Ribosome-Mediated Incorporation of Hydrazinophenylalanine into Modified Peptide and Protein Analogues

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Abstract: (*S*)-α-Hydrazinophenylalanyl-tRNA^{Phe}, an aminoacyl-tRNA derivative containing the unnatural amino acid (*S*)-α-hydrazinophenylalanine, was prepared in an effort to examine the stereochemical requirements of the A-site of the ribosome during in vitro protein synthesis. The (*S*)-α-hydrazinophenylalanine moiety was of interest because it contains two nucleophilic centers, the secondary nitrogen attached to C$^{\alpha}$, which is normally acylated during the course of peptide bond formation, and the sterically less hindered primary nitrogen. To determine the position of acylation, (*S*)-α-hydrazinophenylalanyl-tRNA^{Phe} was tested in an *Escherichia coli* in vitro protein biosynthesizing system lacking elongation factor G, such that only dipeptide products were formed. The dipeptide product mixture was analyzed by HPLC in direct comparison with authentic synthetic standards. The dipeptide assay utilizing (*S*)-α-hydrazinophenylalanyl-tRNA^{Phe} as the A-site tRNA established that the analogue functioned well as an acceptor tRNA; HPLC analysis of the products showed that both dipeptides were formed in approximately equal amounts. When attached to a suppressor tRNA transcript, (*S*)-α-hydrazinophenylalanine was also incorporated into position 27 of dihydrofolate reductase in an *E. coli* protein synthesizing system by readthrough of a nonsense codon. This finding expands the currently accepted model of peptide bond formation at the ribosome and adds to the repertoire of peptide-like products shown to form at the peptidyltransferase center of the ribosome.

The catalysis of peptide bond formation between the aminoacyl moiety of the A-site tRNA and the peptidyl moiety of the P-site tRNA may be regarded as the primary catalytic function of the ribosome.[1] Catalysis occurs at the peptidyltransferase site which is located on the 50S ribosomal subunit in prokaryotes. Many fundamental aspects of peptide bond formation at the peptidyltransferase site remain incompletely defined. This includes the actual nature of catalysis, which could in principle occur either as a result of induced proximity of aminoacyl- and peptidyl-tRNA substrates or by some more active role of the peptidyltransferase center.[2]

Studies of the peptidyltransferase center have utilized probes such as the antibiotic puromycin, which disrupts peptide synthesis by substituting for the A-site tRNA during peptide bond formation.[3] The peptidyltransferase site has also been studied using aminoacyl-tRNA analogues with chemically or photochemically activated components.[2c,4]

One very specific way to probe peptidyltransferase function would be to utilize natural tRNA substrates that contain altered aminoacyl components, i.e., misacylated tRNAs. The first example of the use of misacylated tRNA in protein biosynthesis was reported in 1962 by Chapeville et al.[5] These researchers synthesized alanyl-tRNA^{Cys} from cysteinyl-tRNA^{Cys} by Raney nickel-mediated desulfurization and utilized the resulting misacylated tRNA to incorporate an alanine residue in the place of a cysteine residue in the protein globin. This finding supported the original hypothesis that the fidelity of protein synthesis was controlled largely at the level of tRNA activation by aminoacyl-tRNA synthetases. Fahnestock and Rich also explored the capabilities of peptidyltransferase by the use of a misacylated tRNA obtained via chemical deamination of enzymatically activated phenylalanyl-tRNA^{Phe}.[6] A more general procedure for the elaboration of misacylated tRNA's was developed in our laboratory[7,8] and has been employed by a number of laboratories for the introduction of modified amino acids into peptides[8,9] and proteins.[10]

A commonly used in vitro protein biosynthesizing system for the study of peptidyltransferase activity involves polynucleotide templates (e.g., poly(U)) in the presence of reconstituted ribosomes, elongation factors, GTP, K^+, and Mg^{2+}.[11] This system forms polypeptides (e.g., polyphenylalanine) in response to specific aminoacyl-tRNA's (i.e., phenylalanyl-tRNA^{Phe}). The

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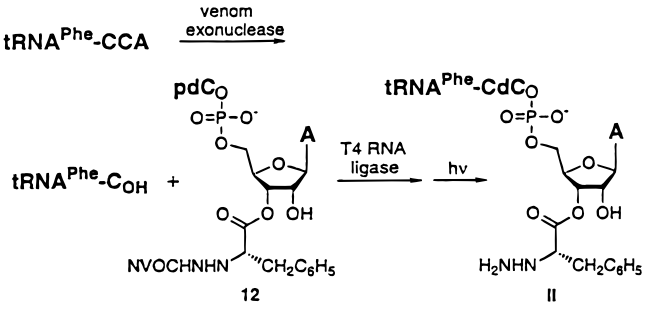
Scheme 1. Preparation of (*S*)-α-Hydrazinophenylalanyl-tRNA^{Phe} (**II**) by T4 RNA Ligase-Mediated Coupling of tRNA^{Phe}-C_{OH} + Chemically Protected (*S*)-α-Hydrazinophenylalanyl-pdCpA



resulting peptide products are of varying length due to the lack of normal stop and start codons. The formation of peptides of varying length does not allow for the precise definition of the effect of altering aminoacyl-tRNA substrates, however, and a modified assay using high-salt washed ribosomes lacking the elongation factor G has been used for this purpose instead.[7f,h,i,8] This modified assay system forms only dipeptide products due to the lack of elongation factor G, allowing for the quantification and structural analysis of the dipeptide products.

Misacylated tRNA's constructed via the strategy outlined in Scheme 1 have been used to study the structural requirements of the peptidyltransferase center using this dipeptide assay. Both A- and P-site aminoacyl-tRNA analogues have been constructed and used.[7h,i,8] P-site aminoacyl-tRNA analogues studied have included those acylated with (*R*)-amino acids, (*R,S*)-β-amino acids, and non-amino acid substrates. (*R)*-Amino acids bound to the ribosome but did not function well in peptide bond formation.[7h] This indicates a binding pocket at the ribosomal P-site sensitive to chirality. (*R,S*)-β-amino acids did function well, however, indicating that no such restriction exists at the β-position. A particularly interesting result was found using *N*-(chloroacetyl)phenylalanyl-tRNA^{Phe} at the P-site.[7f] It was shown that two dipeptide products were formed, one resulting from the expected acylation of the activated ester and one resulting from the S_N2 displacement of the chloro group of the *N*-chloroacetyl moiety. The formation of such a product of altered connectivity indicated a capability for chemical transformations at the peptidyltransferase center not previously anticipated.

The present study was designed to determine whether an activated tRNA whose aminoacyl moiety contained more than one nucleophilic functionality could form products from each when bound at the A-site of the peptidyltransferase center. The specific aminoacyl-tRNA analogue employed contained tRNA activated with (*S*)-2-hydrazino-3-phenylpropionic acid ("(*S*)-α-hydrazinophenylalanine") which contains two nucleophilic N atoms within the amino acid moiety. We demonstrate that this aminoacyl-tRNA forms a product derived from each nucleophilic center, and that (*S*)-α-hydrazinophenylalanyl-tRNA$_{CUA}$ can be used to introduce hydrazinophenylalanine into positions 10 and 27 of *Escherichia coli* dihydrofolate reductase (DHFR) by suppression of a nonsense (UAG) codon at the corresponding positions in DHFR mRNA. This modified amino acid, therefore, must function with reasonable facility in each of the partial reactions of protein biosynthesis.

Results and Discussion

Preparation of (*S*)-α-Hydrazinophenylalanyl-tRNA^{Phe}. The primary technical goal of this study was definition of the ability of an aminoacyl-tRNA containing more than one nucleophilic functionality within the aminoacyl moiety to form a product derived from each at the ribosomal peptidyltransferase center. The aminoacyl-tRNA analogue used for this purpose was (*S*)-α-hydrazinophenylalanyl-tRNA^{Phe} (**II**). As shown in Figure 1, this analogue differs from (*S*)-phenylalanyl-tRNA^{Phe} (**I**) in that it contains two N nucleophiles at C^{α} of the aminoacyl moiety. Thus it seemed logical to think that tRNA **II** might exhibit dual reactivity once bound at the ribosomal A-site. As illustrated in Scheme 2 for a ribosome bearing *N*-acetylphenylalanyl-tRNA^{Phe} at the P-site and (*S*)-α-hydrazinophenylalanyl-tRNA^{Phe} at the A-site, nucleophilic attack of the N-atom connected to C^{α} of α-hydrazinophenylalanine on the activated carboxylate of *N*-acetylphenylalanyl-tRNA^{Phe} (pathway b) would afford dipeptide **1** having connectivity analogous to those in naturally occurring peptides. In contrast, nucleophilic attack by the other N (pathway a) would produce a peptide analogue (**2**) of altered connectivity.

To study the transformations outlined in Scheme 2, we prepared (*S*)-α-hydrazinophenylalanyl-tRNA^{Phe} by the route outlined in Scheme 1. This involved removal of two nucleotides from the 3′-terminus of mature yeast tRNA^{Phe} by limited digestion with venom exonuclease to afford tRNA^{Phe}-C_{OH}, as described previously.[7] Condensation with NVOC-protected (*S*)-α-hydrazinophenylalanyl pdCpA (**12**) via the agency of T4 RNA ligase afforded NVOC-protected (*S*)-α-hydrazinophenylalanyl-tRNA^{Phe}, which was deblocked photochemically as described

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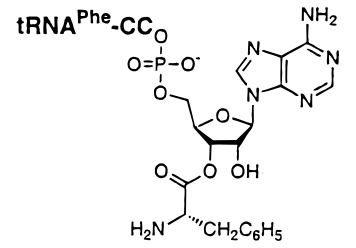
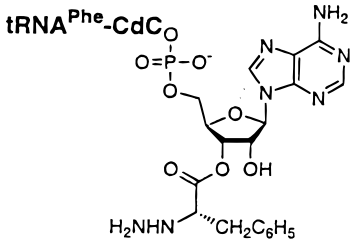
(11) See, e.g., ref 7b and references therein.



S-phenylalanyl-tRNA^{Phe}

I



S-hydrazinophenylalanyl-tRNA^{Phe}

II

Figure 1. Structures of (*S*)-phenylalanyl-tRNA^{Phe} (**I**) and (*S*)-α-hydrazinophenylalanyl-tRNA^{Phe} (**II**).

below. The synthesis of the requisite aminoacylated dinucleotide **12** is outlined in Schemes 3−5 and involved initial synthesis of (*S*)-α-hydrazinophenylalanine (**7**) itself (Scheme 3). (*R*)-phenylalanine (**3**) was converted to (*R*)-2-hydroxy-3-phenylpropionic acid (**4**) with overall retention of stereochemistry in a diazotization/hydrolysis reaction believed to proceed via an intermediate α-lactone (42% yield).[12] The optical rotation of (*R*)-2-hydroxy-3-phenylpropionic acid (**4**) was of essentially the same magnitude but opposite sign relative to the published value for the *S* enantiomer {$[\alpha]^{25}_D$ +29.8° (*c* 1.13, acetone); literature value,[12a] $[\alpha]^{25}_D$ −27.8° (*c* 1.13, acetone)}. Compound **4** was converted to the respective methyl ester (**5**) using diazomethane (80% yield). The enantiomeric purity (>98%) of methyl ester **5** was verified by derivatization as the corresponding Mosher ester.[13] The α-hydroxy moiety was then replaced with a BOC-hydrazino group with inversion of stereochemistry by S_N2 displacement of a trifluoromethanesulfonate intermediate, affording methyl (*S*)-2-[*N*-*tert*-butoxycarbonyl)hydrazino]-3-phenylpropionate (**6**) as a colorless wax in 67% yield.[14] The optical rotation of compound **6** was of the same magnitude but opposite sign relative to the published value for the corresponding *R* enantiomer {$[\alpha]^{25}_D$ −12.2° (*c* 1.19, $CHCl_3$); literature value,[14] $[\alpha]^{25}_D$ +12.0° (*c* 1.0, $CHCl_3$)}. The assignment of the structure of compound **6** as resulting from nucleophilic displacement of the triflate by the unsubstituted nitrogen of *tert*-butylcarbazate ($BocNHNH_2$) was made initially based on the work of Legrel et al.[15] who studied the acylation of *tert*-butylcarbazate. Compound **6** was deprotected by successive treatments with trifluoroacetic acid and NaOH, affording (*S*)-α-hydrazinophenylalanine (**7**) in 52% overall yield.

The conversion of (*S*)-α-hydrazinophenylalanine to the respective *N*-NVOC cyanomethyl ester is outlined in Scheme 4. Treatment of (*S*)-α-hydrazinophenylalanine (**7**) with 2-nitroveratryl chloroformate[16] provided both the mono- and di-NVOC protected compounds **8** and **9** (in 39% and 33% yields, respectively). The assignment of position of monoacylation shown for compound **8** (N^{β}) was based on literature precedent for N^{β}-acylation of α-hydrazino acids.[17] The mono- and diprotected intermediates **8** and **9** were then treated separately with $ClCH_2CN$ to give the corresponding cyanomethyl esters **10** and **11** in 71% and 61% yields, respectively.

Tetrabutylammonium pdCpA[18] was aminoacylated by treatment with monoprotected *N*-NVOC-(*S*)-α-hydrazinophenylalanine (**10**) and diprotected *N*,*N*′-di-NVOC-(*S*)-α-hydrazinophenylalanine (**11**) to give the corresponding aminoacylated dinucleotides **12** and **13**, respectively (Scheme 5). The progress of the reactions was monitored by HPLC, and the compounds were subsequently purified by HPLC. To permit control reactions to be run, *N*-NVOC-(*S*)-phenylalanyl-pdCpA was synthesized by the same method.[19] (*S*)-Phenylalanine was treated with nitroveratryl chloroformate to afford *N*-NVOC-(*S*)-phenylalanine in 79% yield. This intermediate was treated with chloroacetonitrile to afford *N*-NVOC-(*S*)-phenylalanine cyanomethyl ester in 78% yield. Tetrabutylammonium pdCpA was then treated with *N*-NVOC-(*S*)-phenylalanine cyanomethyl ester to give *N*-NVOC-(*S*)-phenylalanyl-pdCpA.[19]

To ensure the successful deprotection of the corresponding *N*-NVOC-aminoacyl-tRNAs, attempts were made to deprotect the *N*-NVOC-aminoacyl-pdCpA dinucleotides. The deprotection reactions were carried out in 1 mM potassium acetate buffer solution, pH 4.5, at a concentration anticipated to be close to that which would be used for the corresponding *N*-NVOC-aminoacyl-tRNA's (28 μM).[7i] Each sample was irradiated with light from a high-pressure mercury lamp (1000 W), and time points were taken every 2.5 min for analysis by HPLC. For *N*-NVOC-(*S*)-α-hydrazinophenylalanyl-pdCpA (**12**) and *N*,*N*′-di-NVOC-(*S*)-α-hydrazinophenylalanyl-pdCpA (**13**), HPLC analysis showed that, after 2.5 min of irradiation, much of the starting material had been consumed and after 7.5 min all of the starting material was gone and many products were present. In comparison, *N*-NVOC-(*S*)-phenylalanyl-pdCpA showed no such behavior; after 5 min of irradiation most of the starting material was consumed and a single product peak had formed. The mechanism of photolytic NVOC removal is thought to involve light-catalyzed intramolecular rearrangement in which the nitro group is reduced to a nitroso function and the oxygen is inserted into the C−H bond in the ortho position.[20] The resulting α-hydroxycarbamate functionality decomposes to give the corresponding nitrosobenzaldehyde product, CO_2, and the free amine. It has been reported that photolytic removal of the NVOC group from amino moieties are occasionally not quantitative because of a side reaction between the released amino functionality and the aldehyde byproduct, which forms the corresponding imine.[21] In the present case, it seemed possible that the released hydrazinophenylalanyl dinucleotide **14** might condense with the benzaldehyde to form the corre-

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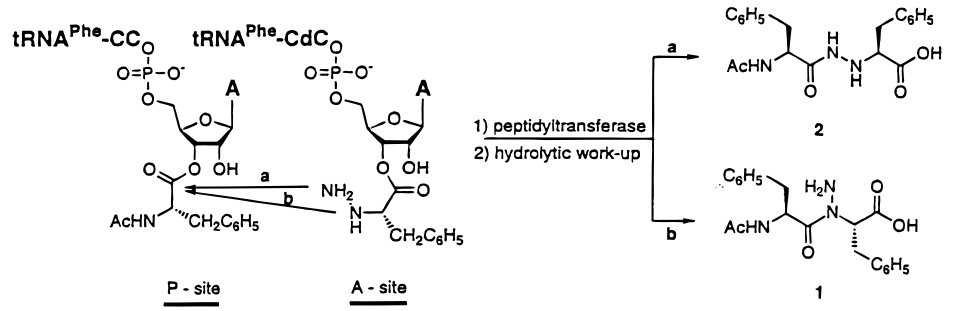
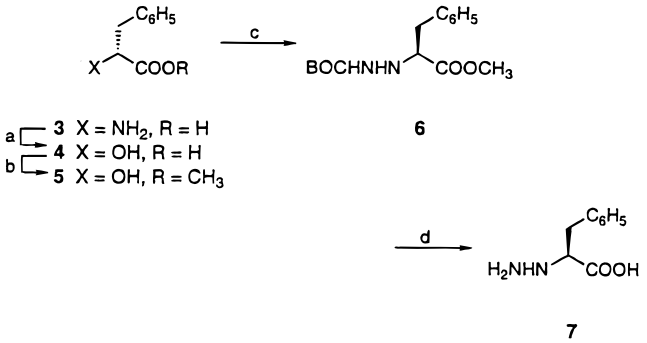
Scheme 2. Ribosomally Mediated Dipeptide Formation Using *N*-Acetyl-(*S*)-phenylalanyl-tRNA^{Phe} and (*S*)-α-Hydrazinophenylalanyl-tRNA^{Phe} as the Donor and Acceptor, Respectively

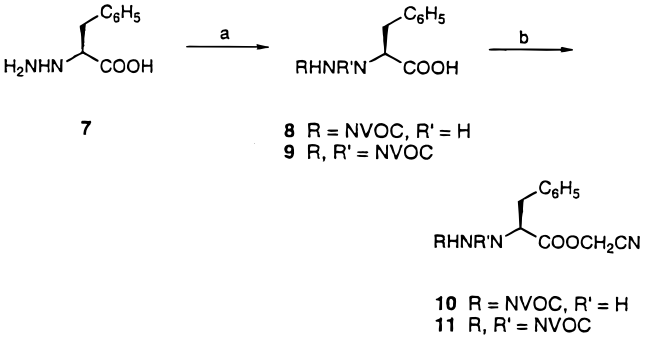


Scheme 3.[a] Chemical Synthesis of (*S*)-α-Hydrazinophenylalanine (**7**)



[a] Reaction Conditions: (a) $NaNO_2$, $H^+{}_3O$; (b) CH_2N_2; (c) Tf_2O and then $BocNHNH_2$; (d) 25% CF_3COOH in CH_2Cl_2 and then NaOH.

Scheme 4.[a] Chemical Synthesis of *N*-NVOC Protected Cyanomethyl Ester Derivatives of (*S*)-α-Hydrazinophenylalanine



[a] Reaction conditions: (a) 2-nitroveratryl chloroformate, Na_2CO_3; (b) $ClCH_2CN$.

sponding hydrazone. The resulting hydrazone might well be subject to decomposition, as hydrazones have been reported to be unstable photochemically.[17c]

It was found that the addition of semicarbazide hydrochloride to the photolysis mixture (as a scavenger for the formed benzaldehyde derivative[21]) led to the formation of a single major product (49%) having a retention time (14.4 min) expected for the desired product **14** under the HPLC conditions employed. To ensure that the addition of semicarbazide hydrochloride was not interfering with NVOC removal per se, a parallel control reaction was run containing NVOC-(*S*)-phenylalanyl-pdCpA. Comparison of the results of the photolysis of NVOC-(*S*)-phenylalanyl-pdCpA in the presence or absence of semicarbazide hydrochloride (200 equiv) showed that the same major product was formed to the same extent in either case.

Dipeptide Formation and Analysis. The aminoacylated dinucleotide *N*-NVOC-(*S*)-α-hydrazinophenylalanyl-pdCpA was

Scheme 5. Synthesis and Photodeprotection of *N*-NVOC-(*S*)-α-hydrazinophenylalanyl-pCpAs

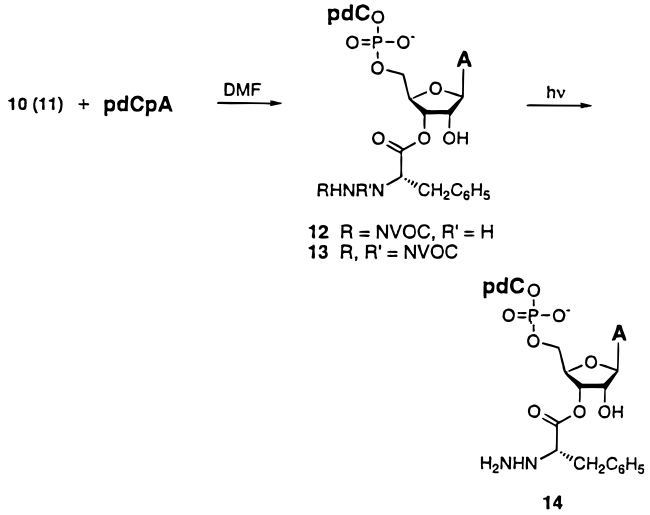


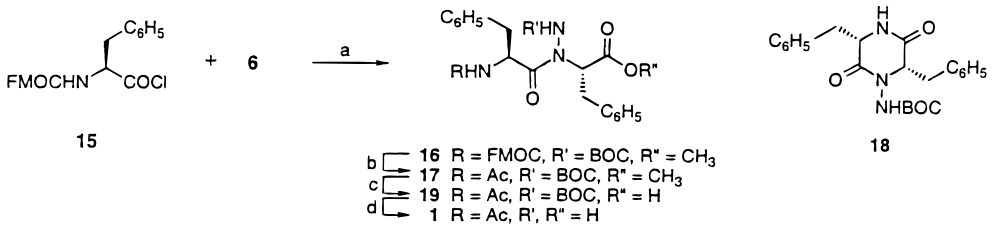
Table 1. Ribosome-Mediated Dipeptide Formation[a]

aminoacyl-tRNA	dipeptide formed (%)[b]
(*S*)-phenylalanyl-tRNA^{Phe} (**I**)[c]	100
(*S*)-phenylalanyl-tRNA^{Phe} (**I**)[d]	58
(*S*)-α-hydrazinophenylalanyl-tRNA^{Phe} (**II**)[d]	74

[a] The peptidyl-tRNA analogue employed in each case was [^{3}H]*N*-acetyl-(*S*)-phenylalanyl-tRNA^{Phe}. [b] Relative yields of dipeptides, based on incorporation of [^{3}H]radiolabel. [c] Prepared by activation of yeast tRNA^{Phe} with (*S*)-phenylalanine in the presence of the cognate aminoacyl-tRNA synthetase. [d] Prepared by T4 RNA ligase-mediated coupling of tRNA^{Phe}-C_{OH} with an aminoacylated pdCpA.

ligated onto the enzymatically abbreviated tRNA (tRNA^{Phe}-C_{OH}; Scheme 1) and deblocked, and the resulting (*S*)-α-hydrazinophenylalanyl-tRNA^{Phe} (**II**) was used as the acceptor tRNA in an in vitro protein biosynthesizing system that contained ribosomes, poly(U) and [^{3}H]-*N*-acetylphenylalanyl-tRNA^{Phe} but no elongation factor G; this ensured that peptide bond formation would be limited to the elaboration of dipeptides. The results of the dipeptide assay are shown in Table 1. (*S*)-α-Hydrazinophenylalanyl-tRNA^{Phe} (**II**) functioned well as an acceptor tRNA, forming dipeptide in slightly higher yield (74%) than (*S*)-phenylalanyl-tRNA^{Phe} constructed via the chemical aminoacylation procedure outlined in Scheme 1 (58% yield). The lower yields of dipeptide obtained with both of these species, relative to enzymatically activated phenylalanyl-tRNA^{Phe}, is consistent with experience and probably reflects damage inflicted on the "chemically aminoacylated" tRNA's during the several steps employed for their preparation. While not investigated in detail, adventitious ribonuclease activities present in the enzymes used for tRNA modification, as well as

Scheme 6.[a] Synthesis of the Dipeptide *N*-Acetylphenylalanyl-α-hydrazinophenylalanine Having Normal Peptide Connectivity



[a] Reaction conditions: (a) 1:1 CH_2Cl_2−10% $NaHCO_3$; (b) DBU, *N*-acetoxysuccinimide; (c) NaOH; (d) 25% CF_3COOH in CH_2Cl_2.

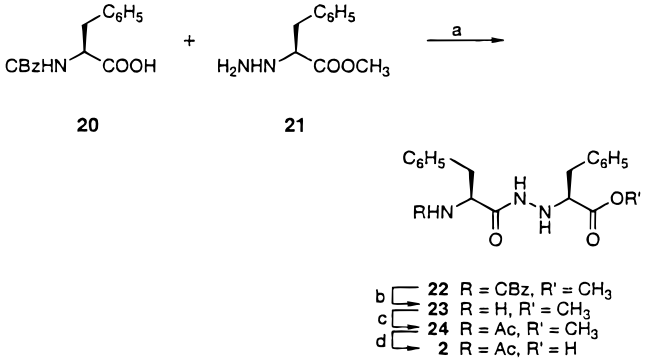
photolysis with a high-intensity light source during NVOC removal, are likely sources of the damage.

The participation of (*S*)-α-hydrazinophenylalanyl-tRNA^{Phe} as an acceptor tRNA having been established, the next consideration was the determination of the connectivity of the dipeptide product(s). Experimentally, this was carried out by HPLC analysis of the ribosomally formed dipeptide(s) using authentic, synthetic dipeptides as standards.

Scheme 6 illustrates the synthesis of the dipeptide acylated at the nitrogen attached to C^{α} of hyrazinophenylalanine, i.e., having connectivity analogous to that in normal peptides. It was felt that protection of the primary amine of α-hydrazinophenylalanine would allow for the regioselective acylation of the other nitrogen. In fact, regioselective acylation of the α-nitrogen of N^{β}-protected hydrazino acid esters (CBz-$N^{\beta}NN^{\alpha}$-$HCH_2COOCH_2CH_3$) has been reported using *N*-FMOC-protected amino acid chlorides.[17c,d] Accordingly, *N*−*t*-BOC-(*S*)-α-hydrazinophenylalanine methyl ester (**6**) and the acid chloride of *N*-FMOC-(*S*)-phenylalanine (**15**)[22] were coupled in high yield to afford putative dipeptide derivative **16**. Conversion of putative **16** to the required dipeptide standard (**1**) involved removal of the FMOC group, acetylation, hydrolysis of the methyl ester, and removal of the *t*-BOC protecting group. Initial attempts to remove the FMOC group from compound **16** under the usual basic conditions using piperidine or DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) did not afford the desired dipeptide product **17** but instead resulted in quantitative formation of diketopiperazine **18**. Since the next step would have been to reacetylate the released amino function in any case, the problem of diketopiperazine side product formation was circumvented by removing the FMOC group in the presence of the acetylating reagent *N*-acetoxysuccinimide.[23] Optimization of the reaction conditions permitted the isolation of the desired product **17** in 40% yield. Dipeptide derivative **17** was then saponified to give the corresponding carboxylic acid **19** in 55% yield. Treatment with trifluoroacetic acid then provided the dipeptide of normal connectivity (**1**) in 88% yield.

Scheme 7 shows the synthetic route used for preparation of the dipeptide acylated at the primary amine of α-hydrazinophenylalanine, i.e., the dipeptide of altered connectivity (**2**). As noted above, it has been reported that under standard peptide coupling conditions, the coupling of α-hydrazino acid esters ($N^{\beta}H_2N^{\alpha}HCHRCOOCH_2CH_3$) with *N*-protected amino acids results primarily in N^{β}-acylation.[17] The strategy used for synthesis of the dipeptide of altered connectivity (**2**) involved the coupling of the deprotected (*S*)-α-hydrazinophenylalanine methyl ester (**21**; accessible from **6** by treatment with trifluoroacetic acid) with *N*-CBz-(*S*)-phenylalanine (**20**) via the agency of DCC and HOBT to afford putative dipeptide derivative **22** in 54% yield. The CBz group was then removed by hydrogenolysis over 10% palladium-on-carbon to afford **23** in 83% yield; **23** showed no propensity toward cyclization. Dipeptide **23** was then *N*-acetylated using *N*-acetoxysuccinimide[23] to afford compound **24** (95% yield). Finally, dipeptide derivative **24** was saponified to give the dipeptide of altered connectivity (**2**).

Scheme 7.[a] Synthesis of the Dipeptide *N*-Acetylphenylalanyl-α-hydrazinophenylalanine Having Altered Connectivity



[a] Reaction Conditions: (a) DCC, HOBt; (b) 10% Pd−C, H_2; (c) *N*-acetoxysuccinimide; (d) NaOH.

Beyond the assignment of connectivity in dipeptides **1** and **2** based on expectations from literature precedent involving the coupling of hydrazino acid esters ($N^{\beta}H_2N^{\alpha}HCHRCOOCH_2CH_3$) with *N*-protected amino acids,[17] the facile formation of diketopiperazine **18** upon removal of the nitrogen protecting group of peptide ester **16** (Scheme 6) constitutes strong inferential evidence that dipeptide **16** was acylated on the α-nitrogen. Diketopiperazine formation from dipeptide esters is a common side reaction in solid-phase peptide synthesis.[24] The *lack* of cyclization of analogous intermediate **23** (which would involve the formation of a seven-membered ring) may be adduced as further support for the assignments.

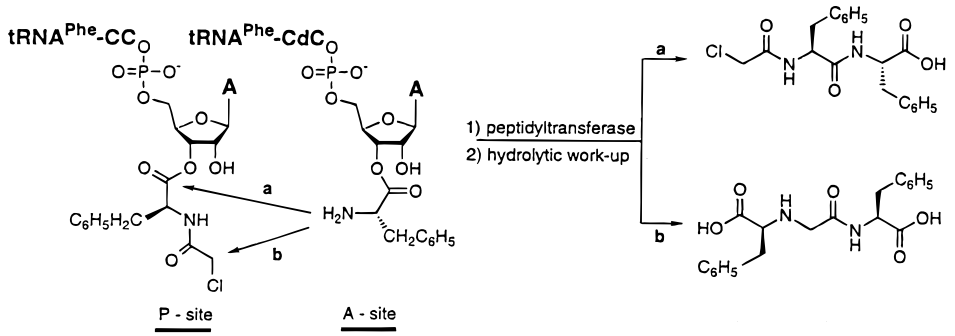
The connectivity of the formed dipeptides was verified using a dipeptide of altered connectivity, *N*-Ac-(*S*)-phenylalanyl-N^{β}-(*S*)-α-hydrazinophenylalanine methyl ester (**24**), for detailed NMR spectral analysis. A COSY spectrum taken in $CDCl_3$ indicated the presence of a cross-peak between the two hydrazino NHs at δ 7.60 and 4.59, corresponding to $N^{\beta}H$ and $N^{\alpha}H$, respectively. The latter exhibited a cross-peak to $C^{\alpha}H$ (δ 3.67) of the hydrazinophenylalanine moiety. Comparable analysis of the NH signals for two dipeptides of normal connectivity, including *N*-Ac-(*S*)-phenylalanyl-N^{α}-(*S*)-α-hydrazinophenylalanine methyl ester (methyl ester of dipeptide **1**; accessible by treatment of **17** with trifluorocetic acid in CH_2Cl_2) revealed a single signal at ~δ 3.9 which had no cross-peak to any other H in the COSY spectrum. The assignment of signals to the hydrazino NH's was supported by the ability of each of these H's to exchange with D_2O; this exchange was fast relative to that of the amide NH's present.

(22) Carpino, L. A.; Cohen, B. J.; Stephens, K. E.; Sadat-Aalaee, S. Y.; Tien, J.-H.; Langridge, D. C. *J. Org. Chem.* **1986**, *51*, 3732.

(23) Rappoport, S.; Lapidot, Y. *Methods Enzymol.* **1974**, *29*, 685.

(24) Bodansky, M. In *Principles of Peptide Synthesis*; Springer-Verlag: Heidelberg, Germany, 1993.

Scheme 8. Products Formed from *N*-chloroacetylphenylalanyl-tRNA^{Phe} and Phenylalanyl-tRNA^{Phe} in the Presence of *E. coli* Ribosomes



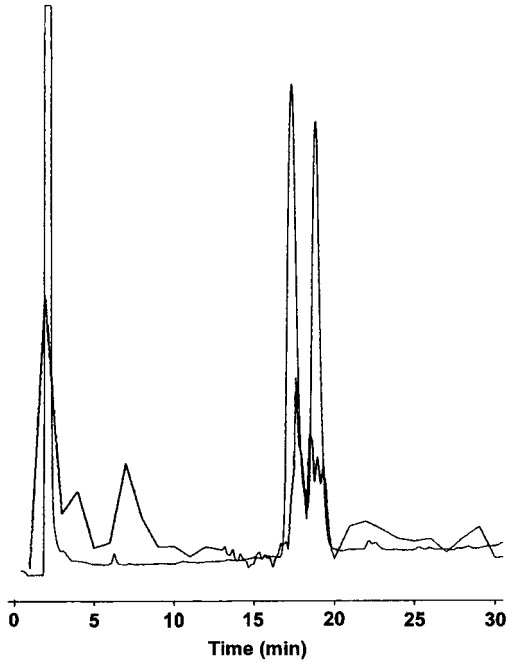


Figure 2. Superposition of the radioactivity and UV HPLC profiles of a sample formed by admixture of authentic **1** and **2** (taller peaks at 17.7 and 19.2 min, respectively) to a dipeptide synthesized ribosomally in response to poly(U) in the presence of [^{3}H]*N*-acetyl-(*S*)-phenylalanyl-tRNA^{Phe} and (*S*)-α-hydrazinophenylalanyl-tRNA^{Phe}.

In preparation for the HPLC analysis of the ribosomal dipeptide mixture, dipeptide standards **1** and **2** were purified by HPLC; optimization of the HPLC conditions to maximize separation of the two was carried out and separation was achieved (data not shown).

HPLC analysis was carried out on a portion of the ribosomally derived dipeptide product(s) obtained using (*S*)-α-hydrazinophenylalanyl-tRNA^{Phe} (**II**) as the acceptor tRNA. An aliquot was frozen, concentrated under diminished pressure, and combined with a 1:1 mixture of dipeptide standards. HPLC analysis was carried out on this mixture. Fractions were taken every 1 min until the region where dipeptide(s) elution was anticipated and then every 10 s. The radioactivity in each fraction was determined, and a graph of time vs radioactivity was constructed. Figure 2 shows the superposition of the UV and radioactivity profiles. Comparison of the two profiles show that compounds containing radioactivity coeluted with the authentic dipeptide standards. The first and second peaks corresponded to the dipeptides of normal (**1**) and altered (**2**) connectivity, respectively; they were formed in a ratio of 1:1.2. On the basis of the amount of [^{3}H]*N*-acetyl-(*S*)-phenylalanyl-tRNA^{Phe} that bound to the ribosomes, the total dipeptide formation reflected in Figure 2 was 72% of theoretical. The yields of dipeptides **1** and **2** were 33 and 39%, respectively.

An analysis of the ribosomal dipeptide solution obtained by using phenylalanyl-tRNA^{Phe} as the acceptor tRNA in the dipeptide assay was carried out as a control. A portion of this dipeptide product was mixed with unlabeled synthetic standard and subjected to HPLC analysis under the same conditions employed for **1** and **2**. The UV profile matched the radioactivity profile closely; *N*-acetyl-(*S*)-phenylalanyl-(*S*)-phenylalanine was formed in 41% of theoretical yield (data not shown).

In an earlier study, we demonstrated that a peptidyl-tRNA analogue (*N*-chloroacetyl-(*S*)-phenylalanyl-tRNA^{Phe}) containing two electrophilic groups was capable of binding to the P-site of *E. coli* ribosomes and reacting with a normal aminoacyl-tRNA in the A-site to provide products derived from reaction at both electrophilic centers (Scheme 8).[7f] The products were identified by comparison with authentic synthetic standards, as well as chemical degradation studies involving intermediates radiolabeled on specific atoms in the amino acids.

The present study extends the earlier findings in that it reinforces the concept that the peptidyltransferase reaction leading to peptide bond formation is likely a direct chemical reaction between the reactive partners at the ribosomal A- and P-sites. As was also evident from our earlier report,[7f] the present study reflects a greater degree of flexibility of ribosomal function than might be anticipated if the peptidyltransferase center were viewed as an active participant in the process of peptide bond formation. Thus, the present findings reinforce the view that the function of the peptidyltransferase center is simply to bring into spatial proximity chemically reactive species intrinsically capable of forming the products that are actually observed. To the extent that the ribosome facilitates peptide bond formation beyond the proximity effect, it seems likely that the microenvironment of the peptidyltransferase center is responsible, rather than a specific set of functional groups that control the motion of the reactive aminoacylated tRNA participants. Aside from its mechanistic implications, this view leads to the prediction that peptide bond formation could be accomplished on any scaffold that can achieve a spatial organization of individual aminoacyl-tRNA's and microenvironment comparable to that which obtains on the ribosome. In fact, the formation of peptide bonds on deproteinized ribosomes,[25] as well as by 23S ribosomal RNA alone,[26] is entirely consistent with this view.

Incorporation of (*S*)-α-Hydrazinophenylalanine into Dihydrofolate Reductase. The foregoing data indicate that (*S*)-α-hydrazinophenylalanyl-tRNA^{Phe} (**II**) functions well as an

(25) Noller, H. F.; Hoffarth, V.; Zimniak, L. *Science* **1992**, *256*, 1416.
(26) (a) Nitta, I.; Ueda, T.; Watanabe, K. In *17th International tRNA Workshop Abstracts*; Chiba, Japan, 1997; p 9−18. (b) Nitta, I. In *22nd Seminar on Frontier Technology Abstracts*; Tokyo, 1997; p 57ff.

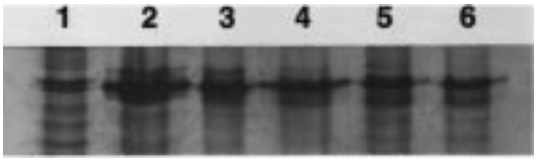


Figure 3. Synthesis of *E. coli* dihydrofolate reductase containing (*S*)-α-hydrazinophenylalanine at position 10 by in vitro suppression of a nonsense codon in the presence of (*S*)-α-hydrazinophenylalanyl-tRNA^{Phe}: lane 1, mRNA from plasmid pTHD10, no suppressor tRNA; lane 2, DHFR elaborated from wild-type mRNA; lanes 3−6, DHFR expressed from pTHD10 mRNA in the presence of suppressor tRNA$^{Phe}_{CUA}$ activated with (lane 3) (*S*)-valine (lane 4) (*S*)-phenylalanine, (lane 5) (*S*)-α-hydrazinophenylalanine (prepared from **12**); lane 6, (*S*)-α-hydrazinophenylalanine (prepared from **13**). A control was also run using the mRNA from plasmid pTHD10 in the presence of full length unacylated tRNA$^{Phe}_{CUA}$. Negligible readthrough was observed.

acceptor in the peptidyltransferase reaction and that both possible dipeptide products resulting from *N*-acylation are formed in approximately equal amounts. Because virtually all misacylated tRNA's tested have functioned as donors in the peptidyltransferase reaction,[7f,h,8] it seemed likely that (acylated) (*S*)-α-hydrazinophenylalanyl-tRNA (**II**) would do so as well. Accordingly, we attempted to incorporate the (*S*)-α-hydrazinophenylalanyl moiety into a single position in a protein. This was carried out by attaching (*S*)-α-hydrazinophenylalanine to a suppressor tRNA$_{CUA}$ transcript, essentially as outlined in Scheme 1, and including the misacylated suppressor tRNA in an *E. coli* protein synthesizing system. Also included was the mRNA for *E. coli* dihydrofolate reductase containing a nonsense (UAG) codon at position 10. As shown in Figure 3, full length DHFR was formed when (*S*)-α-hydrazinophenylalanyl-tRNA$_{CUA}$ was present in the protein synthesizing system. Relative to the elaboration of wild-type DHFR from a plasmid lacking any nonsense codon, (*S*)-α-hydrazinophenylalanine was incorporated to the extent of about 10% when (*S*)-α-hydrazinophenylalanyl-tRNA$_{CUA}$ (**II**) was prepared from aminoacylated dinucleotide **12** (Scheme 1) and in about 3% yield when activated tRNA **II** was prepared using dinucleotide **13** (Scheme 5). In comparison valine was incorporated into position 10 in 53% yield and phenylalanine in 12% yield (Figure 3, lanes 3 and 4, respectively) in this particular experiment. (*S*)-α-Hydrazinophenylalanine was also incorporated into position 27 of DHFR in analogous fashion, albeit in somewhat lower yield (not shown). Critically, the incorporation of hydrazinophenylalanine could be demonstrated reproducibly. No full length DHFR was obtained in the absence of this misacylated tRNA or when the tRNA was not deblocked prior to admixture to the protein synthesizing system.

While numerous amino acid analogues have now been incorporated into peptides[8,9] and proteins,[10] only a few of these have resulted in products of altered connectivity. While no direct analysis of the connectivity of the hydrazinophenylalanine moiety in DHFR has been carried out as yet, it seems not unlikely that both N's have participated in forming the backbone of the derived DHFR. Another interesting facet of hydrazinophenylanyl-tRNA's not addressed in the current studies involves the ability of aminoacyl-tRNA's derived from monoprotected α-hydrazino acids, (e.g. **8**) to participate in peptide bond formation. If successful, these would afford caged proteins[27] of known connectivity; the facile deblocking of such species has recently been demonstrated,[10p] affording catalytically active proteins.

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

An α-hydrazino acid moiety affords the opportunity to introduce an additional substituent in a protein (attached to N^{α} or N^{β}) without alteration of the primary amino acid sequence. Also, an α-hydrazino acid might be used to exert a predefined conformational effect in a peptide; Aubry et al. have reported data on the geometry and conformational influence of the α-hydrazino acid moiety in α-hydrazino acid-containing peptides obtained from solid-state studies (X-ray crystallography) and solution studies (^{1}H NMR, IR spectroscopy).[17c,d,28] The data generally indicate that while the geometry (bond lengths, bond angles) of the amide moiety remains largely unchanged, the presence of the second nitrogen significantly alters the hydrogen bonding characteristics of the peptide and, thus, the overall conformation. Also, acylation of N^{α} or N^{β} showed very different effects on the flexibility of the N−N bond and, thus, the structural rigidity of the peptide.

Experimental Section

General Methods. Elemental analyses were carried out by Atlantic Microlab, Inc. Melting points were taken on a Thomas-Hoover apparatus and are not corrected. TLC R_f values reported were obtained using 0.25 mm Merck glass-backed silica gel TLC plates. Optical rotations were determined on a Perkin-Elmer model 141 polarimeter. ^{1}H NMR spectra were recorded on a 300 MHz spectrophotometer. Chemical shifts are referenced to $CHCl_3$ at 7.26 ppm, CH_3OH at 3.30 ppm, or HOD at 4.80 ppm. The following abbreviations are used; s, singlet; d, doublet; t, triplet; m, multiplet; br, broad; dd, doublet of doublets. Chemical ionization mass spectra (CIMS) were recorded on a Finnigan MAT 4600 mass spectrometer using methane as the carrier gas unless otherwise specified. Electron impact mass spectra (EIMS) were also recorded on a Finnigan MAT 4600 mass spectrometer. Liquid secondary ion mass spectra (LSIMS) were obtained on a Quadrupole-FT mass spectrometer. High-resolution peak matching was carried out at the Michigan State University Mass Spectrometry Facility or at the Nebraska Center for Mass Spectrometry, University of Nebraska.

(*R*)-2-Hydroxy-3-phenylpropionic Acid (4). To a cold (0−5 °C), stirred suspension of 20 g (0.12 mol) of (*R*)-phenylalanine in 150 mL of $CHCl_3$ was added 4.4 mL (5.3 g; 0.15 mol) of concentrated HCl. The solution was stirred at 0 °C for 10 min and concentrated under diminished pressure. The residue was diluted with 350 mL of 5% aqueous H_2SO_4 and cooled in an ice bath. To the cold (0−5 °C) solution was added a solution containing 25 g (0.36 mmol) of $NaNO_2$ in 100 mL of water. The solution was stirred at 0 °C for 6 h and then extracted with three 200-mL portions of ether. The organic extract was dried over Na_2SO_4 and concentrated under diminished pressure. Crystallization from CH_2Cl_2 gave (*R*)-2-hydroxy-3-phenylpropionic acid (**4**) as colorless needles: yield 8.4 g (42%); mp 120−121 °C, lit.[12a] mp 126−127 °C; silica gel TLC R_f 0.83 (4:1:1 *n*-butanol−acetic acid−water); $[\alpha]^{25}_D$ +29.8° (*c* 1.13, acetone), lit.[12a] value for *S* enantiomer, $[\alpha]^{25}_D$ −27.8° (*c* 1.13, acetone); ^{1}H NMR (CD_3OD) δ 2.87 (dd, 1H, J = 14, 8 Hz), 3.08 (dd, 1H, J = 14, 4 Hz), 4.32 (dd, 1H, J = 8, 4 Hz), 4.71 (br s, 1H), and 7.13−7.22 (m, 5H); mass spectrum (chemical ionization) *m*/*z* 167, $(M + H)^+$. Anal. Calcd for $C_9H_{10}O_3$: C, 65.05; H, 6.07. Found: C, 64.98; H, 6.10.

Methyl (*R*)-2-Hydroxy-3-phenylpropionate (5). To a cold (0−5 °C) solution containing 7.0 g (42 mmol) of (*R*)-2-hydroxy-3-phenylpropionic acid (**4**) in 60 mL of dry THF was added diazomethane until a yellow color persisted. Excess diazomethane was destroyed by dropwise addition of 10% aqueous acetic acid until bubbling ceased. The solution was concentrated under diminished pressure, and the residue was dissolved in 30 mL of CH_2Cl_2 and extracted with two 20-mL portions of brine. The organic extract was dried over Na_2SO_4 and concentrated under diminished pressure. Crystallization of the product from ether gave methyl (*R*)-2-hydroxy-3-phenylpropionate (**5**) as colorless needles: yield 6.1 g (80%); mp 43−44 °C; silica gel TLC R_f 0.78 (1:1 ethyl acetate−hexane); $[\alpha]^{25}_D$ +15.5° (*c* 1.0, CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 2.73 (br d, 1H, J = 6 Hz), 2.97 (dd, 1H, J = 14, 7 Hz), 3.13 (dd, 1H, J = 14, 5 Hz), 3.77 (s, 3H), 4.46 (m, 1H), and

7.20−7.33 (m, 5H); mass spectrum (chemical ionization) *m*/*z* 181, (M + H)$^+$. Anal. Calcd for $C_{10}H_{12}O_3$: C, 66.65; H, 6.71. Found: C, 66.50; H, 6.71.

Mosher Ester Derivative of Methyl (*R*)-2-Hydroxy-3-phenylpropionate (5). A solution containing 20 mg (0.11 mmol) of methyl (*R*)-2-hydroxy-3-phenylpropionate (**5**), 23 mg (0.11 mmol) of *N*,*N*′-dicyclohexylcarbodiimide, 29 mg (0.24 mmol) of 4-(dimethylamino)pyridine, and 34 mg (0.15 mmol) of (*R*)-(+)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA) in 0.5 mL of CH_2Cl_2 was stirred at 25 °C for 8 h. The solution was filtered to remove the precipitate, and the filtrate was concentrated under diminished pressure. The product was purified by preparative silica gel TLC (development was with 15% ethyl acetate in hexane) to provide the Mosher ester[13] of methyl (*R*)-2-hydroxy-3-phenylpropionate (**5**) as a colorless oil: yield 9 mg (20%); silica gel TLC R_f 0.54 (25% ethyl acetate in hexane); ^{1}H NMR ($CDCl_3$) δ 3.10 (dd, 1H, J = 14, 8 Hz), 3.22 (dd, 1H, J = 14, 4 Hz), 3.55 (s, 3H), 3.77 (s, 3H), 5.42 (dd, 1H, J = 9, 4 Hz), and 7.05−7.50 (m, 10H); mass spectrum (chemical ionization) *m*/*z* 397, (M + H)$^+$; mass spectrum (electron impact), *m*/*z* 397.126 ($C_{20}H_{20}O_5F_3$ requires *m*/*z* 397.126).

For comparison, compound **5** was prepared in racemic form starting from phenylalanine; the Mosher ester of racemic **5** was synthesized. A solution containing 20 mg (0.11 mmol) of methyl (*R*,*S*)-2-hydroxy-3-phenylpropionate (racemic **5**), 23 mg (0.11 mmol) of *N*,*N*′-dicyclohexylcarbodiimide, 27 mg (0.22 mmol) of 4-(dimethylamino)pyridine, and 33 mg (0.14 mmol) of (*R*)-(+)-MTPA in 0.5 mL of dry CH_2Cl_2 was stirred at 25 °C for 8 h. The solution was filtered to remove precipitate, and the filtrate was concentrated under diminished pressure. The product was purified by preparative silica gel TLC (development with 15% ethyl acetate in hexane). The product was obtained as a colorless oil: yield 21 mg (48%); silica gel TLC R_f 0.54 (25% ethyl acetate in hexane); ^{1}H NMR ($CDCl_3$) δ 3.11 (dd, 1H, J = 14, 9 Hz), 3.22 (dd, 0.5H, J = 14, 4 Hz), 3.33 (dd, 0.5H, J = 14, 4 Hz), 3.34 (s, 1.5H), 3.56 (s, 1.5H), 3.77 (s, 3H), 5.42 (m, 1H), and 7.04−7.50 (m, 10H); mass spectrum (chemical ionization) *m*/*z* 397, (M + H)$^+$; mass spectrum (electron impact) *m*/*z* 397.126 ($C_{20}H_{20}O_5F_3$ requires *m*/*z* 397.126).

Methyl (*S*)-[2-(*N*-*tert*-butoxycarbonyl)hydrazino]-3-phenylpropionate (6). To a cold (0−5 °C) solution of 1.44 g (8 mmol) of methyl (*R*)-2-hydroxy-3-phenylpropionate (**5**) in 5 mL of CH_2Cl_2 under argon was added 2.0 mL (3.39 g, 12 mmol) of trifluoromethanesulfonic anhydride followed by 2.0 mL (1.84 g, 17 mmol) of 2,6-lutidine. The solution was stirred at 0 °C under argon for 1.5 h. To the solution was added 2.11 g (16 mmol) of *t*-BOC-hydrazine. The reaction was stirred at 0 °C for 4 h and then at 25 °C for 15 h. The solution was diluted with CH_2Cl_2, extracted with water and brine, dried over Na_2SO_4, and evaporated to dryness. The crude product was purified by flash chromatography on a silica gel column (12 × 4 cm). Elution with 15% ethyl acetate in hexane provided methyl (*S*)-2-[(*N*-*tert*-butoxycarbonyl)hydrazino]-3-phenylpropionate (**6**) as colorless microcrystals: yield 1.40 g (60%); mp 52−54 °C; silica gel TLC R_f 0.80 (1:1 ethyl acetate−hexane); $[\alpha]^{25}_D$ −12.8° (*c* 1.19, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.40 (s, 9H), 2.96 (dd, 1H, J = 14, 7 Hz), 3.06 (dd, 1H, J = 14, 5 Hz), 3.69 (s, 3H), 3.96 (m, 1H), 4.18 (br s, 1H), 6.17 (br s, 1H), and 7.20−7.30 (m, 5H); mass spectrum (chemical ionization) *m*/*z* 295 (M + H)$^+$. Anal. Calcd for $C_{15}H_{22}O_4N_2$: C, 61.21; H, 7.53. Found: C, 61.23; H, 7.56.

(*S*)-α-Hydrazinophenylalanine (7).[29] To a cold (0−5 °C) solution containing 300 mg (1.02 mmol) of methyl (*S*)-2-[(*N*-*tert*-butoxycarbonyl)hydrazino]-3-phenylpropionate (**6**) in 1.6 mL of dry CH_2Cl_2 under argon was added 0.4 mL of trifluoroacetic acid. The solution was stirred under argon at 0 °C for 2 h and then concentrated under diminished pressure. The residue was triturated with hexane and dried overnight under vacuum and then dissolved in 1.5 mL of CH_3OH and cooled in an ice bath. To the cold (0−5 °C) solution was added 4.0 mL (4 mmol) of 1 N aqueous NaOH. The solution was stirred at 0 °C for 1 h and then at 25 °C for 1 h. The solution was concentrated under diminished pressure, and the resulting precipitate was filtered out and washed with cold water to give (*S*)-hydrazinophenylalanine (**7**) as colorless microcrystals: yield 95 mg (52%); mp 197−200 °C, lit.[29a] mp 196−201 °C; silica gel TLC R_f 0.61 (5:2:3 butanol−acetic acid−water); ^{1}H NMR (D_2O) δ 2.87 (m, 2H), 3.45 (m, 1H), and 7.21−7.32 (m, 5H); mass spectrum (LSIMS) *m*/*z* 181, (M + H)$^+$.

***N*-NVOC-(*S*)-α-hydrazinophenylalanine (8) and *N*,*N*′-di-NVOC-(*S*)-α-hydrazinophenylalanine (9).** To a solution containing 70 mg (0.39 mmol) of (*S*)-α-hydrazinophenylalanine (**7**) and 62 mg (0.58 mmol) of Na_2CO_3 in 1.0 mL of water was added a solution containing 128 mg (0.47 mmol) of 2-nitroveratryl chloroformate[16] in 2 mL of dioxane. The combined solution was stirred at 25 °C for 5 h. The dioxane was removed under diminished pressure, and the aqueous layer was acidified to pH 6.0 with 1 N $NaHSO_4$. The aqueous layer was extracted with three 20-mL portions of CH_2Cl_2. The organic layer was dried over Na_2SO_4 and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 1 cm). Elution with 1% CH_3OH in CH_2Cl_2 gave *N*-NVOC-(*S*)-α-hydrazinophenylalanine (**8**) and *N*,*N*′-di-NVOC-(*S*)-α-hydrazinophenylalanine (**9**) as yellow foams: yield 63 mg (39%) of **8** and 54 mg (33%) of **9**. Compound **8**: silica gel TLC R_f 0.12 (2:98:0.1 CH_3OH−CH_2Cl_2−HOAc); ^{1}H NMR ($CDCl_3$ + CD_3OD) δ 2.90 (dd, 1H, J = 14, 7 Hz), 3.06 (dd, 1H, J = 14, 6 Hz), 3.89 (br s, 7H), 5.43 (s, 2H), 6.99 (s, 1H), 7.21 (m, 6H) 7.65 (br s, 1H), and 7.67 (s, 1H); mass spectrum (chemical ionization) *m*/*z* 420, (M + H)$^+$; mass spectrum (FABMS) *m*/*z* 420.139 ($C_{19}H_{22}N_3O_8$ requires *m*/*z* 420.141). Compound **9**: silica gel TLC R_f 0.40 (2:98:0.1 CH_3OH−CH_2Cl_2−HOAc); ^{1}H NMR (DMSO-d_6) δ 2.95 (m, 1H), 3.12 (m, 1H), 3.69 (br s, 3H), 3.78 (br s, 3H), 3.81 (br s, 6H), 4.87 (br s, 1H), 5.41 (m, 4H), 6.50 (br s, 1H), 6.91 (s, 1H), 6.95 (s, 1H), 7.22 (m, 5H) and 7.64 (s, 2H); mass spectrum (chemical ionization) *m*/*z* 659, (M + H)$^+$; mass spectrum (FAB) *m*/*z* 658.171 ($C_{29}H_{30}N_4O_{14}$ requires *m*/*z* 658.176).

***N*-NVOC-(*S*)-α-hydrazinophenylalanine Cyanomethyl Ester (10).** A solution containing 22 mg (53 μmol) of *N*-NVOC-(*S*)-α-hydrazinophenylalanine (**8**) in 200 μL of 1:1 triethylamine−CH_3CN was cooled in an ice bath and treated under argon with 13 μL (16 mg; 210 μmol) of $ClCH_2CN$. The solution was stirred at 0 °C for 30 min and then at 25 °C for 15 h. The reaction mixture was diluted with 5 mL of CH_2Cl_2 and washed with two 10-mL portions of 1 N aqueous Na_2HSO_4 and two 10-mL portions of water. The organic layer was dried over Na_2SO_4 and concentrated under diminished pressure. The residue was purified by preparative silica gel TLC (development with 1:1 ethyl acetate−hexane) to give *N*-NVOC-(*S*)-α-hydrazinophenylalanine cyanomethyl ester (**10**) as a yellow foam: yield 17 mg (71%); silica gel TLC R_f 0.55 (1:1 ethyl acetate−hexane); ^{1}H NMR ($CDCl_3$) δ 3.00 (dd, 1H, J = 14, 8 Hz), 3.11 (dd, 1H, J = 14, 6 Hz), 3.69 (br s, 1H), 3.95 (s, 3H), 3.97 (s, 3H), 4.07 (m, 1H), 4.73 (s, 2H), 5.50 (s, 2H), 6.47 (br s, 1H), 6.93 (s, 1H), 7.21−7.35 (m, 5H), and 7.70 (s, 1H); mass spectrum (chemical ionization) *m*/*z* 459, (M + H)$^+$; mass spectrum (FAB) *m*/*z* 459.147 ($C_{21}H_{23}N_4O_8$ requires *m*/*z* 459.152).

***N*,*N*′-di-NVOC-(*S*)-α-hydrazinophenylalanine Cyanomethyl Ester (11).** A solution containing 71 mg (0.17 mmol) of *N*,*N*′-di-NVOC-(*S*)-α-hydrazinophenylalanine (**9**) in 400 μL of 1:1 triethylamine−CH_3CN was cooled in an ice bath and treated under argon with 54 μL (64 mg; 0.85 mmol) of $ClCH_2CN$. The solution was stirred at 0 °C for 1 h and then at 25 °C for 15 h. The reaction mixture was diluted with 30 mL of CH_2Cl_2 and washed with two 20-mL portions of 1 N aqueous Na_2HSO_4 and two 20-mL portions of water. The organic layer was dried over Na_2SO_4 and concentrated to dryness. The residue was purified by flash chromatography on a silica gel column (12 × 1 cm). Elution with 2:3 ethyl acetate−hexane gave *N*,*N*′-di-NVOC-(*S*)-α-hydrazinophenylalanine cyanomethyl ester (**11**) as a yellow foam: yield 47 mg (61%); silica gel TLC R_f 0.20 (2:3 ethyl acetate−hexane); ^{1}H NMR ($CDCl_3$) 3.14−3.55 (m, 2H), 3.86 (s, 3H), 3.89 (s, 3H), 3.94 (s,

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6H), 4.01 (m, 1H), 4.58−4.80 (m, 2H), 5.30−5.70 (m, 4H), 6.85 (br s, 2H), 6.95 (br s, 1H), 7.12−7.40 (m, 5H) and 7.69 (br s, 2H); mass spectrum (chemical ionization) *m*/*z* 698, $(M + H)^+$; mass spectrum (FAB) *m*/*z* 697.190 ($C_{31}H_{31}O_{14}N_5$ requires *m*/*z* 697.187).

***N*-NVOC-(*S*)-α-hydrazinophenylalanyl-pdCpA (12).** A solution containing 5.0 mg (11 μmol) of *N*-NVOC-(*S*)-α-hydrazinophenylalanine cyanomethyl ester (**10**) and 2.75 μmol of tris(tetrabutylammonium) pdCpA[18] in 50 μL of dry DMF was stirred under argon at room temperature for 3.5 h. The reaction mixture was treated with 0.95 mL of 1:2 CH_3CN−50 mM NH_4OAc, pH 4.5. The crude product was purified by C_{18} reverse phase HPLC (100 × 4.6 mm column). The column was washed with 1 → 56% CH_3CN in 50 mM NH_4OAc, pH 4.5, over a period of 40 min at a flow rate of 1.0 mL/min (monitoring at 260 nm). The fractions containing the desired product (retention time 21.1 min) were combined and lyophilized to afford *N*-NVOC-(*S*)-α-hydrazinophenylalanyl-pdCpA (**12**) as a colorless solid: yield 17.2 A_{260} units (33%); mass spectrum (FAB) *m*/*z* 1037.236 (M^+) ($C_{38}H_{45}N_{11}O_{20}P_2$ requires *m*/*z* 1037.232).

***N*,*N*′-di-NVOC-(*S*)-α-hydrazinophenylalanyl-pdCpA (13).** A solution containing 6.5 mg (9.3 μmol) of *N*,*N*′-di-NVOC-(*S*)-α-hydrazinophenylalanine cyanomethyl ester (**11**) and 2.75 μmol of tris(tetrabutylammonium) pdCpA[18] in 50 μL of dry DMF was stirred under argon at room temperature for 10 h. The reaction was quenched by the addition of 0.95 mL of 1:2 CH_3CN−50 mM NH_4OAc, pH 4.5, and the crude product was purified by C_{18} reverse phase HPLC (100 × 4.6 mm column). The column was washed with 1 → 64% CH_3CN in 50 mM NH_4OAc, pH 4.5, over a period of 45 min at a flow rate of 1.0 mL/min (monitoring at 260 nm). The fractions containing the desired product (retention time 27.0 min) were combined and lyophilized to afford *N*,*N*′-di-NVOC-(*S*)-α-hydrazinophenylalanyl-pdCpA (**13**) as a colorless solid: yield 20.4 A_{260} units (30%); mass spectrum (LSIMS) *m*/*z* 1275, $(M - H)^+$; mass spectrum (FAB) *m*/*z* 1276.274 (M^+) ($C_{48}H_{54}N_{12}O_{26}P_2$ requires *m*/*z* 1276.275).

(*S*)-α-Hydrazinophenylalanyl-pdCpA (14). The photolyses of *N*-NVOC-(*S*)-α-hydrazinophenylalanyl-pdCpA (**12**) and *N*-NVOC-(*S*)-phenylalanyl-pdCpA were carried out simultaneously and under the same conditions. One A_{260} unit (40 nmol) of *N*-NVOC-(*S*)-α-hydrazinophenylalanyl-pdCpA (**12**) and 1.0 A_{260} unit (40 nmol) *N*-NVOC-(*S*)-phenylalanyl-pdCpA were each dissolved separately in 800 μL of 1 mM KOAc, pH 4.5. To each sample was added 180 μL of a 36 mM solution (6.5 μmol) of semicarbazide hydrochloride in 1 mM KOAc, pH 4.5. The solutions were cooled in an ice bath and irradiated with light from a high-pressure mercury lamp (1000 W). Time points were taken at 0, 5, and 10 min by withdrawing 100-μL aliquots of reaction solution and freezing the aliquots in dry ice. The aliquots were analyzed by C_{18} reverse phase HPLC under the conditions described above for dinucleotide **12**.

HPLC analysis of the photolysis reaction containing *N*-NVOC-(*S*)-α- hydrazinophenylalanyl-pdCpA (**12**) in the presence of semicarbazide hydrochloride showed that the reaction proceeded with the formation of a major product having a retention time of 14.4 min. HPLC analysis of the photolysis reaction of the control compound *N*-NVOC-(*S*)-phenylalanyl-pdCpA in the presence of semicarbazide hydrochloride revealed that the deprotection proceeded as it did in the absence of semicarbazide hydrochloride, with the formation of a single product having a retention time of 13.5 min.

***N*-FMOC-(*S*)-phenylalanyl-N^{β}-*t*-BOC-N^{α}-(*S*)-α-hydrazinophenylalanine Methyl Ester (16).** To a solution containing 496 mg (1.2 mmol) of *N*-FMOC-(*S*)-phenylalanine acid chloride (**15**)[22] in 2.0 mL of CH_2Cl_2 under argon was added successively a solution containing 300 mg (1.02 mmol) of *N*-*t*-BOC-(*S*)-α-hydrazinophenylalanine methyl ester (**6**) in 1.0 mL of CH_2Cl_2 and 3.0 mL of 10% aqueous $NaHCO_3$. The reaction solution was stirred at 25 °C for 45 min and then diluted with CH_2Cl_2 and washed with three 30-mL portions of brine. The organic layer was dried over Na_2SO_4 and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (12 × 1.5 cm). Elution with 25% ethyl acetate in hexane gave *N*-FMOC-(*S*)-phenylalanyl-N^{β}-*t*-BOC-N^{α}-(*S*)-α-hydrazinophenylalanine methyl ester (**16**) as a colorless foam: yield 621 mg (92%); silica gel TLC R_f 0.42 (30% ethyl acetate in hexane); ^{1}H NMR ($CDCl_3$, 50 °C) δ 1.47 (s, 9H), 2.99−3.11 (m, 4H), 3.63 (s, 3H), 4.17−4.36 (m, 3H), 4.90 (br s, 1H), 5.32 (m, 1H), 5.50 (br s, 1H), 6.36 (br s, 1H), 7.13−7.43 (m, 14H), 7.55 (d, 2H), and 7.76 (d, 2H); mass spectrum (chemical ionization, isobutane) *m*/*z* 662, $(M - H)^+$, 590, and 500; mass spectrum (electron impact) *m*/*z* 663.297 ($C_{39}H_{41}N_3O_7$ requires *m*/*z* 663.295).

***N*-Acetyl-(*S*)-phenylalanyl-N^{β}-*t*-BOC-N^{α}-(*S*)-α-hydrazinophenylalanine Methyl Ester (17).** To a solution containing 200 mg (0.30 mmol) of *N*-FMOC-(*S*)-phenylalanyl-N^{β}-*t*-BOC-N^{α}-(*S*)-α-hydrazinophenylalanine methyl ester (**16**) and 615 mg (3.9 mmol) of *N*-acetoxysuccinimide in 2 mL of dry DMF under argon was added successively 135 μL (138 mg; 0.90 mmol) of 1,8-diazabicyclo[5.4.0]undec-7-ene and 84 μL (64 mg; 0.60 mmol) of triethylamine. The reaction mixture was stirred at 25 °C for 15 h and then concentrated under diminished pressure, diluted with 30 mL of CH_2Cl_2, and washed with three 50-mL portions of brine. The organic layer was dried over Na_2SO_4 and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (12 × 2 cm). Elution with 35% ethyl acetate in hexane gave *N*-acetyl-(*S*)-phenylalanyl-N^{β}-*t*-BOC-N^{α}-(*S*)-α-hydrazinophenylalanine methyl ester (**17**) as a yellow oil: yield 58 mg (40%); silica gel TLC R_f 0.25 (1:1 ethyl acetate−hexane); ^{1}H NMR ($CDCl_3$, 50 °C) δ 1.47 (s, 9H), 1.90 (br s, 3H), 3.01−3.09 (m, 4H), 3.62 (s, 3H), 5.08 (br s, 1H), 5.29 (m, 1H), 6.06 (br m, 1H), and 7.19−7.30 (m, 11H); mass spectrum (chemical ionization, isobutane) *m*/*z* 484, $(M + H)^+$; mass spectrum (electron impact), *m*/*z* 484.246, $(M + H)^+$ ($C_{26}H_{34}N_3O_6$ requires *m*/*z* 484.245).

Cyclo((*S*)-phenylalanyl-N^{β}-*t*-BOC-N^{α}-(*S*)-α-hydrazinophenylalanine) (18). To a cold (0−5 °C) solution containing 75 mg (0.11 mmol) of *N*-FMOC-(*S*)-phenylalanyl-N^{β}-*t*-BOC-N^{α}-(*S*)-α-hydrazinophenylalanine methyl ester (**16**) in 0.5 mL of dry CH_2Cl_2 was added 34 μL (29 mg; 0.34 mmol) of piperidine. The reaction mixture was stirred at 0 °C for 2 h and then at 25 °C for 15 h. The reaction mixture was concentrated under diminished pressure, and the residue was purified by flash chromatography on a silica gel column (10 × 1.5 cm). Elution with 1:1 ethyl acetate−hexane gave cyclo((*S*)-phenylalanyl-N^{β}-*t*-BOC-N^{α}-(*S*)-α-hydrazinophenylalanine) (**18**) as a colorless foam: yield 44 mg (94%); silica gel TLC R_f 0.25 (1:1 ethyl acetate−hexane); ^{1}H NMR ($CDCl_3$) δ 1.50 (s, 9H), 2.86 (dd, 1H, J = 13, 3 Hz), 3.29 (m, 2H), 3.98 (br dd, 1H), 4.64 (m, 1H), 5.71 (br s, 1H), and 6.89−7.42 (m, 12H); mass spectrum (chemical ionization, isobutane) *m*/*z* 410, $(M + H)^+$, and 543; mass spectrum (electron impact) *m*/*z* 409.199 ($C_{23}H_{27}N_3O_4$ requires *m*/*z* 409.200).

***N*-Acetyl-(*S*)-phenylalanyl-N^{β}-*t*-BOC-N^{α}-(*S*)-α-hydrazinophenylalanine (19).** To a cold (0−5 °C) solution containing 58 mg (0.12 mmol) of *N*-acetyl-(*S*)-phenylalanyl-N^{β}-*t*-BOC-N^{α}-(*S*)-α-hydrazinophenylalanine methyl ester (**17**) in 0.5 mL of 1:19 H_2O−CH_3OH was added 260 μL (0.26 mmol) of 1 N aqueous NaOH. The reaction mixture was stirred at 0 °C for 1 h and then 25 °C for 1 h. The reaction mixture was neutralized with 10% aqueous citric acid and concentrated under diminished pressure. The residue was dissolved in 15 mL of CH_2Cl_2 and extracted with three 10-mL portions of 5% aqueous $NaHCO_3$. The aqueous layer was then cooled in an ice bath, acidified to pH 6 with 1 N HCl, and extracted with three 10-mL portions of CH_2Cl_2. The organic layer was dried over Na_2SO_4 and concentrated to give *N*-acetyl-(*S*)-phenylalanyl-N^{β}-*t*-BOC-N^{α}-(*S*)-α-hydrazinophenylalanine (**19**) as a colorless foam: yield 31 mg (55%); silica gel TLC R_f 0.55 (10:90:0.1 CH_3OH−CH_2Cl_2−HOAc); ^{1}H NMR ($CDCl_3$, 50 °C) δ 1.43 (s, 9H), 1.86 (br s, 3H), 2.85−3.10 (m, 4H), 3.32 (m, 1H), 3.72 (br s, 1H), 4.94 (br s, 1H), and 7.11−7.26 (m, 11H); mass spectrum (chemical ionization) *m*/*z* 468, $(M-H)^+$; mass spectrum (chemical ionization) *m*/*z* 470.232, $(M + H)^+$ ($C_{25}H_{32}N_3O_6$ requires *m*/*z* 470.229).

***N*-Acetyl-(*S*)-phenylalanyl-N^{α}-(*S*)-α-hydrazinophenylalanine (1).** To a cold (0−5 °C) solution of 31 mg (0.66 mmol) *N*-acetyl-(*S*)-phenylalanyl-N^{β}-*t*-BOC-N^{α}-(*S*)-α-hydrazinophenylalanine (**19**) in 750 μL of dry CH_2Cl_2 under argon was added 250 μL of trifluoroacetic acid. The reaction mixture was stirred at 0 °C for 2 h and then at 25 °C for 30 min. The reaction mixture was concentrated under diminished pressure and coevaporated repeatedly with portions of benzene to afford *N*-acetyl-(*S*)-phenylalanyl-N^{α}-(*S*)-α-hydrazinophenylalanine (**1**) as a colorless foam: yield 28 mg (88%); silica gel TLC R_f 0.42 (15:85:0.1 CH_3OH−CH_2Cl_2−HOAc); ^{1}H NMR ($CDCl_3$, 50 °C) δ 1.87 (s, 3H), 2.85 (dd, 1H, J = 14, 8 Hz), 3.04−3.18 (m, 2H), 3.36 (m, 1H), 5.29

(m, 1H), 5.56 (m, 1H), 6.65 (d, 1H, $J = 8$ Hz), 7.11−7.29 (m, 10H), and 7.77 (br s, 2H); mass spectrum (chemical ionization, isobutane) *m*/*z* 370, $(M + H)^+$; mass spectrum (FAB) *m*/*z* 370.175 ($C_{20}H_{24}N_3O_4$ requires *m*/*z* 370.177).

***N*-Acetyl-(*S*)-phenylalanyl-N^{α}-(*S*)-α-hydrazinophenylalanine Methyl Ester (Methyl Ester of 1).** To a cold solution (0−5 °C) containing 11 mg (24 μmol) of *N*-acetyl-(*S*)-phenylalanyl-N^{β}-t-BOC-N^{α}-(*S*)-α-hydrazinophenylalanine methyl ester (**17**) in 0.5 mL of dry dichloromethane was added 100 μL of trifluoroacetic acid under argon. The reaction mixture was stirred at 0 °C for 2 h and then at 25 °C for 30 min and then concentrated under diminished pressure. The residue was dried under vacuum for 2 h and then dissolved in a minimum amount of dichloromethane and purified by flash chromatography on a silica gel column (15 × 1 cm). Elution with 4% CH_3OH in CH_2Cl_2 gave *N*-acetyl-(*S*)-phenylalanyl-N^{α}-(*S*)-α-hydrazinophenylalanine methyl ester as a colorless oil: yield 11 mg (97%); silica gel TLC R_f 0.21 (4% CH_3OH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 1.87 (s, 3H), 2.92 (dd, 1H, $J = 14$, 6 Hz), 3.09 (dd, 2H, $J = 14$, 8 Hz), 3.35 (m, 1H), 3.75 (s, 3H), 3.99 (br s, 2H), 5.59 (m, 2H), 6.05 (d, 1H, $J = 8$ Hz), and 7.19 (m, 10H); mass spectrum (FAB) *m*/*z* 406.173, $(M + Na)^+$ ($C_{21}H_{25}N_3O_4Na$ requires *m*/*z* 406.174).

***N*-CBz-(*S*)-phenylalanyl-N^{β}-(*S*)-α-hydrazinophenylalanine Methyl Ester (22).** To a cold (0−5 °C) solution of 400 mg (1.36 mmol) of methyl (*S*)-2-BOC-hydrazino-3-phenylpropionate (**6**) in 1.5 mL of CH_2Cl_2 was added 250 μL of trifluoroacetic acid. The solution was stirred at 0 °C for 3.5 h. The solution was concentrated under diminished pressure to give the crude trifluoroacetic acid salt of (*S*)-α-hydrazinophenylalanine methyl ester (**21**) as a yellow oil. The residue was triturated with hexane and dried under vacuum for 15 h.

To a cold (0−5 °C) solution containing 407 mg (1.36 mmol) of *N*-CBz-(*S*)-phenylalanine (**20**) and 184 mg (1.36 mmol) of hydroxybenzotriazole in 0.8 mL of dry DMF under argon was added 280 mg (1.36 mmol) of *N*,*N*′-dicyclohexylcarbodiimide. The solution was stirred under argon at 0 °C for 15 min. To the reaction solution was added a solution containing 264 mg (1.36 mmol) of the trifluoroacetic acid salt of (*S*)-α-hydrazinophenylalanine methyl ester and 380 μL (275 mg; 2.72 mmol) of triethylamine in 1 mL of dry DMF. The reaction mixture was stirred at 0 °C for 2 h and then concentrated under diminished pressure. The residue was dissolved in 30 mL of CH_2Cl_2 and filtered, and then the filtrate was washed with two 30-mL portions of saturated aqueous $NaHCO_3$, two 30-mL portions of brine, and two 30-mL portions of water. The organic layer was dried over Na_2SO_4 and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (12 × 1.5 cm). Elution with 35:65:0.1 ethyl acetate−hexane-triethylamine gave *N*-CBz-(*S*)-phenylalanyl-N^{β}-(*S*)-α-hydrazinophenylalanine methyl ester (**22**) as colorless microcrystals: yield 346 mg (54%); mp 121−124 °C; silica gel TLC R_f 0.47 (1:1 ethyl acetate−hexane); ^{1}H NMR (CD_3OD) δ 2.86 (dd, 2H, $J = 16$, 6 Hz), 2.99 (dd, 2H, $J = 13$, 7 Hz), 3.53 (s, 3H), 3.64 (t, 1H, $J = 7$ Hz), 4.27 (t, 1H, $J = 8$ Hz), 4.99 (s, 2H) and 7.14−7.23 (m, 15H); mass spectrum (chemical ionization) *m*/*z* 476, $(M + H)^+$, and 342; mass spectrum (chemical ionization) *m*/*z* 475.210 (M^+) ($C_{27}H_{29}N_3O_5$ requires *m*/*z* 475.211).

(*S*)-Phenylalanyl-N^{β}-(*S*)-α-hydrazinophenylalanine Methyl Ester (23). A solution containing 250 mg (0.53 mmol) of *N*-CBz-(*S*)-phenylalanyl-N^{β}-(*S*)-α-hydrazinophenylalanine methyl ester (**22**) and 50 mg of 10% palladium-on-carbon in 2 mL of dry DMF was stirred under an atmosphere of H_2 at 25 °C for 2.5 h. The catalyst was removed by filtration, and the filtrate was concentrated under diminished pressure. The residue was purified by flash silica chromatography on a silica gel column (12 × 1.5 cm). Elution with 5% CH_3OH in CH_2Cl_2 gave (*S*)-phenylalanyl-N^{β}-(*S*)-α-hydrazinophenylalanine methyl ester (**23**) as a colorless oil: yield 149 mg (83%); silica gel TLC R_f 0.20 (5% CH_3OH in CH_2Cl_2); ^{1}H NMR (CD_3OD) δ 2.77 (dd, 2H, $J = 13$, 7 Hz), 2.89 (dd, 2H, $J = 12$, 7 Hz), 3.44 (dd, 1H, $J = 7$ Hz), 3.54 (s, 3H), 3.62 (t, 1H, $J = 7$ Hz), and 7.14−7.26 (m, 10H); mass spectrum (chemical ionization) *m*/*z* 342, $(M + H)^+$; mass spectrum (FAB) *m*/*z* 364.164, $(M + Na)^+$ ($C_{19}H_{23}N_3O_3Na$ requires 364.164).

***N*-Acetyl-(*S*)-phenylalanyl-N^{β}-(*S*)-α-hydrazinophenylalanine Methyl Ester (24).** To a cold (0−5 °C) solution containing 143 mg (0.42 mmol) of (*S*)-phenylalanyl-N^{β}-(*S*)-α-hydrazinophenylalanine methyl ester (**23**) in 1 mL of dry DMF under argon was added 66 mg (0.42 mmol) of *N*-acetoxysuccinimide. The reaction mixture was stirred at 0 °C for 15 min and then at 25 °C for 2 h. The reaction mixture was concentrated under diminished pressure, and the residue was purified by flash chromatography on a silica gel column (12 × 1.5 cm). Elution with 4:1:0.1 ethyl acetate−hexane−triethylamine gave *N*-acetyl-(*S*)-phenylalanyl-N^{β}-(*S*)-α-hydrazinophenylalanine methyl ester (**24**) as colorless foam: yield 153 mg (95%); silica gel TLC R_f 0.10 (80:20:0.1 ethyl acetate−hexane−triethylamine); ^{1}H NMR (CD_3OD) δ 1.86 (s, 3H), 2.86 (m, 2H), 2.99 (m, 2H), 3.52 (s, 3H), 3.66 (dd, 1H, $J = 14$, 7 Hz), 4.55 (t, 1H, $J = 8$ Hz), and 7.13−7.22 (m, 10H); mass spectrum (chemical ionization) *m*/*z* 384, $(M + H)^+$; mass spectrum (electron impact) *m*/*z* 383.185 ($C_{21}H_{25}N_3O_4$ requires *m*/*z* 383.185).

***N*-Acetyl-(*S*)-phenylalanyl-N^{β}-(*S*)-α-hydrazinophenylalanine (2).** To a cold (0−5 °C) solution containing 46 mg (0.12 mmol) of *N*-Ac-(*S*)-phenylalanyl-N^{β}-(*S*)-α-hydrazinophenylalanine methyl ester (**24**) in 2.5 mL of 4:1 CH_3OH-H_2O was added 12.5 mg (0.3 mmol) of $LiOH\cdot H_2O$. The reaction was stirred at 25 °C for 2 h. At the end of this time, no reaction had occurred. To this solution was added 6 μL of aqueous 0.02 M NaOH. The solution was stirred at 25 °C for 4 h, at which time no starting material remained. The reaction mixture was concentrated under diminished pressure, diluted with 25 mL water, and extracted with three 20-mL portions of ethyl acetate. The aqueous phase was then acidified to pH 5.0 with 1 N HCl and extracted with three 30-mL portions of ethyl acetate. The ethyl acetate layer was dried over Na_2SO_4 and concentrated under diminished pressure. The residue was purified by flash silica chromatography on a silica gel column (10 × 1 cm). Elution with 8:92:0.1 $CH_3OH-CH_2Cl_2-$HOAc gave *N*-acetyl-(*S*)-phenylalanyl-N$^{\beta}$-(*S*)-α-hydrazinophenylalanine (**2**) as a colorless foam: yield 28 mg (44%); silica gel TLC R_f 0.17 (8:92:0.1 $CH_3OH-CH_2Cl_2-$ HOAc); ^{1}H NMR (CD_3OD) δ 1.85 (s, 3H), 2.85 (m, 2H), 2.97 (m, 2H), 3.63 (dd, 1H, $J = 6$ Hz), 4.51 (dd, 1H, $J = 7$ Hz), and 7.19−7.23 (m, 10H); mass spectrum (chemical ionization) *m*/*z* 370, $(M + H)^+$; mass spectrum (electron impact) *m*/*z* 369.171 ($C_{20}H_{23}N_3O_4$ requires *m*/*z* 369.169).

^{1}H NMR COSY Experiments To Determine Dipeptide Connectivity. DFQ COSY[30] experiments were performed on a Varian Unity 1 INOVA 500 spectrometer (^{1}H frequency was 500.018 MHz). Complex points were collected in the F-2 dimension (1024) and in the F-1 dimension (256). The F-1 dimension was zero-filled to 1024 points. The adaption function used for the DFQ COSY was 45° shifted $\sin^2$ function in both dimensions. COSY experiments were phase-sensitive. The time between scans (recycle time) for the COSY was 2 s.

HPLC Analysis of the Dipeptide Mixture Obtained by Utilizing (*S*)-α-Hydrazinophenylalanyl-tRNA^{Phe} (II) as the Acceptor for Ribosomally Mediated Dipeptide Formation. A portion of the ribosomal dipeptide mixture (one-sixth, 200 μL, 1045 cpm) obtained by utilizing (*S*)-α-hydrazinophenylalanyl-tRNA^{Phe} (**II**) as the acceptor tRNA in an in vitro protein biosynthesis system[7h,i] was frozen and concentrated under diminished pressure to remove the water and ethanol, affording 30 μL of a cloudy solution. The dipeptide standards (0.15 mg each) were added to this solution in 20 μL of 2:1 $CH_2Cl_2-CH_3OH$. The resulting clear solution was concentrated under diminished pressure and then treated with 40 μL of formamide. HPLC analysis was carried out on a C_{18} reverse phase column (3 μ, 100 × 4.6 mm). The column washed with 15 → 25% CH_3CN in 0.1% aqueous CF_3COOH over a period of 10 min at a flow rate of 1.0 mL/min and then with 25 → 42% CH_3CN in 0.1% aqueous CF_3COOH over a period of 24 min at the same flow rate (monitoring at 260 nm).

Fractions were collected in scintillation vials once every 1 min and then once every 10 s over the dipeptide elution range. Scintillation fluid (5 mL) was added to each sample, and the samples were analyzed for tritium content. The profile of elution vs time was compared to the UV profile for the experiment. A comparison of the two profiles showed that compounds containing radioactivity coeluted with the authentic dipeptide standards: 345 cpm for the dipeptide of normal connectivity (**1**) having a retention time of 17.7 min (33%) and 408

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cpm for the dipeptide of altered connectivity (**2**) having a retention time of 19.2 min (39%). These yields are based on the theoretical yield of dipeptide determined from a nitrocellulose filter binding assay that showed the extent of peptidyl-tRNA binding to the ribosomal P-site.[7i]

HPLC Analysis of the Dipeptide Mixture Obtained by Utilizing (*S*)-Phenylalanyl-tRNA^{Phe} (I) as the Acceptor for Ribosomally Mediated Dipeptide Formation. A portion of the ribosomal dipeptide solution obtained by utilizing phenylalanyl-tRNA^{Phe} as the acceptor tRNA in the dipeptide assay (150 μL, 1335 cpm) was frozen, concentrated under diminished pressure to 40 μL, mixed with unlabeled synthetic standard (0.1 mg), and analyzed by C_{18} reverse phase HPLC as described above. The fractions were analyzed for tritium content, and a graph of elution time vs radioactivity was constructed. The UV profile matched the radioactivity profile, with the majority of radioactivity coeluting with the authentic dipeptide standard at a retention time of 20.7 min.

Preparation of Misacylated tRNAs. Chemical misacylation reactions were carried out in 100 μL (total volume) of 50 mM K Hepes, pH 7.5, containing 0.5 mM ATP, 15 mM $MgCl_2$, 50 μg suppressor tRNA-C_{OH}, 0.5 A_{260} unit NVOC-protected aminoacyl-pdCpA (5−10-fold molar excess), 20% dimethyl sulfoxide, and 100 units of T4 RNA ligase (Promega). After incubation at 37 °C for 30 min, the reaction was quenched by the addition of 0.1 volume of 2 M sodium acetate, pH 4.5, and the tRNA was precipitated with 2.5 volumes of ethanol. Deprotection of NVOC-containing aminoacyl-tRNAs was carried out in 5 μL (total volume) of 1 mM potassium acetate, pH 4.5. The aminoacyl-tRNAs were cooled to 1 °C and irradiated with a 500 W mercury−xenon lamp using both Pyrex and water filters. Typically, monosubstituted NVOC aminoacyl-tRNAs were deblocked for 5 min. (*S*)-α-Hydrazinophenylalanyl-tRNAs were deblocked in the presence of 200 equiv of semicarbazide hydrochloride.

In Vitro Synthesis of Dihydrofolate Reductase. An (*S*)-30 extract was prepared from an *E. coli* strain (XAC-RF) containing a temperature sensitive release factor 1. The (*S*)-30 extract was prepared according to Pratt[31] with some modifications.[32]

Dihydrofolate reductase was synthesized in a reaction mixture (28 μL final volume) that contained 2 μg of plasmid DNA (pTHD10, containing the gene for DHFR with a TAG codon at position 10) dissolved in diethyl pyrocarbonate-treated water, 10 μL of premix (35 mM Tris-acetate, pH 7.0, 190 mM potassium glutamate, 30 mM ammonium acetate, 2 mM dithiothreitol, 11 mM magnesium acetate, 0.8 mg/mL *E. coli* tRNA, 2 mM isopropyl-β-D-thiogalactopyranoside, 35 mg/mL poly(ethylene glycol) 6000, 20 μg/mL folinic acid, 20 mM ATP and GTP, 5 mM CTP and UTP, and 10 mM cAMP),[33] 100 μM of amino acids minus methionine, 10 μCi of [^{35}S]-(*S*)-methionine, and 7.5 μL of (*S*)-30 extract. Suppression reactions (100 μL) contained 50 μg of deblocked misacylated suppressor tRNA dissolved in 10 μL of diethylpyrocarbonate-treated water and were incubated at 37 °C for 2 h. Protein was precipitated from the reaction mixtures by the addition of 5 volumes of acetone, followed by centrifugation. The protein pellets were dried and resuspended in sodium dodecyl sulfate loading buffer prior to polyacrylamide gel electrophoresis.[34] Dried gels were visualized and analyzed using a Molecular Dynamics Phosphorimager.

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