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SITE-SPECIFIC INCORPORATION OF NON-NATURAL RESIDUES INTO PEPTIDES: EFFECT OF RESIDUE STRUCTURE ON SUPPRESSION AND TRANSLATION EFFICIENCIES

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ABSTRACT—A systematic survey of the structural requirements for biosynthetic incorporation of non-natural residues into a polypeptide is presented. Relative translation efficiencies for a series of 12 semi-synthetic acylated suppressor tRNAs ranged from 0 to 91% depending on the structure of the residue incorporated.

Site-specific mutagenesis is one of the most important experimental tools available for protein research, serving as the cornerstone for selective structural modification in both basic mechanistic enzymology and commercial genetic engineering. Despite its tremendous significance, this technique suffers from the limitation that amino acid substitutions are restricted to the twenty primary amino acids. This prerequisite normally excludes the direct site-specific introduction into proteins of "designer" amino acids intended to modify function or activity in a predictable way. For instance, it might be desirable to introduce a unique detection residue (e.g., a fluorescent amino acid) into an enzyme in order to follow a single metabolic pathway, or to insert a novel catalytic residue in the active site in order to modify its activity. For other applications, a linking residue would be useful for enhancement of thermal stability or selective post-translational modification. Another possibility would be to introduce cleaving residues that would allow the chemical equivalent of proenzyme-to-enzyme conversion. All of these modifications would require an exceptionally high degree of chemoselectivity if they were to be carried out by modification of the enzyme itself. Such post-translational chemical modification has resulted in a few notable successes,[1] but a truly general method of site-specific protein modification has been elusive. It seems likely that a successful approach would require intervention during protein biosynthesis, i.e., during translation, if the innate selectivity problems associated with post-translational chemical modification were to be avoided. One method for accomplishing this goal via semi-synthetic aminoacylated tRNA suppressors[2] has recently been developed independently by the Schultz group[3] and by us.[4] In this paper, a new rapid assay developed by our group[5] has been utilized to systematically survey the structural requirements for incorporation of 12 non-natural amino acids into a peptide.

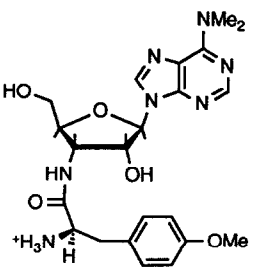
RESULTS AND DISCUSSION

A Historical Overview. Methods to manipulate protein structure during biosynthesis have centered around incorporation of non-natural residues via an exogenous source of a "misacylated" tRNA or tRNA analogue, which have been prepared by various combinations of chemical and enzymatic methods. One approach relies upon aminoacylation of normal tRNA with its cognate synthetase,[6] but the array of non-natural residues that can be introduced by this direct method is severely limited by the remarkable substrate specificity of these enzymes. Alternatively, chemical alteration of 2'(3')-O-acylated tRNAs or tRNA analogs does not suffer directly from this limitation because the normal, highly specific aminoacylation step is bypassed. However, it is clearly limited to the non-natural residues that can be produced by chemical transformation of available aminoacyl-tRNAs. A third,

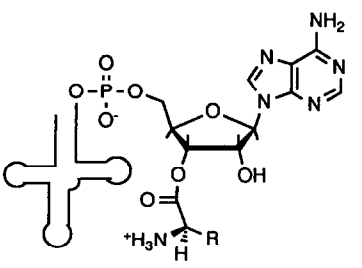
more general strategy is one in which the 2'(3')-O-acyl bond is formed chemically. Although this bond cannot be formed selectively with an intact tRNA, the indirect methods described below allow an essentially unlimited choice of acyl groups to be introduced.

Previous studies have lead to a rudimentary understanding of the interaction of misacylated tRNAs with the ribosome—the RNA/enzyme complex that mediates protein biosynthesis. One of the seminal experiments in this area was the insertion of alanine at a cysteine codon by conversion of cysteinyl-tRNA^{Cys} to alanyl-tRNA^{Cys} through reductive desulfhydration with Raney Nickel,[7] which was the first direct test of the "adaptor hypothesis"[8] and clearly established that recognition of each aminoacyl-tRNA by the ribosome is not dependent upon the amino acid itself, but rather upon structural elements of the tRNA to which it is attached, specifically the anticodon. Since the number of non-natural residues that have been incorporated into peptides by this method is quite small, the limits of ribosome compatibility with non-natural aminoacylated tRNAs have not been well delineated. Furthermore, at least two binding sites on the ribosome have been defined: the A-site, which accepts the aminoacyl-tRNA carrying the next amino acid residue (the nucleophile for peptide formation) to be incorporated into the growing peptide chain, and the P-site, which accommodates the tRNA acylated with the growing chain itself after peptide bond formation. Thus, for successful incorporation of any residue, the aminoacyl-tRNA must be able to bind at each of these sites, as well as function as a nucleophile in the A-site and as an electrophile in the P-site. Accordingly, any specific misacylated tRNA can be categorized as either a donor (compatible with the P-site), as an acceptor (compatible with the A-site), or both.

Misacylated tRNAs have not only been used to prepare proteins with altered amino acid side-chains, but also with modified backbones. For instance, polyphenyllactyl ester was produced by deamination of phenylalanyl-tRNA^{Phe} with nitrous acid to yield the α-hydroxyacyl analog, phenyllactyl-tRNA^{Phe}.[9] Further evidence to support the notion that ribosomes could be employed for ester formation was established in two different studies. In one, peptidyl transferase—the enzymatic component of the ribosome responsible for peptide formation—catalyzed the transesterification reaction involving nucleophilic attack of methionyl-tRNA^{fMet} by ethanol to produce ethyl N-formylmethionate.[10] The second exploited the ability of puromycin (**1**; which bears a close resemblance to the 3'-terminus of an acylated tRNA, **2**) to participate at the acceptor site of the ribosome. Analogues of puromycin in which the α-amino group was replaced by a hydroxyl group resulted in ester formation through ribosome-catalyzed condensation with methionyl-tRNA^{fMet}.[11] A similar strategy was employed to produce the thioester N-acetylphenylalanyl-L-thiopuromycin.[12] These examples illustrate that the entire tRNA is not necessary for productive interaction with the ribosome, and have spurred interest in the study of dramatically truncated aminoacyl-tRNA species substrates for ribosome-catalyzed condensations. Both a thioamide[13] and phosphinoamide[14] have been produced by employing N-acetylthioleucyl-pCpA and (N-acetylmethionyl-aminomethyl)methylphosphinyl-pA, respectively, as donors with the acceptor phenylalanyl-tRNA.



Puromycin (1)



Aminoacyl-tRNA (2)

Subsequent to these elegant studies, which relied on relatively specialized methods of preparing misacylated tRNAs, a general aminoacylation strategy was pioneered by Hecht and co-workers.[15] Several novel tRNAs were produced by utilizing T4 RNA ligase to couple N-protected 2'(3')-O-acylated pCpA derivatives with tRNAs lacking the 3'-terminal cytidine and adenosine moieties, resulting in acylated tRNAs capable of dipeptide formation.[15a,16] Further innovations based upon this strategy were reported by Brunner[17] and by Hecht,[15d] who prepared misacylated tRNAs containing a free amino group. Such derivatives were shown to function normally in

the ribosomal A-site, resulting in the biosynthetic incorporation of the respective amino acid residue into proteins. The former example, conducted with a $tRNA^{Phe}$ misacylated with L-4'-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenylalanine, is particularly interesting because it resulted in the incorporation of this non-natural amino acid into the translation product.

Misacylated tRNAs thus can be utilized for the incorporation of non-natural amino acids into proteins, but this strategy lacks generality. For instance, if the codon recognized by the tRNA happens to occur more than once in the mRNA, then the non-natural amino acid would be inserted at multiple sites in the translation product. Additionally, the misacylated tRNA and the corresponding wild-type tRNA both recognize the same mRNA codon, so that either amino acid could be incorporated at each site. Such a competition would occur for all codons except the three termination codons (UAG, UGA, and UAA), for which there are normally no corresponding tRNAs. Because these codons function to signal termination of translation, a point mutation that inserts any of them at an inappropriate site in a vital gene (a "nonsense mutation") leads to truncated, nonfunctional products and cellular death.[18] However, in some cases suppressor tRNAs have arisen that specifically recognize the misplaced termination codon (i.e., nonsense suppression site), resulting in amino acid incorporation—rather than termination—thereby allowing the production of some functional protein.

With this well-known biological mechanism in mind, a site-specific method of incorporating non-natural residues into proteins is clear: engineer into a gene a termination codon that would signal the desired position of incorporation, and provide the translation system with the corresponding semi-synthetic suppressor tRNA charged with the non-natural residue to be incorporated. This strategy[19] is in fact a known biological mechanism, observed in *Escherichia coli*, by which selenocysteine (which is not one of the twenty primary amino acids) is incorporated during translation of formate dehydrogenase.[20] Recently, Schultz and co-workers reported the incorporation of several modified phenylalanine residues into ß-lactamase by suppression of a UAG stop codon with chemically misacylated $tRNA^{Phe}_{CUA}$ prepared by anticodon loop replacement.[3,21] Our group has independently assessed a similar strategy in a simplified test system designed to unambiguously determine site-specificity and suppression efficiencies.[4] A combination of chemical synthesis and run-off transcription was employed to prepare a semi-synthetic, non-hypermodified $tRNA^{Gly}_{CUA}$ nonsense suppressor acylated with L-3-[^{125}iodo]tyrosine.[22] The presence of this synthetic tRNA during *in vitro* translation of mRNA containing a nonsense suppression site (e.g. a UAG termination codon) results in the incorporation of the non-natural residue L-3-iodotyrosine into the polypeptide exclusively at the position corresponding to that site (see Figure). This simple polypeptide was used as an initial target to allow for the rigorous and unambiguous analysis of the translation product while avoiding the possible problems associated with drawing conclusions based on catalytic activity of enzymatic end-products.[23] The site-specificity of incorporation was unambiguously demonstrated by careful analysis of the translation product, which was purified and sequenced. In addition, suppression due to the synthetic tRNA was quantified in relation to read-through—suppression by endogenous aminoacyl-tRNAs during in vitro translation—verifying that the observed suppression was due entirely to the added synthetic suppressor.

A Rapid Assay Method for Determining Suppression and Translation Efficiencies A simple method for determining suppression efficiency (defined as the percentage of suppression product relative to the total of suppression plus termination products) of a wide variety of synthetic acylated suppressors would be highly desirable in order to rapidly obtain a better understanding of the steric and electronic requirements involved in protein biosynthesis. The non-natural amino acid chosen for the initial experiments was L-3-[^{125}iodo]tyrosine, mainly because it can be easily synthesized with a specific activity high enough for detection in cell-free translation using rabbit reticulocyte lysate.[24] Additionally, this system was also designed to provide suppression efficiencies in subsequent studies without the need to radiolabel each non-natural residue to be tested. In order to do so, translation in the rabbit reticulocyte lysate is conducted as it was in the initial experiments, except that *unlabeled* acylated tRNA is used, in conjunction with added L-[^{35}S]-methionine and L-[^{3}H]-leucine. As before,

the termination product is an 8-mer polypeptide, while the suppression product is 16-mer. Suppression efficiency can then be determined after selective precipitation of the peptides simply by measuring the ratio of radiolabeled methionine to leucine: since the 8-mer contains three leucines and the a 16-mer contains six, 0% suppression results in an ^{35}S:^{3}H ratio of 1:3 (corrected for specific activities), while 100% suppression would give a 1:6 ratio. Intermediate levels can be determined by simple interpolation. As the most rigorously studied example to date, L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA (**3**) served to calibrate the assay. Within experimental error, the two assay methods give the same result, and thus the rapid assay was employed in all of the studies described below.[25]

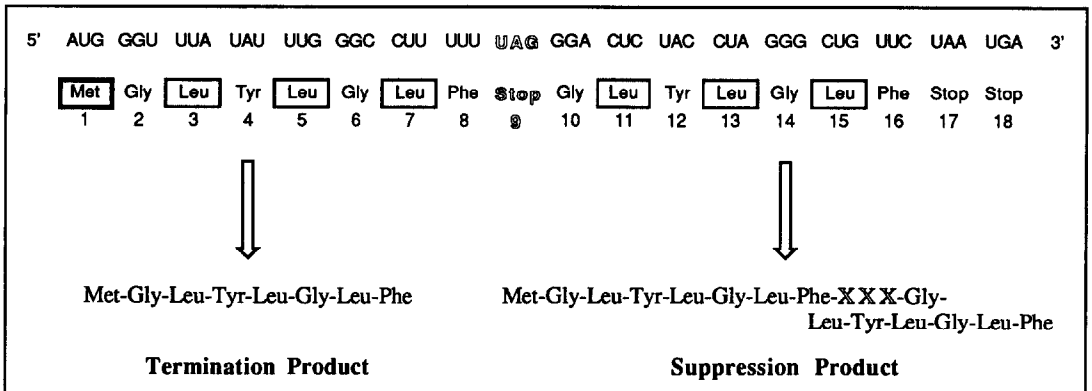
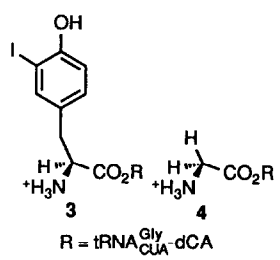


Figure. Termination and suppression products obtained from translation of mRNA. Details of the translation reactions are described in the experimental section.

Suppression efficiency is dependent upon the ability of the aminoacyl-tRNA to effectively suppress the UAG codon relative to normal termination. The experimental values for suppression efficiency are therefore a measure of the compatibility of the entire aminoacyl-tRNA structure with the ribosome. In order to focus on the specific effect of the non-natural residues, i.e., factor out effects due to the tRNA structure, the experimental values were also compared with one for which no residue incompatibility exists, i.e., glycyl-tRNA$^{Gly}_{CUA}$-dCA (**4**).

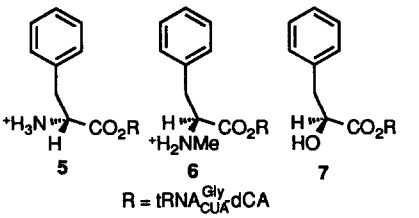


All suppression values determined for non-natural residues were therefore compared with that observed for the glycine derivative—normalized to 100%—to give relative translation efficiencies. In this way factors other than residue structure are eliminated, and the relative translation efficiency represents a direct measure of the effect of residue structure on ribosome compatibility. This normalization also allows a direct comparison of the results of this study with previous work where wild-type tRNAs rather than nonsense suppressors were employed. Rapid assay analysis of glycyl-tRNA$^{Gly}_{CUA}$-dCA (**4**) suppression gave a value of 79% under optimized conditions,[5] which is normalized to 100%. The previously determined suppression efficiency of 65% determined for L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA (**3**) then yields a relative translation efficiency of 82%. We attribute this ~20% difference between **3** and **4** to the effect of a slightly unfavorable interaction of the iodotyrosyl residue in the ribosome.

Initial Studies to Determine the Basic Steric and Electronic Requirements of Amino Acids in Protein Biosynthesis. The non-natural amino acids tested to date, including L-3-iodotyrosine, deviate structurally from natural amino acids only marginally. Extension of these experiments to a much wider range of structures would provide a better understanding of the steric and electronic constraints imposed by the ribosome. Our initial series was designed to probe to steric requirements with respect to the backbone geometries and functionalities by employing the acylated tRNAs **5**, **6**, and **7** shown below. The first residue evaluated in our expression system

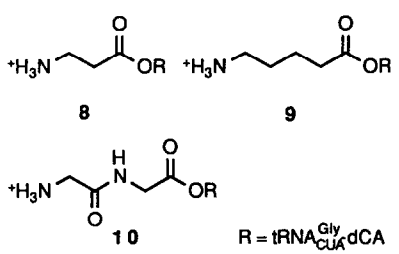
was D-phenylalanyl-tRNA$^{Gly}_{CUA}$-dCA (**5**). No suppression product was observed above background levels, which is in accord with earlier attempts to incorporate this residue during *in vitro* protein biosynthesis from *Escherichia coli* extracts.[3] Previous analysis of the donor and acceptor components of **5**, respectively, revealed only marginal activity in the former[15f] and none in the latter.[15e]

One reason for attempting to incorporate a D-amino acid would be the possibility of generating modified proteins which are resistant to enzymatic degradation. Another way to achieve this goal would be to incorporate N-alkylated residues. Additionally, such substituted residues might be employed to rotationally bias particular conformations within a particular protein. N-Methyl-L-phenylalanyl-tRNA$^{Gly}_{CUA}$-dCA (**6**) was therefore tested, and proved to be second only to glycine in relative translation efficiency (91%).

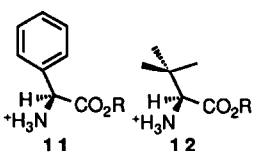


Another application would be to incorporate *cleaving residues*, that would allow the chemical equivalent of proenzyme-to-enzyme conversion from primary translation products. One possibility for site-specific cleavage would be to incorporate an α-hydroxy acid analogue during protein biosynthesis, which would permit the selective hydrolysis of the resultant ester linkage along the polyamide backbone. The ribosome compatibility of the α-hydroxy acid L-phenyllactyl-tRNA$^{Gly}_{CUA}$-dCA (**7**) was therefore evaluated. Previous experiments have clearly established the ability of ribosomes to catalyze ester formation,[9,10,11] so that it was not surprising to find that **7** functioned with good relative translation efficiency (58%). As expected, the HPLC-isolated 16-mer produced in this experiment was cleanly converted into the corresponding 8-mer by simple alkaline hydrolysis.[5]

It was also of interest to determine the "allowable" distance between the carboxyl and amino groups of the residue. The homologous series of aminoacyl-tRNAs **8**, **9**, and **10** were assayed. Previous experiments with ß-phenylalanyl-tRNA^{Phe} (not a nonsense suppressor) had established this acylated tRNA to have very efficient donor activity (110%),[16b] but a rather low acceptor activity (8%).[15f] One possible explanation posed for the low acceptor activity was that the side-chain interfered with the stereoelectronic geometry required for attack of the ß-phenylalanine amino group upon the P-site aminoacyl-tRNA. The structurally simpler 3-aminopropionyl-tRNA$^{Gly}_{CUA}$-dCA (**8**) was therefore employed in our studies to evaluate only the ß-amino acid component with respect to translation efficiency. Site-specific incorporation of this residue into our model peptide resulted in a relative translation efficiency of only 11%, while the second example in this series, 5-aminovaleryl-tRNA$^{Gly}_{CUA}$-dCA (**9**), resulted in an even lower translation efficiency (6%), which is near the sensitivity limit of our assay (± 5%).[26] As a more conformationally rigid example of the same chain length, the dipeptide glycylglycyl-tRNA$^{Gly}_{CUA}$-dCA (**10**) was also assayed. Not surprisingly, no suppression product was detected within the sensitivity of the assay.

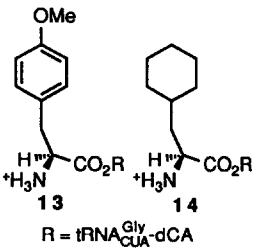


Another aspect of ribosome compatibility pertinent to these studies was the effect of greater steric bulk at the ß-position of individual residues, and L-phenylglycyl-tRNA$^{Gly}_{CUA}$-dCA (**11**) and L-2-amino-3,3-dimethylbutyryl-tRNA$^{Gly}_{CUA}$-dCA (**12**) were chosen to examine this issue. Evaluation of ribosome compatibility for **11** by the rapid assay method revealed a relative translation efficiency (65%), which is entirely consistent with previous studies that showed phenylglycyl-tRNA^{Phe} to have reasonable acceptor activity (70%)[15f] and excellent donor activity (93%).[15e] Still greater steric bulk at the ß-position, as represented by *tert*-butyl glycine **12**, resulted in a more dramatic decrease in translation efficiency to only 9%.

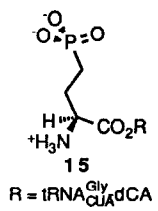


The final two examples in this series were examined as steric probes further from the α-carbon of the residue. Incorporation of O-methyl-L-tyrosyl-tRNA$^{Gly}_{CUA}$-dCA (**13**) resulted in a relative translation

efficiency of 68%, which compares favorably with independent results obtained by the Hecht and co-workers, who have conducted studies to determine the acceptor activity (70%) of O-methyl-L-tyrosyl-tRNA^{Phe} in the formation of a dipeptide.[15f] The aminoacyl-tRNA L-2-cyclohexylalanyl-tRNA$^{Gly}_{CUA}$-dCA (**14**) contains the fully saturated form of phenylalanine, which was predicted to be compatible with the ribosome based on previous studies of an analogue of puromycin (**1**) in which a cyclohexane ring replaced the normal aromatic substituent of the antibiotic.[27] Consistent with this expectation, rapid assay analysis of **14** resulted in an relative translation efficiency of 75%. The successful biosynthetic incorporation of **14** paves the way for more detailed experiments with specifically substituted cyclohexyl groups in order to probe the steric and electronic environment of the ribosomal side chain pockets.



2-Amino-4-Phosphonobutyric Acid: A Non-Hydrolizable Synthon of O-Phosphorylserine. A final example, 2-amino-4-phosphonobutyryl-tRNA$^{Gly}_{CUA}$-dCA (**15**), is an interesting one that represents a non-hydrolizable synthon of O-phosphorylserine. Reversible covalent modification of proteins is a universal mechanism through which many of the molecular events which govern cellular function are regulated. One such common alteration is the enzymatic transfer of phosphate onto site-specific residues (e.g., serine) at the surface of particular target proteins.[28] Phosphorylation events are often transient and are tightly regulated by phosphatases, which makes the study of individual steps in this process difficult to monitor. This problem could be easily circumvented by employing a non-hydrolizable version of a phosphorylated serine residue at the site of interest which would provide a definitive means to explore the consequences of non-reversible phosphorylation in a protein controlled by this type of covalent modification. As an initial phase in such an experiment, it was first necessary to determine the efficacy of site-specific incorporation of **15** into our model expression system. Experiments with the phosphonate analogue **15** clearly demonstrate this residue to be compatible with the ribosome, yielding an average translation efficiency of 77%.



ENTRY NO.	PARENT RESIDUE COMPONENT OF ACYLATED tRNA$^{Gly}_{CUA}$-dCA (**2**)	[^{3}H] IN DPM[a]	[^{35}S] IN DPM[a]	SUPPRESSION EFFICIENCY (%)[b]	RELATIVE TRANSLATION EFFICIENCY (%)[c]
1	Control 1: No Added tRNA	259616	146501	—	—
2	Control 2: Nonacylated tRNA	224665	124433	—	—
3	L-3-Iodotyrosine (**3a**)	449824	150238	**65**	82
4	Glycine (**4a**)	446216	137663	79	**100**
5	D-Phenylalanine (**5a**)	338340	158895	0	0
6	N-Methyl-L-phenylalanine (**6a**)	466512	122843	72	91
7	L-Phenyllactic acid (**7a**)	499356	159766	46	58
8	3-Aminopropionic acid (**8a**)	297293	150990	9	11
9	5-Aminovaleric acid (**9a**)	255807	134490	5	6
10	Glycylglycine (**10a**)	251509	140818	0	0
11	L-Phenylglycine (**11a**)	387360	141415	51	65
12	L-2-Amino-3,3-dimethylbutyric acid (**12a**)	263976	136123	7	9
13	O-Methyl-L-tyrosine (**13a**)	379503	135809	54	68
14	L-2-Cyclohexylalanine (**14a**)	404234	140811	59	75
15	2-Amino-4-phosphonobutyric acid (**15a**)	391398	133921	61	77

[a]Values given are an average of three trials. Counts per min have been converted into decays per min (dpm) and are corrected for background and quenching for each individual trial prior to averaging. [b]Values shown are taken from the average suppression value of individual trials, which were derived from the ratio of [^{35}S]-methionine to [^{3}H]-leucine divided by the [^{35}S]:[^{3}H] ratio obtained from Control 2. L-3-Iodotyrosine was employed as an internal standard in each assay to normalize inter-assay variances. [c]Values are relative to glycine, for which a suppression efficiency of 79% has been normalized to 100%.

CONCLUSION

Whether or not a particular non-natural residue will be successfully incorporated by the methodology described in this paper is determined in part by limitations imposed by the ribosome. While the suppression efficiency values reported here undoubtedly reflect not only the specific target peptide chosen, but also the characteristics of the in vitro translation system employed, the experiments presented are intended to begin providing a basis for predicting the range of non-natural residue structures that can be accommodated by both binding sites in the ribosome. Some of the residues tested were intended to probe steric requirements of the ribosome without themselves being of particular interest once incorporated. From the these examples, it is clear that even minor changes relative to the primary amino acids cause a small but measurable decrease in the efficiency of suppression. It also appears that D-amino acids are unlikely to be incorporated, and that increased steric bulk at the ß-carbon reduces incorporation dramatically, but not to zero. Attempts to "extend" the peptide backbone with ß- and δ-amino acids either gave very low levels of incorporation or failed completely, but modifications such as ester and N-methyl amide linkages are tolerated well. Finally, an analogue that mimics O-phosphorylserine is incorporated efficiently in an experiment that represents a prelude to some of the intriguing possibilities this methodology may allow in the future.

The potential of this technique for the design and *in vitro* expression of unique enzymes and proteins is high. However, broad applicability of these procedures would require advances in two important areas. First, a general *in vitro* method needs to be developed that would eliminate or reduce the number of laborious chemical or enzymatic steps required to produce the acylated tRNAs. Secondly, the issue of scale must be addressed. The necessity to produce larger amounts of translation product for many applications will ultimately require *in vivo* protein expression, which would allow large fermentation-scale reactions and eliminate the μmolar scale limit imposed by current methods. We currently are addressing these problems by testing a number of other more highly functionalized residues, as well as attempting to simplify the overall process and develop an *in vivo* translation system.

EXPERIMENTAL

Materials

The following biological and chemical reagents were purchased: Sephadex G-25 Select-D columns (5301-730608/725608, 5 Prime⟶3 Prime, Inc), T4 RNA ligase (New England Biolabs), L-(3,4,5-^{3}H)-leucine (143 Ci/mmol), L-[^{35}S]-methionine (>800 Ci/mmol) and rabbit reticulocyte lysate (N.90, Amersham), benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (Advanced ChemTech), and sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (Calbiochem-Behring).

Solvents and reagents were dried prior to use when necessary. Acetonitrile was dried by heating at reflux over K_2CO_3 (5 g/L) for 2 h, distilled, and stored over 3 Å molecular sieves for at least 7 days prior to use. N,N-Dimethlyformamide was dried by stirring overnight at room temperature over CaH_2 (5 g/L) and distilled under diminished pressure. 1-Hydroxybenzotriazole hydrate (Aldrich) was dried before use for 72 h at 50 °C over P_2O_5 *in vacuo* (WARNING: at higher temperatures 1-hydroxybenzotriazole may explode). All other solvents employed were of the highest commercial grade obtainable.

The following compounds were prepared by the methods referenced: N-(9-fluorenylmethyloxy)carbonyl-L-glycylglycine and N-(9-fluorenylmethyloxy)carbonyl-D-phenylalanine,[29] 2-Chlorophenyl N^4-[(9-fluorenylmethyloxy)carbonyl]-5'-O-[bis(2-chlorophenyl)phosphoryl]-2'-deoxycytidylyl (3'-5')-[N^6-[(9-fluorenylmethyloxy)carbonyl]-2'-O-(tetrahydropyranyl)adenosine], L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA (**3**), glycyl-tRNA$^{Gly}_{CUA}$-dCA

(**4**), D-phenylalanyl-tRNA$^{Gly}_{CUA}$-dCA (**5**), N-methyl-L-phenylalanyl-tRNA$^{Gly}_{CUA}$-dCA (**6**) and L-phenyllactyl-tRNA$^{Gly}_{CUA}$-dCA (**7**),[30] and mRNA and peptide standards (for translation experiments).[4,5,30]

General Methods

Melting points (mp) were taken on a Laboratory Devices melting-point apparatus and are reported uncorrected. Nuclear magnetic resonance spectra (^{1}H NMR or ^{13}C NMR) were obtained on either a General Electric QE-300 (300 MHz, FT) or General Electric GN-500 (500 MHz, FT) spectrometer as specified. Spectra are reported in ppm from internal tetramethylsilane on the δ scale. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, app = apparent, br = broad), coupling constant (Hz), and integration. Infrared spectra (IR) were recorded on a Perkin-Elmer 1600 FT-IR spectrophotometer. Ultraviolet spectra (UV) were obtained on either a Perkin-Elmer Lambda 3B UV/vis spectrophotometer or a Perkin-Elmer 4A UV/vis spectrophotometer controlled by an IBM PC using Softways UV 428 software. Low resolution mass spectra (LRMS), electron impact (EI), chemical ionization (CI) and fast atom bombardment (FAB), were determined on a Finnigan 9610 spectrometer. High resolution mass spectra (HRMS) were determined on a Vacuum Generators analytical 7070E double sector spectrometer. Elemental analyses were performed by Desert Analytics, Tucson, Arizona.

Thin-layer chromatography (TLC) was performed on 0.25 mm E. Merck precoated silica gel plates (60 F-254). Flash chromatography was performed on ICN 200-400 mesh silica gel as described by Still *et al.* Small and medium scale purifications (20-1500 mg) were alternatively accomplished by radial chromatography using a Harrison Research Chromatotron. Radial silica gel plates of 1, 2, or 4 mm thickness were used consisting of Merck silica gel (60 PF_{254}) containing gypsum. High-performance liquid chromatography (HPLC) was performed on a Waters system consisting of two 6000A pumps and U6K injector, a reverse phase Vydac C-4 preparative column (5 μm packing, 10 mm ID x 250 mm length), an in-line Applied Biosystems 1000S Diode Array Detector, and a LKB 2112 Redirac Fraction Collector.

Representative Procedure for Synthesis of N-(9-Fluorenylmethyloxy)carbonyl-Protected Amino Acids. 3-[N-(9-Fluorenylmethyloxy)carbonyl]aminopropionic acid.

A stirred solution containing 3-aminopropionic acid (**8a**; 344 mg, 3.87 mmol), 10% aqueous Na_2CO_3 (10.2 mL), and dioxane (5 mL) in a round bottom flask (50 mL) was cooled in an ice bath and 9-fluorenylmethyl chloroformate (1.00 g, 3.87 mmol) dissolved in dioxane (10 mL) was added in one portion over a 2 min period. The mixture was stirred in the ice bath for 1 h and then allowed to warm to room temperature. The solution was subsequently stirred for an additional 3 h, poured into H_2O (200 mL) and extracted with Et_2O (3 x 50 mL). The aqueous layer was cooled in an ice bath and the product acidified with concentrated HCl, followed by extraction with EtOAc (3 x 50 mL). The organic layers were combined, dried over $MgSO_4$, and concentrated *in vacuo* to a white foam. Crystallization from CH_3CN gave 1.17 g (97%) as a white crystalline solid: mp 164-166 °C; ^{1}H NMR (300 MHz, DMSO) δ 12.50-11.75 (br s, 1 H), 7.89 (d, J = 7.3 Hz, 2 H), 7.69 (d, J = 7.3 Hz, 2 H), 7.42 (t, J = 7.3 Hz, 2 H), 7.36-7.31 (m, 3 H), 4.30 (d, J = 6.6 Hz, 2 H), 4.21 (t, J = 6.6 Hz, 1 H), 3.30-3.18 (m, 2 H), 2.40 (t, J = 6.8 Hz, 2 H); ^{13}C NMR (75 MHz, DMSO) δ 172.8, 156.0, 143.9, 140.7, 127.6, 127.1, 125.2, 120.1, 65.3, 46.7, 36.5, 34.1; IR (KBr) 3550-2450 (br), 3330, 3053, 3013, 2944, 1688, 1536, 1448, 1265, 1147, 992, 757, 738 cm^{-1}; LRMS (CI, isobutane), *m/e* (relative intensity) 312 (MH^+, 8), 179 (8), 134 (100), 116 (21), 90 (73); HRMS (CI, isobutane) calcd for $C_{18}H_{17}NO_4$ (+1.0078) 312.1236, found 312.1227. Anal. calcd for $C_{18}H_{17}NO_4$: C, 69.44; H, 5.50; N, 4.70, found C, 69.55; H, 5.49; N, 5.08.

5-[N-(9-Fluorenylmethyloxy)carbonyl]aminovaleric acid.

Addition of 9-fluorenylmethyl chloroformate (1.00 g, 3.87 mmol) dissolved in dioxane (10 mL) to a solution containing 5-aminovaleric acid (**9a**; 453 mg; 3.87 mmol), 10% aqueous Na_2CO_3 (10.2 mL), and dioxane (5.0 mL) gave 1.23 g (93%) as a white crystalline solid after recrystallization from CH_3CN: mp 130-132

°C; ^{1}H NMR (300 MHz, DMSO) δ 12.25-11.75 (br s, 1 H), 7.87 (d, *J* = 7.3 Hz, 2 H), 7.67 (d, *J* = 7.3 Hz, 2 H); 7.40 (t, *J* = 7.3 Hz, 2 H), 7.31 (t, *J* = 7.3 Hz, 2 H), ~7.27 (partially obscured d, *J* = 6.7 Hz, 2 H), 4.29 (d, *J* = 6.7 Hz, 2 H), 4.19 (t, *J* = 6.7 Hz, 1 H), 2.97 (q, *J* = 5.9 Hz, 2 H), 2.20 (t, *J* = 6.7 Hz, 2 H), 1.50-1.40 (m, 4 H); ^{13}C NMR (75 MHz, DMSO) δ 174.3, 156.1, 143.9, 140.7, 127.5, 127.0, 125.1, 120.1, 65.1, 46.8, 33.3, 28.9, 21.7; IR (KBr) 3510-2430 (br), 3346, 3066, 3018, 2948, 2883, 1696, 1546, 1450, 1259, 1138, 1110, 1020, 920 cm^{-1}; LRMS (CI, isobutane), *m/e* (relative intensity) 340 (MH^+, 18), 179 (60), 178 (17), 162 (63), 144 (15), 118 (100), 100 (61); HRMS (CI, isobutane) calcd for $C_{20}H_{21}NO_4$ (+1.0078) 340.1548, found 340.1539. Anal. calcd for $C_{20}H_{21}NO_4$: C, 70.78; H, 6.24; N, 4.13, found C, 70.58; H, 6.24; N, 4.36.

N-(9-Fluorenylmethyloxy)carbonyl-L-phenylglycine.

Addition of 9-fluorenylmethyl chloroformate (816 mg, 3.15 mmol) dissolved in dioxane (5 mL) to a solution containing L-phenylglycine (**11a**; 500 mg, 3.31 mmol), 5% aqueous $NaHCO_3$ (13.7 mL), and dioxane (5 mL) gave 933 mg (80%) as a white foam after radial chromatography (silica gel, step gradient: 0 to 1% AcOH in 1:1 hexanes/Et_2O): mp 177.0-178.5 °C; ^{1}H NMR (300 MHz, DMSO) δ 13.25-12.50 (br s, 1 H), 8.23 (d, *J* = 8.0 Hz, 1 H), 7.88 (d, *J* = 7.5 Hz, 2 H), 7.75 (d, *J* = 7.4 Hz, 2 H), 7.41-7.27 (m, 8 H), 5.16 (d, *J* = 8.0 Hz, 1 H), 4.29-4.18 (m, 3 H); ^{13}C NMR (75 MHz, DMSO) δ 172.0, 155.8, 143.8, 143.7, 140.7, 137.2, 128.4, 127.9, 127.8, 127.6, 127.1, 125.4, 120.1, 65.9, 58.1, 46.6; IR (KBr) 3650-2500 (br), 3398, 1732, 1531, 1230, 735 cm^{-1}; LRMS (FAB, DMSO/*p*-nitrobenzyl alcohol), *m/e* (relative intensity) 374 (MH^+, 23), 328 (23), 273 (24), 228 (11), 217 (14), 195 (12), 180 (20), 179 (100), 178 (83), 167 (10), 165 (23), 152 (21), 150 (10), 139 (13), 138 (19), 137 (34); HRMS (FAB, CH_2Cl_2/*p*-nitrobenzyl alcohol) calcd for $C_{23}H_{19}NO_4$ (+1.0078) 374.1392, found 374.1407. Anal. calcd for $C_{23}H_{19}NO_4 \cdot \frac{1}{2} H_2O$: C, 72.23; H, 5.27; N, 3.66, found C, 72.26; H, 5.00 N, 3.73.

L-2-[N-(9-Fluorenylmethyloxy)carbonyl]amino-3,3-dimethylbutyric acid.

Addition of 9-fluorenylmethyl chloroformate (241 mg, 0.93 mmol) dissolved in dioxane (5 mL) to a solution containing 2-L-amino-3,3-dimethylbutyric acid (**12a**; 111 mg, 0.85 mmol), 5% aqueous $NaHCO_3$ (10 mL), and dioxane (5 mL) gave 252 mg (84%) as a white foam after radial chromatography (silica gel, step gradient: 0 to 1% AcOH in 1:1 hexanes/Et_2O): mp 60.0-62.0 °C; ^{1}H NMR (300 MHz, DMSO) δ 7.88 (d, *J* = 7.3 Hz, 2 H), 7.76 (d, *J* = 7.3 Hz, 2 H), 7.58 (d, *J* = 9.2 Hz, 1 H), 7.41 (t, *J* = 7.3 Hz, 2 H), 7.32 (t, *J* = 7.3 Hz, 2 H), 4.27-4.21 (m, 3 H), 3.85 (d, *J* = 9.2 Hz, 1 H), 0.97 (s, 9 H); ^{13}C NMR (75 MHz, DMSO) δ 172.6, 143.8, 140.7, 127.6, 127.0, 125.4, 120.1, 65.8, 62.7, 46.6, 33.4, 26.7; IR (KBr) 3700-2500 (br), 2963, 1718, 1522, 1450, 1330, 1226, 1059, 160, 740 cm^{-1}; LRMS (CI, isobutane), *m/e* (relative intensity) 354 (MH^+, 10), 180 (12), 179 (81), 176 (26), 158, (81), 132 (42), 130 (100), 86 (27), 85 (22), 83 (18), 81 (28), 79 (13), 71 (34), 70 (27); HRMS (FAB, CH_2Cl_2/*p*-nitrobenzyl alcohol) calcd for $C_{21}H_{23}NO_4$ (+1.0078) 354.1705, found 354.1698. Anal. calcd for $C_{21}H_{23}NO_4$: C, 71.37; H, 6.56; N, 3.96, found C, 71.69; H, 6.54; N, 4.08.

N-(9-Fluorenylmethyloxy)carbonyl-O-methyl-L-tyrosine.

Addition of 9-fluorenylmethyl chloroformate (298 mg, 1.15 mmol) dissolved in dioxane (5 mL) to a solution containing O-methyl-L-tyrosine (**13a**; 204 mg, 1.04 mmol), 5% aqueous $NaHCO_3$ (10 mL), and dioxane (5 mL) gave 401 mg (92%) as a white foam after radial chromatography (silica gel, step gradient: 0 to 1% AcOH in 1:1 hexanes/Et_2O): mp 163.0-163.5 °C; ^{1}H NMR (300 MHz, DMSO) δ 13.00-12.50 (br s, 1 H), 7.85 (d, *J* = 7.5 Hz, 2 H), 7.67-7.60 (m, 3 H), 7.38 (t, *J* = 7.5 Hz, 2 H), 7.27 (q, *J* = 7.5 Hz, 2 H), 7.15 (d, *J* = 8.5 Hz, 2 H), 6.79 (d, *J* = 8.5 Hz, 2 H), 4.22-4.05 (m, 4 H), 3.66 (s, 3 H), 2.98 (dd, *J* = 13.9, 4.4 Hz, 1 H), 2.77 (dd, *J* = 13.9, 10.6 Hz, 1 H); ^{13}C NMR (75 MHz, DMSO) δ 173.3, 157.8, 155.9, 143.7, 140.6, 130.1, 129.7, 127.6, 127.0, 125.3, 121.9, 120.1, 113.5, 65.6, 55.7, 54.9, 46.5, 40.3, 35.6; IR (KBr) 3700-2800 (br), 3420, 1721, 1696, 1516, 1447, 1401, 1298, 1245, 1176, 1060, 760, 739 cm^{-1}; LRMS (CI, isobutane), *m/e* (relative intensity) 418 (MH^+, 10), 355 (10), 194 (16), 180 (20), 179 (100); HRMS (FAB, DMSO/*p*-nitrobenzyl alcohol)

calcd for $C_{25}H_{23}NO_5$ (+1.0078) 418.1654, found 418.1628. Anal. calcd for $C_{25}H_{23}NO_5$: C, 71.93; H, 5.55; N, 3.36, found C, 71.83; H, 5.40; N, 3.29.

N-(9-Fluorenylmethyloxy)carbonyl-L-2-cyclohexylalanine.

L-Phenylalanine (**14a**; 201 mg, 1.22 mmol), 5% rhodium on alumina (185 mg), and 70% AcOH (4 mL) were added to a round bottom flask (10 mL). The reaction flask was flushed with nitrogen and hydrogen introduced into the system at 1 atm. The reaction was terminated after 48 h when 121 mL of hydrogen (5.40 mmol) were absorbed. The reaction flask was flushed with nitrogen to remove excess hydrogen, and the rhodium filtered off and washed with H_2O (3 x 1 mL). The H_2O washes were combined with the filtered reaction mixture into a round bottom flask (25 mL) and the solution lyophilized to a white solid. Addition of 9-fluorenylmethyl chloroformate (348 mg, 1.34 mmol) dissolved in dioxane (5 mL) to a solution containing the lyophilized solid, 10% aqueous $NaHCO_3$ (10 mL), and dioxane (5 mL) gave 252 mg (53%) as a white foam after radial chromatography (silica gel, step gradient: 0 to 1% AcOH in 1:1 hexanes/Et_2O): mp 50.0-52.0 °C; ^1H NMR (300 MHz, DMSO) δ 12.75-12.25 (br s, 1 H), 7.88 (d, J = 7.3 Hz, 2 H), 7.71 (d, J = 7.3 Hz, 2 H), 7.62 (d, J = 8.2 Hz, 1 H), 7.41 (t, J = 7.3 Hz, 2 H), 7.31 (t, J = 7.3 Hz, 2 H), 4.34-4.19 (m, 3 H), 4.03-3.95 (m, 1 H), 1.71-0.76 (m, 13 H); ^{13}C NMR (75 MHz, DMSO) δ 174.4, 156.1, 143.7, 140.7, 127.6, 127.0, 125.2, 120.1, 65.5, 51.4, 46.6, 33.5, 33.2, 31.3, 26.0, 25.8, 25.6; IR (KBr) 3600-2500 (br), 2924, 1718, 1522, 1449, 1233, 1046, 759, 739 cm^{-1}; HRMS (CI, isobutane) calcd for $C_{24}H_{27}NO_4$ (+1.0078) 394.2018, found 394.2015. Anal. calcd for $C_{24}H_{27}NO_4$: C, 73.26; H, 6.92; N, 3.56, found C, 72.98; H, 6.95; N, 3.55.

2-[N-(9-Fluorenylmethyloxy)carbonyl]amino-4-phosphonobutyric acid.

A stirred solution containing 2-amino-4-phosphonobutyric acid (**15a**; 46 mg, 0.25 mmol) and hexamethyldisilazane (5mL) in a flame-dried round bottom flask under argon and fitted with a spiral condenser was heated at reflux for 1 h. Excess hexamethyldisilazane was removed *in vacuo* to yield a yellow oil which was dissolved in CH_2Cl_2 (2 mL) containing 1-methylimidazole (34 mg, 0.417 mmol). Addition of 9-fluorenylmethyl chloroformate (72 mg, 0.28 mmol) in CH_2Cl_2 (2 mL) was followed by stirring for 3 h at room temperature. The reaction was quenched by the addition of H_20 (20 mL), and extracted with CH_2Cl_2 (3 x 20mL). The H_2O layer was acidified with concentrated HCl and extraction with EtOAc (3 x 25 mL). The EtOAc layers were combined, dried over $MgSO_4$, and concentrated *in vacuo* to a white foam (75 mg, 74%) which was employed in the next step without further purification: ^1H NMR (300 MHz, DMSO) δ 7.88 (d, J = 7.3 Hz, 2 H), 7.77 (d, J = 8.1 Hz, 1 H), 7.73 (d, J = 7.3 Hz, 2 H), 7.41 (t, J = 7.3 Hz, 2 H), 7.32 (t, J = 7.3 Hz, 2 H), 4.24-4.21 (m, 3 H), 4.05-4.00 (m, 1 H), 1.97-1.81 (m, 2 H), 1.62-1.54 (m, 2 H); ^{13}C NMR (75 MHz, DMSO) δ 173.4, 156.1, 143.8, 140.7, 127.7, 127.1, 125.3, 120.1, 65.7, 59.8, 46.6, 20.8, 14.1.

General Procedure for Formation and Deprotection of Aminoacyl-Dinucleotide Derivatives. 5'-O-Phosphoryl-2'-deoxycytidylyl(3'-5')-[2'(3')-O-(aminoacyl)adenosine].

The (9-flourenylmethyloxy)carbonyl amino acid (0.030 mmol), benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (0.030 mmol), 1-hydroxybenzotriazole (0.030 mmol), and dry DMF (100 μL) were added to a vial (0.5 mL) under argon and allowed to stand at room temperature for 20 min. The protected dinucleotide 2-chlorophenyl N^4-[(9-fluorenylmethyloxy)carbonyl]-5'-O-[bis(2-chlorophenyl)phosphoryl]-2'-deoxycytidylyl (3'-5')-[N^6-[(9-fluorenylmethyloxy)carbonyl]-2'-O-(tetrahydropyranyl)adenosine] (0.005 mmol) and 1-methylimidazole (0.015 mmol) dissolved in dry DMF (100 μL) were added to the solution. After 2 h, the reaction was quenched by addition of saturated aqueous NaCl (300 μL) and the resultant white precipitate isolated by centrifugation. The solvent was decanted, the pellet dissolved into CH_3CN (3 mL) and H_2O (1 mL), and the layers separated. The H_2O layer was extracted with CH_3CN (2 x 3 mL), the organic layers combined, and concentrated *in vacuo*. A freshly prepared solution of 1,1,3,3-tetramethylguanidine (0.33 mmol) and 4-nitrobezaldoxime (0.38 mmol) in dry CH_3CN (1 mL) was added and allowed to stir for 2 h. The solution was added to Et_2O (30 mL) and isolated by centrifugation. The pellet was dissolved in 80% HCO_2H (2.5 mL

precooled to 0 °C in ice) and allowed to stand for 30 min at 0 °C, followed by precipitation by pouring the solution with stirring into Et_2O (10 mL). The product was isolated by centrifugation, dissolved in 80% HCO_2H (150 μL), and injection onto a Vydac C-4 column equilibrated in 80% 5 mM ammonium formate (pH 4.5)/CH_3CN. HPLC: RP C4 prep column. Run 10-40% CH_3CN linear gradient over 30 min. Buffer was 5 mM ammonium formate (pH 4.5). Collected eluents (retentions times for all the acylated dinucleotides were between 23-27 min; the nonacylated dinucleotide starting material was recovered at a retention time of 15 min) were lyophilized; dissolved in 0.1 N formic acid and lyophilized again. followed by addition of H_2O and lyophilization to a white solid (yield: 100-300 μg of the desired acylated dinucleotide). HRMS (FAB, DMSO/*p*-nitrobenzyl alcohol) calcd for parent residue components: **8a** $C_{22}H_{31}N_9O_{14}P_2$ (+1.0078) 708.1544, found 708.1562; **9a** $C_{24}H_{35}N_9O_{14}P_2$ (+1.0078) 736.1857, found 736.1853; **10a** $C_{23}H_{32}N_{10}O_{15}P_2$ (+1.0078) 751.1602, found 751.1596; **11a** $C_{27}H_{33}N_9O_{14}P_2$ (+1.0078) 770.1700, found 770.1684; **12a** $C_{25}H_{37}N_9O_{14}P_2$ (+1.0078) 750.2013, found 750.2018; **13a** $C_{29}H_{37}N_9O_{15}P_2$ (+1.0078) 814.1962, found 814.1971; **14a** $C_{28}H_{41}N_9O_{14}P_2$ (+1.0078) 790.2326, found 790.2325; **15a** $C_{23}H_{34}N_9O_{17}P_3$ (+1.0078) 802.1363, found 802.1374

General Preparation of Chemically Misacylated tRNAs. Construction of L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA.

Synthesis of L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA (**3**) was accomplished by ligation of tRNA$^{Gly}_{CUA}$-C_{OH} (20 μg) with 10 μg of 5'-O-phosphoryl-2'-deoxycytidylyl(3'-5')-[2'(3')-O-(L-3-iodotyrosyl)adenosine] in a 40 μL reaction containing 55 mM Na^+-Hepes, pH 7.5/15 mM $MgCl_2$/250 μM ATP/8 μg bovine serum albumin/10% DMSO, with 15 units of T4 RNA ligase. The mixture was incubated for 10 min at 37 °C and the reaction terminated by addition of 100 μL of a 250 mM NaOAc, pH 4.5/5 M NaCl/50 mM $MgCl_2$ buffer, followed by extraction once with phenol/$CHCl_3$/isoamyl alcohol (25:24:1), once with $CHCl_3$/isoamyl alcohol (24:1), precipitation with 2.5 volumes of EtOH. The precipitate was dissolved in 10 mM NaOAc, pH 4.5/1 mM EDTA/100 mM NaCl (50 μL) and filtered through a Sephadex G-25 Select D column equilibrated with the same buffer. The appropriate fractions were combined and the tRNA EtOH-precipitated (2.5 volumes), washed with 70% EtOH, suspended in H_2O, lyophilized, and stored under argon at -80 °C as a fluffy white powder (7 μg).

Rapid Screening of Unlabeled Non-Natural Residues.

Translations with rabbit reticulocyte lysate were performed with a slightly modified procedure from manufacturer's instructions (Amersham). Magnesium ion and mRNA concentrations were determined as per instructions. A typical reaction (10 μL) contained lysate (9 μL), L-[^{35}S]-methionine (15 μCi), L-[3,4,5-^{3}H]-leucine (5 μCi), mRNA (2.0 μM), chemically misacylated tRNA (20 μM). The mixture was incubated for 1 h at 30 °C followed by addition of synthetically prepared polypeptide standards, corresponding to the expected 8-mer and 16-mer products from the translation (10 μL of a 0.5 mM solution in 77% formic acid). The solution was immediately quenched with H_2O (1.0 mL), and the resulting precipitate was centrifuged and the solvent decanted. A cycle of resuspension in 77% formic acid (10 μL) followed by precipitation with H_2O (1.0 mL) was repeated twice. The resulting precipitate was dissolved in 77% formic acid (100 μL) followed by radioisotope detection by scintillation counting.

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1. For example, see Hilvert, D.; Hatanaka, Y.; Kaiser, E. T. *J. Am. Chem. Soc.* **1988**, *110*, 682-689.
2. The following nomenclature has been employed throughout this manuscript: X-tRNA^Y_Z designates a tRNA normally aminoacylated with Y *in vivo*, containing the anticodon Z, and acylated at the 3'-terminus with the residue X. If Z is not present, the anticodon of the wild-type tRNA is employed. Those tRNAs containing a

deoxycytidine residue coupled to an adenosine moiety on the 3'-terminus rather than the normal all-ribose sequence are represented by the following: X-tRNA$_Y^Z$-dCA.

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25. For an in-depth discussion and experimental details of the rapid assay, see reference 4.
26. The value of ± 5% represents the variance between individual translation experiments as calculated from control experiments.
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