In Vitro Site-Specific Incorporation of Fluorescent Probes into β-Galactosidase

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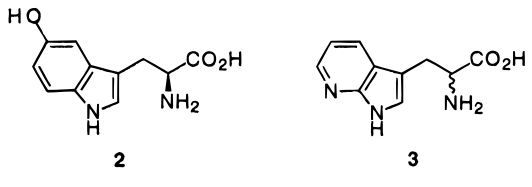
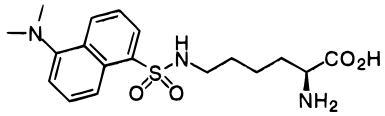
Abstract: Fluorescence spectroscopy is a powerful biophysical technique for studying protein structure, function, dynamics, and intermolecular interactions. Such studies are often conducted using intrinsic probes, such as tryptophan residues, or extrinsic probes introduced by post-translational modification, such as dansyl. Specificity, however, is often a concern since many proteins contain more than one tryptophan and chemical modification often will occur at more than one site. Herein we report the *in vitro*, site-specific incorporation of three fluorescent amino acid analogues, 5-hydroxytryptophan, 7-azatryptophan, and ϵ-dansyllysine, each of which was incorporated into β-galactosidase at a single designated site.

Protein fluorescence spectroscopy is a powerful biophysical technique for studying protein structure, function, dynamics, and intermolecular interactions in solution.[1] Covalent extrinsic fluorophores such as dansyl, or intrinsic fluorophores such as tryptophan, are commonly used for this purpose, each with serious limitations, however. The use of extrinsic probes requires post-translational modification, which is often nonspecific and/or inefficient, although reactive lysine or cysteine residues can sometimes be introduced or removed, as needed, by site-directed mutagenesis.[2] The use of intrinsic tryptophan residues is problematic when multiple tryptophans are present, although the fluorescence spectra can be simplified by the conservative replacement of all but one tryptophan residue with phenylalanine or tyrosine using site-directed mutagenesis.[3] This strategy always carries the risk, however, that changing multiple residues will have unanticipated effects on protein structure and/or function.[4]

The introduction of noncoded amino acids into proteins by *in vitro* suppression of termination codons[5] offers a unique solution to these limitations, allowing the site-specific introduction of a single fluorescent probe whose spectral window is distinct from all endogenous amino acid residues. In this paper we report the incorporation of three noncoded amino acids with useful spectral properties into β-galactosidase: the tryptophan analogues 5-hydroxytryptophan (5-OHTrp, **2**), 7-azatryptophan (7-azaTrp, **3**), and ϵ-dansyllysine (dnsLys, **4**). Both tryptophan





analogues have been incorporated into proteins by *in vivo* methods that uniformly replace all of the tryptophan residues.[6] Dansyl has been introduced post-translationally as a sulfonyl halide or isothiocyanate, but it generally modifies nucleophilic amino groups indiscriminately.[7,8] We chose to incorporate these amino acids into β-galactosidase because it is a difficult case with multiple tryptophan and lysine residues (37 and 20, respectively, per subunit)[9] and because incorporation of noncoded residues is readily monitored, as described below.

Suppressor tRNA aminoacylated with 7-azaTrp was prepared by our standard methodology.[5a,b] Boc-7-azaTrp was prepared

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(6) (a) This has been accomplished with Trp deficient bacterial auxotrophs. See Ross, J. B. A.; Szabo, A. G.; Hogue, C. W. V. *Methods Enzymol.*, in press, and references cited therein. (b) The site-specific incorporation of amino acid **3** into T4 lysozyme by suppression techniques has also previously been reported: Cornish, V. W.; Benson, D. R.; Altenbach, C. A.; Hideg, K.; Hubbell, W. L.; Schultz, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2910−2914.

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essentially as described by Rich et al.[10] and then coupled with pdCpA (CDI) to give Boc-7-azaTrp−pdCpA. The *tert*-butyl group was removed by treatment with 90:5:3:2 TFA/thioanisole/ethanedithiol/anisole for 10 min at ambient temperature, followed by ligation of the fully deprotected aminoacyl dinucleotide to $tRNA^{Gly}_{CUA}$-C_{OH} to give the desired suppressor, 7-azaTrp-$tRNA^{Gly}_{CUA}$-dCA. The ϵ-dnsLys suppressor was prepared in a similar fashion with $^{\alpha}$*N*-Boc-$^{\epsilon}$*N*-dansylLys in place of the tryptophan derivative; full details are given in the Experimental Section.

Preparation of the 5-OHTrp-$tRNA^{Gly}_{CUA}$-dCA suppressor proved to be considerably more difficult. Initially, aminoacylation of the dinucleotide was attempted by the standard CDI acylation method; however, the major product recovered from this reaction was the acyl imidazole derivative resulting from competitive reaction of the (unprotected) 5-OH group. Additives such as pentachlorophenol that reportedly suppress esterification of the indole hydroxyl group in solid-phase peptide synthesis[11] failed to resolve the problem in this case. Acylation reactions with the indole hydroxyl group protected as the tetrabutyldimethyl silyl ether (TBS) gave no desired product, while *in situ* protection of the hydroxyl as the trimethylsilyl (TMS) ether followed by CDI activation and coupling to pdCpA gave the desired product in only 1% yield.

We finally resorted to blocking the α-amine with the photolabile Nvoc protecting group, which allows the acylation to be conducted in dry organic solvent and avoids the necessity of treating the sensitive tryptophan analogue with TFA in the nitrogen deprotection step. The Nvoc-5-OHTrp was prepared from 5-OHTrp essentially as described for other amino acids,[12] followed by triethylsilylation of the 5-hydroxy group. Activation of the carboxyl group as the cyanomethyl ester, coupling with the tetrabutylammonium salt of the dinucleotide in dry DMF, and *in situ* desilylation with aqueous acetic acid gave the 2′- and 3′-isomers in 15% yield after reverse-phase HPLC purification. This aminoacyl dinucleotide was then ligated to $tRNA^{Gly}_{CUA}$-C_{OH}, and the Nvoc group was photolytically removed as described in the Experimental Section to yield the desired suppressor, 5-OHTrp-$tRNA^{Gly}_{CUA}$-dCA.

With the three synthetic suppressors in hand, we turned to determining their suppression efficiencies in protein synthesis. As a test case we chose β-galactosidase, which we routinely use as an initial screen for the biosynthetic incorporation of noncoded amino acids by suppression of amber stop codons. The plasmid construct pT7lac-7amb, which contains a DNA sequence that corresponds to the *lacZ* gene with an inserted TAG (amber) stop codon at position 7, serves as the DNA template for incorporation of noncoded amino acids into β-galactosidase. Transcription and translation of the DNA template in the presence of synthetic suppressor tRNA[5a] charged with the noncoded amino acid of interest yields full-length mutant β-galactosidase modified at position 7. Since mutations in this region are known not to affect enzymatic activity,[13] production of β-galactosidase is monitored very conveniently by the well-established colorimetric assay for enzymatic activity.[5a,14]

Low protein yield, compared to *in vivo* expression methods, is a major drawback to *in vitro* protein synthesis. The yields of even wild-type proteins are relatively low in S30 lysates, and suppression mutagenesis lowers the yields even more. The reduced levels of full-length protein are in part a result of the competition for binding to the termination codon between the suppressor tRNAs and the endogenous release factors (RFs) that normally mediate termination of translation. We demonstrated previously that when noncoded amino acids are incorporated by expansion of the genetic code, i.e., using a non-natural codon that is not recognized by RFs, the level of suppression is increased.[15] While this method of expanding the genetic code is not amenable to the synthesis of large proteins, the results did suggest that we might be able to garner the same benefits by deactivating or removing a release factor present in the S30 lysate. We therefore prepared lysate from a mutant strain (US477)[16] of *E. coli* that produces a faulty RF1 (which is the release factor competing with our suppressors for binding to the AUG codon), hypothesizing that heat shock treatment of this lysate prior to the translation reaction might thermally denature RF1 and lead to increased suppression efficiencies.

Preliminary experiments showed that, as predicted, heat-shocked S30 extracts prepared from this strain lead to increased amounts of suppression product compared to extracts from standard *Escherichia coli* strains,[17] so that subsequent suppression reactions were run with US477 S30 lysates containing the DNA template pT7lac-7amb and amber suppressor tRNA charged with amino acids **1**−**4**. Upon completion, the crude reactions were checked for β-galactosidase activity via the standard *o*-(nitrophenyl)-β-D-galactopyranoside hydrolysis assay.[5a,14] The control amino acid, phenylalanine (**1**), and all three noncoded amino acids were found to be incorporated well above background levels (Table 1). Control reactions containing (i) no template and no suppressor tRNA, (ii) template but no suppressor tRNA, and (iii) template with non-acylated suppressor tRNA produced little or no active β-galactosidase. Therefore, any β-galactosidase produced above background levels within the suppression reactions is due to suppression of the termination codon by aminoacyl suppressor tRNA, resulting in incorporation of the charged amino acid into β-galactosidase. On the basis of previous incorporation studies with both *E. coli* auxotrophs[6a] and suppressors,[6b] it was not surprising that analogues **2** and **3** were incorporated efficiently. We were gratified to find that **4** was also incorporated well above

(8) It should be mentioned that other amino acids modified with fluorescent reagents have also been incorporated biosynthetically, but either in systems that do not allow site-specificity or in systems that are more limited. See: (a) Crowley, K. S.; Liao, S.; Worrell, V. E.; Reinhart, G. D.; Johnson, A. E. *Cell* **1994**, *78*, 461−471. (b) Crowley, K. S.; Reinhart, G. D.; Johnson, A. E. *Cell* **1993**, *73*, 1101−1115. (c) Picking, W. D.; Picking, W. L.; Odom, O. W.; Hardesty, B. *Biochemistry* **1992**, *31*, 2368−2375. (d) Picking, W. D.; Odom, O. W.; Hardesty, B. *Biochemistry* **1992**, *31*, 12565−12570 and references cited therein.

(9) The active enzyme is a homotetramer comprised of 116 kDa (1023 amino acid) monomers. See: (a) Fowler, A. V.; Zabin, I. *J. Biol. Chem.* **1978**, *253*, 5521−5525. (b) Kalnins, A.; Otto, R.; Ruther, U.; Muller-Hill, B. *Embo J.* **1983**, *2*, 593−597.

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(12) Ellman, J.; Mendel, D.; Anthony-Cahill, S.; Noren, C. J.; Schultz, P. G. *Methods Enzymol.* **1991**, *202*, 301−336.

(13) Studies of *lacZ* gene fusions have shown that up to 26 amino acids can be removed from the amino terminus and can be substituted by other amino acid sequences with little or no effect on the specific activity: Fowler, A. V.; Zabin, I. *J. Biol. Chem.* **1983**, *258*, 14354−14358. This allows noncoded amino acids to be screened efficiently without fear that decreased activity is due to structural changes. Additionally, since termination competes with suppression to give only a hexapeptide, there is no possibility of the termination product having enzymatic activity.

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(15) Bain, J. D.; Switzer, C.; Chamberlin, A. R.; Benner, S. A. *Nature* **1992**, *356*, 537−539.

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(17) A detailed comparision is underway.

Table 1. Yields for 50 μL Lysate Reactions

DNA template[a]	suppressor	μg of β-gal[b]
pT7lac (wild type)		2.71 ± 0.21
pT7lac-7amb	Phe-tRNA$^{Gly}_{CUA}$-dCA	0.780 ± 0.075
pT7lac-7amb	5-OHTrp-tRNA$^{Gly}_{CUA}$-dCA	0.353 ± 0.015
pT7lac-7amb	7-azaTrp-tRNA$^{Gly}_{CUA}$-dCA	0.227 ± 0.006
pT7lac-7amb	ϵ-dnsLys-tRNA$^{Gly}_{CUA}$-dCA	0.089 ± 0.014
pT7lac-7amb	tRNA$^{Gly}_{CUA}$-dCA[c]	0.014 ± 0.003
pT7lac-7amb	*d*	0.003 ± 0.001
e		0.001 ± 0.001

[a] DNA templates were either the plasmid pT7lac containing the *lacZ* gene for full-length wild-type β-galactosidase or the plasmid pT7lac-7amb containing the *lacZ* gene with an amber stop codon at position 7. [b] Yields are determined as described in the text and are averages for three assays of crude lysate reaction. [c] Non-acylated suppressor tRNA. [d] Control reaction without suppressor tRNA. [e] Control reaction lacking plasmid and suppressor tRNA.

background levels, albeit with lower efficiency than **1**−**3**, despite literature reports to the contrary for a related translation system.[6b,18]

Amounts of 5-OHTrp- and ϵ-dnsLys-β-galactosidase sufficient for fluorescence analysis were purified from *in vitro* suppression reactions by affinity chromatography.[19] The mutant proteins were assayed for activity and their approximate concentrations determined.[5a,14] Relative concentrations were determined by fluorescence, based on comparing the dominant tryptophan emission after excitation at 280 and 295 nm (the amounts of protein were too small to quantify accurately by absorption). As controls, wild-type β-galactosidase was purified from lysate to which each fluorescent amino acid analogue had been added post-reaction, and Phe-β-galactosidase was prepared from the corresponding phenylalanyl suppressor, purified, and characterized in parallel with the other mutant proteins.

It should be noted that dansyl and 5-OHTrp also are excited at 280 and 295 nm, which could affect this quantitation method. Dansyl fluorescence would not interfere directly because it is well-separated from that of tryptophan; however, the spectral overlap of the dansyl absorption with tryptophan fluorescence does allow the possibility of resonance energy transfer, which would cause quenching of the tryptophan emission. In the case of 5-OHTrp, its fluorescence and that of tryptophan do occur at similar wavelengths, and therefore, the analogue will contribute to the total emission. While it is difficult to predict *a priori* the fluorescence yield of any fluorophore in a protein environment, it is useful to consider what might be expected for two different fluorophores given similar environments. For example, in water at pH 7, the free amino acids tryptophan and 5-OHTrp have average fluorescence lifetimes of approximately 3 and 3.7 ns.[6a,20] With these considerations in mind, it should be recalled that β-galactosidase has 37 tryptophan residues per subunit; assuming to a first-order approximation that the fluorescence yields for a tryptophan and a 5-OHTrp residue in the protein are equivalent, the analogue would be expected to make only a ca. 3% contribution to the total emission. Conversely, dansyl will tend to quench the nearest tryptophan residues, and while the magnitude of dansyl quenching is impossible to predict accurately, the net result would most likely be a less than 5% reduction in the emission, considering the size of the protein and the number of typtophan residues.

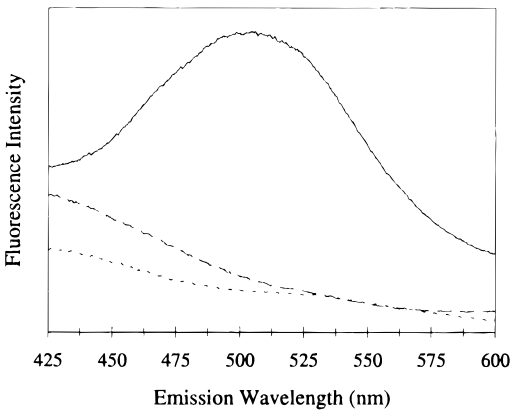

Figure 1. Fluorescence spectra (uncorrected) of dnsLys-β-galactosidase (13.6 nM, —), Phe-β-galactosidase (21.4 nM, — —), and wild-type β-galactosidase purified from ϵ-dnsLys (10.5 nM, - - -); excitation at 340 nm.

Finally, since the experimental estimates of concentration by fluorescence and by enzymatic activity agree within 20%, it seems reasonable to conclude that the amount of quenching by a single dansyl, or the incremental increase in fluorescence due to a single 5-OHTrp, would affect the total protein emission by only a few percent. There would, of course, be much greater uncertainty in this regard if the protein had only three or four tryptophan residues, which is another advantage of employing a tryptophan-rich enzyme such as β-galactosidase for these initial studies.

We demonstrated previously that red-edge excitation at 315 nm could be used to selectively excite the fluorescence of 5-OHTrp in the protein λ *c*I repressor, which has only three tryptophan residues.[20] In the case of 5-OHTrp-β-galactosidase, even with this mode of excitation, the fluorescence yield of the incorporated tryptophan analogue was too low compared to that of the 37 tryptophan residues to clearly resolve the analogue emission. On the other hand, the dansyl group has a lower energy absorption band that allows excitation at 340 nm, and we were able to obtain a well-resolved emission spectrum from dnsLys-β-galactosidase (Figure 1). The spectrum shows a fluorescence emission maximum at ca. 500 nm, which is shifted from that of ϵ-dnsLys in water[21] and corresponds well with published emission maxima for proteins that have been non-specifically modified post-translationally with dansyl chloride.[22] As anticipated, the fluorescence spectrum of the control mutant Phe-β-galactosidase reveals no fluorescence in the emission region of a dansyl group. The fluorescence spectrum of dnsLys-β-galactosidase in conjunction with the enzymatic activity confirms the incorporation of ϵ-dnsLys into a full-length, functional protein.

The results described in this paper clearly illustrate the ability to site-specifically engineer spectrally enhanced proteins with minimal structural perturbations. We are particularly interested in developing *in vitro*, site-specific incorporation of fluorescence probes to investigate the functional interactions of proteins

(18) This difference could be due to different proteins or different codon context, as well as the use of the temperature-sensitive RF strain.

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involved in blood coagulation and regulation of transcription; however, we anticipate that this approach would be general for fluorescence spectroscopy of any protein that can be expressed successfully *in vitro*.

Experimental Section

General Methods. Unless otherwise noted, reagents were obtained from commercial suppliers and were used without further purification. L-5-Hydroxytryptophan, D,L-7-azatryptophan, ε*N*-dansyl-L-lysine, Dowex 50W-X8-200 ion exchange resin, and the β-galactosidase affinity resin *p*-aminobenzyl 1-thio-β-D-galactopyranoside supported on 4% beaded agarose were purchased from Sigma, Boc-L-phenylalanine was from BaChem, and tetrabutylammonium hydroxide, [40% (w/w)] was from Aldrich. Acetonitrile (CH_3CN) and dimethylformamide (DMF) were dried over activated 4 Å molecular sieves. Triethylamine (Et_3N) was purified by distillation from calcium hydride. Trifluoroacetic acid (TFA) was dried by distillation from P_2O_5. All moisture-sensitive reactions were performed under positive pressure of nitrogen in flame- or oven-dried glassware. All aqueous solutions were prepared from water filtered through a Millipore Nanopure purification system. ^{1}H-NMR spectra were obtained on an Omega 500 (500 MHz) or a General Electric QE-300 (300 MHz) spectrometer. Spectral data are reported in ppm relative to the solvent peak. Data are reported as follows: chemical shift, multiplicity (app = apparent, par obsc = partially obscured, ovrlp = overlapping, s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m = multiplet, br = broad), coupling constant(s) in Hz, and integration. Infrared (IR) spectra were recorded as thin films with a Perkin-Elmer Model 1600 series FTIR spectrophotometer. Fast atom bombardment mass spectra (FAB-MS) were recorded at the U.C. Irvine Mass Spectral Laboratory on a Fisions VG AutoSpec. Absorbance spectra were recorded on a Kontron Instruments UVIKON 9410 UV−vis spectrophotometer. Fluorescence spectra were recorded on Hitachi F-4500 and SLM-4800 fluorescence spectrophotometers. Thin-layer chromatography (TLC) was performed on 0.25 mm Merck precoated silica gel plates (60 F-254), and flash chromatography was performed using ICN 200−400 mesh silica gel. Reverse-phase high-performance liquid chromatography (rpHPLC) was performed on a Rainin Dynamax system consisting of two model SD-200 pumps and an in-line Rainin Dynamax UV-1 absorbance detector interfaced with a Macintosh LC II. Analytical HPLC was performed with a Microsorb-MV C-18 analytical column (5 μm packing, 4.6 mm i.d. × 250 mm length) at a flow rate of 1.0 mL/min, and semiprepartive HPLC was perfomed with a Waters Nova-Pak C-18 semipreparative column (5 μm packing, 10 mm i.d. × 250 mm length) at a flow rate of 5 mL/min.

The dinucleotide, 5′-*O*-phosphoryl-2′-deoxycytidylyl-(3′-5′)-adenosine (pdCpA), was synthesized as described,[5a,b] and the truncated 74-mer tRNA ($tRNA^{Gly}_{CUA}$-C_{OH}) was prepared by runoff transcription of plasmid pJDB2, as reported previously.[5a,b] Photodeprotections were accomplished with a Pyrex-jacketed Hanovia 300 W mercury lamp.

Preparation of the Tetrabutylammonium Salt of pdCpA. The tetrabutylammonium salt of the dinucleotide was prepared with 50W-X8−200 ion exchange resin in the tetrabutylammonium form. A column containing ca. 5 mL swelled volume of resin was equilibrated with 1% tetrabutylammonium hydroxide until the eluant was basic (pH 12−13), and the column was then washed extensively with H_2O until the pH of the eluant was neutral. Dinucleotide pdCpA (100 mg) was dissolved in 0.5 mL of H_2O, loaded onto the column, and eluted with H_2O. Fractions (1−2 mL) were collected, and elution was followed by UV absorbance of material spotted on TLC plates. After elution, 0.2 equiv of tetrabutylammonium hydroxide (40% w/w) was added to the dinucleotide.[23] The sample was then lyophilized to dryness and stored at −20 °C.

5′-*O*-Phosphoryl-2′-deoxycytidylyl-(3′-5′)-2′(3′)-*O*-[*N*-(Boc)-L-phenylalanyl]adenosine (1a). Aminoacylation was carried out according to the method of Gottikh,[24] with minor modifications. To a stirred solution of 45.7 mg (0.17 mmol) of Boc-L-phenylalanine in 0.25 mL of dry CH_3CN at ambient temperature was added 37.4 mg (0.23 mmol) of carbonyl diimidazole. After 20 min, 11.1 mg (0.017 mmol) of pdCpA dissolved in 0.25 mL of 100 mM NaH_2PO_4 was added, resulting in formation of a white precipitate that dissolved after ca. 10 min of vigorous strirring. The reaction was stirred for 1 h and then was extracted with 0.75 mL of EtOAc. The organics were back-extracted with 10 mM AcOH (2 × 0.2 mL). The combined aqueous extracts were acidified with glacial AcOH (pH ≤ 5), yielding a small amount of precipitate that was dissolved by the addition of CH_3CN to 10% (v/v). The sample was filtered and purified by semipreparative reverse phase HPLC using a gradient of 5:95 → 60:40 CH_3CN/10 mM AcOH over 55 min [retention time (rt) = 28.6 and 29.7 min][25] to afford 0.002 mmol (11% yield) of **1a**. The purified product was lyophilized and stored at −75 °C. High-resolution MS calcd for $C_{33}H_{44}N_9O_{16}P_2$ ($M + H^+$) 884.2381, found 884.2402 (FAB, 3:1 glycerol/thioglycerol).

L-Phenylalanyl-$tRNA^{Gly}_{CUA}$-dCA (1b). Deprotection of aminoacyl dinucleotide **1a** and ligation to 74-mer $tRNA^{Gly}_{CUA}$-C_{OH} was carried out as previously reported, with the following modifications.[5a,b] Immediately following termination of the ligation reaction with 3 M NaOAc, 40 μg of glycogen was added and **1b** was precipitated by the addition of 3 vol of absolute EtOH and incubation at −75 °C for 90 min. The precipitate was pelleted by centrifugation at 14000*g* for 30 min at 4 °C. The pellet was washed (2×) with 70:30 EtOH/10 mM AcOH and dried *in vacuo* with a SpeedVac system. The dry pellet was resuspended in 150 μL of 10 mM AcOH, and insoluble material was removed by centrifugation. The cleared supernatant was quantified by UV absorbance, lyophilized to dryness, and stored at −75 °C until use.

***N*-(Nvoc)-L-5-Hydroxytryptophan (2a).** To a stirred solution of 750 mg (2.93 mmol) of L-5-hydroxytryptophan·dihydrate in 15 mL of a 2:1 mixture of 0.3 M aqueous Na_2CO_3/dioxane at ambient temperature was added dropwise [(6-nitroveratryl)oxy]carbonyl chloride[26] as a suspension in dioxane (7 mL). The reaction was stirred for 2 h. The reaction was made basic by addition of 20 mL of saturated Na_2CO_3 and was extracted with CH_2Cl_2 (3 × 20 mL). The aqueous layer was acidified to pH 2 with 1 M HCl and extracted with EtOAc (3 × 30 mL). The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated *in vacuo* to yield a yellow oil. The oil was resuspended in EtOAc, adsorbed onto SiO_2, and purified by flash chromatography on SiO_2 (1.8:98:0.2 MeOH/CH_2Cl_2/AcOH → 5.8:94:0.2 MeOH/CH_2Cl_2/AcOH) to give 863.1 mg (67.6% yield) of **2a** as a yellow oil: ^{1}H-NMR (300 MHz, CD_3OD) δ 7.68 (s, 1H) 7.14 (d, J = 8.6 Hz, 1H), 7.05 (s, 1H), 7.00 (s, 1H), 6.95 (d, J = 2.0, 1H), 6.64 (dd, J = 2.0, 8.6 Hz, 1H), 5.40 (d, J = 2.5 Hz, 2H), 4.48 (dd, J = 8.7, 4.5 Hz, 1H), 3.86 (s, 3H), 3.73 (s, 3H), 3.29 (ovrlp m, 1H), 3.07 (dd, J = 8.7, 14.9 Hz, 1H); IR (thin film) 3385, 2933, 2361, 1700, 1582, 1522, 1456, 1439, 1326, 1278, 1220, 1071 cm^{-1}; high-resolution MS calcd for $C_{21}H_{21}N_3O_9$ (M^+) 459.1278, found 459.1271 (FAB, *m*-nitrobenzyl alcohol).

***N*-(Nvoc)-5-[(Triethylsilyl)oxy]-L-tryptophan (2b).** To a stirred solution of 455 mg (0.99 mmol) of **2a** and 0.61 mL (5.27 mmol) of lutidine in 10 mL of anhydrous CH_3CN at 0 °C was added dropwise 0.60 mL (2.64 mmol) of triethylsilyl triflate. After 2 h, the reaction was diluted with 30 mL of EtOAc, extracted with H_2O (2 × 10 mL), and washed with brine (10 mL). The organics were dried over $MgSO_4$, filtered, and concentrated *in vacuo* to yield a yellow oil. Purification by flash chromatography on SiO_2 (2:98 MeOH/CH_2Cl_2 → 8:92 MeOH/CH_2Cl_2) gave 274 mg (48% yield) of **2b** as a yellow oil: ^{1}H-NMR (300 MHz, CD_3OD) δ 8.19 (br s, 1H), 7.69 (s, 1H), 7.19 (d, J = 8.7 Hz, 1H), 7.02 (d, J = 1.4 Hz, 1H), 6.98 (s, 1H), 6.89 (s, 1H), 6.77 (dd, J = 1.4, 8.7 Hz, 1H), 5.60 (d, J = 15.3 Hz, 1H), 5.44 (d, J = 15.3 Hz, 1H), 5.39 (par obsc m, 1H), 4.74 (m, 1H), 3.93 (s, 3H), 3.82 (s, 3H), 3.31 (d, J = 5.1 Hz, 2H), 0.98 (t, J = 7.8 Hz, 9H), 0.72 (q, J = 7.8 Hz, 6 H); IR (thin film) 3388, 2956, 2881, 2360, 1714, 1581, 1520, 1476, 1328, 1278, 1220, 1073, 956 cm^{-1}; high-resolution MS calcd for $C_{27}H_{35}N_3O_9Si$ (M^+) 573.2142, found 573.2151 (FAB, *m*-nitrobenzyl alcohol).

(23) Robertson, S. A.; Ellman, J. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 2722−2729.

(24) See, for example: (a) Gottikh, B. P.; Krayevsky, A.; Tarussova, N. B.; Purygin, P. P.; Tsilevich, T. L. *Tetrahedron* **1970**, *26*, 4419−4433. (b) Tarusova, N. B.; Mazurova, V. V.; Kraevskii, A. A.; Gottikh, B. P. *Bull. Acad. Sci. USSR, Div. Chem. Sci.* **1971**, 1630−1633 and preceeding paper in the series.

(25) The two peaks correspond to the 2′- and 3′- isomers.

(26) Amit, B.; Sehavi, V.; Patchornik, A. *J. Org. Chem.* **1974**, *39*, 192−196.

***N*-(Nvoc)-5-[(triethylsilyl)oxy]-L-tryptophan Cyanomethyl Ester (2c).** To a solution of 190 mg (0.331 mmol) of amino acid **2b** in 0.3 mL of dry DMF at ambient temperature were added 0.38 mL (2.75 mmol) of triethylamine and 0.255 mL (4.0 mmol) of chloroacetonitrile. The reaction was stirred overnight. The reaction was diluted with 25 mL of CH_2Cl_2, extracted with H_2O (2 × 25 mL), and washed with brine (25 mL). The organics were dried over $MgSO_4$, filtered, and concentrated *in vacuo* to a yellow oil. The oil was diluted with CH_2Cl_2, adsorbed onto SiO_2, and purified by flash chromatography on SiO_2 (1:2 EtOAc/hexanes → 1:1 EtOAc/hexanes) to give 167.4 mg (82.6% yield) of **2c**: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.06 (br s, 1H), 7.70 (s, 1H), 7.22 (d, J = 8.7 Hz, 1H), 7.03 (d, J = 2.2 Hz, 1H), 6.98 (s, 1H), 6.90 (s, 1H), 6.79 (dd, J = 2.2, 8.7 Hz, 1H), 5.61 (d, J = 15.4 Hz, 1H), 5.42 (ovrlp d, J = 15.4 Hz, 1H), 5.37 (d, J = 8.9 Hz, 1H), 4.79 (par obsc m, 1H), 4.79 (d, J = 15.6 Hz, 1H), 4.65 (d, J = 15.6 Hz, 1H), 3.94 (s, 3H), 3.89 (s, 3H), 3.31 (d, J = 5.4 Hz, 2H), 0.99 (t, J = 7.8 Hz, 9H), 0.73 (q, J = 7.8 Hz, 6H); IR (thin film) 3388, 2956, 1760, 1715, 1581, 1520, 1476, 1329, 1278, 1220, 1170, 1073 cm^{-1}; high-resolution MS calcd for $C_{29}H_{37}N_4O_9Si$ (M^+) 612.2251, found 612.2247 (FAB, *m*-nitrobenzyl alcohol).

5′-*O*-Phosphoryl-2′-deoxycytidylyl-(3′-5′)-2′(3′)-*O*-[*N*-(Nvoc)-L-5-hydroxytryptophanyl]adenosine (2d). A 1 dram vial containing a stir bar and 51 mg (0.084 mmol) of the activated ester **2c** and 21.2 mg (0.018 mmol) of the tetrabutylammonium salt of pdCpA was dried overnight in a vacuum desiccator containing P_2O_5. To the dried reagents was added 0.5 mL of dry DMF, and the reaction was stirred at ambient temperature for 3.5 h. The reaction was quenched with 1 mL of 10 M aqueous AcOH, yielding a precipitate that was solubilized by addition of 0.3 mL of CH_3CN. The reaction was stirred at ambient temperature for 40 min, and then 50 mM NH_4OAc (pH 5.0) was added until a small amount of precipitate formed. The precipitate was removed by centrifugation in a microcentrifuge tube. The cleared supernatant was filtered and partially purified in four semipreparative reverse-phase HPLC runs with a gradient of 25:75 → 80:20 CH_3CN/50 mM NH_4OAc (pH 5.0) over 55 min. Products eluting between 6 and 14 min were combined and lyophilized. The partially purified material was resuspended in 20:80 CH_3CN/10 mM AcOH. The sample was desalted and purified by semipreparative HPLC with a gradient of 15:85 → 60:40 CH_3CN/10 mM AcOH (rt = 16, 19 and 21 min). FAB-MS analysis revealed that product eluting at 16 min contained tetrabutylammonium ion. This material was lyophilized and desalted by reverse-phase HPLC with the same CH_3CN/10 mM AcOH gradient. The purified products were combined and lyophilized to give 2.9 mg (15.2% yield) of **2d**. Product was stored at −75 °C as a lyophilized powder. MS calcd for $C_{40}H_{46}N_{11}O_{21}P_2$ ($M + H^+$) 1078, found 1078 (FAB, 3:1 glycerol/thioglycerol).

L-5-Hydroxytryptophanyl-tRNA$^{Gly}_{CUA}$-dCA (2e). Aminoacyl dinucleotide **2d** was ligated to tRNA$^{Gly}_{CUA}$-C_{OH} as described for **1b**, with the following modification. The ligation reaction was incubated for 20 min at 37 °C. The precipitated material was resuspended, quantified, and diluted to 1 μg/μL with 10 mM AcOH. The Nvoc group was removed essentially as described.[27] The diluted sample was irradiated for 30 min at 4 °C, lyophilized to dryness, and stored at −75 °C until use.

***N*-(Boc)-D,L-7-azatryptophan (3a).** Amino acid **3a** was prepared as described by Rich et al., with minor modifications.[10] To a stirred solution of 502 mg (2.25 mmol) of **3** in 2.25 mL of a 2:1 H_2O/dioxane mixture at ambient temperature was added 0.5 mL (3.61 mmol) of triethylamine. To the stirring solution was added 582 mg (2.67 mmol) of di-*tert*-butyldicarbonate in 0.75 mL of dioxane. The resultant thick, milky paste was stirred vigorously and became clear after ca. 40 min. The reaction was stirred for 5.5 h and was worked up as described to yield 99.6 mg (72% yield) of **3a** as a white powder. Spectra were identical to those reported.

5′-*O*-Phosphoryl-2′-deoxycytidylyl-(3′-5′)-2′(3′)-*O*-[*N*-(Boc)-D,L-7-azatryptophanyl]adenosine (3b). To a stirred solution of 80.6 mg (0.36 mmol) of **3a** in 0.5 mL of dry CH_3CN at ambient temperature was added 75.6 mg (0.47 mmol) of carbonyldiimidazole. After 20 min, 20.2 mg (0.032 mmol) of pdCpA in 0.5 mL of 100 mM NaH_2PO_4 was added, resulting in a thick yellow paste. The reaction was stirred for 1 h and then was extracted with 1 mL of EtOAc. The organics were back-extracted with 10 mM AcOH (2 × 0.2 mL). The combined aqueous extracts were acidified with glacial AcOH (pH ≤ 5), yielding a precipitate that was dissolved by the addition of CH_3CN to 10% (v/v). The sample was filtered and purified by semipreparative reverse-phase HPLC using a gradient of 10:90 → 60:40 CH_3CN/10 mM AcOH over 50 min (rt = 22.5 and 23.1 min) to afford 0.74 μmol (2.3% yield) of **3b**. The purified product was lyophilized and stored at −75 °C. High-resolution MS calcd for $C_{34}H_{43}N_{11}O_{16}P_2Na$ ($M + Na^+$) 946.2262, found 946.2253 (FAB 3:1 glycerol/thioglycerol).

D,L-7-Azatryptophanyl-tRNA$^{Gly}_{CUA}$-dCA (3c). Aminoacyl dinucleotide **3b** was Boc-deprotected immediately prior to ligation as described,[5a] with the following modifications. Lyophilized **3b** was resuspended in 200 μL of reagent R (90:5:3:2 TFA/thioanisole/ethanedithiol/anisole) and incubated at ambient temperature for 10 min. The deprotected material was precipitated by addition of 2 mL of Et_2O and pelleted by centrifugation (14000*g*). The pellet was resuspended in 10 mM AcOH (100 μL), concentrated to dryness *in vacuo* on a SpeedVac system, and resuspended and concentrated a second time. The deprotected material was then ligated to 74-mer tRNA$^{Gly}_{CUA}$-C_{OH} as described.[5a]

$^{\alpha}$*N*-(Boc)-$^{\epsilon}$*N*-(dansyl)-L-lysine (4a). To a stirred solution of 150 mg (0.39 mmol) of **4** and 330 mg (2.39 mmol) of K_2CO_3 in 1.5 mL of 1:2 H_2O/dioxane cooled to 0 °C was added di-*tert*-butyldicarbonate in 0.5 mL of dioxane. After 1 h the reaction was allowed to warm to ambient temperature and was stirred overnight. The reaction was acidified to pH 4.0 with saturated citric acid and was extracted with EtOAc (3 × 5 mL). The combined organics were washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The fluorescent oil was dissolved in EtOAc, adsorbed onto SiO_2, and purified by flash chromatography on SiO_2 (29:70:1 EtOAc/hexanes/AcOH → 59:40:1 EtOAc/hexanes/AcOH) to yield 181 mg (97% yield) of **4a** as a fluorescent yellowish-green oil: ^{1}H-NMR (500 MHz, $CDCl_3$) δ 8.52 (d, J = 8.4 Hz, 1H), 8.29 (d, J = 8.4 Hz, 1H), 8.22 (d, J = 7.2 Hz, 1H), 7.55−7.49 (m, 2H), 7.18 (d, J = 7.6 Hz, 1H), 5.22 (s, 1H), 5.13 (d, J = 7.6 Hz, 1H), 4.20 (d, J = 5.2 Hz, 1H), 2.88 (app s, 8H), 1.69−1.66 (br, 2H), 1.55−1.53 (br, 2H), 1.43 (s, 9H), 1.35−1.28 (br, 2H); IR (thin film) 3287, 2939, 2871, 2354, 1712, 1694, 1568, 1506, 1452, 1392, 1367, 1315, 1232, 1160, 1145, 1073 cm^{-1}; high-resolution MS calcd for $C_{23}H_{33}N_3O_6S$ (M^+) 479.2090, found 479.2080 (FAB, *m*-nitrobenzyl alcohol).

5′-*O*-Phosphoryl-2′-deoxycytidylyl-(3′-5′)-2′(3′)-*O*-[$^{\alpha}$*N*-(Boc)-$^{\epsilon}$*N*-(dansyl)-L-lysinyl]adenosine (4b). To a stirred solution of 29.1 mg (0.06 mmol) of **4a** in 0.1 mL of dry CH_3CN at ambient temperature was added 12.9 mg (0.08 mmol) of carbonyldiimidazole. After 20 min 3.9 mg (0.006 mmol) of pdCpA in 0.5 mL of 100 mM NaH_2PO_4 was added, causing a yellow precipitate to form. The precipitate dissolved after 10 min of vigorous stirring. The reaction was stirred an additional 50 min and then was extracted with 1 mL of EtOAc. The organics were back-extracted with 10 mM AcOH (2 × 0.2 mL). The combined aqueous extracts were acidified with glacial AcOH (pH ≤ 5), yielding a precipitate that was dissolved by addition of CH_3CN to 10% (v/v). The sample was filtered and purified by semipreparative reverse-phase HPLC with a gradient of 16:84 to 60:40 CH_3CN/10 mM AcOH over 44 min (rt = 24.5 and 25.3 min) to afford 0.00035 mmol (6% yield) of **4b**. The purified product was lyophilized and stored at −75 °C. MS calcd for $C_{42}H_{58}N_{11}O_{18}P_2S$ (M^+) 1098, found 1098 (FAB, 3:1 glycerol/thioglycerol).

$^{\epsilon}$*N*-(dansyl)-L-lysinyl-tRNA$^{Gly}_{CUA}$-dCA (4c). Aminoacyl tRNA **4c** was prepared as described for **1b**.

S30 Lysate Reactions for the Synthesis of β-Galactosidase. Plasmids pT7lac and pT7lac-7amb were purified by standard methods.[5a] The S30 extract for *in vitro* protein production was prepared with minor modifications to the published procedure.[5a] The *in vitro* wild-type and suppression reactions were run according to established procedures.[5a]

Purification of β-Galactosidase. Purification of wild-type and modified β-galactosidase was based on a protocol for purification of the enzyme from *E. coli* and *B. megaterium*.[28] The minicolumns

(27) Ellman, J.; Mendel, D.; Anthony-Cahill, S.; Noren, C. J. *Methods Enzymol.* **1991**, *202*, 301−336.

(28) Steers Jr., E.; Cuatrecasas, P. *Methods Enzymol.* **1974**, *34*, 350−358.

consisted of disposable 1 mL poly(propylene) syringe barrels plugged with 100% acrylic yarn and containing a luer-lock stopcock. The columns were loaded with 100 μL of the suspended affinity resin (ca. 50 μL packed volume)[29] and were then packed and equilibrated with buffer A (50 mM Tris pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 100 mM β-mercaptoethanol) by gravity flow at 4 °C. The lysate reactions were diluted 2-fold with buffer A and loaded onto the pre-equilibrated columns. The columns were washed extensively with buffer A until the background A_{280} reading returned to baseline.[30] The β-galactosidase was eluted with 0.1 M sodium borate [pH 10.0 (200−500 μL)]. One 50 μL Phe suppression reaction was purified, and seven 50 μL dnsLys suppression reactions were combined and purified.

As a control for hydrophobic binding of ε-dnsLys, wild-type β-galactosidase was incubated with the free amino acid **4** and then was purified. Wild-type β-galactosidase from the crude lysate reaction (0.6 μg, 11 μL) was diluted to 350 μL (14.8 nM) with buffer A, corresponding to the approximate concentration of dnsLys-β-galactosidase produced in seven combined 50 μL lysate reactions (14.6 nM assuming 0.085 μg/50 μL lysate reaction). Then ε-dnsLys (**4**) was added to a final concentration of 19.0 μM, corresponding to the concentration that would be present if 100% of **4c** hydrolyzed within the lysate reaction. The β-galactosidase/ε-dnsLys mixture was incubated at 37 °C for 10 min, then was diluted 2-fold with buffer A and purified as described above.

***β*-Galactosidase Assay.** The 50 μL S30 lysate reactions were quenched by addition of 750 μL of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol). The reaction mixtures were then vortexed to suspend insoluble material, and the desired amount was removed and diluted to 800 μL with Z buffer.[31] The diluted reaction mixture was equilibrated in a shaking H_2O bath at 37 °C. The β-galactosidase assay was initiated by addition of 200 μL of ONPG (4 mg/mL in 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, pre-equilibrated at 37 °C), followed by immediate vortexing and incubation at 37 °C. The time that ONPG was added was recorded. Upon the appearance of yellow color the assay was terminated by addition of 500 μL of 1 M Na_2CO_3 and immediate vortexing. The time of quenching was recorded. Assay mixtures were clarified by centrifugation at 12000*g* for 10 min at 4 °C, and the absorbance at 420 nm (A_{420}) was determined.

In order to quantify the assays, the A_{420} reading was divided by the picomolar extinction coefficient of ONP (4.5×10^{-9} pM^{-1} cm^{-1}) and divided again by the pathlength (1 cm) to yield the picomolar concentration of ONP. The pM concentration of ONP was multiplied by the final assay volume (1.5×10^{-3} L), divided by the assay time (min), and multiplied by the amount the 800 μL quenched reaction was diluted[22] to yield picomoles of ONP produced per minute per 50 μL S30 reaction (pmol of ONP/(min/50 μL S30 reaction)). In order to convert pmol of ONP/min to μg of β-galactosidase, it was assumed that the enzyme has the specific activity of pure β-galactosidase. The specific activity of pure β-galactosidase is ca. 300 000 pmol of ONP/(min/μg of β-galactosidase) at 28 °C,[32] and the enzyme is ca. 1.4-fold more active at 37 °C.[33] Thus pmol of ONP/min was divided by 1.4 and then divided again by 300 000 pmol of ONP/(min/μg) to yield μg of β-galactosidase/50 μL S30 reaction.

Fluorescence Analysis. The fluorescence emission spectra in Figure 1 were recorded with the Hitachi-4500. Excitation and emission slit widths were 10 and 20 nm, respectively, and the PMT voltage was 950 V. Emission was scanned from 350 to 750 nm at a scan rate of 240 nm/min. A background spectrum of 1:2 Z buffer/0.1 M sodium borate (pH 10.0) showed no significant emission.

If necessary, purified β-galactosidase samples eluting from the columns were diluted to 350 μL with Z buffer. The fluorescence spectra were then recorded. The concentrations of the samples were determined on the basis of enzymatic activity by removing 50 μL from the fluorescence sample, diluting with 750 μL of Z buffer, and assaying for β-galactosidase activity as described above.

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(29) Binding capacity reported by Sigma: 2−8 mg of β-galactosidase/mL of resin.

(30) Background readings normally returned to baseline after washing with 2 mL of buffer; however, each column was washed with a minimum of 5 mL.

(31) The assay can be run on the 800 μL quenched reaction; however, in order for the assay to be accurate, the quenched reaction should be diluted such that the formation of yellow color resulting from hydrolysis of ONPG should take at least 10 min. Thus high yielding reactions must be diluted several-fold. The amount of dilution is determined experimentally but is generally within the 2−100-fold range (400−8 μL, respectively, of the quenched reaction).

(32) One unit of β-galactosidase is defined as the amount of enzyme that will hydrolyze 10^{-9} mol of ONPG/min at 28 °C. Pure β-galactosidase has an activity of ca. 300 000 units/mg. See ref 14.

(33) Determined experimentally; data not reported. Since the S30 lysate reactions were equilibrated at 37 °C, the assay reactions were run at 37 °C for convenience.