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Structurally Modified Firefly Luciferase. Effects of Amino Acid Substitution at Position 286

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Abstract: Ser was replaced at position 286 of firefly luciferase (*Luciola mingrelica*) by a series of naturally occurring and unnatural amino acids. The effect of these substitutions on the properties of luciferase, such as thermostability, pH dependence, and color of light emitted, was investigated. For these purposes, the Ser286 codon (AGT) was replaced by an amber stop codon (TAG) within the luciferase gene and transformed into *Escherichia coli* strains producing specific amber suppressor tRNA's to express luciferase with different substitutions at this position. The incorporation of Leu, Lys, Tyr, or Gln at this position reduced the thermostability of mutated luciferases. The color of emitted light changed upon substitution from yellow-green (λ_{max} 582 nm) for the wild-type enzyme having Ser286 to, for example, red (λ_{max} 622 nm) for luciferase having Leu286. For further evaluation of the structural relationship between the amino acid position at 286 and the wavelength of emitted light, we used the method of *in vitro* incorporation of unnatural amino acids, which involves readthrough of a nonsense (UAG) codon by a misacylated suppressor tRNA. The amino acids incorporated at position 286 in this fashion included *O*-glucosylated serine, serine phosphonate, tyrosine phosphate, and tyrosine methylenephosphonate. The wavelength of light emitted by the luciferase analogues was measured. While the introduction of serine phosphonate and glucosylated serine did not change the λ_{max} of light produced by luciferase, the incorporation of tyrosine phosphate and tyrosine methylenephosphonate into position 286 altered the spectra of emitted light compared with those of Ser286 and Tyr286. The pH dependence of the wavelength of light emitted by the luciferases containing the negatively charged phosphorylated Tyr analogues was demonstrated and could be rationalized in terms of the pK_a's of the phosph(on)ate oxygens.

Introduction

Firefly luciferase catalyzes the conversion of luciferin to oxyluciferin in the presence of ATP, Mg^{2+}, and molecular oxygen.[1] Due to its high sensitivity and narrow specificity for ATP, luciferase is used to determine the amount of ATP in biological samples.[2] The firefly luciferase gene is also widely used as reporter gene in many molecular biological systems.[3]

Although firefly luciferases all use the same firefly luciferin substrate, luciferases isolated from different firefly species, or even from different anatomical parts of a single firefly, can emit light having different colors.[4] Moreover, the λ_{max} of light emitted by luciferase can be affected by temperature, pH, and metal ions.[4] Light production has been suggested to involve two excited species of oxyluciferin responsible for the color of emitted light (Figure 1).[5] The presently accepted chemical mechanism of the light reaction postulates the formation of an enzyme-bound oxyluciferin intermediate in an electronically

[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1997.

(1) DeLuca, M.; McElroy, W. D. *Methods Enzymol.* **1978**, *57*, 3.
(2) Ludin, A. In *Bioluminescence and Chemiluminescence*; DeLuca, M., McElroy, W. D., Eds.; Academic Press: New York, 1981; p 187.
(3) Szalay, A. A., Kricka, L. J., Stanley, P., Eds. *Bioluminescence and Cheimiluminescence*; John Wiley & Sons Ltd.: Chichester, 1993.
(4) (a) Seliger, H. H.; McElroy, W. D. *Proc. Natl. Acad. Sci. U.S.A.* **1964**, *52*, 75. (b) McElroy, W. D.; Seliger, H. H.; White, E. H. *Photochem. Photobiol.* **1969**, *10*, 153. (c) Wood, K. V.; Lam, Y. A.; Seliger, H. H.; McElroy, W. D. *Science* **1989**, *244*, 700. (d) Wood, K. V.; Lam, Y. A.; McElroy, W. D. *J. Biolumin. Chemilumin.* **1989**, *4*, 289.

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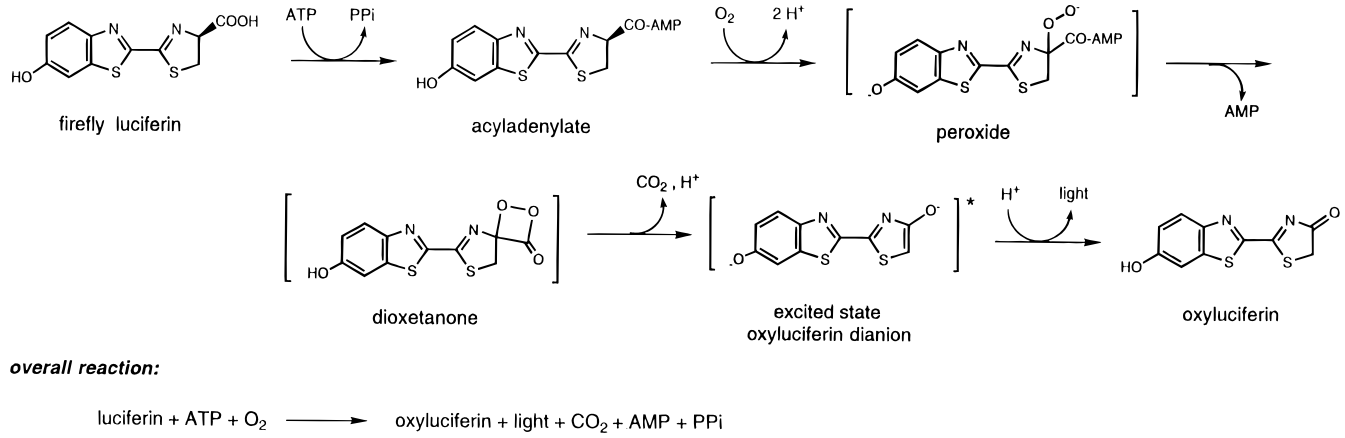


Figure 1. Partial reactions leading to light production by firefly luciferase.

excited state. Under physiological conditions, a proton is abstracted from C-5 of oxyluciferin, resulting in the formation of an enolate dianion and subsequent yellow-green bioluminescence upon its relaxation to the ground state. Alternatively, at lower pH, the oxyluciferin monoanion is thought to emit red light.[5] It has not been clear, however, whether alteration of the wavelength of emitted light must involve changes in properties of a particular amino acid(s) that interacts directly with the luciferin or can result from more global changes in the structure of luciferase. It seems likely, though, that recent advances in the structural characterization of firefly luciferase at high resolution[6] will permit this issue to be addressed experimentally.

Kajiyama and Nakano have identified five sites in *Luciola cruciata* luciferase at which alteration of a single amino acid effected significant alteration of the wavelength at which light was emitted.[7] These five sites are widely separated throughout primary sequence of the protein, which provides at least superficial support for the conclusion that numerous alterations of protein structure can influence the emission wavelength. The same authors have found that substitutions of amino acids at position 217, of both *L. cruciata* and *Luciola lateralis* luciferases, by hydrophobic amino acids such as Leu, Ile, or Val leads to thermostability.[8] It may be noted that some researchers argue against the ability of a single amino acid residue to contribute to color determination and suggest that oligo- or polypeptide fragments are actually responsible sites for the observed changes.[9]

In order to evaluate the relationship between the structure of amino acid residues at one of the positions important for color determination and consequent alterations of the wavelength of light produced by firefly luciferase, we have studied *Luciola mingrelica* luciferase, a thermolabile species.[10] A methodology that allows site-specific incorporation of amino acids into proteins by suppression of a TAG stop codon was employed.[11] Using misacylated suppressor tRNA's[12] a series of natural amino acids and unnatural amino acids, such as glycosyl and methylenephosphonate derivatives of Ser and both phosphate and methylenephosphonate derivatives of Tyr, have been incorporated at position 286 of *L. mingrelica* luciferase, both *in vivo* and *in vitro*.[13] Presently, we provide a complete account of our studies involving replacement of Ser286 in *L. mingrelica* luciferase, including the syntheses of the requisite amino acid analogues, their attachment to a suitable suppressor tRNA, and incorporation into protein to afford luciferase analogues. Also reported is the purification of a number of the derived luciferases, their light emission properties, and the effects of pH on light emission, as well as the thermostability of representative analogues.

Results

Synthesis of Amino Acid Derivatives. Although synthesis of bis(2-nitrobenzyl)-*N*,*N*-diisopropylphosphoramidite (**2**) in analogy with the general method of Bannwarth and Trzeciak[14] proved to be problematic, treatment of dichloro(diisopropylamino)phosphine (**1**) with 2-nitrobenzyl alcohol in THF gave **2** in 72% yield as a colorless solid.

The preparation of tyrosine phosphate derivative **5** began from tyrosine (Scheme 1). The amino group was protected with 2-nitroveratryl chloroformate (NVOCCl) in the presence of Na_2CO_3, giving **3** in 93% yield. Conversion to the respective

(5) (a) DeLuca, M.; Leonard, N. J.; Gates, B. J.; McElroy, W. D. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 1664. (b) White, E. H.; Steinmetz, M. G.; Miano, J. D.; Wides, P. D.; Morland, R. J. *J. Am. Chem. Soc.* **1980**, *102*, 3199. (c) Rosendahl, M. S.; Leonard, N. J.; DeLuca, M. *Photochem. Photobiol.* **1982**, *35*, 857. (d) Wood, K. V. *Photochem. Photobiol.* **1985**, *62*, 662.

(6) (a) Baldwin, T. O. *Structure* **1996**, *4*, 233. (b) Conti, E.; Franks, N. P.; Brick, P. *Structure* **1996**, *4*, 287.

(7) Kajiyama, N.; Nakano, E. *Protein Eng.* **1991**, *4*, 691.

(8) (a) Kajiyama, N.; Nakano, E. *Biochemistry* **1993**, *32*, 13795. (b) Kajiyama, N.; Nakano, E. *Biosci., Biotech. Biochem.* **1994**, *58*, 1170.

(9) Ohmiya, Y.; Hirano, T.; Ohashi, M. *FEBS Lett.* **1996**, *384*, 83.

(10) Devine, J. H.; Kutuzova, G. D.; Green, V. A.; Ugarova, N. N.; Baldwin, T. O. *Biochim. Biophys. Acta* **1990**, *1173*, 121.

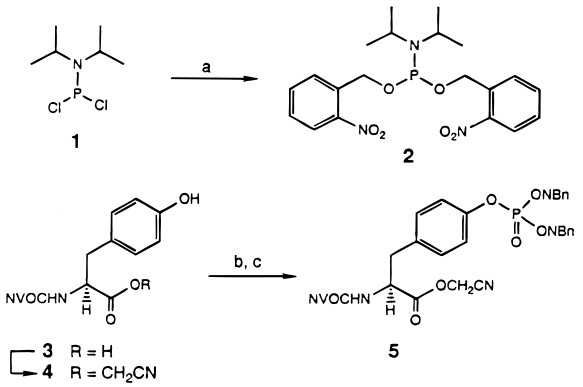
(11) (a) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182. (b) Ellman, J. A.; Mendel, D.; Anthony-Cahill, S. J.; Noren, C. J.; Schultz, P. G. *Methods Enzymol.* **1992**, *202*, 301. (c) Bain, J. D.; Wacker, D. A.; Kuo, E. E.; Chamberlin, A. R. *Tetrahedron* **1991**, *47*, 2389. (d) Bain, J. D.; Diala, E. S.; Glabe, C. G.; Wacker, D. A.; Lyttle, M. J. D.; Dix, T. A.; Chamberlin, A. R. *Biochemistry* **1991**, *30*, 5411. (e) Bain, J. D.; Switzer, C.; Benner, S. A.; Chamberlin, A. R. *Nature* **1992**, *356*, 537. (f) Cornish, V. W.; Mendel, D.; Schultz, P. G. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 621. (g) Karginov, V. A.; Mamaev, S. V.; An, H.; Van Cleve, M. D.; Hecht, S. M.; Komatsoulis, G. A.; Abelson, J. N. *J. Am. Chem. Soc.* **1997**, *119*, 8166.

(12) (a) Hecht, S. M.; Alford, B. L.; Kuroda, Y.; Kitano, S. J. *J. Biol. Chem.* **1978**, *253*, 4517. (b) Baldini, G.; Martoglio, B.; Zugliani, C.; Brunner, J. *Biochemistry* **1988**, *27*, 7951. (c) Bain, J. D.; Glabe, C. G.; Dix, T. A.; Chamberlin, A. R.; Diala, E. S. *J. Am. Chem. Soc.* **1989**, *111*, 8013. (d) Roesser, J. R.; Xu, C.; Payne, R. C.; Surratt, C. K.; Hecht, S. M. *Biochemistry* **1989**, *28*, 5185. (e) Robertson, S. A.; Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Nucleic Acids Res.* **1989**, *17*, 9649. (f) Hecht, S. M. *Acc. Chem. Res.* **1992**, *25*, 545. (g) Lodder, M.; Golovine, S.; Hecht, S. M. *J. Org. Chem.* **1997**, *62*, 778.

(13) Mamaev, S. V.; Laikhter, A. L.; Arslan, T.; Hecht, S. M. *J. Am. Chem. Soc.* **1996**, *118*, 7243.

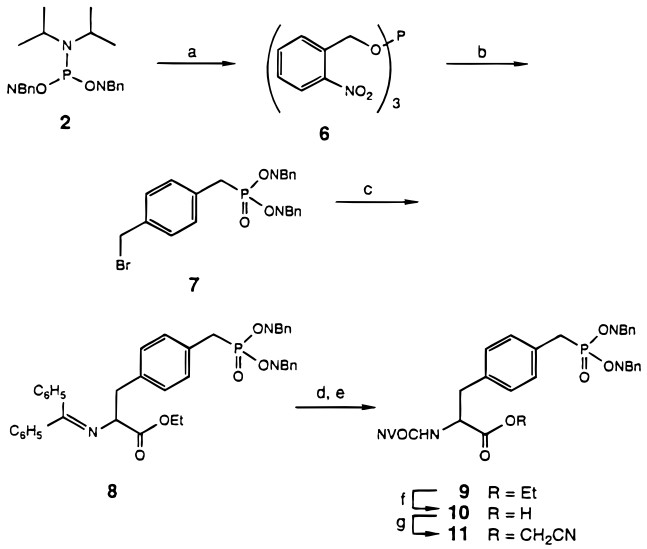
(14) Bannwarth, B; Trzeciak, A. *Helv. Chim. Acta* **1987**, *70*, 175.

Scheme 1[a]



[a] (a) 2-$NO_2C_6H_5CH_2OH$, $EtN(iPr)_2$, 70%; (b) **2**, tetrazole; (c) Bu_4NIO_4.

Scheme 2[a]



[a] (a) 2-$NO_2C_6H_5CH_2OH$, tetrazole; (b) dibromo-*p*-xylene, 100 °C, 20 min; (c) $Ph_2C{=}NCH_2COOEt$, KHMDS, −78 °C, 3 h; (d) 1 N HCl; (e) NVOCCl, Na_2CO_3; (f) 0.1 N NaOH; (g) ICH_2CN, $EtN(iPr)_2$.

cyanomethyl ester[15] **4** was achieved in 83% yield using iodoacetonitrile. The coupling reaction between **2** and **4** was then accomplished by activation of the phosphoramidite reagent with tetrazole to give the corresponding phosphite. This intermediate was not isolated, but was oxidized directly with tetrabutylammonium periodate to afford tyrosine phosphate derivative **5** in 61% overall yield for the two steps.

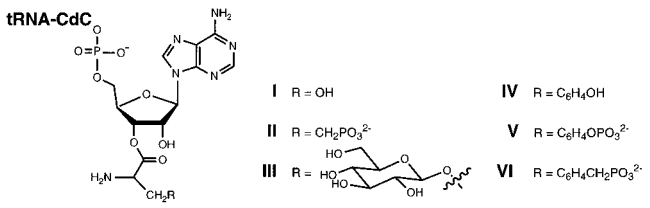
Also prepared for incorporation into luciferase was the respective methylenephosphonate derivative of tyrosine. Since ribosomal protein synthesizing systems exhibit good selectivity for (*S*)-amino acids, even when present in a racemic mixture,[16] racemic tyrosine methylenephosphonate was prepared rather than the (*S*)-isomer. The strategy employed for the synthesis of the methylenetyrosine phosphonate derivative employed the well-established Arbuzov reaction,[17] but under modified conditions compatible with the stability of the benzylic bromide functionality.

As shown in Scheme 2, phosphoramidite **2** was employed as the starting material and was treated with 2-nitrobenzyl alcohol in the presence of tetrazole to give tris(2-nitrobenzyl) phosphite (**6**) as a thick oil. This intermediate was used immediately for the next step to preclude oxidation. Compound **6** was mixed with dibromo-*p*-xylene and stirred at 100 °C for 20 min to give phosphonate **7** in 30% yield (for two steps) as a colorless powder. The coupling reaction between **7** and the protected (*N*-diphenylmethylene)glycine using potassium bis(trimethylsilyl)amide as a base afforded tyrosine methylenephosphonate **8** in 58% yield.[18] The amino group of **8** was deprotected in ethereal HCl; subsequent treatment with 2-nitroveratryl chloroformate (NVOCCl) afforded fully protected tyrosine methylenephosphonate analogue **9** in 66% overall yield. The ethyl ester moiety of **9** was removed selectively by treatment with 0.5 N NaOH in THF in the presence of pyridine; free carboxylate derivative **10** was obtained as a colorless solid in 75% yield. Conversion to the respective cyanomethyl ester **11** was accomplished in 92% yield.

Preparation of Aminoacyl pdCpA Esters. The coupling reaction between several activated amino acids and the tris(tetrabutylammonium) salt of pdCpA was investigated under conditions that included variation of solvents, reaction times, activated ester:dinucleotide ratios and the addition of a base as catalyst. In general, the coupling reactions proceeded to the maximal extent within one hour; longer reaction times did not result in higher yields. Bases such as Et_3N or KF failed to increase the coupling yields, but the addition of an excess of the activated ester did promote complete consumption of pdCpA to form the desired esters. Cyanomethyl esters **5** and **11** were coupled with the tris(tetrabutylammonium) salt of the pdCpA in DMF at 25 °C to give **12** and **13**, respectively (Scheme 3). The progress of the reactions was followed by HPLC; both were complete within 1 h. The products were purified by preparative HPLC. The photochemical deprotection reaction was studied using aminoacylated dinucleotides **12** and **13**; the reactions were complete within 8 min, as judged by HPLC analysis and the UV spectra of the derived products (Supporting Information, Figures 1−9).

Preparation of Misacylated tRNA's. The misacylated suppressor tRNA's required for the protein synthesis experiments were prepared by T4 RNA ligase-mediated attachment of *N*-protected 2′(3′)-*O*-aminoacyl-pdCpA derivatives to tRNA's or tRNA transcripts lacking the last two nucleotides.[12] The transformation is exemplified in Scheme 3 for the attachment of tyrosyl-pdCpA derivatives **12** and **13** to a truncated yeast suppressor $tRNA^{Phe}_{CUA}$ transcript lacking the 3′-terminal CA sequence.[11a,12]

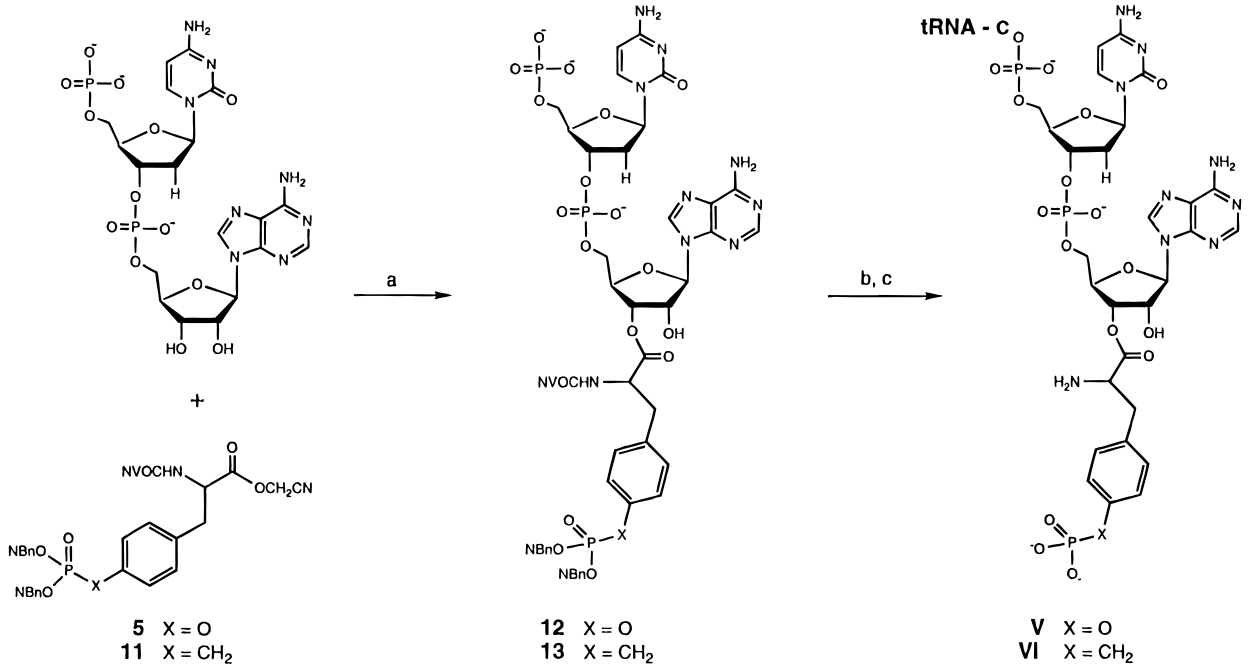
A number of misacylated tRNA's were prepared in this fashion, notably tRNA's **I**−**III** and **IV**−**VI**, involving derivatives of serine and tyrosine, respectively. The ligation yields were determined by polyacrylamide gel electrophoresis, carried out in 0.1 M NaOAc, pH 5.0, by modification of the method of Varshney et al.[19] (Figure 1, Supporting Information).



***In Vivo* Substitutions of Ser286 in Luciferase by Other Naturally Occurring Amino Acids.** The luciferase gene of

(15) Bodanszky, M.; Bodanszky, A. In *The Practice of Peptide Synthesis*, 2nd revised ed.; Springer-Verlag: Berlin, 1994; p 96.
(16) Heckler, T. G.; Roesser, J. R.; Xu, C.; Chang, P.-I.; Hecht, S. M. *Biochemistry* **1988**, *27*, 7254.
(17) Kosolapoff, G. M. *J. Am. Chem. Soc*. **1944**, *66*, 109.
(18) Hamilton, R.; Shute, R. E.; Travers, J.; Walker, B.; Walker, B. J. *Tetrahedron Lett*. **1994**, *35*, 3597.
(19) Varshney, U.; Lee, C.-P.; RajBhandary, U. L. *J. Biol. Chem*. **1991**, *266*, 24712.

Scheme 3[a]



[a] (a) DMF, 25 °C; (b) tRNA-COH, T4 RNA ligase, pH 7.5; (c) *hν*.

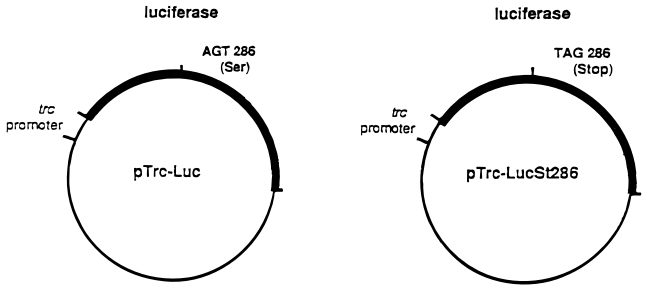


Figure 2. Plasmid constructs used for expression of the luciferase gene (*L. mingrelica*).

L. mingrelica was cloned into expression vector pTrc-99A under control of a strong *trc* promoter. Site-specific mutagenesis was used to change Ser codon AGT at position 286 to an amber stop codon TAG (Figure 2). For *in vivo* incorporation of natural amino acids, *Escherichia coli* strains, each containing a specific suppressor tRNA recognized by a different endogenous amino-acyl-tRNA synthetase, were used.[20] Following the transformation of these strains with plasmid pTrcLuc-St286, the spectrum of light emitted by each of the elaborated luciferases was measured. It was found that luciferases with Leu, Lys, Tyr, or Gln at position 286 produced light with λ_{max} shifted toward longer wavelengths than luciferase having Ser286 (λ_{max} 582).[13]

Effect of pH on the Bioluminescence of Luciferases Altered at Position 286. The cell extracts containing the luciferases with substitutions at position 286 were prepared as described in the Experimental Section and used directly in luciferase assays that employed 0.1 M MOPS−MES buffer, 5 mM $MgSO_4$, 0.5 mM luciferin, 1.5 mM ATP, and 1 mM EDTA. Spectra were measured at pH values from pH 5.2 to 8.0. The results of this experiment are presented in Table 1.

As is clear from Table 1, the five luciferases studied exhibited somewhat different behavior as a function of pH. Wild-type luciferase (Ser286) exhibited a change in the λ_{max} of emitted light from 581 nm at pH 8.0 to 618 nm at pH 5.2, with a significant change apparent at pH 6.6. This pH dependence is very similar to that observed for the luciferases from other sources.[4a] In comparison, the luciferase containing Leu286 did not exhibit significant alteration in the wavelength of emitted light at any pH from 8.0 to 5.2, consistent with the interpretation that this analogue may be able to support only the oxyluciferin monoanion species under both physiological and more acidic conditions. The luciferases containing Gln, Lys, and Tyr at position 286 were intermediate in their behavior, although it is interesting that the pH values at which the greatest changes in emission wavelength occurred were not the same for each of these luciferase analogues.

Table 1. Emission Characteristics of Luciferase Analogues as a Function of pH

amino acid at position 286	wavelength of emitted light (nm)				
	pH 5.2	pH 6.1	pH 6.6	pH 7.0	pH 8.0
Ser	618	611	590	582	581
Tyr	623	623	622	619	613
Lys	*a*	619	615	613	608
Leu	622	622	621	620	619
Gln	*a*	614		612	609

[a] Not detected.

Comparison of the Thermostability of Wild-Type Luciferase with Luciferase Analogues Altered at Position 286. Comparison of the thermostabilities of individual luciferases was carried out in cell free extracts after expression *in vivo* and also after purification of individual enzymes on Ni-NTA agarose. The purified luciferases were incubated at 22 or 37 °C (pH 7.8). The residual luciferase activity was assayed at pH 7.8 and the spectrum of emitted light was measured at various times. As shown in Figure 3, wild-type luciferase (Ser286) exhibited the greatest thermostability when incubated at 22 °C. The luciferase analogue containing Tyr286 had the next greatest thermostability at 22 °C and was actually more stable than the wild-type enzyme when both were incubated at 37 °C. In parallel with its apparent inability to support the formation of the excited state oxyluciferin dianion (*vide supra*),

(20) Promega Corporation Interchange *in vivo* system; see: (a) Kleina, L. G.; Masson, J.-M.; Normanly, J.; Abelson, J.; Miller, J. H. *J. Mol. Biol.* **1990**, *212*, 705. (b) Normanly, J.; Kleina, L. G.; Masson, J.-M.; Abelson, J.; Miller, J. H. *J. Mol. Biol.* **1990**, *212*, 719.

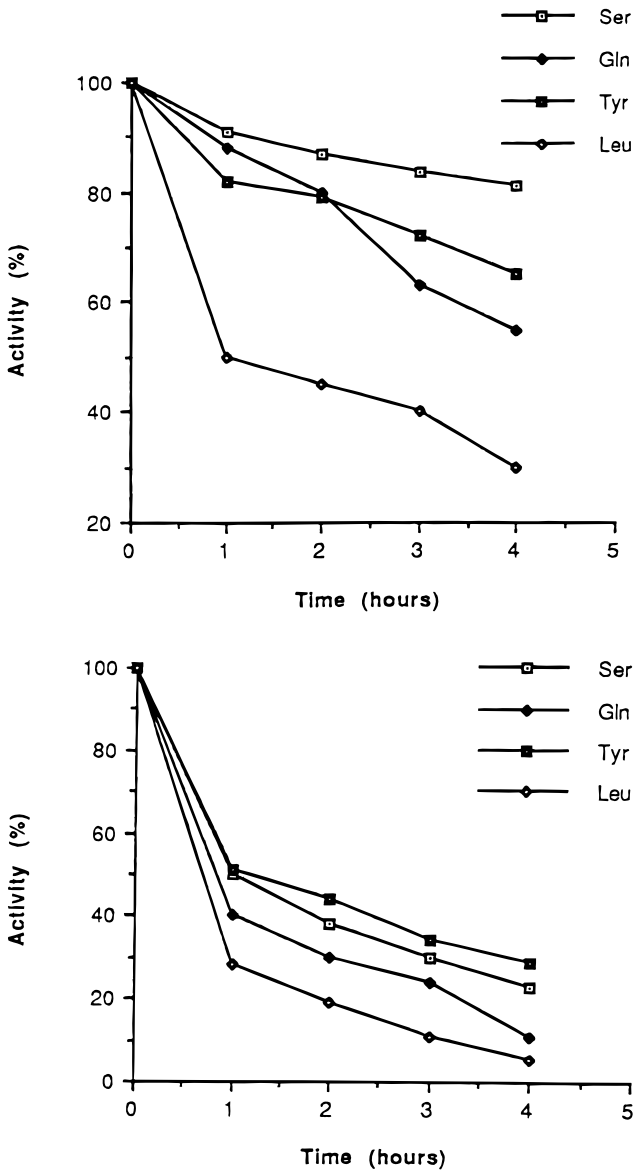


Figure 3. Thermostability of the Ni-NTA-purified luciferase analogues. Luciferases were incubated at 22 °C (upper panel) and 37 °C (lower panel) in 100 mM K phosphate buffer, pH 7.8, containing 1 mM $MgSO_4$, 1 mM EDTA, and 15% glycerol. The luciferase activities were determined after 1, 2, 3, and 4 h, and shown as a percentage of the activity measured after maintenance of the enzyme at 0 °C.

the luciferase analogue containing Leu286 was also the least thermostable of those species studied, both at 22 and 37 °C.

None of the luciferase analogues studied was noted to alter the wavelength of emitted light at any time during the course of the thermostability study (Supporting Information, Table 1).

***In Vitro* Synthesis of Luciferase Analogues.** The general strategy employed for the synthesis of luciferase analogues *in vitro* is illustrated in Scheme 4. In common with the approach employed *in vivo*, this scheme uses misacylated suppressor tRNA's for readthrough of nonsense codons placed at predetermined sites in the mRNA of interest. However, because the misacylated tRNA's prepared *in vitro* can include amino acid analogues for which there is no aminoacyl-tRNA synthetase, the derived proteins can have a much greater variety of amino acids at the position of interest.

Each *N*-protected misacylated tRNA was deprotected by *hν* irradiation and the misacylated suppressor tRNA having a deblocked amino acid was included in an *E. coli* S30 coupled transcription−translation system containing [^{35}S]methionine to effect readthrough of the UAG codon at codon position 286. Aliquots of the translation mixture were used directly to measure the spectrum and intensity of the light produced (Figure 4). SDS−PAGE analysis of [^{35}S]methionine-labeled luciferase was used for estimation of suppression efficiency. It was found that luciferases obtained after *in vitro* translation produced light with the same spectral characteristics as luciferases obtained from *E. coli* cells (cf. Tables 1−3).[13] The suppression efficiency is defined as the ratio of the amount of luciferase produced by suppressing a gene containing a stop codon relative to the amount produced from wild-type plasmid pTrcLuc.

Purification of a number of the luciferase analogues was accomplished by translation of a fusion protein that had a dodecapeptide at the C-terminus which included a hexahistidine sequence. These fusion proteins could be purified on Ni-NTA agarose columns[11g] and were shown to have unaltered properties of light emission as a consequence of introduction of the dodecapeptide.

Incorporation of Unnatural Amino Acids into Position 286 of Luciferase. The several protected pdCpA derivatives of Ser and Tyr were ligated to yeast $tRNA^{Phe}_{CUA}$(-CA) in typical yields of about 60−70%, as evaluated by PAGE analysis at acid pH (Supporting Information, Figure 1). After photochemical deprotection (5 min for GlcSer, 2 min for Val, and 8 min for Tyr derivatives), the deprotected aminoacyl tRNA's were introduced into an *E. coli* S30 translation system containing plasmid pTrcLuc-St286 DNA and [^{35}S]methionine. Protein synthesis was carried out at 22 °C for 2 h. Aliquots of the reaction mixtures were used for assay of light production and for SDS−PAGE analysis.

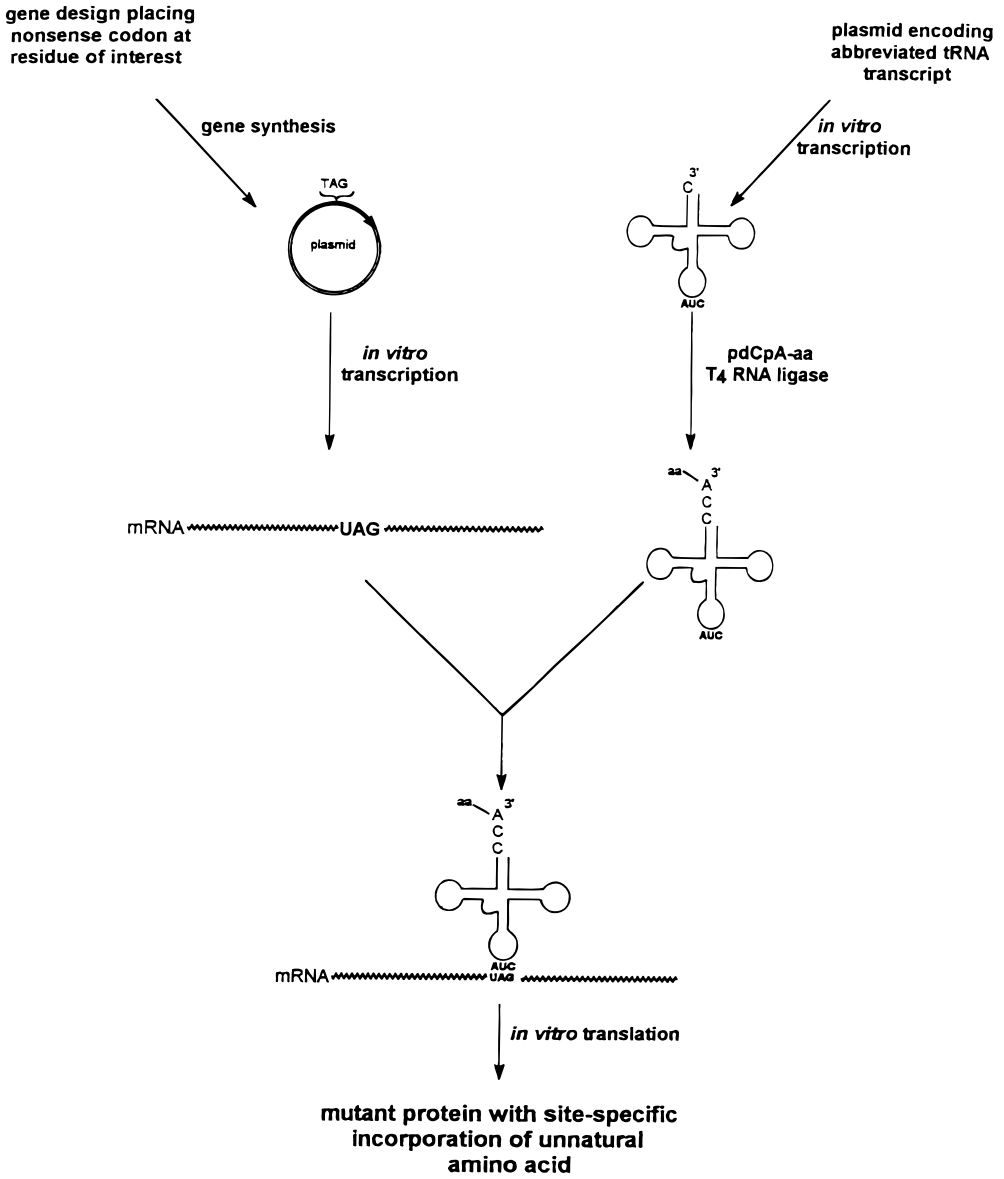
Representative results from the electrophoretic analysis of [^{35}S]methionine-labeled luciferases are shown in Figure 5. Lane 2 illustrates luciferase production from wild-type luciferase mRNA. Lane 3 shows that *E. coli* $tRNA^{Tyr}_{CUA}$ can efficiently suppress the nonsense codon at position 286 (cf. lane 1, which uses the same mRNA but lacks an activated suppressor tRNA). Lanes 4 and 5 illustrate the production of luciferase analogues containing tyrosine phosphonate and tyrosine phosphate, respectively. Protein production levels were determined by phosphorimager analysis of the band corresponding to luciferase; this permitted a calculation of the specific activities of the individual luciferase analogues. A summary of the enzymatic activities and wavelengths of emitted light for several luciferase analogues is given in Table 2.

As shown in the table, wild-type luciferase (elaborated from pTrcLuc in the absence of a suppressor tRNA) had the same specific activity and emission wavelength as luciferase prepared by suppression of the stop codon at position 286 with seryl-$tRNA^{Phe}_{CUA}$. Luciferase analogues containing Phe286 and Val286 exhibited lower specific activities and emission maxima at 608 and 621 nm, respectively. Interestingly, substitution of Ser286 with *O*-glucosylated serine or serine methylenephosphonate had no effect on the emission wavelength, although the efficiency of light production was diminished somewhat (Figure 6 and Table 2).

Substitution of position 286 with tyrosine, tyrosine phosphate and tyrosine phosphonate afforded luciferases all of which emitted light at different wavelengths. In the belief that the differences in emission wavelength for the latter two might reflect differences in the pK_a's for tyrosine phosphate (pK_{a2} 6.22) and tyrosine methylenephosphonate (pK_{a2} 7.72),[21] the light

(21) (a) Smyth, M. S.; Ford, H., Jr.; Burke, T. R., Jr. *Tetrahedron Lett.* **1992**, *33*, 4137. (b) Burke, T. R., Jr.; Smyth, M. S.; Nomizu, M.; Otaka, A.; Roller, P. P. *J. Org. Chem.* **1993**, *58*, 1336.

Scheme 4. General Strategy Employed for the Synthesis of Proteins Containing Synthetic Amino Acids at Single, Predetermined Sites



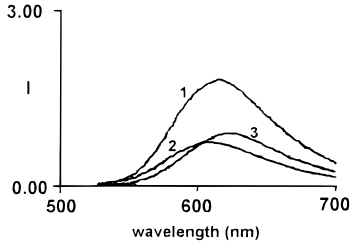


Figure 4. Emission spectra of *L. mingrelica* luciferase in which Ser286 has been replaced by tyrosine (curve 1), lysine (curve 2), and valine (curve 3).

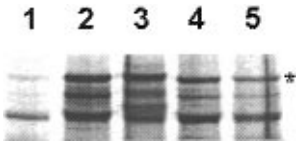


Figure 5. Phosphorimager analysis of a 10% polyacrylamide gel containing crude *in vitro* suppression products labeled with [^{35}S]-methionine and synthesized using pTrcLuc-St286 plasmid DNA and $tRNA^{Phe}_{CUA}$ activated with the following amino acids: lane 1, plasmid pTrcLuc-St286 + unactivated $tRNA^{Phe}_{CUA}$; lane 2, plasmid pTrcLuc + no suppressor tRNA; lane 3, plasmid pTrcLuc-St286 + *E. coli* $tRNA^{Tyr}_{CUA}$; lane 4, plasmid pTrcLuc-St286 + phosphonotyrosyl-$tRNA^{Phe}_{CUA}$; lane 5, pTrcLuc-St286 + phosphorotyrosyl-$tRNA^{Phe}_{CUA}$. The asterisk indicates the bands corresponding to full length luciferases.

emission characteristics of these luciferases were measured at pH 8.5, as well as pH 7.8. As shown in Table 3, light emission from luciferases having Ser286, Tyr286, and PhTyr286 was not altered at the higher pH. However, the luciferase containing PhnTyr286 emitted light at shorter wavelength at pH 8.5 than at pH 7.8, undoubtedly reflecting deprotonation of this amino acid at the higher pH value. This strongly suggests a real dependence of the wavelength of emitted light on the extent of negative charge on the tyrosine moiety at this site.

Discussion

The luciferases from *L. mingrelica* and *L. cruciata* are about 80% identical in amino acid sequence, although they do exhibit differences in thermostability and in the spectrum of emitted light.[10] On the basis of the finding of Kajiyama and Nakano[7] that a single amino acid change at any of five sites in *L. cruciata* luciferase could alter the wavelength of light produced, we sought to alter the structure of luciferase systematically at single

Table 2. Emission Characteristics of Luciferases Synthesized in a Cell Free System[a]

DNA	(misacylated) suppressor tRNA	suppression (%)	specific activity[b] (%)	emission wavelength (nm)	color
pTrcLuc		*c*	100	583	yellow-green
pTrcLuc-St286	$tRNA_{CUA}$	*d*	*d*	*d*	
pTrcLuc-St286	$tRNA_{CUA}$-Ser (**I**)	11	109	584	yellow-green
pTrcLuc-St286	$tRNA_{CUA}$-PhnSer (**II**)	7.5	29	584	yellow-green
pTrcLuc-St286	$tRNA_{CUA}$-GlcSer (**III**)	6	36	584	yellow-green
pTrcLuc-St286	$tRNA_{CUA}$-Phe	27	27	608	orange
pTrcLuc-St286	$tRNA_{CUA}$-Val	25	21	621	red-orange
pTrcLuc-St286	$tRNA^{Tyr}_{CUA}$-Tyr (**IV**)	85	14	613	orange
pTrcLuc-St286	$tRNA_{CUA}$-PhTyr (**V**)	6	20	593	yellow
pTrcLuc-St286	$tRNA_{CUA}$-PhnTyr (**VI**)	11	17	603	orange

[a] Transcription and translation were carried out in an *E. coli* S30 system, as described in the Experimental Section. [b] Relative to that of wild type. [c] Not applicable. The amount of luciferase protein was determined by phosphorimager analysis of the appropriate band on a SDS−PAGE gel and calibrated by reference to a purified luciferase standard of known specific activity. [d] No detectable light production.

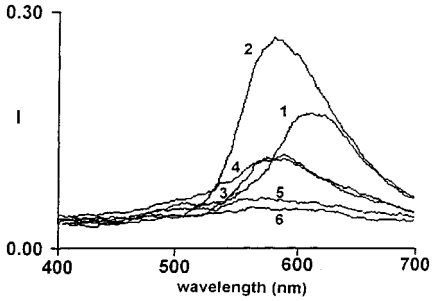


Figure 6. Emission spectra of *L. mingrelica* luciferases produced by readthrough of a UAG codon at position 286: curve 1, Val286; curve 2, Ser286; curve 3, GlcSer286; curve 4, PhnSer286. Curve 5 was recorded for luciferase produced in the presence of deacylated suppressor tRNA; curve 6 in the absence of a suppressor tRNA.

Table 3. pH Dependence of Light Emission by Luciferases with Tyrosine Derivatives at Position 286

amino acid at position 286	wavelength of emitted light (nm)	
	pH 7.8	pH 8.5
serine	581	582
tyrosine	611	612
tyrosine phosphate	590	590
tyrosine phosphonate	601	596

sites to define the basis for alterations in the wavelength of emitted light. The luciferases from *L. cruciata* and *L. mingrelica* are homologous at four of the five sites shown to be capable of controlling the wavelength of light emitted by the former species. One of these sites, Ser286, was chosen for initial modification in *L. mingrelica* luciferase.[13]

As shown in Tables 1 and 2 and Figure 4, alteration of Ser286 in *L. mingrelica* luciferase can also result in a change in the wavelength of emitted light. Interestingly, the largest changes were noted for the luciferase analogues containing the lipophilic amino acids Leu and Val at position 286. Amino acids such as Leu and Val might be thought to promote conformational changes through the introduction of new hydrophobic interactions, inducing alterations in luciferase structure by changing the protein environment around the bound luciferase substrate. Consistent with this thesis, it was found that luciferase [Val^{286}]-[13] and [Leu^{286}]luciferase (Figure 3) exhibited the poorest thermostability of any of the analogues studied. Measurement of the pH dependence of the emission wavelength for several luciferase analogues differing only at position 286 revealed that most of these species emitted light at longer wavelengths as the pH decreased. This is consistent with light emission by the putative excited stated oxyluciferin monoanion at lower pH. In contrast, luciferase Leu286 emitted light at virtually the same wavelength (619−622 nm) at all pH values, suggesting that this enzyme may be altered structurally in a fashion that precludes the generation of an excited state oxyluciferin dianion even at higher pH values. In this context it is important to note that the introduction of the polar amino acids Gln and Tyr, or positively charged Lys at position 286, did not decrease thermostability dramatically, although they all resulted in changes in the wavelength of emitted light (Figure 3 and Supporting Information, Table 1). Thus the factors that control the wavelength of light emission may well include, but need not be limited to, alterations of protein structure linked to conformational stability.

It is interesting to consider the foregoing results in the context of observations made for other luciferases. All known firefly luciferases are hydrophobic proteins.[22] Although 60% of their amino acid residues are hydrophobic, the overall hydrophobicity of the *Photinus pyralis* luciferase was judged to be only moderate, suggesting that a significant portion of the hydrophobic residues are buried within the protein matrix.[22] Unlike the results obtained by substitution of Leu or Val at position 286, Kajiyama and Nakano[8] have shown that substitution of hydrophobic amino acids at position 217 of *L. cruciata* and *L. lateralis* luciferases (for the threonine normally present) actually increased the thermostability of these enzymes. Thus the presence of hydrophobic interactions in luciferases is well-documented and need not have a deleterious effect on protein function.

In addition to the data obtained in the present study, the results obtained from analysis of five click beetle *Pyrophorus plagiophthalamus* luciferases[4c] and five *L. cruciata* luciferases obtained after random mutagenesis[7] indicate that alteration of the color of light produced could be induced by changing amino acid residues widely distributed throughout the sequence of the proteins. This is in sharp contrast to the reports from the laboratories which posit the involvement of larger structural elements in luciferase as responsible for determining the color of emitted light. These were defined as the fragments 223−247[23] within click beetle luciferase and 208−318 in *Hataria parvula* and *Pyrocoelia miyako* luciferases.[9] To some extent this may be regarded as a semantic issue since changes at a single site can effect larger structural elements within a protein. However, the present results, as well as earlier studies employing other luciferases,[4c,7] clearly indicate that alteration of a single amino acid is sufficient to change the spectrum of emitted light, even if the global change to protein structure resulting from that substitution extends well beyond the specific amino acid altered.

(22) Hlady, V.; Yeh, P.; Andrade, J. D. *J. Fluorescence* **1991**, *1*, 47.
(23) Morozov, V. M. In *Bioluminescence and Chemiluminiscence: Fundamental and Applied Aspects*; Campbell, A. K., Krica, L. J., Stanley, P. E., Eds.; Wiley Interscience: Chichester, 1994; p 536.

Among the most interesting modifications of luciferase structure in the present study are those involving the introduction of a glucosylated amino acid, as well as a phosphorylated amino acid (and two methylenephosphonate analogues). Such modifications are widespread in nature and are believed to be introduced post-translationally. They are strongly associated with the biological properties of the modified proteins; for example the carbohydrates in glycoproteins are thought to be essential for elements of biological recognition.[24] Likewise, the phosphorylation of proteins is involved in biological processes such as receptor activation, carcinogenesis, and protein synthesis.[25] Phosphorylation is also involved in regulation of the activity of certain enzymes, including DNA topoisomerase I,[26] and can play a role as a recognition element in peptides.[27] Clearly, the ability to introduce such modifications at single sites in proteins can have an enormous facilitating effect on the study of normal modification processes and on the mechanisms by which the modified species function, as well as providing a vehicle for the elaboration of fundamentally new species for investigation. It is particularly encouraging that modifications, such as the introduction of glucosylserine or serine phosphonate into a specific site in luciferase, could be made without alteration of the primary function of the enzyme (Table 2).

A more sobering perspective is provided by introduction of tyrosine phosphate and tyrosine phosphonate into position 286 of luciferase. Nonhydrolyzable analogues of phosphorylated amino acids[28] have been widely employed due both to the greater ease of handling such species and to the potential for probing biochemical events that normally result in dephosphorylation. It is frequently assumed in such studies that the phosphorylated amino acids and their phosphonate analogues are essentially equivalent since they involve only a small structural change in a large molecule. The results shown in Tables 2 and 3 indicate clearly that this need not be true. While the differences noted for luciferases with PhTyr286 and PhnTyr286 very likely are due to differences in pK_a values, as described above, these results nonetheless argue for caution in the interpretation of results obtained with structural analogues of the phosphorylated amino acids.

Recently the crystal structure of firefly luciferase from *Photinus pyralis* has been determined.[29] According to this structure Ser284 in *Photinus pyralis* luciferase (corresponding to Ser286 in *Luciola mingrelica* luciferase) is located in one of the β strands inside the large domain of luciferase. As argued previously,[13] it seems likely that this residue is not part of the active site of luciferase but may well play an important role in maintaining the native structure of luciferase. In the context of the changes in emission wavelength resulting from the introduction of hydrophobic amino acids into position 286 in *L. mingelica* luciferase (*vide supra*), it is interesting to note that several amio acids (notably Phe286, Phe273, Leu287, Leu291, Phe295, and Leu309) within reasonable proximity to Ser284 in *P. pyralis* luciferase form what appears to be a hydrophobic pocket. In any case, the availability of a crystal structure for a firefly luciferase should prove extraordinarily valuable in guiding the dissection of those structure elements that contribute to luciferase function.

(24) (a) Baenziger, J. U. *FASEB J.* **1994**, *8*, 1019. (b) Lasky, L. A. *Annu. Rev. Biochem.* **1995**, *64*, 113.

(25) (a) Pawson, T.; Schlessinger, J. *Current Biol.* **1993**, *3*, 434. (b) Panayotou, G.; Waterfield, M. D. *Bioassays* **1993**, *15*, 171. (c) Pawson, T. *FASEB J.* **1994**, *8*, 1113. (d) Crabtree, G. R.; Clipstone, N. A. *Annu. Rev. Biochem.* **1994**, *63*, 1045. (e) Hunter, T. *Cell* **1995**, *80*, 225.

(26) Pommier, Y.; Kerrigan, D.; Hartman, K. D.; Glazer, R. I. *J. Biol. Chem.* **1990**, *265*, 9418.

(27) (a) Escobedo, J. A.; Kaplan, D. R.; Kavanaugh, W. M.; Turck, C. W.; Williams, L. T. *Mol. Cell Biol.* **1991**, *11*, 1125. (b) Thatcher, G. R. J.; Campbell, S. A. *J. Org. Chem.* **1993**, *58*, 2272.

(28) Burke, Jr., T. R.; Smyth, M. S.; Otaka, A.; Nomizu, M.; Roller, P. P.; Wolf, G.; Case, R.; Shoelson, S. E. *Biochemistry* **1994**, *33*, 6490.

(29) Conti, E.; Franks, N. P.; Brick, P. *Structure* **1996**, *4*, 287.

Experimental Section

General Methods. Oligonucleotides were obtained from Cruachem, Inc. Restriction enzymes and T4 RNA ligase were purchased from New England Biolabs. *E. coli* S30 coupled transcription−translation system, Interchange *in vivo* amber suppression mutagenesis system, luciferase assay system, beetle luciferin, and pGEM-5Zf(+) vector were purchased from Promega Corporation; pTrc 99A vector was from Pharmacia Biotech. Acrylamide, *N,N*-methylenebisacrylamide, urea, Tris base, and dNTPs were obtained from Sigma Chemicals. Yeast extract and bacto tryptone were from Difco.

Plasmid DNAs were isolated using a Wizard Minipreps purification system (Promega) or QIAGEN tip 100 columns (QIAGEN) according to the protocols provided. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis was carried out using the standard Laemmli procedure.[30] Gels were visualized and quantified utilizing a Molecular Dynamics 400E phosphorimager with ImageQuant version 3.2 software. Sequence analysis was performed using a Sequenase version 2.0 DNA sequencing kit (USB). All procedures involving water employed distilled, deionized water from a Milli-Q system.

Experiments requiring anhydrous conditions were performed under an argon atmosphere. Reactions were done at 25 °C unless otherwise indicated. Solvents were J.T Baker p.a. and were used without further purification unless mentioned otherwise. THF was distilled from potassium/benzophenone. Melting points were measured on a Thomas Hoover apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR were recorded on a General Electric QE-300; all δ values are given in ppm relative to tetramethylsilane and J values are in Hz. Thin layer chromatography (TLC) was run on Merck silica gel F_{245} precoated plates; spots were visualized by dipping the plates in a Ce−Mo staining reagent. For column chromatography, Fluka silica gel 60, mesh size 230−400, was used.

Synthesis of the Unnatural Amino Acids. Bis(2-nitrobenzyl) *N,N*-Diisopropylphosphoramidite (2). Dichloro(diisopropylamino)phosphine (**1**) (5.0 g, 24.7 mmol) was dissolved in 20 mL of THF and the resulting solution was added slowly to a solution containing 12.8 mL (73 mmol) of *N,N*-diisopropylethylamine and 7.56 g (49.4 mmol) of 2-nitrobenzyl alcohol in 50 mL of THF at 0 °C. The reaction mixture was stirred at 0 °C for 30 min and then at 25 °C for another 30 min. The colorless precipitate was isolated by filtration and the solid was washed with 100 mL of ethyl acetate. The organic phase was washed successively with 50-mL portions of saturated $NaHCO_3$ and saturated NaCl and then dried ($MgSO_4$) and concentrated under diminished pressure at 25 °C. The residue was precipitated from ethyl acetate−hexane, affording bis(2-nitrobenzyl) *N,N*-diisopropylphosphoramidite (**2**) as a colorless solid: yield 7.6 g (72%); mp 71−73 °C; ^{1}H NMR ($CDCl_3$) δ 1.24 (d, 12 H, J = 7 Hz), 3.72 (m, 2 H), 5.10 (dd, 2 H, J = 7.5, 16 Hz), 5.11 (dd, 2 H, J = 7, 16 Hz), 7.43 (m, 2 H), 7.65 (m, 2 H), 7.86 (d, 2 H, J = 8 Hz), and 8.10 (d, 2 H, J = 8.5 Hz); ^{13}C NMR ($CDCl_3$) δ 25.1, 25.2, 43.8, 43.9, 62.9, 63.0, 125.1, 128.2, 129.0, 134.2, 136.5, 136.6, and 147.3. Anal. Calcd for $C_{20}H_{26}O_6N_3P$: C, 55.14; H, 4.74. Found: C, 54.94; H, 4.91.

***N*-[(2-Nitroveratryl)oxy]carbonyl]-(*S*)-tyrosine (3).** Tyrosine (181 mg, 1.0 mmol) was dissolved in 3 mL of water and treated with 210 mg (2.0 mmol) of Na_2CO_3. To this solution was added 303 mg (1.1 mmol) of 2-nitroveratryl chloroformate in 5 mL of dioxane at 0 °C. The mixture was stirred at 25 °C for 1 h. The reaction mixture was then treated with 30 mL of 1 N $NaHSO_4$ and the aqueous phase was extracted with ethyl acetate. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm); elution with 10:10:1 ethyl acetate−hexane−acetic acid afforded NVOC tyrosine derivative **3** as a yellow powder: yield 390 mg (93%); silica gel TLC R_f 0.25 (10:10:1 ethyl acetate−hexane−acetic acid); mp 133−

(30) Laemmli, U. K. *Nature* **1970**, *227*, 680.

140 °C; ^{1}H NMR ($CDCl_3$) δ 2.82 (dd, 1 H, J = 9.5, 14 Hz), 3.11 (dd, 1 H, J = 4.5, 14 Hz,), 3.84 (s, 3 H), 3.87 (s, 3 H), 4.33 (m, 1 H), 5.37 (d, 1 H, J = 15.5 Hz), 5.42 (d, 1 H, J = 15.5 Hz), 6.67 (d, 2 H, J = 8.5 Hz), 7.03 (d, 2 H, J = 8.5 Hz), 7.04 (s, 1 H), 7.50 (d, 1 H, J = 8.5 Hz), and 7.71 (s, 1 H). Anal. Calcd for $C_{19}H_{20}O_9N_2$: C, 54.28; H, 4.79. Found: C, 54.47; H, 4.97.

***N*-[[(2-Nitroveratryl)oxy]carbonyl]-(*S*)-tyrosine Cyanomethyl Ester (4).** Tyrosine derivative **3** (80 mg, 0.190 mmol) was dissolved in 5 mL of CH_3CN and treated with 132 μL (0.76 mmol) of *N*,*N*-diisopropylethylamine followed by 27.5 μL (0.38 mmol) of iodoacetonitrile. The reaction mixture was stirred at 25 °C for 12 h and then treated with 75 mL of ethyl acetate. The organic phase was washed successively with 25-mL portions of saturated $NaHCO_3$ and saturated NaCl and then dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 3 cm). Elution with ethyl acetate afforded cyanomethyl ester **4** as a colorless powder: yield 72 mg (83%); silica gel TLC R_f 0.70 (ethyl acetate); mp 148−152 °C; ^{1}H NMR ($CDCl_3$) δ 3.07 (m, 2 H), 3.95 (s, 6 H), 4.55 (m, 1 H), 4.68 (d, 1 H, J = 15.5 Hz), 4.82 (d, 1 H, J = 15.5 Hz), 4.90 (s, 1 H), 5.22 (d, 1 H, J = 8 Hz), 5.46 (d, 1 H, J = 15 Hz), 5.56 (d, 1 H, J = 15 Hz), 6.77 (d, 2 H, J = 8.5 Hz), 6.92 (s, 1 H), 7.01 (d, 2 H, J = 8.5 Hz), and 7.70 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 37.3, 49.5, 55.4, 56.8, 56.9, 64.6, 108.7, 110.5, 114.3, 116.3, 126.8, 128.0, 130.8, 140.0, 148.6, 154.1, 155.9, and 171.0. Anal. Calcd for $C_{21}H_{21}O_9N_3$: C, 54.88; H, 4.60. Found: C, 55.05; H, 4.66.

***N*-[[(2-Nitroveratryl)oxy]carbonyl]-*O*-[bis[(2-nitrobenzyl)oxy]-phosphoryl]-(*S*)-tyrosine Cyanomethyl Ester (5).** Tyrosine cyanomethyl ester **4** (72 mg, 0.156 mmol) was dissolved in 10 mL of THF and treated with 105 mg (0.241 mmol) of bis(2-nitrobenzyl) *N*,*N*-diisopropylphosphoramidite (**2**) and 22 mg (0.285 mmol) of tetrazole. The solution was stirred at 25 °C for 30 min under an argon atmosphere. Tetrabutylammonium periodide (100 mg, 0.23 mmol) was added in 3 mL of THF. The combined solution was stirred for 10 min at 25 °C and then treated with 75 mL of ethyl acetate. The organic phase was washed successively with 25-mL portions of saturated $NaHCO_3$ and saturated NaCl and then dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (30 × 3 cm). Elution with 1:1 ethyl acetate−hexane afforded cyanomethyl ester **5** as a colorless powder: yield 77 mg (61%); silica gel TLC R_f 0.40 (1:1 ethyl acetate−hexane); mp 83−87 °C; ^{1}H NMR ($CDCl_3$) δ 3.14 (m, 2 H), 3.94 (s, 3 H), 3.96 (s, 3 H), 4.69 (d, 1 H, J = 15.5 Hz), 4.73 (m, 1 H), 4.82 (d, 1 H, J = 15.5 Hz), 5.28 (d, 1 H, J = 8.5 Hz), 5.45 (d, 1 H, J = 14.5 Hz), 5.57 (d, 1 H, J = 14.5 Hz), 5.63 (d, 4 H, J = 7.5 Hz), 6.95 (s, 1 H), 7.12 (d, 2 H, J = 8.5 Hz), 7.19 (d, 2 H, J = 8.5 Hz), 7.50 (m, 2 H), 7.70 (m, 5 H), and 8.12 (d, 2 H, J = 8.5 Hz); ^{13}C NMR ($CDCl_3$) δ 37.4, 49.5, 55.1, 56.9, 57.0, 64.6, 67.3, 67.4, 108.7, 110.6, 114.2, 120.8, 120.9, 125.6, 129.0, 129.6, 131.2, 132.2, 132.8, 134.7, 147.2, 150.2, 154.1, 155.7, and 170.7. Anal. Calcd for $C_{35}H_{32}O_{16}N_5P$: C, 51.91; H, 3.98. Found: C, 52.27; H, 4.11.

Bis(2-nitrobenzyl) 4-(Bromomethyl)benzylphosphonate (7). Phosphoramidite reagent **2** (435 mg, 1 mmol) in 3 mL of CH_2Cl_2 was added to a solution containing 140 mg (2 mmol) of tetrazole and 153 mg (1 mmol) of 2-nitrobenzyl alcohol in 10 mL of CH_2Cl_2 under an argon atmosphere. The reaction mixture was stirred at 25 °C for 50 min and then treated with 25 mL of CH_2Cl_2 and washed with 10 mL of saturated NaCl. The organic phase was dried (Na_2SO_4) and concentrated under diminished pressure at 25 °C. Crude product **6**, isolated as a thick oil, was used directly in the next step to preclude oxidation. Crude **6** and dibromo-*p*-xylene (263 mg, 1 mmol) were mixed and stirred together under an argon atmosphere at 100 °C for 20 min. The solution was permited to cool to room temperature and the product was purified by flash chromatography on a silica gel column (35 × 3 cm). Elution with 5:2:1 ether−hexanes−ethyl acetate gave phosphonate **7** as a colorless powder: yield 160 mg (30% for two steps); silica gel TLC R_f 0.60 (5:2:1 ether−hexanes−ethyl acetate); mp 110 °C; ^{1}H NMR ($CDCl_3$) δ 3.31 (s, 1 H), 3.38 (s, 1 H), 4.480 (s, 1 H), 4.483 (s, 1 H), 5.39 (s, 2 H), 5.41 (s, 2 H), 7.28−7.68 (m, 10 H), and 8.07 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 32.9, 33.5, 34.7, 65.0, 65.1, 125.4, 129.1, 129.4, 129.9, 130.7, 130.8, 132.8, 132.9, 134.5, 137.4, and 147.3. Anal. Calcd for $C_{22}H_{20}O_7N_2PBr$: C, 49.36; H, 3.76. Found: C, 49.50; H, 3.68.

***N*-(Diphenylmethylene)-4-[[bis(2-nitrobenzyl)phosphono]methyl]-(*R*,*S*)-phenylalanine Ethyl Ester (8).** Phosphonate **7** (140 mg, 0.262 mmol) was dissolved in 5 mL of THF and the solution was added slowly to a solution containing 84 mg (0.315 mmol) of ethyl *N*-(diphenylmethylene)glycine and 60 mg (0.315 mmol) of potassium bis(trimethylsilyl)amide in 10 mL of THF at −78 °C. The combined solution was stirred at −78 °C for an additional 3 h and then permitted to warm to 25 °C. Ethyl acetate (100 mL) was added. The organic phase was washed successively with 30-mL portions of saturated $NaHCO_3$ and saturated NaCl and then dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (40 × 3 cm). Elution with 5:2:1 CH_2Cl_2−hexane−ethyl acetate afforded *N*-(diphenylmethylene)-4-[[bis(2-nitrobenzyl)phosphono]methyl]-(*R*,*S*)-phenylalanine ethyl ester (**8**) as a yellowish oil: yield 110 mg (58%); silica gel TLC R_f 0.30 (5:2:1 CH_2Cl_2−hexane−ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.24 (t, 3 H, J = 7 Hz), 3.22 (m, 3 H), 3.34 (s, 1 H), 4.09−4.25 (m, 3 H), 5.22−5.43 (m, 4 H), 6.99−7.56 (m, 20 H), and 8.04 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 14.6, 32.7, 34.5, 39.7, 61.5, 64.8, 64.9, 67.6, 125.4, 125.6, 128.4, 128.7, 128.9, 129.0, 129.1, 129.3, 129.4, 129.6, 130.0, 130.1, 130.7, 132.8, 132.9, 133.0, 134.4, 134.6, 136.5, 137.7, 137.8, 139.7, 147.2, 171.3, and 172.1; mass spectrum (FAB) *m/z* 722 $(M + H)^+$; (FAB) *m/z* 722.226 $(M + H)^+$ ($C_{39}H_{36}O_9N_3P$ requires 722.226).

***N*-[[(2-Nitroveratryl)oxy]carbonyl]-4-[[bis(2-nitrobenzyl)phosphono]methyl]-(*R*,*S*)-phenylalanine Ethyl Ester (9).** Amino acid **8** (110 mg, 0.153 mmol) was dissolved in 10 mL of ether and the solution was treated with 2 mL of 1 N HCl. The reaction mixture was stirred at 25 °C for 30 min. The pH of the aqueous phase was then adjusted to 7 by addition of 1 N NaOH. The reaction mixture was treated with 10 mL of hexane and the organic phase was separated. The aqueous phase containing the amino acid analogue was treated with 25.8 mg (0.25 mmol) of Na_2CO_3, then with 51 mg (0.19 mmol) of NVOCCl in 5 mL of dioxane at 0 °C. The mixture was stirred at 25 °C for 2 h and then treated with 100 mL of ethyl acetate. The organic phase was washed with 30-mL portions of saturated $NaHCO_3$ and then with saturated NaCl. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 3 cm); elution with 3:1 ethyl acetate−hexane afforded ethyl ester (**9**) as a yellowish powder: yield 80 mg (66% for two steps); silica gel TLC R_f 0.50 (3:1 ethyl acetate−hexanes); mp 139−142 °C; ^{1}H NMR ($CDCl_3$) δ 1.24 (t, 3 H, J = 7 Hz), 3.13−3.48 (m, 4 H), 3.92 (s, 3 H), 3.93 (s, 3 H), 4.17 (q, 2 H, J = 7 Hz), 4.68 (m, 1 H), 5.20−5.59 (m, 7 H), 6.99 (s, 1 H), 7.14 (d, 2 H, J = 8 Hz), 7.21 (d, 2 H, J = 8 Hz), 7.46−7.69 (m, 6 H), 7.68 (s, 1 H), and 8.04 (d, 2 H, J = 8 Hz); ^{13}C NMR ($CDCl_3$) δ 14.6, 32.8, 34.7, 38.1, 55.3, 56.8, 56.9, 62.1, 64.2, 65.0, 108.8, 110.3, 125.4, 128.5, 129.1, 130.2, 130.5, 132.8, 134.4, 147.3, 148.5, 154.1, 155.7, and 171.8.

***N*-[[(2-Nitroveratryl)oxy]carbonyl]-4-[[bis(2-nitrobenzyl)phosphono]methyl]-(*R*,*S*)-phenylalanine (10).** Tyrosine phosphonate derivative **9** (80 mg, 0.10 mmol) was dissolved in 3 mL of THF and treated with 200 μL of pyridine and 515 μL of 0.5 N NaOH. The reaction mixture was stirred at 25 °C for 30 min and the pH was adjusted to 4 by the addition of 20 mL of 1 N $NaHSO_4$. The reaction mixture was extracted twice with 50-mL portions of ethyl acetate, and the combined organic extract was dried ($MgSO_4$) and concentrated. The residue was precipitated from ethyl acetate−hexane, affording *N*-[[(2-nitroveratryl)-oxy]carbonyl]-4-[[bis(2-nitrobenzyl)phosphono]methyl]-(*R*,*S*)-phenylalanine (**10**) as a colorless solid: yield 58 mg (75%); silica gel TLC R_f 0.10 (ethyl acetate); mp 96−98 °C; ^{1}H NMR ($CDCl_3$) δ 3.12 (m, 2 H), 3.30 (s, 1 H), 3.37 (s, 1 H), 3.94 (s, 3 H), 3.96 (s, 3 H), 4.78 (d, 1 H, J = 16 Hz), 4.74 (m, 1 H), 4.80 (d, 1 H, J = 15.5 Hz), 5.30−5.59 (m, 7 H), 6.96 (s, 1 H), 7.11 (d, 2 H, J = 8 Hz), 7.27−7.64 (m, 8 H), 7.69 (s, 1 H), and 8.06 (d, 2 H, J = 8 Hz). Anal. Calcd for $C_{34}H_{33}O_{15}N_4P$: C, 53.13; H, 4.32. Found: C, 53.02; H, 4.39.

***N*-[[(2-Nitroveratryl)oxy]carbonyl]-4-[[bis(2-nitrobenzyl)phosphono]methyl]-(*R*,*S*)-phenylalanine Cyanomethyl Ester (11).** Tyrosine phosphonate derivative **10** (58 mg, 0.075 mmol) was dissolved in 5 mL of CH_3CN and treated with 70 μL (0.376 mmol) of *N*,*N*-diisopropylethylamine followed by 50 μL (0.19 mmol) of iodoacetonitrile. The reaction mixture was stirred at 25 °C for 12 h and then treated with 25 mL of ethyl acetate. The organic phase was washed successively with 10-mL portions of saturated $NaHCO_3$ and saturated

NaCl and then dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with ethyl acetate afforded cyanomethyl ester **12** as a colorless powder: yield 56 mg (92%); silica gel TLC R_f 0.80 (ethyl acetate); mp 83−85 °C; ^{1}H NMR ($CDCl_3$) δ 3.12 (m, 2 H), 3.30 (s, 1 H), 3.37 (s, 1 H), 3.94 (s, 3 H), 3.96 (s, 3 H), 4.68 (d, 1 H, J = 16 Hz), 4.74 (m, 1 H), 4.80 (d, 1 H, J = 15.5 Hz), 5.50−5.59 (m, 7 H), 6.96 (s, 1 H), 7.11 (d, 2 H, J = 8 Hz), 7.27−7.64 (m, 8 H), 7.69 (s, 1 H), and 8.06 (d, 2 H, J = 8 Hz). Anal. Calcd for $C_{36}H_{34}O_{15}N_5P$: C, 53.53; H, 4.24. Found: C, 53.84; H, 4.40.

***N*-[[(2-Nitroveratryl)oxy]carbonyl]-*O*-[bis[(2-nitrobenzyl)oxy]-phosphoryl]-(*S*)-tyrosine pdCpA Ester (12).** A solution of the tris-(tetrabutylammonium) salt of pdCpA (4.1 mg, 3 μmol) dissolved in 50 μL of freshly distilled DMF was added to a flame-dried conical vial containing 20 mg (25 μmol) of cyanomethyl ester **5**. The reaction mixture was stirred at 25 °C and monitored by HPLC. Four-microliter aliquots were removed periodically and diluted with 40 μL of 50 mM NH_4OAc, pH 4.5; 10 μL of each diluted aliquot was analyzed on a 3 μm C_{18} reverse phase HPLC column (100 × 4.6 mm). The column was washed with 1% → 65% CH_3CN−50 mM NH_4OAc, pH 4.5, over 45 min at a flow rate of 1.0 mL/min [detection at 260 nm; the desired product had a retention time of 30.2 min (Supporting Information, Figures 2 and 3)]. The remaining reaction mixture was diluted with 600 μL of 1:1 CH_3CN−50 mM NH_4OAc, pH 4.5, and purified by C_{18} reverse phase HPLC on a semipreparative column (250 × 10 mm) using the same gradient described above at a flow rate of 4.0 mL/min. Dinucleotide derivative **12** was recovered from the appropriate fractions by lyophilization as a colorless solid: yield (3.2 mg, 77%); mass spectrum (FAB) *m/z* 1389 $(M + H)^+$; (FAB) *m/z* 1389.255 $(M + H)^+$ ($C_{52}H_{56}O_{28}N_{12}P_3$ requires 1389.254).

***N*-[[(2-Nitroveratryl)oxy]carbonyl]-4-[[bis[(2-nitrobenzyl)oxy]-phosphono]methyl]-(*R,S*)-phenylalanine pdCpA Ester (13).** A solution of the tris(tetrabutylammonium) salt of pdCpA (4.1 mg, 3 μmol) dissolved in 50 μL of freshly distilled DMF was added to a flame-dried conical vial containing 20 mg (25 μmol) of cyanomethyl ester **11**. The reaction mixture was stirred at 25 °C and monitored by HPLC. Four-microliter aliquots were removed periodically and diluted with 40 μL of 50 mM NH_4OAc, pH 4.5; 10 μL of each diluted aliquot was analyzed on a 3 μm C_{18} reverse phase HPLC column (100 × 4.6 mm). The column was washed with 1% → 65% 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 [detection at 260 nm; the desired product had a retention time of 28.7 min (Supporting Information, Figures 4 and 5)]. The remaining reaction mixture was diluted with 600 μL of 1:1 CH_3CN−50 mM NH_4OAc, pH 4.5, and purified by C_{18} reverse phase HPLC on a semipreparative column (250 × 10 mm) using the same gradient described above at a flow rate of 4.0 mL/min. Dinucleotide derivative **13** was recovered from the appropriate fractions by lyophilization as a colorless solid: yield (3.4 mg, 82%); mass spectrum (FAB) *m/z* 1387 $(M + H)^+$; (FAB) *m/z* 1387.273 $(M + H)^+$ ($C_{53}H_{58}O_{27}N_{12}P_3$ requires 1387.275).

Photochemical Deprotection of pdCpA Esters 12 and 13. The photochemical deprotection of aminoacyl-pdCpA derivatives **12** and **13** was carried out following literature precedent[31] by use of a 500 W mercury−xenon lamp using Pyrex and water filters. The samples were irradiated for 8 min. The deblocking of **12** and **13** was monitored by C_{18} reverse phase HPLC as described above. Retention time (t_R) of **12** was 30.2 min (Supporting Information, Figures 2 and 3) and the t_R of the corresponding fully deprotected aminoacyl pdCpA was 9.7 min (Supporting Information, Figures 6 and 7). The t_R of **13** was 28.7 min (Supporting Information, Figures 4 and 5) and the t_R of the corresponding fully deprotected aminoacyl pdCpA was 6.9 min (Supporting Information, Figures 8 and 9) under the same conditions. The UV spectra reflected complete deprotection.

Construction of the Plasmid for Expression of Luciferase Gene. pGK3 plasmid[10] was kindly provided by Dr. G. Kutuzova. The luciferase gene was subcloned into the pGEM-5Zf(+) vector (Promega) using the *Nco*I−*Mam*I fragment of pGK3 containing the luciferase gene and *Nco*I−*Eco*RV-digested pGEM-5Zf(+) plasmid DNA. The luciferase gene was isolated from intermediate plasmid pGEM5-luc after digestion by *Nco*I−*Pst*I and ligated with the pTrc 99A vector that had been digested by the same restriction enzymes. The cloning of the luciferase gene into the pTrc 99A expression vector was confirmed by restriction analysis and the luciferase assay. A TAG codon at position 286 was introduced into pTrcLuc using a T7-Gen *in vitro* mutagenesis kit (USB). The mutation was confirmed by sequencing by the dideoxy chain termination method of Sanger.[32]

Expression of pTrcLuc-St286 in *E. coli* Strains Containing Suppressor tRNAs. *E. coli* strains carrying suppressor tRNA's for Ser, Leu, and Gln and a control strain without any suppressor tRNA were transformed by the DNA of plasmid pTrcLuc-St286. Individual colonies obtained after transformation were cultured overnight at 22 °C in 100 mL of Luria−Bertani broth containing 50 μg/mL ampicillin and 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation, resuspended in 1 mL of lysis buffer (100 mM potassium phosphate, pH 7.8, containing 1 mM EDTA and 1 mg/mL lysozyme), incubated on ice for 30 min, and then frozen on dry ice. The frozen pellets were allowed to thaw at 25 °C and the DNA was disrupted by sonication. Debris was removed by centrifugation for 15 min at 14 000 rpm using a microcentrifuge. Glycerol was added to the extracts to 17% v/v concentration, and the extracts were stored at −20 °C.

Incorporation of a Fusion Dodecapeptide Containing Hexahistidine onto the C Terminus of Luciferase. A 36-base pair (bp) synthetic oligonucleotide was ligated into *Spe*I−*Pst*I sites of plasmids pTrcLuc and pTrcLuc-St286, introducing a sequence of Tyr codon (instead of the TAG stop codon) and the codons encoding hexahistidine at the 3′-end of the luciferase gene, followed by the TAA stop codon and a *Xho*I site. Incorporation of the hexahistidine sequence was confirmed by the appearance of the *Xho*I site and by dideoxy sequencing analysis. The resulting plasmids [pTrcluc(H) and pTrcLuc-St286(H)] were used for expression in *E. coli* strains of the Interchange system and for *in vitro* translation. Luciferase assays carried out using luciferases substituted at position 286 (obtained both *in vivo* and *in vitro*) have demonstrated that the incorporation of the hexahistidine fusion peptide at the C-terminus of the *Luciola mingrelica* luciferase did not change properties of the enzyme such as emission wavelength or specific activity.

Construction of a Plasmid for Runoff Transcription of Yeast $tRNA^{Phe}_{CUA}$(-CA). The DNA fragment containing the T7 promoter, a sequence corresponding to yeast $tRNA^{Phe}_{CUA}$, *Kpn*I, *Hin*dIII, *Bst*NI, and *Fok*I cleavage sites was constructed using two overlapping synthetic oligonucleotides. The oligonucleotides were purified on a 8% denaturing polyacrylamide gel; 5 μg of each oligonucleotide was combined and annealed in 100 μL of 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 1 mM DTT, and 50 mg/mL of bovine serum albumin. Transcription was carried out in the presence of 1 mM each dNTP and 5 units of DNA polymerase I (Klenow) at 37 °C for 30 min. The double-stranded DNA was purified on a 6% polyacrylamide gel, digested with restriction endonucleases *Kpn*I and *Hin*dIII, and ligated to plasmid pUC19 that had been digested with the same restriction endonucleases. The structure of the resulting plasmid (pYRNA) was confirmed by restriction digest analysis and dideoxy sequencing.

Runoff Transcription of Suppressor tRNA(-CA). Plasmid pYRNA DNA was digested with *Fok*I and then transcribed using an AmpliScribe T7 transcription kit (Epicentre Technologies) in a 500-μL reaction mixture at 37 °C for 4 h according to the protocol supplied with the kit. After incubation, 500 μL of 80% formamide containing 0.02% xylene cyanol and bromophenol blue was added and mixture was applied to an 8% denaturing polyacrylamide gel (400 × 200 × 2 mm). After electrophoresis for 3 h at 800 V, the RNA band was visualized by UV shadowing,[33] excised from the gel, and cut into small pieces. The RNA was eluted from the gel by treatment with 5 mL of 100 mM sodium acetate, pH 4.6, containing 1 mM EDTA and 0.01% SDS at 4 °C for 12 h. The tRNA was precipitated with two volumes of cold ethanol, dried, and then dissolved in RNase-free water and divided into aliquots and stored at −80 °C.

Synthesis of Misacylated tRNAs. Ligation reactions were carried out in reaction mixtures containing per 50 μL: 0.5 A_{260} unit of

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

(32) Sanger, F.: Nicklen, S.; Coulson, A. R. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5463.

(33) Hassur, S. M.; Whitlock, H. W. *Anal. Biochem.* **1974**, *59*, 162.

aminoacyl pdCpA, 10 μg $tRNA^{Phe}_{CUA}$(-CA), 50 mM Hepes buffer, pH 7.5, 15 mM $MgCl_2$, 10−20% DMSO and 100 units of T4 RNA ligase. Reactions were incubated at 37 °C for 25 min and then quenched by addition of 0.1 volume of 3 M sodium acetate, pH 4.5. The tRNA was precipitated by the addition of two volumes of cold ethanol and collected by centrifugation. The tRNA was washed with 70% ethanol, dried, and dissolved in 1 mM potassium acetate solution to a concentration of 1 mg/mL. Photodeprotection was carried out using a 500 W mercury−xenon lamp with Pyrex and water filters. The aminoacyl-tRNA solution in a transparent microfuge tube was placed into a glass container with cooling water circulated at 2 °C. Irradiation was carried out for 2 min for Val and Phe, 5 min for Ser and glucosylated Ser, and 8 min for Ser phosphonate, as well as Tyr phosphate and phosphonate derivatives. Deprotected aminoacyl-tRNAs were used in *in vitro* suppression experiments immediately after irradiation.

***In Vitro* Suppression.** *In vitro* synthesis of luciferase were carried out in an *E. coli* S30 translation system for circular DNA. Four micrograms of plasmid DNA and 5 μg of misacylated tRNA were typically used in a 50-μL reaction mixture (total volume), which also contained amino acids (100 μM final conc) 20 μL of S30 premix,[34] 10 μCi of [^{35}S]methionine (1200 Ci/mmol), and 15 μL of S30 extract. The reaction mixture was maintained at 22 °C for 1−2 h. The reaction was stopped by immersion in an ice bath for 5 min. Ten microliters of this translation mixture was used for luciferase assay and 5-μL aliquots were used for 10% SDS−PAGE analysis after acetone precipitation.

Purification of Luciferase Containing a Hexahistidine Fusion Peptide. The luciferases were expressed in Interchange *E. coli* strains and cell free extracts were prepared as described previously.[13] The extracts were applied to 0.5-mL Ni-NTA (QIAGEN) columns. The columns were washed successively with 5 mL of 100 mM K phosphate buffer, pH 7.8, and then with 5 mL of the same buffer containing 60 mM imidazole. The luciferase fusion proteins were eluted from the column with 4 mL of 100 mM K phosphate buffer, pH 7.8, containing 120 mM imidazole; 1-mL fractions were collected. The fractions containing luciferase were determined using an assay for light production. The fractions containing highest luciferase activity (usually 2 and 3) were pooled and dialyzed against 100 mM K phosphate, pH 7.8, containing 1 mM $MgSO_4$, 1 mM EDTA, and 15% glycerol v/v. The concentrations of the purified luciferases were estimated using SDS−PAGE followed by Coomassie Brillant Blue staining.

Luciferase Assay. Luciferase assays were typically carried out using a commercial luciferase assay system (Promega). After translation, 10 μL of the translation reaction mixture was added to 100 μL of the luciferase assay reagent and light emission was measured immediately using an Hitachi F2000 fluorescence spetrophotometer. Experiments monitoring pH dependence of the spectra of light emitted were carried out using 0.1 M MOPS−MES buffer, 5 mM $MgCl_2$, 0.5 mM luciferin, 1.5 mM ATP, and 1 mM EDTA.

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Supporting Information Available: A table outlining the thermostability of the luciferase analogues and PAGE, UV, and HPLC analysis of some analogues (11 pages). See any current masthead page for ordering and Internet access instructions.

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(34) Lesley, S. A.; Brow, M. A. D.; Burgess, R. R. *J. Biol. Chem.* **1991**, *266*, 2632.