*-*β*-methylaspartic acid; lane 6, wild-type mRNA, no $tRNA_{CUA}$.

Table 3. Efficiency of Suppression by Aspartyl-$tRNA_{CUA}$s at Position 27 of Dihydrofolate Reductase

amino acid	suppression efficiency[a]
aspartic acid	26 (11)
β,*β*-dimethylaspartic acid	20 (16)
erythro-*β*-methylaspartic acid	35 (7)
threo-*β*-methylaspartic acid	26 (10)

[a] Yields in parentheses were obtained in the absence of side chain protection.[16]

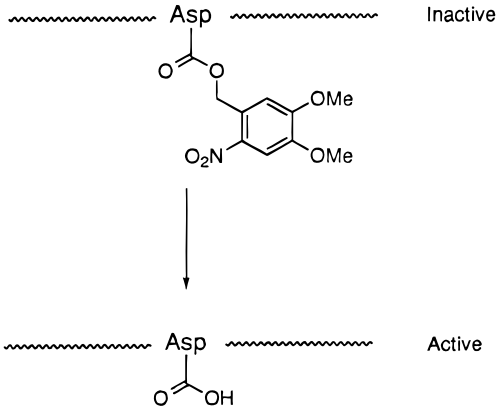


Figure 4. Irradiation of side-chain-protected (inactive) analogues of DHFR for 30 min yields catalytically active proteins by removing the side chain protecting group.

Activation of Modified Proteins. The derived DHFR analogues containing a side chain protecting group on Asp-27 had essentially no activity in the reduction of dihydrofolate to tetrahydrofolate. Irradiation of the modified proteins for 30 min with a 500 W mercury−xenon lamp provided deblocked DHFRs (Figure 4) which had activity comparable to those of DHFRs prepared without side chain protection. Enzymatic activity assays were carried out as described previously.[20] The relative catalytic efficiencies of the modified DHFRs containing *erythro*-*β*-methylaspartic acid, *threo*-*β*-methylaspartic acid, and *β*,*β*-dimethylaspartic acid were about 85%, 75%, and 75%, respectively, of that determined for DHFR containing Asp-27.

Discussion

The availability of a variety of misacylated tRNAs has permitted the study of the nature of the peptidyltransferase reaction[3c−f,h,21] and the elaboration of modified peptides[3j,21,22] and proteins.[16,23] Because the most versatile method for the preparation of such misacylated tRNAs involves the T4 RNA ligase-mediated condensation of an abbreviated tRNA ($tRNA\text{-}C_{OH}$) with an *N*-protected aminoacyl-pdCpA derivative,[3−5,7] improvements in the nature of the protecting group may contribute importantly to further development of this technology.

A protecting group that can be removed by treatment with aqueous iodine is very attractive because the conditions under which this reaction occurs are very mild. The deprotection reaction is believed to proceed via iminolactone formation, followed by hydrolysis of the formed intermediate (Scheme 1). All of the pentenoyl derivatives tested, except compounds **4**−**6**, could be removed by treatment with 3 equiv of iodine in aqueous THF. Contrary to what was expected, none of the derivatives was removed more efficiently than the unmodified pentenoyl group. It was anticipated, for example, that the dimethyl pentenoyl derivative would exhibit more facile cyclization based on the "*gem*-dimethyl" effect.[24] It has been shown that *gem*-dialkyl derivatives undergo faster cyclization in this type of reaction than unsubstituted derivatives. The absence of any change in the rate of the reaction suggests that the rate-determining step in the deprotection reaction may not be the cyclization per se, but rather the hydrolysis of the formed intermediate. This conclusion is also supported by the fact that none of the diphenyl derivatives (**7**−**10**) was removed faster than the unsubstituted pentenoyl derivative. In previous studies[25] on diphenyl derivatives of pentenoic acid, an increase in the rate of the reaction was observed. The additional trans double bonds in compounds **4** and **5** clearly did not have a facilitating effect on the rate of deblocking. The fact that

(20) Baccanari, D.; Phillips, A.; Smith, S.; Sinski, D.; Burchall, J. *Biochemistry* **1975**, *14*, 5267.
(21) Killian, J. A.; Van Cleve, M. D.; Shayo, Y. F., Hecht, S. M. Manuscript in preparation.
(22) (a) Bain, J. D.; Glabe, C. G.; Dix, T. A.; Chamberlin, A. R.; Diala, E. S. *J. Am. Chem. Soc.* **1989**, *111*, 8013. (b) Bain, J. D.; Wacker, D. A.; Kuo, E. E.; Chamberlin, A. R. *Tetrahedron* **1991**, *47*, 2389. (c) Bain, J. D.; Switzer, C.; Benner, S. A.; Chamberlin, A. R. *Nature* **1992**, *356*, 537.
(23) (a) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182. (b) Ellman, J.; Mendel, D.; Anthony-Cahill, S.; Noren, C. J.; Schultz, P. G. *Methods Enzymol.* **1991**, *202*, 301. (c) Ellman, J. A.; Mendel, D.; Schultz, P. G. *Science* **1992**, *255*, 197. (d) Ellman, J. A.; Volkman, B. F.; Mendel, D.; Schultz, P. G.; Wemmer, D. E. *J. Am. Chem. Soc.* **1992**, *114*, 7959. (e) Mendel, D.; Ellman, J. A.; Chang, Z.; Veenstra, D. L.; Kollman, P. A.; Schultz, P. G. *Science* **1993**, *256*, 1798. (f) Judice, J. K.; Gamble, T. R.; Murphy, E. C.; deVos, A. M.; Schultz, P. G. *Science* **1993**, *261*, 1578. (g) Chang, H.-H.; Benson, D. R.; Schultz, P. G.. *Science* **1993**, *259*, 806. (h) Mendel, D.; Ellman, J. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1993**, *115*, 4359. (i) Cornish, V. W.; Benson, D. R.; Altenbach, C. A.; Hideg, K.; Hubbell, W. L.; Schultz, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2910. (j) Thorson, J.; Chapman, E.; Murphy, E. C.; Schultz, P. G., Judice J. K. *J. Am. Chem. Soc.* **1995**, *117*, 1157. (k) Cornish, V. W.; Mendel, D.; Schultz, P. G. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 621. (l) Nowak, M. W.; Kearney, P. C.; Sampson, J. R.; Saks, M. E.; Labarca, C. G.; Silverman, S. K.; Zhong, W.; Thorson, J.; Abelson, J. N.; Davidson, N.; Schultz, P. G.; Dougherty, D. A.; Lester, H. A. *Science* **1995**, *268*, 439. (m) Mamaev, S. V.; Laikhter, A. L.; Hecht, S. M. *J. Am. Chem. Soc.* **1996**, *118*, 7243. (n) Saks, M. E.; Sampson, J. R.; Nowak, M. W.; Kearney, P. C.; Du, F.; Abelson, J. N.; Lester, H. A.; Dougherty, D. A. *J. Biol. Chem.* **1996**, *271*, 23169. (o) Steward, L. E.; Collins, C. S.; Gilmore, M. A.; Carlson, J. E.; Ross, J. B. A.; Chamberlin, A. R. *J. Am. Chem. Soc.* **1997**, *119*, 6. (p) Arslan, T.; Mamaev, S. V.; Mamaeva, N. V.; Hecht, S. M. *J. Am. Chem. Soc.* **1997**, *119*, 10877−10887.
(24) (a) Beesley, R. M.; Ingold, C. K.; Thorpe, J. F. *J. Chem. Soc.* **1915**, 1080. (b) Ingold, C. K. *J. Chem. Soc.* **1921**, 305. (c) Capon, B.; McManus, S. P. *Neighboring Group Participation*; Plenum: New York, 1976; Vol. 1, pp 58−70. (d) Hill, E. A.; Link, D. C.; Donndelinger, P. *J. Org. Chem.* **1981**, *46*, 1177.
(25) do Amaral, L.; Melo, S. C. *J. Org. Chem.* **1973**, *38*, 800.

compound **6** did not yield the free amine upon treatment with iodine may be explained by the aromatic nature of the thiophene ring.

The unsubstituted 4-pentenoyl group proved to be very useful for the protection of aminoacyl-tRNAs. The protecting group could be introduced efficiently by a simple *N*,*N*′-dicyclohexylcarbodiimide-mediated coupling. Earlier studies[9,10] employed 4-pentenoyl anhydride, but direct coupling of the acid resulted in the same yields without having to prepare the anhydride first. Removal of the 4-pentenoyl group proceeded smoothly within 5−10 min. Deprotection of the more complex *N*-(4-pentenoyl)aminoacyl dinucleotides did not take any longer than deprotection of a simple amino acid. Control experiments with *N*-(NVOC)aminoacyl-pdCpAs showed that both methods yield the same product upon deprotection. The removal of the 4-pentenoyl group of *N*-(4-pentenoyl)-(*S*)-methionyl-pdCpA by treatment with iodine provides further confirmation of the mild nature of the deprotection conditions, because no oxidation of the sensitive amino acid was observed under these conditions.

After ligation of the *N*-(4-pentenoyl)aminoacyl-pdCpAs to tRNA transcripts lacking the 3′-terminal pCpA moiety, the protecting group could be removed under the same conditions that had been used for the pdCpA derivatives. Under these conditions, the *N*-pentenoyl group was removed within 5−10 min, as judged by the yield of the derived proteins. A major advantage of the strategy described herein is the wherewithal to effect differential protection of the N^{α} and side chain functional group of the aminoacyl moiety. In the present case this was accomplished for four aspartyl-pdCpA analogues by using the 4-pentenoyl group for N^{α} protection and a photochemically removable nitroveratryl ester for side chain protection.

Previously, it has been noted that misacylated suppressor tRNAs bearing polar amino acids tend to function poorly in readthrough of nonsense codons.[16,23k] The combination of N^{α} and side chain protecting groups employed here for the aspartyl-$tRNA_{CUA}$s permitted side chain protection to be retained during protein synthesis and thereby resulted in a significant increase in the yields of DHFR produced in the cell-free protein synthesizing system by nonsense codon suppression.

In addition to the increased yields of proteins accessible by in vitro synthesis, another interesting aspect of the orthogonal protection strategy is the possibility of creating caged proteins.[12] Before removal of the side chain protecting group, a full length protein is obtained which, in the case of DHFR, lacks any catalytic activity. Irradiation affords a fully functional protein with the same enzymatic activity as obtained before.[16] A related approach to caged proteins has been described before, employing a BPOC group for the protection of the α-amino group and a nitrobenzyl group for the protection of the side chain carboxylate of aspartic acid.[26] The BPOC group, however, is labile under acidic conditions and had to be removed before ligation of the aminoacylated dinucleotide to the truncated tRNA, thereby greatly diminishing the effectiveness of the protection strategy.

Experimental Section

General Methods. Melting points were taken on a capillary melting point apparatus and are not corrected. Moisture-sensitive reactions were conducted under argon in oven-dried glassware. 4-Pentenoic acid, 2,2-dimethyl-4-pentenoic acid, 2,4-pentadienoic acid, 2,4-hexadienoic acid, and 3-thiopheneacetic acid were purchased from Aldrich Chemicals. 2,2-Diphenyl-4-pentenoic acid, 3-methyl-2,2-diphenyl-4-pentenoic acid, 4-methyl-2,2-diphenyl-4-pentenoic acid, and 9-allylfluorene-9-carboxylic acid were prepared as described.[27] All other 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. Triethylamine was distilled from P_2O_5. 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.

Nuclease-treated rabbit reticulocyte lysate was purchased from Promega Corp.; T4 RNA ligase was from New England Biolabs. DEAE Sepharose CL-4B, NADPH, and dihydrofolic acid were obtained from Sigma Chemicals. Kits for plasmid isolation and for purification of proteins on Ni−NTA agarose were purchased from QIAGEN Inc. (Chatsworth, CA). AmpliScribe transcription kits were from Epicenter Technologies (Madison, WI). [^{35}S]Methionine (1000 Ci/mmol) was obtained from Amersham Corp.

Phosphorimager analysis was performed using a Molecular Dynamics 300E Phosphorimager equipped with Image-Quant software.

***N*-(4-Pentenoyl)-(*S*)-valine Benzyl Ester (2).** To a cooled (0−5 °C) solution containing 200 mg (0.53 mmol) of (*S*)-valine benzyl ester *p*-toluenesulfonate, 60 μL (58 mg, 0.58 mmol) of 4-pentenoic acid, 58 μL (54 mg, 0.53 mmol) of *N*-methylmorpholine, and 78 mg (0.58 mmol) of hydroxybenzotriazole in 6 mL of CH_2Cl_2 was added 120 mg (0.58 mmol) of *N*,*N*′-dicyclohexylcarbodiimide. The reaction was allowed to warm to room temperature and was then stirred for 18 h. The reaction mixture was filtered, diluted with 15 mL of CH_2Cl_2, and washed successively with two 25-mL portions of 0.5 N 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 (2 × 25 cm); elution with 2% MeOH in CH_2Cl_2 afforded *N*-(4-pentenoyl)-(*S*)-valine benzyl ester (**2**) as a colorless oil: yield 134 mg (87%); silica gel TLC R_f 0.57 (4% MeOH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 0.86 (d, 3H, J = 7 Hz), 0.91 (d, 3H, J = 7 Hz), 2.12−2.23 (m, 1H), 2.31−2.42 (m, 4H), 4.62−4.66 (m, 1H), 4.99−5.10 (m, 2H), 5.14 (d, 1H, J = 12 Hz), 5.20 (d, 1H, J = 12 Hz), 5.76−5.88 (m, 1H), 5.93 (d, 1H, J = 8.5 Hz), and 7.36 (s, 5H); ^{13}C NMR ($CDCl_3$) δ 18.2, 19.5, 30.0, 31.8, 36.2, 57.4, 67.5, 116.2, 128.9, 129.0, 129.1, 135.8, 137.5, 172.6, and 172.8; mass spectrum (chemical ionization, methane) m/z 290 $(M + H)^+$. Anal. Calcd for $C_{17}H_{23}NO_3$: C, 70.56; H, 8.01. Found: C, 70.48; H, 7.99.

***N*-(2,2-Dimethyl-4-pentenoyl)-(*S*)-valine Benzyl Ester (3).** To a cooled (0−5 °C) solution containing 200 mg (0.53 mmol) of (*S*)-valine benzyl ester *p*-toluenesulfonate, 75 μL (74 mg, 0.58 mmol) of 2,2-dimethyl-4-pentenoic acid, 58 μL (54 mg, 0.53 mmol) of *N*-methylmorpholine, and 78 mg (0.58 mmol) of hydroxybenzotriazole in 6 mL of CH_2Cl_2 was added 120 mg (0.58 mmol) of *N*,*N*′-dicyclohexylcarbodiimide. The reaction was allowed to warm to room temperature and was then stirred for 18 h. Workup and purification as described for compound **2** afforded 2,2-dimethyl-*N*-(4-pentenoyl)-(*S*)-valine benzyl ester (**3**) as a colorless oil: yield 128 mg (76%); silica gel TLC R_f 0.70 (4% MeOH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 0.85 (d, 3H, J = 7 Hz), 0.91 (d, 3H, J = 7 Hz), 1.20 (s, 6H), 2.15−2.20 (m, 1H), 2.22−2.30 (m, 2H), 4.59−4.63 (m, 1H), 5.04−5.09 (m, 2H), 5.12 (d, 1H, J = 12 Hz), 5.22 (d, 1H, J = 12 Hz), 5.68−5.82 (m, 1H), 6.12 (d, 1H, J = 8.5 Hz), and 7.36

(26) Mendel, D.; Ellman, J. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 2758.

(27) (a) Arnold, R. T.; Searles, S. *J. Am. Chem. Soc.* **1949**, *71*, 1150. (b) Arnold, R. T.; Parham, W. E.; Dodson, R. M. *J. Am. Chem. Soc.* **1949**, *71*, 2439.

(s, 5H); ^{13}C NMR ($CDCl_3$) δ 18.2, 19.5, 25.6, 25.7, 31.8, 42.7, 45.6, 57.4, 67.5, 118.6, 128.8, 128.9, 129.1, 134.8, 135.9, 172.5, and 177.6; mass spectrum (chemical ionization, methane) *m/z* 318 $(M + H)^+$. Anal. Calcd for $C_{19}H_{27}NO_3$: C, 71.89; H, 8.57. Found: C, 71.65; H, 8.59.

***N*-(2,4-Pentadienoyl)-(*S*)-valine Benzyl Ester (4).** To a cooled (0−5 °C) solution containing 200 mg (0.53 mmol) of (*S*)-valine benzyl ester *p*-toluenesulfonate, 65 mg (0.66 mmol) of 2,4-pentadienoic acid, and 222 μL (161 mg, 1.59 mmol) of Et_3N in 10 mL of CH_2Cl_2 was added 168 mg (0.66 mmol) of BOP-Cl. The reaction was allowed to warm to room temperature and was then stirred for 18 h. Workup and purification as described for compound **2** afforded *N*-(2,4-pentadienoyl)-(*S*)-valine benzyl ester (**4**) as a colorless oil: yield 85 mg (56%); silica gel TLC R_f 0.60 (4% MeOH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 0.88 (d, 3H, J = 7 Hz), 0.93 (d, 3H, J = 7 Hz), 2.16−2.25 (m, 1H), 4.71−4.75 (m, 1H), 5.15 (d, 1H, J = 12 Hz), 5.20 (d, 1H, J = 12 Hz), 5.45 (d, 1H, J = 10 Hz), 5.58 (d, 1H, J = 17 Hz), 5.95 (d, 1H, J = 15 Hz), 6.02 (d, 1H, J = 8.5 Hz), 6.38−6.50 (m, 1H), 7.18−7.29 (m, 1H), and 7.36 (s, 5H); ^{13}C NMR ($CDCl_3$) δ 18.2, 19.5, 32.1, 57.6, 67.6, 124.7, 125.2, 128.9, 129.0, 129.1, 135.2, 135.8, 142.3, 166.2, and 172.6; mass spectrum (chemical ionization, methane) *m/z* 288 $(M + H)^+$. Anal. Calcd for $C_{17}H_{21}NO_3$: C, 71.06; H, 7.37. Found: C, 70.79; H, 7.50.

***N*-(2,4-Hexadienoyl)-(*S*)-valine Benzyl Ester (5).** To a cooled (0−5 °C) solution containing 200 mg (0.53 mmol) of (*S*)-valine benzyl ester *p*-toluenesulfonate, 74 mg (0.66 mmol) of 2,4-hexadienoic acid (**5**), and 222 μL (161 mg, 1.59 mmol) of Et_3N in 10 mL of CH_2Cl_2 was added 168 mg (0.66 mmol) of BOP-Cl. The reaction was allowed to warm to room temperature and was then stirred for 18 h. Workup and purification as described for compound **2** afforded *N*-(2,4-hexadienoyl)-(*S*)-valine benzyl ester (**5**) as a colorless solid: yield 102 mg (64%); mp 59−61 °C; silica gel TLC R_f 0.62 (4% MeOH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 0.87 (d, 3H, J = 7 Hz), 0.93 (d, 3H, J = 7 Hz), 1.84 (d, 3H, J = 5 Hz), 2.15−2.26 (m, 1H), 4.71−4.75 (m, 1H), 5.14 (d, 1H, J = 12 Hz), 5.20 (d, 1H, J = 12 Hz), 5.81 (d, 1H, J = 15 Hz), 5.94 (d, 1H, J = 8 Hz), 6.04−6.21 (m, 2H), 7.16−7.25 (m, 1H), and 7.36 (s, 5H); ^{13}C NMR ($CDCl_3$) δ 18.2, 19.1, 19.5, 32.1, 57.5, 67.6, 121.5, 128.9, 129.0, 129.1, 130.1, 135.8, 138.7, 142.5, 166.7, and 172.6; mass spectrum (chemical ionization, methane) *m/z* 302 $(M + H)^+$; mass spectrum (electron impact) *m/z* 301.167 (M^+) ($C_{18}H_{23}NO_3$ requires 301.168).

***N*-(3-Thiopheneacetyl)-(*S*)-valine Benzyl Ester (6).** To a cooled (0−5 °C) solution containing 200 mg (0.53 mmol) of (*S*)-valine benzyl ester *p*-toluenesulfonate, 82 mg (0.58 mmol) of 3-thiopheneacetic acid, 58 μL (54 mg, 0.53 mmol) of *N*-methylmorpholine, and 78 mg (0.58 mmol) of hydroxybenzotriazole in 6 mL of CH_2Cl_2 was added 120 mg (0.58 mmol) of *N*,*N*′-dicyclohexylcarbodiimide. The reaction was allowed to warm to room temperature and was then stirred for 18 h. Workup and purification as described for compound **2** afforded *N*-(3-thiopheneacetyl)-(*S*)-valine benzyl ester (**6**) as a colorless solid: yield 140 mg (80%); mp 69−70 °C; silica gel TLC R_f 0.62 (4% MeOH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 0.74 (d, 3H, J = 7 Hz), 0.85 (d, 3H, J = 7 Hz), 2.08−2.19 (m, 1H), 3.65 (s, 2H), 4.57−4.62 (m, 1H), 5.09 (d, 1H, J = 12 Hz), 5.17 (d, 1H, J = 12 Hz), 5.91 (d, 1H, J = 8.5 Hz), 7.01 (d, 1H, J = 5 Hz), 7.16 (s, 1H), and 7.30−7.39 (m, 6H); ^{13}C NMR ($CDCl_3$) δ 18.0, 19.5, 31.7, 38.6, 57.5, 67.5, 123.9, 127.2, 128.9, 129.0, 129.1, 135.1, 135.8, 170.9, and 172.1; mass spectrum (chemical ionization, methane) *m/z* 332 $(M + H)^+$. Anal. Calcd for $C_{18}H_{21}NO_3$: C, 65.23; H, 6.39. Found: C, 65.34; H, 6.46.

***N*-(2,2-Diphenyl-4-pentenoyl)-(*S*)-valine Benzyl Ester (7).** To a cooled (0−5 °C) solution containing 200 mg (0.53 mmol) of (*S*)-valine benzyl ester *p*-toluenesulfonate, 167 mg (0.66 mmol) of 2,2-diphenyl-4-pentenoic acid, and 222 μL (161 mg, 1.59 mmol) of Et_3N in 10 mL of CH_2Cl_2 was added 168 mg (0.66 mmol) of BOP-Cl. The reaction was allowed to warm to room temperature and was then stirred for 18 h. Workup and purification as described for compound **2** afforded 2,2-diphenyl-*N*-(4-pentenoyl)-(*S*)-valine benzyl ester (**7**) as a colorless oil: yield 153 mg (65%); silica gel TLC R_f 0.61 (1% MeOH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 0.60 (d, 3H, J = 7 Hz), 0.74 (d, 3H, J = 7 Hz), 2.08−2.14 (m, 1H), 3.20−3.25 (m, 2H), 4.57−4.61 (m, 1H), 4.91−5.00 (m, 2H), 5.08 (d, 1H, J = 12 Hz), 5.16 (d, 1H, J = 12 Hz), 5.68−5.77 (m, 1H), 5.98 (d, 1H, J = 8 Hz), and 7.23−7.34 (m, 15H); ^{13}C NMR ($CDCl_3$) δ 17.8, 19.5, 31.5, 43.8, 57.9, 61.3, 67.5, 118.3, 127.6, 128.8, 128.85, 129.0, 129.1, 129.5, 129.7, 135.7, 135.9, 143.0, 143.7, 172.0, and 174.5; mass spectrum (chemical ionization, methane) *m/z* 442 $(M + H)^+$. Anal. Calcd for $C_{29}H_{31}NO_3$: C, 78.88; H, 7.08. Found: C, 78.79; H, 7.07.

***N*-(2,2-Diphenyl-3-methyl-4-pentenoyl)-(*S*)-valine Benzyl Ester (8).** To a cooled (0−5 °C) solution containing 200 mg (0.53 mmol) of (*S*)-valine methyl ester *p*-toluenesulfonate, 176 mg (0.66 mmol) of 2,2-diphenyl-3-methyl-4-pentenoic acid, and 222 μL (161 mg, 1.59 mmol) of Et_3N in 10 mL of CH_2Cl_2 was added 168 mg (0.66 mmol) of BOP-Cl. The reaction was allowed to warm to room temperature and was then stirred for 18 h. Workup and purification as described for compound **2** afforded *N*-(2,2-diphenyl-3-methyl-4-pentenoyl)-(*S*)-valine benzyl ester (**8**) as a colorless oil (1:1 mixture of diastereomers): yield 171 mg (71%); silica gel TLC R_f 0.61 (1% MeOH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 0.52 (d, 1.5H, J = 7 Hz), 0.58 (d, 1.5H, J = 7 Hz), 0.59 (d, 1.5H, J = 7 Hz), 0.63 (d, 1.5H, J = 7 Hz), 0.88 (d, 1.5H, J = 6.5 Hz), 0.92 (d, 1.5H, J = 6.5 Hz), 2.02−2.06 (m, 1H), 3.93−3.98 (m, 1H), 4.52−4.58 (m, 1H), 4.92−4.98 (m, 2H), 5.04−5.18 (m, 2H), 5.65−5.82 (m, 1H), 5.97 (d, 0.5H, J = 8 Hz), 6.06 (d, 0.5H, J = 8 Hz), and 7.22−7.44 (m, 15H); ^{13}C NMR ($CDCl_3$) δ 16.7, 17.4, 17.6, 17.8, 19.4, 31.7, 31.8, 41.2, 57.8, 65.8, 65.9, 67.4, 116.1, 116.5, 127.4, 127.6, 128.1, 128.35, 128.4, 128.6, 128.7, 128.9, 129.1, 129.6, 129.7, 130.5, 131.15, 131.2, 135.9, 139.9, 140.2, 140.7, 141.2, 141.9, 142.3, 172.1, and 174.0; mass spectrum (chemical ionization, methane) *m/z* 456 $(M + H)^+$. Anal. Calcd for $C_{30}H_{33}NO_3$: C, 79.09; H, 7.30. Found: C, 79.03; H, 7.29.

***N*-(2,2-Diphenyl-4-methyl-4-pentenoyl)-(*S*)-valine Benzyl Ester (9).** To a cooled (0−5 °C) solution containing 200 mg (0.53 mmol) of (*S*)-valine benzyl ester *p*-toluenesulfonate, 176 mg (0.66 mmol) of 2,2-diphenyl-4-methyl-4-pentenoic acid, and 222 μL (161 mg, 1.59 mmol) of Et_3N in 10 mL of CH_2Cl_2 was added 168 mg (0.66 mmol) of BOP-Cl. The reaction was allowed to warm to room temperature and was then stirred for 18 h. Workup and purification as described for compound **2** afforded *N*-(2,2-diphenyl-4-methyl-4-pentenoyl)-(*S*)-valine benzyl ester (**9**) as a colorless oil: yield 176 mg (73%); silica gel TLC R_f 0.61 (1% MeOH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 0.62 (d, 3H, J = 7 Hz), 0.72 (d, 3H, J = 7 Hz), 1.40 (s, 3H), 2.04−2.13 (m, 1H), 3.14 (d, 1H, J = 14 Hz), 3.28 (d, 1H, J = 14 Hz) 4.46 (s, 1H), 4.58−4.61 (m, 1H), 4.68 (s, 1H), 5.09 (d, 1H, J = 12 Hz), 5.16 (d, 1H, J = 12 Hz), 6.12 (d, 1H, J = 8 Hz), and 7.21−7.41 (m, 15H); ^{13}C NMR ($CDCl_3$) δ 17.8, 19.5, 25.3, 31.6, 46.5, 58.0, 62.0, 67.4, 116.1, 127.5, 128.6, 128.62, 128.88, 128.9, 129.1, 129.6, 130.0, 135.9, 143.0, 144.0, 172.1, and 174.1; mass spectrum (chemical ionization, methane) *m/z* 456 $(M + H)^+$. Anal. Calcd for $C_{30}H_{33}NO_2$: C, 79.09; H, 7.30. Found: C, 79.10; H, 7.35.

***N*-(9-Allylfluorenyl-9-carbonyl)-(*S*)-valine Benzyl Ester (10).** To a cooled (0−5 °C) solution containing 200 mg (0.53 mmol) of (*S*)-valine benzyl ester *p*-toluenesulfonate, 146 mg (0.58 mmol) of 9-allylfluorenyl-9-carboxylic acid, 58 μL (54 mg, 0.53 mmol) of *N*-methylmorpholine, and 78 mg (0.58 mmol) of hydroxybenzotriazole in 6 mL of CH_2Cl_2 was added 120 mg (0.58 mmol) of *N*,*N*′-dicyclohexylcarbodiimide. The reaction was allowed to warm to room temperature and was then stirred for 18 h. Workup and purification as described for compound **2** afforded *N*-(9-allylfluorenyl-9-carbonyl)-(*S*)-valine benzyl ester (**10**) as a colorless oil: yield 221 mg (94%); silica gel TLC R_f 0.64 (1% MeOH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 0.50 (d, 3H, J = 7 Hz), 0.66 (d, 3H, J = 7 Hz), 1.93−1.99 (m, 1H), 3.09−3.12 (m, 2H), 4.43−4.48 (m, 1H), 4.75−4.88 (m, 2H), 4.98 (d, 1H, J = 12 Hz), 5.06 (d, 1H, J = 12 Hz), 5.20−5.34 (m, 1H), 5.68 (d, 1H, J = 8.5 Hz), and 7.19−7.77 (m, 13H); ^{13}C NMR ($CDCl_3$) δ 17.8, 19.4, 31.7, 41.4, 57.6, 62.8, 67.4, 118.9, 120.8, 120.9, 125.1, 125.4, 128.2, 128.4, 128.8, 128.9, 129.0, 129.1, 133.7, 135.8, 141.2, 141.5, 145.5, 146.1, 171.6, and 172.8; mass spectrum (chemical ionization, methane) *m/z* 440 (M +

H)$^+$. Anal. Calcd for $C_{29}H_{29}NO_3$: C, 79.24; H, 6.65. Found: C, 79.02; H, 6.69.

General Procedure for Deprotection of Pentenoyl Derivatives. To a solution of 0.20 mmol of protected (*S*)-valine benzyl ester in 2 mL of 1:1 THF−H_2O was added 3 equiv of I_2. The reaction mixture was stirred at room temperature for 5 min, quenched with solid $Na_2S_2O_3$, and concentrated under diminished pressure. The residue was applied to a silica gel column (1 × 20 cm); elution with 5% MeOH in CH_2Cl_2 gave (*S*)-valine benzyl ester as a colorless oil. The deprotected product was then converted to the *p*-toluenesulfonic acid salt. ^{1}H NMR data and melting points were the same as those of the authentic valine benzyl ester *p*-toluenesulfonate salt.

During the treatment of compound **10** with iodine the formation of iodohydrin **10a** was observed. Compound **10a**: ^{1}H NMR ($CDCl_3$) δ 0.44 (d, 1.5 H, J = 7 Hz), 0.51 (d, 1.5 H, J = 7 Hz), 0.59 (d, 1.5 H, J = 7 Hz), 0.70 (d, 1.5 H, J = 7 Hz), 1.94−2.12 (m, 2 H), 2.88−2.97 (m, 1 H), 3.06−3.20 (m, 2 H), 3.38−3.51 (m, 1 H), 4.41−4.46 (m, 1 H), 4.92−5.09 (m, 2 H), 5.61 (t, 1 H, J = 8 Hz) and 7.17−7.81 (m, 13 H); mass spectrum (FAB) m/z 584.3 (M + H)$^+$.

***N*-(4-Pentenoyl)-(*S*)-valine Methyl Ester (12a).** To a cooled (0−5 °C) solution containing 500 mg (2.98 mmol) of (*S*)-valine methyl ester hydrochloride (**11a**), 335 μL (328 mg, 3.28 mmol) of pentenoic acid, 328 μL (301 mg, 2.98 mmol) of *N*-methylmorpholine, and 443 mg (3.28 mmol) of hydroxybenzotriazole in 20 mL of CH_2Cl_2 was added 677 mg (3.28 mmol) of *N*,*N*′-dicyclohexylcarbodiimide. The reaction was allowed to warm to room temperature and was then stirred for 18 h. The reaction mixture was filtered, diluted with 30 mL of CH_2Cl_2, and washed successively with two 50-mL portions of 0.5 N 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 crude product was applied to a silica gel column (2 × 40 cm); elution with 1−2% MeOH in CH_2Cl_2 afforded *N*-(4-pentenoyl)-(*S*)-valine methyl ester (**12a**) as a colorless oil: yield 575 mg (90%); silica gel TLC R_f 0.36 (4% MeOH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 0.90 (d, 3H, J = 7 Hz), 0.93 (d, 3H, J = 7 Hz), 2.10−2.18 (m, 1H), 2.31−2.42 (m, 4H), 3.74 (s, 3H), 4.56−4.61 (m, 1H), 5.01−5.12 (m, 2H), 5.77−5.85 (m, 1H), and 5.93 (d, 1H, J = 8.5 Hz); mass spectrum (electron impact) m/z 213.136 ($C_{11}H_9NO_3$ requires 213.136).

***N*-(4-Pentenoyl)-(*S*)-phenylalanine Methyl Ester (12b).** To a cooled (0−5 °C) solution containing 500 mg (2.32 mmol) of (*S*)-phenylalanine methyl ester hydrochloride (**11b**), 260 μL (255 mg, 2.55 mmol) of pentenoic acid, 255 μL (235 mg, 2.32 mmol) of *N*-methylmorpholine, and 345 mg (2.55 mmol) of hydroxybenzotriazole in 20 mL of CH_2Cl_2 was added 526 mg (2.55 mmol) of *N*,*N*′-dicyclohexylcarbodiimide. The reaction was allowed to warm to room temperature and was then stirred for 18 h. Workup and purification as described for **12a** afforded *N*-(4-pentenoyl)-(*S*)-phenylalanine methyl ester (**12b**) as a colorless solid: yield 505 mg (83%); mp 40−41 °C; silica gel TLC R_f 0.46 (4% MeOH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 2.24−2.36 (m, 4H), 3.05−3.19 (m, 2H), 3.73 (s, 3H), 4.87−4.97 (m, 1H), 5.00−5.06 (m, 2H), 5.71−5.83 (m, 1H), 5.91 (d, 1H, J = 8.5 Hz), and 7.07−7.32 (m, 5H); mass spectrum (chemical ionization, methane) m/z 262 (M + H)$^+$. Anal. Calcd for $C_{15}H_{19}NO_3$: C, 68.94; H, 7.33. Found: C, 68.81; H, 7.34.

***N*-(4-Pentenoyl)-(*S*)-methionine Methyl Ester (12c).** To a cooled (0−5 °C) solution containing 500 mg (2.50 mmol) of (*S*)-methionine methyl ester hydrochloride (**11c**), 281 μL (275 mg, 2.75 mmol) of pentenoic acid, 275 μL (253 mg, 2.50 mmol) of *N*-methylmorpholine, and 372 mg (2.75 mmol) of hydroxybenzotriazole in 20 mL of CH_2Cl_2 was added 567 mg (2.75 mmol) of *N*,*N*′-dicyclohexylcarbodiimide. The reaction was allowed to warm to room temperature and was then stirred for 18 h. Workup and purification as described for **12a** afforded *N*-(4-pentenoyl)-(*S*)-methionine methyl ester (**12c**) as a colorless oil: yield 514 mg (84%); silica gel TLC R_f 0.38 (4% MeOH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 1.94−2.04 (m, 1H) 2.10 (s, 3H), 2.12−2.23 (m, 1H), 2.31−2.60 (m, 6H), 3.76 (s, 3H), 4.70−4.77 (m, 1H), 5.01−5.12 (m, 2H), 5.76−5.88 (m, 1H), and 6.15 (d, 1H, J = 7 Hz); mass spectrum (chemical ionization, methane) m/z 246 (M + H)$^+$. Anal. Calcd for $C_{11}H_{19}NO_3S$: C, 53.85; H, 7.81. Found: C, 53.71; H, 7.82.

***N*-(4-Pentenoyl)-(*S*)-alanine Methyl Ester (12d).** To a cooled (0−5 °C) solution containing 500 mg (3.58 mmol) of (*S*)-alanine methyl ester hydrochloride (**11d**), 402 μL (394 mg, 3.94 mmol) of pentenoic acid, 3.94 μL (362 mg, 3.58 mmol) of *N*-methylmorpholine, and 532 mg (3.94 mmol) of hydroxybenzotriazole in 20 mL of CH_2Cl_2 was added 813 mg (3.94 mmol) of *N*,*N*′-dicyclohexylcarbodiimide. The reaction was allowed to warm to room temperature and was then stirred for 18 h. Workup and purification as described for compound **12a** afforded *N*-(4-pentenoyl)-(*S*)-alanine methyl ester (**12d**) as a colorless oil: yield 422 mg (64%); silica gel TLC R_f 0.34 (4% MeOH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 1.40 (d, 3H, J = 7 Hz), 2.17−2.45 (m, 4H), 3.76 (s, 3H), 4.56−4.66 (m, 1H), 5.00−5.11 (m, 2H), 5.77−5.90 (m, 1H), and 6.02 (br, 1H); mass spectrum (chemical ionization, methane) m/z 186 (M + H)$^+$. Anal. Calcd for $C_9H_{15}NO_3$: C, 58.36; H, 8.16. Found: C, 58.42; H, 8.13.

***N*-(4-Pentenoyl)-(*S*)-valine Cyanomethyl Ester (13a).** To a solution containing 570 mg (2.67 mmol) of compound **12a** in 15 mL of THF was added 336 mg (8.02 mmol) of LiOH in 10 mL of H_2O. The reaction mixture was stirred at room temperature for 3 h, and 50 mL of ether and 50 mL of saturated aqueous $NaHCO_3$ were then added. The layers were separated, and the ether layer was washed with 25 mL of saturated aqueous $NaHCO_3$. The combined aqueous layer was acidified with concentrated HCl and extracted with three 25-mL portions of CH_2Cl_2. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was dissolved in 10 mL of CH_3CN, and 1.59 mL (1.16 g, 11.42 mmol) of Et_3N and 433 μL (516 mg, 6.84 mmol) of chloroacetonitrile were added. The reaction mixture was stirred at room temperature for 20 h and concentrated. The residue was dissolved in 50 mL of CH_2Cl_2 and washed with two 50-mL portions of 1 N $NaHSO_4$ and then with 50 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 (2 × 40 cm); elution with 1:1 ethyl acetate−hexanes afforded *N*-(4-pentenoyl)-(*S*)-valine cyanomethyl ester (**13a**) as a colorless solid: yield 490 mg (77%); mp 45−46 °C; silica gel TLC R_f 0.43 (1:1 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 0.95 (d, 3H, J = 7 Hz), 0.98 (d, 3H, J = 7 Hz), 2.16−2.23 (m, 1H), 2.33−2.42 (m, 4H), 4.59−4.64 (m, 1H), 4.69 (d, 1H, J = 15 Hz), 4.85 (d, 1H, J = 15 Hz), 5.03−5.13 (m, 2H), and 5.77−5.85 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 18.4, 19.4, 29.9, 31.4, 35.9, 49.2, 57.4, 114.6, 116.2, 137.3, 171.3 and 173.2; mass spectrum (chemical ionization, methane) m/z 239 (M + H)$^+$. Anal. Calcd for $C_{12}H_{18}N_2O_2$: C, 60.49; H, 7.61. Found: C, 60.23; H, 7.53.

***N*-(4-Pentenoyl)-(*S*)-phenylalanine Cyanomethyl Ester (13b).** Treatment of compound **12b** (460 mg, 1.76 mmol) as described for **12a** gave *N*-(4-pentenoyl)-(*S*)-phenylalanine cyanomethyl ester (**13b**) as a colorless solid: yield 380 mg (75%); mp 57−58 °C; silica gel TLC R_f 0.33 (1:1 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 2.25−2.36 (m, 4H), 3.13−3.16 (m, 2H), 4.68 (d, 1H, J = 15 Hz), 4.80 (d, 1H, J = 15 Hz), 4.90−5.06 (m, 3H), 5.70−5.82 (m, 2H), and 7.12−7.36 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 29.7, 35.8, 38.0, 49.4, 53.5, 114.5, 116.3, 128.0, 129.4, 129.7, 135.7, 137.2, 171.0, and 172.8; mass spectrum (chemical ionization, methane) m/z 287 (M + H)$^+$. Anal. Calcd for $C_{16}H_{18}N_2O_2$: C, 67.12; H, 6.34. Found: C, 67.18; H, 6.36.

***N*-(4-Pentenoyl)-(*S*)-methionine Cyanomethyl Ester (13c).** Treatment of compound **12c** (405 mg, 1.65 mmol) as described for **12a** gave *N*-(4-pentenoyl)-(*S*)-methionine cyanomethyl ester (**13c**) as a colorless solid: yield 257 mg (58%); mp 58−59 °C; silica gel TLC R_f 0.29 (1:1 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 1.99−2.09 (m, 1H), 2.11 (s, 3H), 2.15−2.26 (m, 1H), 2.32−2.43 (m, 4H), 2.55 (t, 2H, J = 7 Hz), 4.73 (d, 1H, J = 16 Hz), 4.77−4.88 (m, 1H), 4.84 (d, 1H, J = 16 Hz), 5.02−5.13 (m, 2H), 5.77−5.88 (m, 1H), and 6.15 (d, 1H, J = 8.5 Hz); ^{13}C NMR ($CDCl_3$) δ 15.9, 29.8, 30.4, 31.1, 35.8, 49.6, 51.6, 114.6, 116.3, 137.2, 171.3, and 173.2; mass spectrum (chemical ionization, methane) m/z 271 (M + H)$^+$. Anal. Calcd for $C_{12}H_{18}N_2O_3S$: C, 53.31; H, 6.71. Found: C, 53.23; H, 6.67.

***N*-(4-Pentenoyl)-(*S*)-alanine Cyanomethyl Ester (13d).** Treatment of compound **12d** (370 mg, 2.0 mmol) as described for **12a** gave *N*-(4-pentenoyl)-(*S*)-alanine cyanomethyl ester (**13d**) as a colorless solid: yield 50 mg (12%); mp 48−49 °C; silica gel TLC R_f 0.24 (1:1 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 1.46 (d, 3H, J = 7.5 Hz), 2.30−2.44 (m, 4H), 4.61−4.68 (m, 1H), 4.72 (d, 1H, J = 16 Hz), 4.84 (d, 1H, J = 16 Hz), 5.01−5.13 (m, 2H), and 5.77−5.88 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 18.2, 29.8, 35.8, 48.2, 49.5, 114.5, 116.3, 137.2, 172.2, and 172.7; mass spectrum (chemical ionization, methane) *m*/*z* 211 $(M + H)^+$. Anal. Calcd for $C_{10}H_{14}N_2O_3$: C, 57.13; H, 6.71. Found: C, 57.22; H, 6.67.

***N*-(4-Pentenoyl)-(*S*)-valine pdCpA Ester (14a).** To a conical vial containing 4.1 mg (19 μmol) of *N*-4-pentenoyl-(*S*)-valine cyanomethyl ester (**13a**) was added a solution of 4.8 mg (3.5 μmol) of the tris(tetrabutylammonium) salt of pdCpA 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 microliters of the diluted aliquot was analyzed by HPLC on a C_{18} reverse phase column (10 × 250 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 1:2 CH_3CN−50 mM NH_4OAc, pH 4.5, and purified using the same semipreparative C_{18} reverse phase column [retention times 15.3 and 15.7 min, for the two positional (2′,3′) isomers]. After lyophilization of the appropriate fractions, *N*-(4-pentenoyl)-(*S*)-valyl-pdCpA (**14a**) was obtained as a colorless solid: yield 2.7 mg (94%); λ_{max} 260 nm (pH 4.5); mass spectrum (FAB) *m*/*z* 840.209 $(M+Na)^+$ ($C_{29}H_{41}N_9O_{15}P_2Na$ requires 840.209).

***N*-(4-Pentenoyl)-(*S*)-phenylalanyl-pdCpA (14b).** To a conical vial containing 4.5 mg (15.7 μmol) of *N*-(4-pentenoyl)-(*S*)-phenylalanine cyanomethyl ester (**13b**) was added a solution of 3.8 mg (2.8 μmol) of the tris(tetrabutylammonium) salt of pdCpA in 50 μL of DMF. The reaction mixture was stirred at room temperature for 3 h. HPLC purification (retention times 18.9 and 19.3 min) as described for **14a** afforded *N*-(4-pentenoyl)-(*S*)-phenylalanyl-pdCpA (**14b**) as a colorless solid: yield 1.4 mg (58%); mass spectrum (FAB) *m*/*z* 866.230 $(M + H)^+$ ($C_{33}H_{42}N_9O_{15}P_2$ requires 866.228).

***N*-(4-Pentenoyl)-(*S*)-methionyl-pdCpA (14c).** To a conical vial containing 4.1 mg (15.1 μmol) of *N*-(4-pentenoyl)-(*S*)-methionine cyanomethyl ester (**13c**) was added a solution of 4.1 mg (3.0 μmol) of the tris(tetrabutylammonium) salt of pdCpA in 50 μL of DMF. The reaction mixture was stirred at room temperature for 3 h. HPLC purification (retention times 16.0 and 16.5 min) as described for **14a** afforded *N*-(4-pentenoyl)-(*S*)-methionyl-pdCpA (**14c**) as a colorless solid: yield 2.1 mg (82%); mass spectrum (FAB) *m*/*z* 850.200 $(M + H)^+$ ($C_{29}H_{42}N_9O_{15}P_2S$ requires 850.200).

***N*-4-Pentenoyl-(*S*)-alanyl-pdCpA (14d).** To a conical vial containing 3.5 mg (16.6 μmol) of *N*-4-pentenoyl-(*S*)-alanine cyanomethyl ester (**13d**) was added a solution of 4.5 mg (3.3 μmol) of the tris(tetrabutylammonium) salt of pdCpA in 50 μL of DMF. The reaction mixture was stirred at room temperature for 3 h. HPLC purification (retention times 12.8 and 13.3 min) as described for **14a** afforded *N*-4-pentenoyl-(*S*)-alanyl-pdCpA (**14d**) as a colorless solid: yield 1.2 mg (46%); mass spectrum (FAB) *m*/*z* 790.193 $(M + H)^+$ ($C_{27}H_{38}N_9O_{15}P_2$ requires 790.196).

(*S*)-Valyl-pdCpA. To a solution containing 1 A_{260} unit of *N*-(4-pentenoyl)-(*S*)-valyl-pdCpA (**14a**) in 50 μL of H_2O was added 0.1 mg (∼6 equiv) of I_2. After 5 min the reaction was quenched by the addition of 50 μL of 0.1 N $Na_2S_2O_3$ and analyzed by C_{18} reverse phase HPLC using the same conditions as described above for compound **14a**. Deblocked (*S*)-valyl pdCpA ester had the same retention time (11.2 min) as a sample of (*S*)-valine pdCpA ester derived from *N*-(6-nitroveratryloxycarbonyl)-(*S*)-valyl-pdCpA.

(*S*)-Methionyl-pdCpA. To a solution containing 1 A_{260} unit of *N*-(4-pentenoyl)-(*S*)-methionyl-pdCpA (**14c**) in 50 μL of H_2O was added 0.1 mg (∼6 equiv) of I_2. After 10 min the reaction was quenched by the addition of 50 μL of 0.1 N $Na_2S_2O_3$ and analyzed by C_{18} reverse phase HPLC using the same conditions as described above for compound **14a**. Deblocked (*S*)-methionyl-pdCpA had the same retention time (10.3 min) as a sample of (*S*)-methionyl-pdCpA derived from *N*-(6-nitroveratryloxycarbonyl)-(*S*)-methionyl-pdCpA.[28]

Dimethyl *N*-(4-Pentenoyl)-(*S*)-aspartate (18). To a cooled (0−5 °C) solution containing 1.0 g (5.06 mmol) of dimethyl (*S*)-aspartate hydrochloride (**17**), 0.57 mL (558 mg, 5.57 mmol) of pentenoic acid, 0.56 mL (512 mg, 5.06 mmol) of *N*-methylmorpholine, and 0.75 g (5.57 mmol) of hydroxybenzotriazole in 20 mL of CH_2Cl_2 was added 1.15 g (5.57 mmol) of *N*,*N*′-dicyclohexylcarbodiimide. The reaction was allowed to warm to room temperature and was then stirred for 18 h. The reaction mixture was filtered, diluted with 30 mL of CH_2Cl_2, and washed successively with two 50-mL portions of 0.5 N 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 crude product was applied to a silica gel column (2 × 40 cm); elution with 1:1 ethyl acetate−hexanes afforded dimethyl *N*-(4-pentenoyl)-(*S*)-aspartate (**18**) as a colorless oil: yield 1.10 g (89%); silica gel TLC R_f 0.33 (1:1 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 2.30−2.41 (m, 4H), 2.84 (dd, 1H, J = 17.5, 4 Hz), 3.04 (dd, 1H, J = 17.5, 4 Hz), 3.69 (s, 3H), 3.76 (s, 3H), 4.84−4.89 (m, 1H), 4.99−5.10 (m, 2H), 5.75−5.86 (m, 1H), and 6.51 (d, 1H, J = 9 Hz); ^{13}C NMR ($CDCl_3$) δ 29.8, 35.9, 36.5, 48.8, 52.4, 53.2, 116.0, 137.2, 171.7, 172.0, and 172.6; mass spectrum (chemical ionization, methane) *m*/*z* 244 $(M + H)^+$. Anal. Calcd for $C_{11}H_{17}NO_5$: C, 54.31; H, 7.04. Found: C, 54.15; H, 7.14.

Dinitroveratryl *N*-(4-Pentenoyl)-(*S*)-aspartate (19). To a solution containing 203 mg (0.83 mmol) of dimethyl *N*-(4-pentenoyl)-(*S*)-aspartate (**18**) in 2 mL of THF was added 25 mL of 0.1 N NaOH. After stirring at room temperature for 3 h the reaction mixture was acidified to pH 2 with 10% aqueous $NaHSO_4$ and extracted with three 30-mL portions of ethyl acetate. The combined organic extract was dried (Na_2SO_4) and concentrated under diminished pressure. The residue was dissolved in 2 mL of DMF and 429 mg (2.82 mmol) of CsF was added, followed by 276 mg (1.0 mmol) of nitroveratryl bromide.[17] The reaction mixture was stirred at room temperature for 6 h, diluted with 30 mL of ethyl acetate, and washed with two 10-mL portions of water. The organic layer was dried (Na_2SO_4) and concentrated under diminished pressure. The crude product was applied to a silica gel column (2 × 25 cm); elution with 1:3 ethyl acetate−hexanes afforded dinitroveratryl *N*-(4-pentenoyl)-(*S*)-aspartate (**19**) as a pale yellow solid: yield 200 mg (40%); mp 167.5−168.5 °C; silica gel TLC R_f 0.22 (1:1 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 2.32−2.38 (m, 4H), 2.95 (dd, 1H, J = 17.5, 4.5 Hz), 3.19 (dd, 1H, J = 17.5, 4.5 Hz), 3.95 (s, 3H), 3.96 (s, 3H), 3.98 (s, 3H), 3.99 (s, 3H), 4.96−5.08 (m, 3H), 5.44−5.59 (m, 4H), 5.72−5.83 (m, 1H), 6.57 (d, 1H, J = 8.5 Hz), 6.96 (s, 1H), 6.98 (s, 1H) 7.69 (s, 1H), and 7.71 (s, 1H); mass spectrum (chemical ionization, methane) *m*/*z* 606 $(M + H)^+$. Anal. Calcd for $C_{27}H_{31}N_3O_{13}$: C, 53.55; H, 5.16. Found: C, 53.44; H, 5.18.

γ-Nitroveratryl *N*-(4-Pentenoyl)-(*S*)-aspartate Cyanomethyl Ester (20). To a cooled (0−5 °C) solution containing 200 mg (0.33 mmol) of dinitroveratryl *N*-(4-pentenoyl)-(*S*)-aspartate (**19**) in 2 mL of THF was added a solution of 21 mg (0.50 mmol) of LiOH in 2 mL of water. The reaction mixture was stirred for 4 h, diluted with 5 mL of water, and extracted with three 10-mL portions of CH_2Cl_2. The aqueous layer was then acidified with 10% $NaHSO_4$ and extracted with three 15-mL portions of ethyl acetate. The combined organic layer was dried (Na_2SO_4) and concentrated under diminished pressure. The residue was dissolved in 2 mL of acetonitrile and 68 μL (0.48 mmol) of Et_3N was added, followed by 152 μL (2.4 mmol) of chloroacetonitrile. The reaction mixture was stirred at room temperature for 6 h and the solvent was concentrated under diminished pressure. The crude product was applied to a silica gel column (2 × 20 cm); elution with 1:1 ethyl acetate−hexanes

(28) Shayo, Y. F.; Hecht, S. M. Unpublished data. We thank Mr. Yuda Shayo for the authentic sample.

gave γ-nitroveratryl *N*-(4-pentenoyl)-(*S*)-aspartate cyanomethyl ester (**20**) as a pale yellow oil: yield 68 mg (46%); R_f 0.25 (1:1 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 2.34−2.38 (m, 4H), 2.96 (dd, 1H, *J* = 17.5, 4.5 Hz), 3.16 (dd, 1H, *J* = 17.5, 4.5 Hz), 3.97 (s, 3H), 4.01 (s, 3H), 4.70−4.81 (m, 2H), 4.99−5.11 (m, 3H), 5.46 (d, 1H, *J* = 14 Hz), 5.55 (d, 1H, *J* = 13.5 Hz), 5.77−5.90 (m, 1H), 6.50 (d, 1H, *J* = 8.5 Hz), 6.97 (s, 1H), and 7.74 (s, 1H); mass spectrum (electron impact) *m*/*z* 449.142 (M^+) ($C_{20}H_{23}N_3O_9$ requires 449.143).

γ-Nitroveratryl *N*-(4-Pentenoyl)-(*S*)-aspartyl-pdCpA (21). A solution containing 4 mg (2.94 μmol) of the tris(tetrabutylammonium) salt of pdCpA and 10 mg (22.3 μmol) of γ-nitroveratryl *N*-(4-pentenoyl)-(*S*)-aspartate cyanomethyl ester (**20**) in 50 μL of DMF was stirred at room temperature for 4 h. HPLC purification as described for compound **14a** afforded compound **21** as a pale yellow foam: yield 1.1 mg (36%); mass spectrum (FAB) *m*/*z* 1029.238 $(M + H)^+$ ($C_{37}H_{47}N_{10}O_{21}P_2$ requires 1029.239).

γ-Nitroveratryl *N*-(4-Pentenoyl)-*threo*-β-methyl-(*S*)-aspartyl-pdCpA (22a). A solution containing 10 mg (7.35 μmol) of the tris(tetrabutylammonium) salt of pdCpA and 25 mg (53.9 μmol) of γ-nitroveratryl *N*-(4-pentenoyl)-*threo*-γ-methyl-(*S*)-aspartate cyanomethyl ester[19] in 100 μL of DMF was stirred at room temperature for 16 h. HPLC purification as described for compound **14a** afforded compound **22a** as a pale yellow solid: yield 4.5 mg (59%); mass spectrum (FAB) *m*/*z* 1043.254 $(M + H)^+$ ($C_{38}H_{49}N_{10}O_{21}P_2$ requires 1043.255).

γ-Nitroveratryl *N*-(4-Pentenoyl)-*erythro*-β-methyl-(*S*)-aspartyl-pdCpA (22b). A solution containing 10 mg (7.35 μmol) of the tris(tetrabutylammonium) salt of pdCpA and 25 mg (53.9 μmol) of γ-nitroveratryl *N*-(4-pentenoyl)-*erythro*-β-methyl-(*S*)-aspartate cyanomethyl ester[19] in 100 μL of DMF was stirred at room temperature for 6 h. HPLC purification as described for compound **14a** afforded compound **22b** as a pale yellow solid: yield 4.0 mg (52%); mass spectrum (FAB) *m*/*z* 1043.254 $(M + H)^+$ ($C_{38}H_{49}N_{10}O_{21}P_2$ requires 1043.255).

γ-Nitroveratryl *N*-(4-Pentenoyl)-β,β-dimethyl-(*S*)-aspartyl-pdCpA (22c). A solution containing 4.4 mg (3.24 μmol) of the tris(tetrabutylammonium) salt of pdCpA and 8.0 mg (16.8 μmol) of γ-nitroveratryl *N*-(4-pentenoyl)-β,β-dimethyl-(*S*)-aspartate cyanomethyl ester[19] in 100 μL of DMF was stirred at room temperature for 6 h. HPLC purification as described for compound **14a** afforded compound **22c** as a pale yellow solid: yield 2.1 mg (61%); mass spectrum (FAB) *m*/*z* 1057.271 $(M + H)^+$ ($C_{39}H_{51}N_{10}O_{21}P_2$ requires 1057.271).

Synthesis and Deprotection of *N*-(4-pentenoyl)aminoacyl-tRNAs. An incubation mixture having 0.5 A_{260} unit (20 nmol) of a *N*-(4-pentenoyl)aminoacyl-pdCpA (**14**) and 5 μg (0.2 nmol) of a $tRNA_{CUA}$ transcript lacking the 3′-terminal pCpA moiety in 50 μL of 50 mM Hepes buffer, pH 7.5, containing 15 mM $MgCl_2$, 0.75 mM ATP, 10% DMSO (v/v), and 100 units of T4 RNA ligase was incubated at 37 °C for 60 min. The incubation mixture was treated with 3 M NaOAc, pH 5.3, to a final salt concentration of 0.3 M and then with 2.5 volumes of cold ethanol. The precipitated *N*-(4-pentenoyl)-aminoacyl-tRNA (**15**) was collected by centrifugation. The pellet was washed with ethanol, dried, and then dissolved in H_2O to a final concentration of 1 μg/μL.

Deprotection was accomplished by admixture of 0.25 volume of 25 mM I_2 in 1:1 THF−H_2O. The combined solution was maintained at 25 °C for 5−60 min, and the aminoacyl-tRNA (**16**) was recovered by centrifugation following successive additions of 0.1 volume of 3 M NaOAc, pH 5.3, and 2.5 volumes of cold ethanol. The tRNA pellet was washed with ethanol, dried, and then dissolved in H_2O for the translation experiments.

Synthesis and Deprotection of *N*-(Nitroveratryloxycarbonyl)-(*S*)-valyl-tRNA. The protected aminoacyl-tRNA was prepared as described above for *N*-(4-pentenoyl)aminoacyl-tRNAs.

Deprotection was accomplished by irradiation of a cold (2−2.5 °C) aqueous solution of the NVOC-valyl-tRNA (at a concentration of 1 μg/μL) with a 500 W mercury−xenon lamp for 5 min using Pyrex and water filters.

In Vitro Protein Synthesis. In vitro protein synthesis reactions (typically 25 μL final volume) contained 17.5 μL of rabbit reticulocyte lysate, 1 μg of the DHFR mRNA, 40 μM amino acids with or without 40 μM methionine, 20 μCi [^{35}S]-methionine (1000 Ci/mmol), and 2.5 μg of aminoacyl-$tRNA_{CUA}$. Reactions were incubated at 30 °C for 60 min and quenched by cooling to 0 °C. One-microliter aliquots were analyzed by 20% SDS−polyacrylamide gel electrophoresis. For quantitation of the suppression efficiency after electrophoresis, the gel was fixed in 40% methanol−10% acetic acid, dried, and analyzed on a phosphorimager.

Quantitation of DHFR Activity. The enzymatic activity of DHFR was determined as previously described,[16] with one modification. Before enzymatic assay, samples of purified DHFRs containing nitroveratryl-protected derivatives of aspartic acid were irradiated for 30 min at 2 °C with a 500 W mercury−xenon lamp using Pyrex and water filters. No enzymatic activity was detected without irradiation. After irradiation the activities of the DHFRs containing modified aspartic acid analogues were comparable to those described.[16]

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Supporting Information Available: ^{1}H NMR spectra of compounds **5**, **10a** and **20** (3 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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