DOI: 10.1002/cbic.201000436

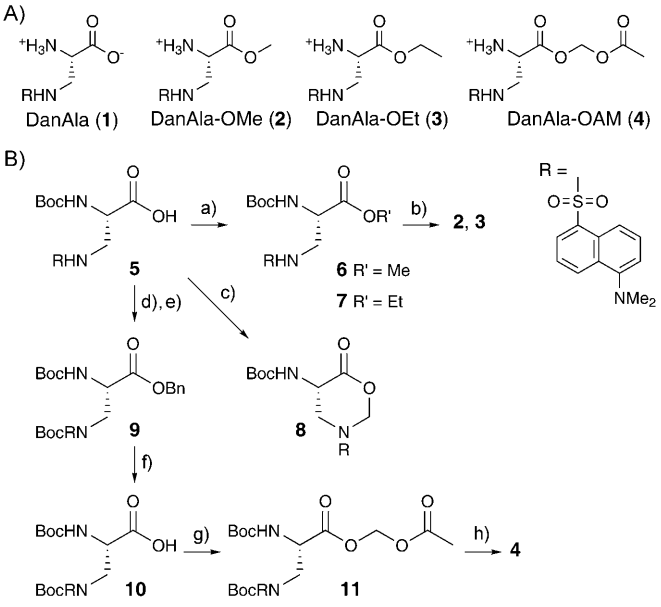
Esterification of an Unnatural Amino Acid Structurally Deviating from Canonical Amino Acids Promotes Its Uptake and Incorporation into Proteins in Mammalian Cells

Jeffrey K. Takimoto, Zheng Xiang, Ji-Yong Kang, and Lei Wang*[a]

Genetically encoded unnatural amino acids (UAAs) enable novel chemical and physical properties to be selectively introduced into proteins directly in live cells; this provides great potential for addressing biological questions at the molecular and cellular level in native settings.[1–3] UAAs have been genetically incorporated into proteins in mammalian cells by using orthogonal tRNA-codon–synthetase sets,[4–6] yet the current low incorporation efficiency hinders their effective application. Efforts to improve efficiency have focused on optimizing the expression and activity of the orthogonal tRNA and synthetase,[5–7] whereas the bioavailability of the UAA inside mammalian cells, a prerequisite for incorporation, has not been addressed. In addition, there are many UAAs, such as glycosylated and phosphorylated amino acids, that have not been genetically incorporated into proteins successfully. These amino acids can be invaluable for studying the contribution of post-translational modifications of protein function and the role of a target protein in cellular signal transduction. Among many reasons, the inability of the UAA to enter cells prevents the evolution of a mutant synthetase specific for the UAA by using cell-based selections or screens.[2] Here, we show that an UAA that deviates structurally from the canonical amino acids in side chain could not be efficiently transported into mammalian cells, but that masking the carboxyl group of the UAA as an ester greatly increased the rate of cellular uptake and intracellular concentration of the UAA. This resulted in a significant increase in the incorporation of this UAA into proteins in mammalian cells. Among three esters tested, acetoxymethyl ester (AME) yielded the highest UAA incorporation efficiency, with a concomitant reduction in the UAA required in the growth medium.

UAAs with side chains similar to canonical amino acids can be transported into cells by endogenous amino acid transporters,[3,8] which are relatively nonspecific for substrates.[9] However, UAAs that deviate significantly from canonical amino acids in side chain structure might not be recognized by these transporters. Cell membranes are more permeable to neutral than charged molecules. As zwitterions, UAAs have only a very small proportion present in the neutral form at physiological pH, thereby making it difficult for them to cross the cell membrane. Esters have been widely used in prodrugs to derivatize carboxyl, hydroxyl, and thio functionalities so as to make the parent drugs more membrane permeable.[10] In particular, Tsien et al. pioneered the use of AME to enhance the cellular uptake of various charged molecules, such as carboxylate-containing Ca^{2+} chelators and phosphate-containing second messengers.[11–12] We reasoned that masking the carboxyl group of the UAA with an ester would convert the UAA into a protonated weak base, which has a higher percentage of neutral form and increased lipophilicity for crossing the membrane. Once inside the cell, intracellular esterases can cleave the ester to regenerate the original UAA for incorporation.

We demonstrated this strategy with the UAA 2-amino-3-(5-(dimethylamino)naphthalene-1-sulfonamide)propanoic acid (DanAla, **1**), which does not resemble and is bulkier in size than any canonical amino acid. Its fluorescence property also makes it an attractive candidate to develop optical reporters for imaging protein activities in live cells. We synthesized the methyl, ethyl, and acetoxymethyl esters of DanAla by using the methods shown in Scheme 1B. Compound **5** was synthe-



Scheme 1. A) Structures of DanAla and DanAla esters. B) Synthetic approach to DanAla esters. Reagents and conditions: a) MeI, DIPEA, DMF, 12 h, 87% for **6**; EtI, DIPEA, DMF, 12 h, 75% for **7**; b) TFA, CH_2Cl_2, 100%; c) bromomethyl acetate, DIPEA, CH_3CN, 81%; d) BnBr, DIPEA, DMF, 0 °C to RT, 76%; e) Boc_2O, DMAP, Et_3N, CH_2Cl_2, 97%; f) Pd/C, H_2, MeOH; g) bromomethyl acetate, DIPEA, CH_3CN, 84% over two steps; h) TFA, CH_2Cl_2, 100%.

[a] J. K. Takimoto,[+] Dr. Z. Xiang,[+] J.-Y. Kang, Prof. L. Wang
The Jack H. Skirball Center for Chemical Biology and Proteomics
The Salk Institute for Biological Studies
10010 N. Torrey Pines Road, La Jolla, CA 92037 (USA)
Fax: (+1) 858-597-0855
E-mail: lwang@salk.edu

[+] These authors contributed equally to this work.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201000436.

sized from Boc-Dap-OH and dansyl chloride according to the known procedure.[13] Alkylation of **5** with iodomethane and iodoethane gave intermediates **6** and **7**, which afforded DanAla-OMe (**2**) and DanAla-OEt (**3**), respectively, after deprotection. However, in the presence of bromomethyl acetate and *N*,*N*-diisopropylethylamine (DIPEA), compound **5** was transformed to the undesired cyclization product **8**, instead of Boc-DanAla-OAM. Therefore, the carboxyl group and the sulfonamide group were protected with the benzyl group and the Boc group,[14] respectively. After debenzylation, carboxylic acid **10** was transformed into acetoxymethyl ester **11**,[15] which furnished DanAla-OAM (**4**) after removal of the Boc groups.

To determine if the DanAla esters could enhance the incorporation of DanAla into proteins in mammalian cells, we used an in cellulo fluorescence assay to evaluate the incorporation efficiency of DanAla.[6–7] The incorporation of DanAla into green fluorescent protein (GFP) was measured by using a stable clonal HeLa cell line, into which a GFP gene containing a premature UAG stop codon at a permissive site (Tyr182) was integrated. The cells were transfected with the orthogonal tRNA–synthetase pair specific for DanAla,[13] and the DanAla or DanAla ester was added to the growth medium. The incorporation of DanAla at the UAG182 position results in full-length, fluorescent GFP, whereas no incorporation results in a truncated, nonfluorescent protein. The total fluorescence intensity of cells was measured by flow cytometry analysis. As shown in Figure 1 A, the cell fluorescence intensity increased when more DanAla was added to the growth medium, but reached a plateau from 0.25 to 1.00 mM. The plateau suggests that either the concentration of DanAla inside the cells has saturated the synthetase or that the cellular availability of DanAla is limiting. When DanAla-OMe was used, the cell fluorescence intensity doubled in comparison to that of cells incubated with the same concentrations of DanAla; this indicates that the cellular availability of DanAla was the limiting factor. Similar results were also obtained for DanAla-OEt. There was no significant increase in cell fluorescence intensity when the concentration of DanAla-OMe or DanAla-OEt was increased from 0.10 to 0.25 mM. In contrast, DanAla-OAM doubled the fluorescence intensity at 0.10 mM and quadrupled it at 0.25 mM. In comparison to 1.00 mM of DanAla, the amount often used in UAA incorporation, DanAla-OAM increased the cell fluorescence intensity fourfold in addition to requiring 75% less compound in the growth medium (0.25 mM). Western blot analysis of the cell lysates confirmed the increase in the amount of GFP produced by the AME modification (Figure 1 B). These results suggest that all three ester modifications were able to increase DanAla incorporation into proteins with a concomitant reduction in the extracellular supply of UAA. Furthermore, we identified DanAla-OAM as the most effective out of the three esters tested.

To understand how DanAla-OAM increases DanAla incorporation, we first monitored the uptake of DanAla and DanAla-OAM into cells by using fluorescence microscopy. We added 0.10 mM of DanAla or DanAla-OAM to the media of HEK293T cells, and measured the cytosolic fluorescence intensity of cells at sequential time points by using confocal microscopy (Figure 2 A). For cells incubated with DanAla, the cytosolic fluorescence intensity was almost flat in the range of 620–750 AU. A striking difference was observed in the DanAla-OAM incubated sample. Within 5 min, the intracellular fluorescence intensity rapidly rose to 1100 AU, a 48% increase from that of the DanAla sample. The intensity increased for 3 h peaking at 1613 AU, which was twice the DanAla peak intensity. The intensity of DanAla-OAM treated cells then gradually dropped, possibly due to the depletion of the extracellular DanAla-OAM in combination with the equilibration of converted DanAla in and outside of cells. The AME modification thus significantly accelerates the cellular uptake rate of the compound.

Next, we quantified the intracellular concentrations of DanAla and DanAla-OAM by using HPLC. HEK293T cells were incubated with 0.10 mM of the compounds for 1 h, and cell contents were extracted. Small molecules in the cell extracts were separated by HPLC, and peaks corresponding to DanAla

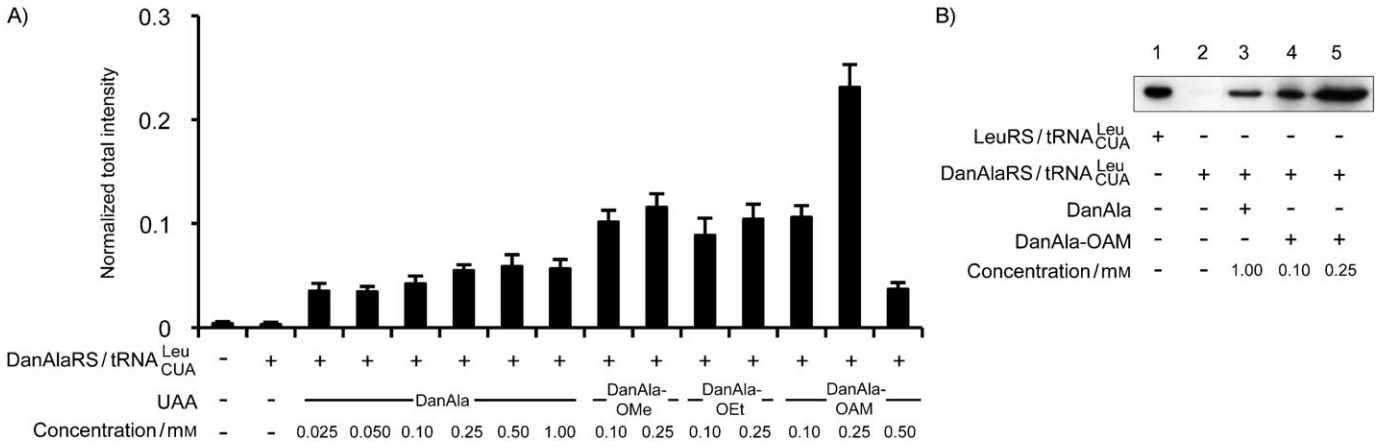


Figure 1. A) Flow cytometric analysis of the incorporation efficiency of DanAla into GFP with different compounds added in the growth medium. Reporter cells transfected with the orthogonal suppressor tRNA and the wild-type LeuRS were used as the positive control to normalize the total fluorescence intensity. Error bars represent standard error of mean (SEM, $n = 3$). B) Western blot analysis of the GFP protein with a GFP-specific antibody. The same number of cells were used in lanes 2–5.

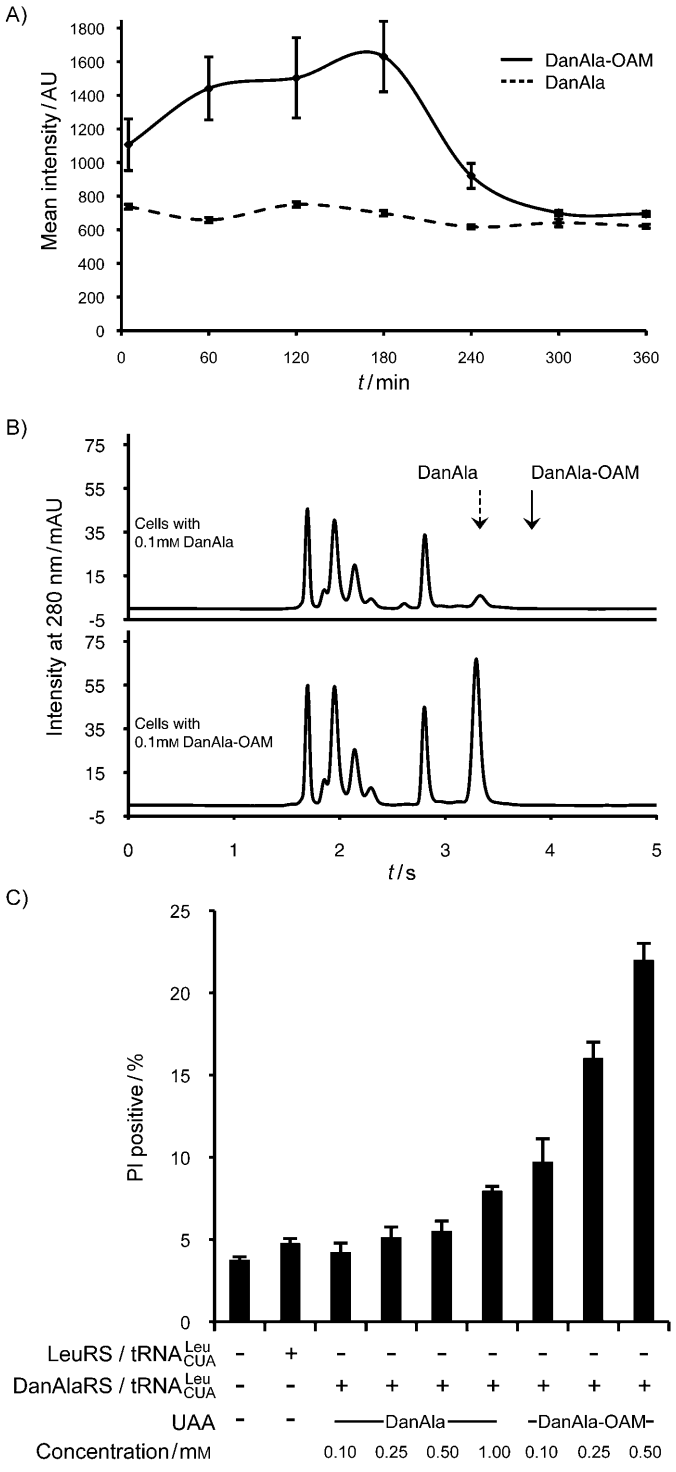


Figure 2. A) Cytosolic fluorescence intensity of cells incubated with 0.10 mM DanAla or DanAla-OAM. Error bars represent standard deviation (SD). B) HPLC analysis of cell extracts from cells incubated with 0.10 mM DanAla or DanAla-OAM. Arrows indicate the peak positions for DanAla and DanAla-OAM, as determined by using pure compounds. C) PI staining analysis of cell health at indicated conditions. Error bars represent SEM, $n = 3$.

and DanAla-OAM were verified by using pure compounds and mass spectrometry. Surprisingly, after only 1 h of incubation, cells incubated with DanAla-OAM showed no peak for DanAla-OAM but a large peak for DanAla (Figure 2B); this indicates that all intracellular DanAla-OAM had been hydrolyzed into DanAla. The peak area for DanAla was used to determine its intracellular concentration. Cells incubated with DanAla and DanAla-OAM had 0.28 and 8.9 mM intracellular concentration of DanAla, respectively. The AME modification dramatically increased the intracellular concentration of DanAla by 31-fold.

When the concentration of DanAla-OAM was further increased to 0.50 mM, the cell fluorescence intensity decreased unexpectedly (Figure 1A). We used propidium iodide (PI) staining to assess the health of cells incubated with DanAla or DanAla-OAM (Figure 2C). For cells treated with 0.10 to 0.50 mM DanAla, the percentage of PI-positive cells was similar to that of cells without UAA. A slight increase of PI-positive cells was observed at 1.00 mM of DanAla. In contrast, cells treated with DanAla-OAM showed a significantly higher percentage of PI-positive cells, especially at the concentration of 0.50 mM. DanAla-OAM hydrolysis releases formaldehyde, which will negatively affect cell health at high concentration.[11] High intracellular concentration of DanAla could be toxic to cells as well. Therefore, an optimal concentration of the AME ester should be used to achieve highest UAA incorporation efficiency with minimal negative effect to cell health.

In summary, we have demonstrated that masking the carboxyl group of DanAla with AME increased the uptake rate and intracellular concentration of the unnatural amino acid in mammalian cells. This resulted in a higher DanAla incorporation efficiency accompanied by the added benefit of reducing the amount of UAA in the growth medium. Efficient UAA incorporation could prove valuable for the effective application of genetically encoded UAAs to studying various biological processes in mammalian cells. Modification of UAAs with AME could be generally applicable to other UAAs that have trouble entering mammalian cells. To test this hypothesis, more UAAs need to be modified, and their incorporation efficiency determined. However, there is currently no orthogonal tRNA–synthetase available for the incorporation of these candidate UAAs because a synthetase cannot be evolved for an UAA that cannot readily enter the cell. We are applying the esterification strategy to highly polar amino acids, such as glycosylated and phosphorylated amino acids, so as to overcome this problem with the final goal of genetically incorporating these biologically important amino acids into proteins.

Experimental Section

Synthesis of DanAla and DanAla esters: All reactions were carried out under nitrogen with dry solvents in anhydrous conditions, unless otherwise noted. Dry *N*,*N*-dimethylformamide (DMF), methanol, methylene chloride, and acetonitrile were obtained by passing commercially available predried, oxygen-free formulations through activated alumina columns. Yields refer to chromatographically homogeneous materials. Reagents were purchased from Sigma–Aldrich and used without further purification. Reactions were monitored by TLC, and carried out on 0.25 mm Silicycle silica gel plates (60F-254) by using UV light for visualization and an ethanolic solution of phosphomolybdic acid and cerium sulfate for developing under heat. Silicycle silica gel (60, particle size 0.040–0.063 mm) was used for flash column chromatography (FCC). NMR spectra

were recorded on a Varian Oxford AS500 instrument and calibrated by using residual undeuterated solvent or tetramethylsilane as an internal reference. LCMS experiments were performed on an Agilent 1100 Series LC/MSD instrument with a Phenomenex Synergi 4u Fusion-RP 80 A column (150×4.6 mm). DanAla was synthesized as described.[13]

NMR (^{1}H and ^{13}C) and HRMS data for compounds are given in the Supporting Information.

Compound 5: Boc-Dap-OH (2.0 g, 9.8 mmol) was added in one portion to a stirring solution of dansyl chloride (2.4 g, 8.9 mmol) and Et_3N (2.6 mL) in CH_2Cl_2 (50 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature. After being stirred for 12 h, the reaction mixture was concentrated under reduced pressure. The residue was purified by FCC (MeOH/CH_2Cl_2 7:93, *v/v*) to give compound **5** (3.675 g, 94%) as a yellow solid. $R_f = 0.22$ (SiO_2, MeOH/$CHCl_3$ 1:8, *v/v*).

Compound 6: Compound **5** (218.8 mg, 0.50 mmol) was dissolved in DMF (2 mL), and the mixture was cooled to 0 °C. DIPEA (0.096 mL, 0.55 mmol) and MeI (0.062 mL, 1.00 mmol) were added dropwise to the solution. The reaction mixture was allowed to warm to room temperature and stirred for 12 h. Water (20 mL) was added, and the solution was extracted with ethyl acetate (3× 20 mL). The organic phase was washed with brine, dried with sodium sulfate, and concentrated under reduced pressure. The residue was purified by FCC (ethyl acetate/hexanes 3:7, *v/v*) to give compound **6** (197.4 mg, 87%) as a yellow solid.

Compound 7: Compound **5** (218.8 mg, 0.50 mmol) was dissolved in DMF (2 mL), and the mixture was cooled to 0 °C. DIPEA (0.096 mL, 0.55 mmol) and EtI (0.080 mL, 1.00 mmol) were added dropwise to the solution. The reaction mixture was allowed to warm to room temperature and stirred for 12 h. Water (20 mL) was added, and the solution was extracted with ethyl acetate (3× 20 mL). The organic phase was washed with brine, dried with sodium sulfate, and concentrated under reduced pressure. The residue was purified by FCC (ethyl acetate/hexanes 3:7, *v/v*) to give compound **7** (174.1 mg, 75%) as a yellow solid.

Compound 2: Compound **6** (45.2 mg, 0.10 mmol) was dissolved in CH_2Cl_2 (0.9 mL), and the mixture was cooled to 0 °C. Trifluoroacetic acid (TFA; 0.3 mL) was added dropwise to the solution. The reaction mixture was allowed to warm to room temperature and stirred for 4 h, then concentrated under reduced pressure. After being under vacuum for 48 h, the residue was dissolved in dimethyl sulfoxide (DMSO; 1.0 mL) for use.

Compound 3: Compound **7** (46.6 mg, 0.10 mmol) was dissolved in CH_2Cl_2 (0.9 mL), and the mixture was cooled to 0 °C. TFA (0.3 mL) was added dropwise to the solution. The reaction mixture was allowed to warm to room temperature and stirred for 4 h, then concentrated under reduced pressure. After being under vacuum for 48 h, the residue was dissolved in DMSO (1.0 mL) for use.

Compound 9: Compound **5** (569.4 mg, 1.30 mmol) was dissolved in DMF (10 mL), and the mixture was cooled to 0 °C. DIPEA (0.25 mL, 1.43 mmol) and benzyl bromide (0.31 mL, 2.60 mmol) were added dropwise to the solution. The reaction mixture was allowed to warm to room temperature and stirred for 24 h. Water (70 mL) was added and the solution was extracted with ethyl acetate (50 mL). The organic phase was washed with brine, dried with sodium sulfate, and concentrated under reduced pressure. The residue was purified by FCC (ethyl acetate/hexanes 1:3–1:2, *v/v*) to give Boc-DanAla-OBn (522.1 mg, 76%) as a yellow solid. $R_f = 0.25$ (SiO_2, ethyl acetate/hexanes 1:2, *v/v*). Boc-DanAla-OBn (466.7 mg, 0.885 mmol) and 4-dimethylaminopyridine (DMAP; 21.6 mg, 0.177 mmol) were dissolved in CH_2Cl_2 (4 mL), and the mixture was cooled to 0 °C. Et_3N (0.136 mL, 0.974 mmol) and Boc_2O (289.8 mg, 1.328 mmol) in CH_2Cl_2 (2 mL) were added dropwise to this stirring mixture. The reaction mixture was allowed to warm to room temperature and stirred for 4 h. Saturated aqueous NH_4Cl solution was added to the mixture. The aqueous phase was separated and washed with CH_2Cl_2 (3×10 mL). The combined organic phase was washed with brine, dried with sodium sulfate, and concentrated under reduced pressure. The residue was purified by FCC (ethyl acetate/hexanes 1:3, *v/v*) to give compound **9** (538.6 mg, 97%). $R_f = 0.34$ (SiO_2, ethyl acetate/hexanes 1:2, *v/v*).

Compound 10: Pd/C (87.3 mg) was added to a solution of compound **9** (515.0 mg, 0.82 mmol) in methanol. The reaction mixture was stirred under hydrogen for 2 h at room temperature. The reaction mixture was passed through a short plug of celite and eluted with ethyl acetate. The filtrate was concentrated to give compound **10** (467.3 mg) as a yellow-green solid. $R_f = 0.22$ (SiO_2, MeOH/$CHCl_3$ 1:10, *v/v*).

Compound 11: Compound **10** (434.9 mg, 0.809 mmol) was dissolved in CH_3CN, and the mixture was cooled to 0 °C. DIPEA (0.56 mL, 3.236 mmol) and bromomethyl acetate (0.24 mL, 2.427 mmol) were added dropwise to the solution. The reaction mixture was allowed to warm to room temperature and stirred for 12 h, then concentrated under reduced pressure. The residue was purified by FCC (ethyl acetate/hexanes 1:2, *v/v*) to give compound **11** (389.5 mg, 84% over 2 steps) as yellow-green solid. $R_f = 0.31$ (SiO_2, ethyl acetate/hexanes 1:2, *v/v*).

Compound 4: Compound **11** (61.0 mg, 0.10 mmol) was dissolved in CH_2Cl_2 (0.9 mL), and the mixture was cooled to 0 °C. TFA (0.3 mL) was added dropwise to the solution. The reaction mixture was allowed to warm to room temperature and stirred for 6 h, then concentrated under reduced pressure. After being under vacuum for 48 h, the residue was dissolved in DMSO (1.0 mL) for use.

Cell lines and culture conditions: HEK293T cells were used for determining the UAA intracellular concentration and imaging experiments. A stable clonal HeLa cell line containing the GFP gene with Tyr182UAG mutation was previously established in this lab and was used here for assaying UAA incorporation efficiency.[6] HEK293T and HeLa-GFP(Tyr182UAG) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Mediatech) supplemented with 10% fetal bovine serum (FBS).

Fluorescence imaging: HEK293T cells (3×10^5) were seeded into an imaging dish supplemented with DMEM with 10% FBS, but without phenol red. After 18–24 h, DanAla or DanAla-OAM was added to the medium to a final concentration of 0.10 mM. Cells were immediately imaged with an Olympus IX81 spinning disc microscope with a Hamamatsu EM-CCD under the same conditions, $\lambda_{ex} = 360 \pm 30$ nm, $\lambda_{em} = 535 \pm 20$ nm. After each time point, cells were placed back into the incubator. Images were analyzed in Slidebook 4.2 by using the masking tool and average intensity function. Cells not treated with any compound were used as control to determine background fluorescence, which was subtracted from the measured intensities of samples to yield the final net intensities.

HPLC analysis of intracellular concentration: HEK293T cells (8.5×10^6) were seeded into a 10 cm tissue culture dish supplemented with DMEM containing 10% FBS. After 18–24 h, DanAla or DanAla-OAM was added to the medium to a final concentration of 0.1 mM. The compounds were incubated with cells for 1 h. Cells were then washed with phosphate-buffered saline (PBS; 1 mL) several times.

After trypsinization with trypsin (1 mL, 0.05%; Mediatech), cells were centrifuged, and the medium was removed. Cells were washed again with water (1 mL) and centrifuged. The supernatant was removed, and cells were resuspended in water (250 μL). Toluene (50 μL) was added, and the cells were placed in a shaking incubator at 37 °C for 30 min. Cells were centrifuged at 20000 relative centrifugal force (RCF) for 10 min. The aqueous layer was placed into a Microcon Ultracel YM10 column (Millipore) and spun for 30 min at 12000 RCF. The flow-through was analyzed by HPLC-MS (Agilent 1100 Series LC/MSD instrument): and aliquot (5 μL) of the aqueous layer was separated on a Phenomenex Synergi 4u Fusion-RP 80 A column (150×4.6 mm) with a gradient of aqueous acetonitrile/0.1% formic acid (75:25 to 25:75) over 10 min. DanAla and DanAla-OAM were identified by extracting their MW (+1) from the total ion mass spectrum. Pure DanAla and DanAla-OAM were also added into the flow-though prepared from cells not treated with compounds so as to verify the peak positions of DanAla (3.3 min) and DanAla-OAM (3.7 min). Calculation of DanAla concentrations in each sample was based on the extracted ion area of DanAla and a standard curve. The standard curve was made by extracting the area of DanAla in solutions with different concentrations (0.10, 0.20, 0.30, 0.40, 0.50, 0.60, 0.70, 0.80, 0.90, and 1.00 mM). Cells not treated with any compound were used to measure the background area at 3.3 and 3.7 min. The intracellular concentration was given by the total molar amount of DanAla divided by the total volume of cells, by using the volume of a single cell as in ref. [16].

Flow cytometry: Stable HeLa–GFP(Tyr182TAG) clonal cells were seeded in a 3.5 cm culture dish and transfected with pELcua-LeuRS or pELcua-DanAlaRS (5 μg) plasmid DNA by using Lipofectamine 2000.[6] HeLa–GFP(Tyr182TAG) cells that were not transfected with a tRNA–synthetase pair were used as a negative control. DanAla and DanAla-OAM were added 24 h post transfection, and flow cytometry was carried out 24 h after the addition of the compounds. Cells were trypsinized and washed with PBS twice. Samples were centrifuged and resuspended in PBS (1.0 mL) and PI (5 μL). Samples were analyzed with a FACScan (Becton, Dickinson and Company, Franklin Lakes, USA). The excitation wavelength was 488 nm, and the emission filter was 530/30 nm. The excitation light (488 nm) excites GFP but not DanAla. For each sample, the total fluorescence intensity of 30000 cells was recorded, and was normalized to the total fluorescence intensity of cells transfected with pELcua-LeuRS. PI (5 $\mu g\,mL^{-1}$, Sigma–Aldrich) was used to determine cell viability by flow cytometry. Cells were trypsinized and incubated with PI for 15 min at room temperature, and then analyzed by using FACScan (Becton, Dickinson and Company).

Western blot: HeLa–GFP(Tyr182TAG) clonal cells were transfected and incubated with the appropriate DanAla or DanAla ester, as previously described for flow cytometry analysis. Cells were trypsinized and washed with PBS, and cell number was counted by using a hemocytometer. Samples were centrifuged and resuspended with PBS (20 μL) containing ethylendiaminetetraacetate-free protease inhibitor cocktail (Roche) and DNase I (Roche). These samples were lysed by flash freezing in liquid nitrogen, and thawed by sonication. The lysis procedure was repeated three times to ensure complete lysis. Loading buffer was added, and the samples were boiled. Prepared samples were loaded onto a 12% SDS-PAGE gel. For the pELcua-LeuRS sample, 10^4 cells were loaded, while 7.5×10^4 cells were loaded for all other samples. The primary antibody (Anti-GFP Monoclonal Antibody 7G9, BioPioneer, San Diego, USA) and horseradish peroxidase (HRP) conjugated secondary antibody (Goat Anti-Mouse IgG-HRP Conjugate, Santa Cruz Biotechnology) were used to detect GFP proteins.

Acknowledgements

We thank Dr. Michael Burkart for help with the HRMS. This work was supported by the Ray Thomas Edwards Foundation, Beckman Young Investigator Program, March of Dimes Foundation (5-FY08-110), California Institute for Regenerative Medicine (RN1-00577-1, and National Institutes of Health (1DP2OD004744-01).

Keywords: amino acids · cellular uptake · enzymes · fluorescence · genetic code expansion

[1] L. Wang, A. Brock, B. Herberich, P. G. Schultz, *Science* **2001**, *292*, 498–500.
[2] L. Wang, P. G. Schultz, *Angew. Chem.* **2004**, *117*, 34–68; *Angew. Chem. Int. Ed.* **2004**, *44*, 34–66.
[3] Q. Wang, A. R. Parrish, L. Wang, *Chem. Biol.* **2009**, *16*, 323–336.
[4] K. Sakamoto, A. Hayashi, A. Sakamoto, D. Kiga, H. Nakayama, A. Soma, T. Kobayashi, M. Kitabatake, K. Takio, K. Saito, M. Shirouzu, I. Hirao, S. Yokoyama, *Nucleic Acids Res.* **2002**, *30*, 4692–4699.
[5] W. Liu, A. Brock, S. Chen, S. Chen, P. G. Schultz, *Nat. Methods* **2007**, *4*, 239–244.
[6] W. Wang, J. K. Takimoto, G. V. Louie, T. J. Baiga, J. P. Noel, K. F. Lee, P. A. Slesinger, L. Wang, *Nat. Neurosci.* **2007**, *10*, 1063–1072.
[7] J. K. Takimoto, K. L. Adams, Z. Xiang, L. Wang, *Mol. BioSyst.* **2009**, *5*, 931–934.
[8] L. Wang, J. Xie, P. G. Schultz, *Annu. Rev. Biophys. Biomol. Struct.* **2006**, *35*, 225–249.
[9] M. S. Malandro, M. S. Kilberg, *Annu. Rev. Biochem.* **1996**, *65*, 305–336.
[10] J. Rautio, H. Kumpulainen, T. Heimbach, R. Oliyai, D. Oh, T. Jarvinen, J. Savolainen, *Nat. Rev. Drug Discovery* **2008**, *7*, 255–270.
[11] R. Y. Tsien, *Nature* **1981**, *290*, 527–528.
[12] C. Schultz, M. Vajanaphanich, A. T. Harootunian, P. J. Sammak, K. E. Barrett, R. Y. Tsien, *J. Biol. Chem.* **1993**, *268*, 6316–6322.
[13] D. Summerer, S. Chen, N. Wu, A. Deiters, J. W. Chin, P. G. Schultz, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 9785–9789.
[14] B. R. Neustadt, *Tetrahedron Lett.* **1994**, *35*, 379–380.
[15] M. M. Meijler, R. Arad-Yellin, Z. I. Cabantchik, A. Shanzer, *J. Am. Chem. Soc.* **2002**, *124*, 12666–12667.
[16] P. Thomas, T. G. Smart, *J. Pharmacol. Toxicol. Methods* **2005**, *51*, 187–200.

Received: July 30, 2010
Published online on September 24, 2010

