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Cellular routines in the synthesis of cyclic peptide probes

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Abstract—For even the most primitive microbes, the activation and regulation of biosynthesis is guided by material transport about the cell. It is within these transactions that secondary metabolite biosynthesis orchestrates a key set of chemical transformations. Cellular factors are often as important to the regulation of biosynthesis as the structures of their metabolites and the mechanism of their biosynthesis. In accord with this issue on biologically-inspired synthesis, this manuscript evaluates the adaptation of cells as tools to direct the synthesis of fluorescent nonribosomal peptide-based probes.

1. Introduction

One of the principle differences between biosynthesis and chemical synthesis can be attributed to the reaction vessel.[1] In the lab, synthetic operations are conducted in a flask,[2] on a resin,[3] or on a surface[4]. Once operations are completed, the products of these reactions are extracted from their vessel, purified, and screened against a battery of molecular, cellular, and physiological assays.[5] Data from these assays are then pooled and processed through chemoinformatic systems in order to develop a bioactivity profile.[6] While this method operates at the center of drug discovery, its process is radically different from that used in nature.

In nature, secondary metabolism is translated through a complex network of interplay between synthetic operations and biochemical activity. For the producer, these events must be simultaneously optimized for performance at the level of molecule, cell, organism, and ecosystem.[7] Entry into biosynthesis is carefully timed to provide an optimal relay between synthesis and transmission of bioactivity. The logic contained within these regulatory events has yet to be adapted to laboratory synthesis.

2. Results and discussion

2.1. Model system

Conventionally, cells are used as an endpoint to screen the optimal molecular probe. The development of these probes is often accelerated by targeting a molecule or class of molecules, such as natural products. For this study, peptides and their cyclic variants were chosen as targets, as: (1) their synthesis can be addressed in a modular fashion;[8] (2) they are readily displayed in a combinatorial manner;[9] (3) they can be synthesized by variety of methods;[4] (4) their biosynthesis is understood;[10] and (5) they are metabolized in both eukaryotic and prokaryotic cells.[11]

For an organism, events such as subcellular trafficking and metabolism are scored according to the structural features of individual components within a given library of compounds. Metabolism often participates in activating the signals transposed by these materials as illustrated by the oxidative cascades on actinomycin[12] or the appendage of glycosides such as in vancomycin.[13] The mimicry of metabolic processes has been extensively reviewed with regards to the development of prodrugs[14] and metabolic engineering.[15] While peptide and cyclic peptide prodrugs have been shown to increase the activity and facilitate delivery,[16] cells still appear as an endpoint within these investigations.

By moving the position of the cell in a synthetic scheme one can advance the biological-sophistication of a molecular probe. Through these new positions, metabolic processes are effectively encoded within a probe through a few chemical transitions. This manuscript illustrates how probe development and metabolism can be simultaneously engineered into peptide-based probes to prepare materials whose intracellular localization can be trafficked between the endoplasmic reticulum (ER) and nucleus (N) without an extracellular stimulus. To model such regulation, our studies focused on examining the macrocyclization of peptides, the macrolactonization of depsipeptides, and their respective hydrolytic reversion.

2.2. Synthesis of a linear peptide pool

A pool of linear pentapeptides **5** was prepared on Wang resin using a protocol comparable to that described by Ellman[17]

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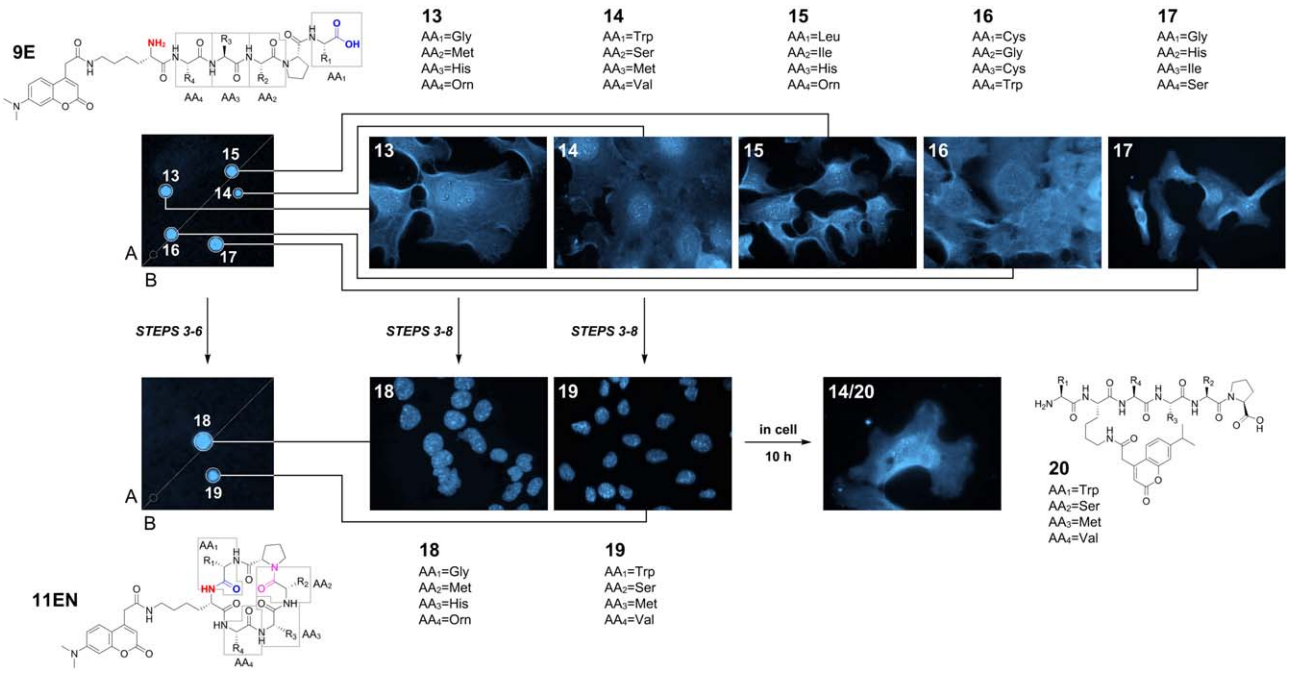


Figure 1. Cell-directed synthesis of cyclic peptide probes. Images depicting the cellular localization of five linear peptides **13**–**17** from sets **9E** denotes the affinity to the ER. Each of these probes was purified by pTLC purification and the images shown were developed from individual TLC spots containing single materials (left). Peptides **13**–**17** were cyclized according to steps 3–6 in Scheme 2 to provide two new cyclic peptides **18**–**19**. The uptake and localization of these peptides was developed using the methods in steps 7–8 in Scheme 2. After incubation at 37 °C for 10 h, cyclic peptide **19** is metabolized in HeLa cells to return peptide **14** and deliver a new linear peptide **20**. For the pTLC analysis (A) denotes acid elution and (B) basic elution.

(Fig. 1). The synthesis was conducted in a parallel-fashion by targeting two pools of peptides, one with a hydroxyl terminus **8** and another with an amine terminus **9**. A pool was developed with 20 sets of peptides from resins **3** containing 0.4–0.6 mmol/g of single amino acid (see Section 4.2). In turn, resins **3** were coupled to Fmoc-proline in a parallel-manner to provide **4**. Resin **5** was prepared from 20 individual dipeptide resins **4** by three sequential couplings with an isokinetic mixture of 20 Fmoc-protected amino acids (see Section 4.2). Resins **5** represented a single set, theoretically containing 8000 peptides. In a parallel-fashion, peptide sets **5** were terminated by capping either with fluorescent-*O*-Fmoc-α-hydroxyacid **1** or fluorescent-Fmoc-lysine **2** providing the respective resins **6** and **7**. Cleavage and deprotection were accomplished by treatment with 20% piperidine in DMF followed by 95% TFA, 2.5% triisopropylsilane, and 2.5% water to afford the corresponding linear peptides **8** and **9**.

2.3. Macrocyclization

Cyclization conditions were identified by screening a series of peptides **8** (AA_1=Ile, AA_2=Ala, AA_3=Ala, AA_4=Ala), **8** (AA_1=Ala, AA_2=Ile, AA_3=Ala, AA_4=Ala), **8** (AA_1=Ala, AA_2=Ala, AA_3=Ile, AA_4=Ala), and **8** (AA_1=Ala, AA_2=Ala, AA_3=Ala, AA_4=Ile). Each peptide was displayed on 256 welled PTFE Teflon plate and screened for macrocyclization with reaction conditions containing combinations of the following reagents: *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC), (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBt), 1-hydroxy-7-azabenzotriazole (HOAt), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)uronium tetrafluoroborate (TDBTU), *N*,*N*,*N*′,*N*″-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU), EtN^iPr_2, *sym*-collidine, dimethylaminopyridine (DMAP), and DL-dithiothreitol (DTT). After extensive analysis, a mixture of 5 equiv of HATU with 0.1 equiv of DTT and 10 equiv of *sym*-collidine in DMF routinely delivered the desired depsipeptide in 40–68% yield. The same conditions also provided an effective conversion of the corresponding series of isoleucine and alanine containing peptides **9** (AA_1=Ile, AA_2=Ala, AA_3=Ala, AA_4=Ala), **9** (AA_1=Ala, AA_2=Ile, AA_3=Ala, AA_4=Ala), **9** (AA_1=Ala, AA_2=Ala, AA_3=Ile, AA_4=Ala), and **9** (AA_1=Ala, AA_2=Ala, AA_3=Ala, AA_4=Ile) providing the desired cyclic peptides in 35–78% yield after purification.

The interference from side chain residues was examined by completing an aspartic acid and lysine scan, using the respective sets of peptides; *set A* [**8** (AA_1=Asp, AA_2=Gly, AA_3=Gly, AA_4=Gly), **8** (AA_1=Gly, AA_2=Asp, AA_3=Gly, AA_4=Gly), **8** (AA_1=Gly, AA_2=Gly, AA_3=Asp, AA_4=Gly), and **8** (AA_1=Gly, AA_2=Gly, AA_3=Gly, AA_4=Asp)], *set B* [**8** (AA_1=Asp-(OAllyl), AA_2=Gly, AA_3=Gly, AA_4=Gly), **8** (AA_1=Gly, AA_2=Asp-OAllyl, AA_3=Gly, AA_4=Gly), **8** (AA_1=Gly, AA_2=Gly, AA_3=Asp-OAllyl, AA_4=Gly), and **8** (AA_1=Gly, AA_2=Gly, AA_3=Gly, AA_4=Asp-OAllyl)], *set C* [**8** (AA_1=Lys, AA_2=Gly, AA_3=Gly, AA_4=Gly), **8** (AA_1=Gly, AA_2=Lys, AA_3=Gly, AA_4=Gly), **8** (AA_1=Gly, AA_2=Gly, AA_3=Lys, AA_4=Gly), and **8** (AA_1=Gly, AA_2=Gly, AA_3=Gly, AA_4=Lys)], and *set D* [**8** (AA_1=Lys-N-Alloc, AA_2=Gly, AA_3=Gly, AA_4=Gly), **8** (AA_1=Gly, AA_2=Lys-N-Alloc, AA_3=Gly, AA_4=Gly), **8**

(AA_1=Gly, AA_2=Gly, AA_3=Lys-N-Alloc, AA_4=Gly), and **8** (AA_1=Gly, AA_2=Gly, AA_3=Gly, AA_4=Lys-N-Alloc)]. Even under optimal macrocyclization conditions, HPLC analysis indicated that without protection the aspartic acid residue in *set A* and the lysine residue in *set C* participated in amide bond formation, thereby reducing the yield of the desired cyclic peptide from 50 to 70% in *sets B/D* from 0.1to 8.5% in *sets A/C*. Comparable complications are also expected to interfere with the cyclization of Arg and Gly peptides, while His, Tyr, and Phe containing peptides may exhibit reactivity within their side chains to a lesser extent. Instead of altering our synthetic scheme, we included these materials in the peptide pools.

2.4. Probe processing

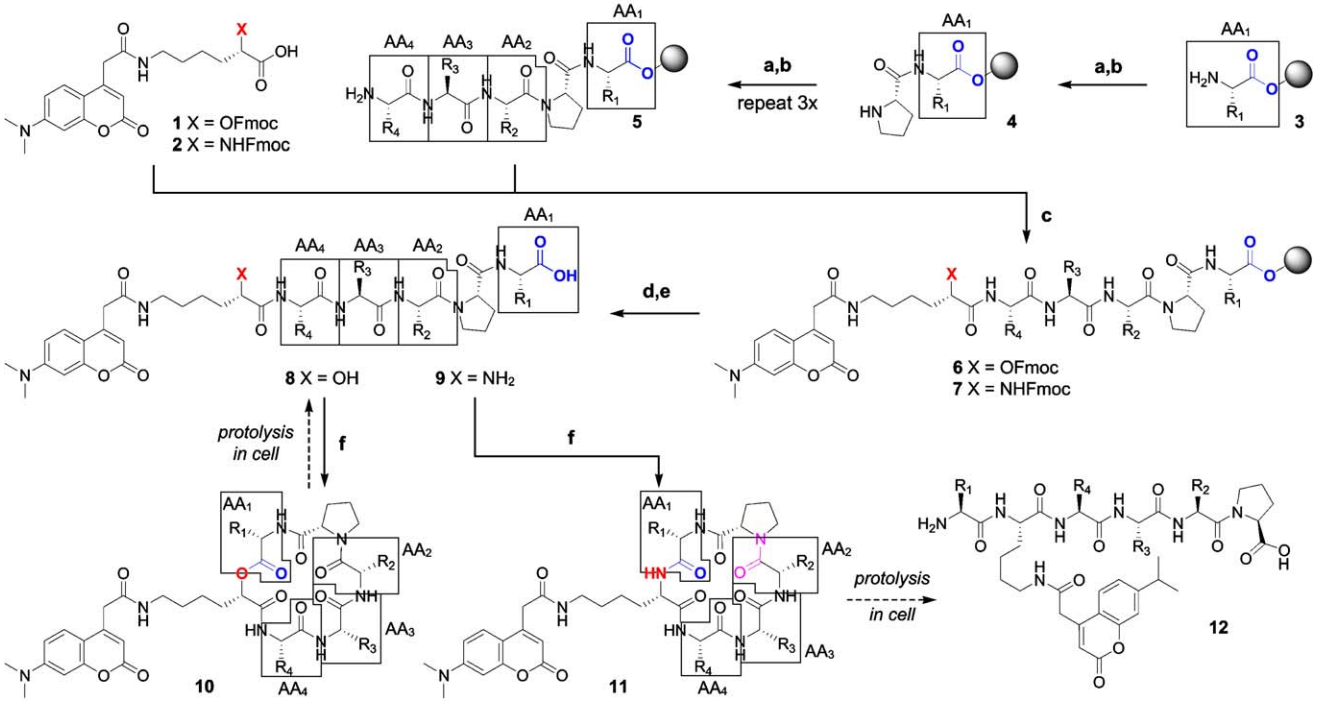
In the cell, depsipeptides **10** and cyclic peptides **11** are subject to proteolysis.[20] For depsipeptides, cleavage arises rapidly at the lactone bond resulting in reversion of **10** to **8**. Cyclic peptides[11] on the other hand can undergo proteolysis at any of the six amide bonds providing reversion to **9** or the formation of a new linear peptide as illustrated by **12** (Scheme 1). The question remained as to the function that can be encoded by the conversion between linear peptides **8**, **9** and their cyclic variants **10**, **11**, respectively. For this study, we were interested in identifying analogs of **10** and **11** that could be trafficked between the ER and nucleus. The procedure outlined in Scheme 2 was selected after examining several approaches.

The process began by evaluating two peptide pools **8** and **9** where **8** was designed to screen depsipeptides **10**, and **9** evaluated the corresponding cyclic peptides **11**. Pools were constructed with 20 sets of peptides as described in Scheme 1. Samples of the sets of peptide **8** or **9** in DMSO were presented to HeLa cells and their fluorescent uptake was imaged (step 1).

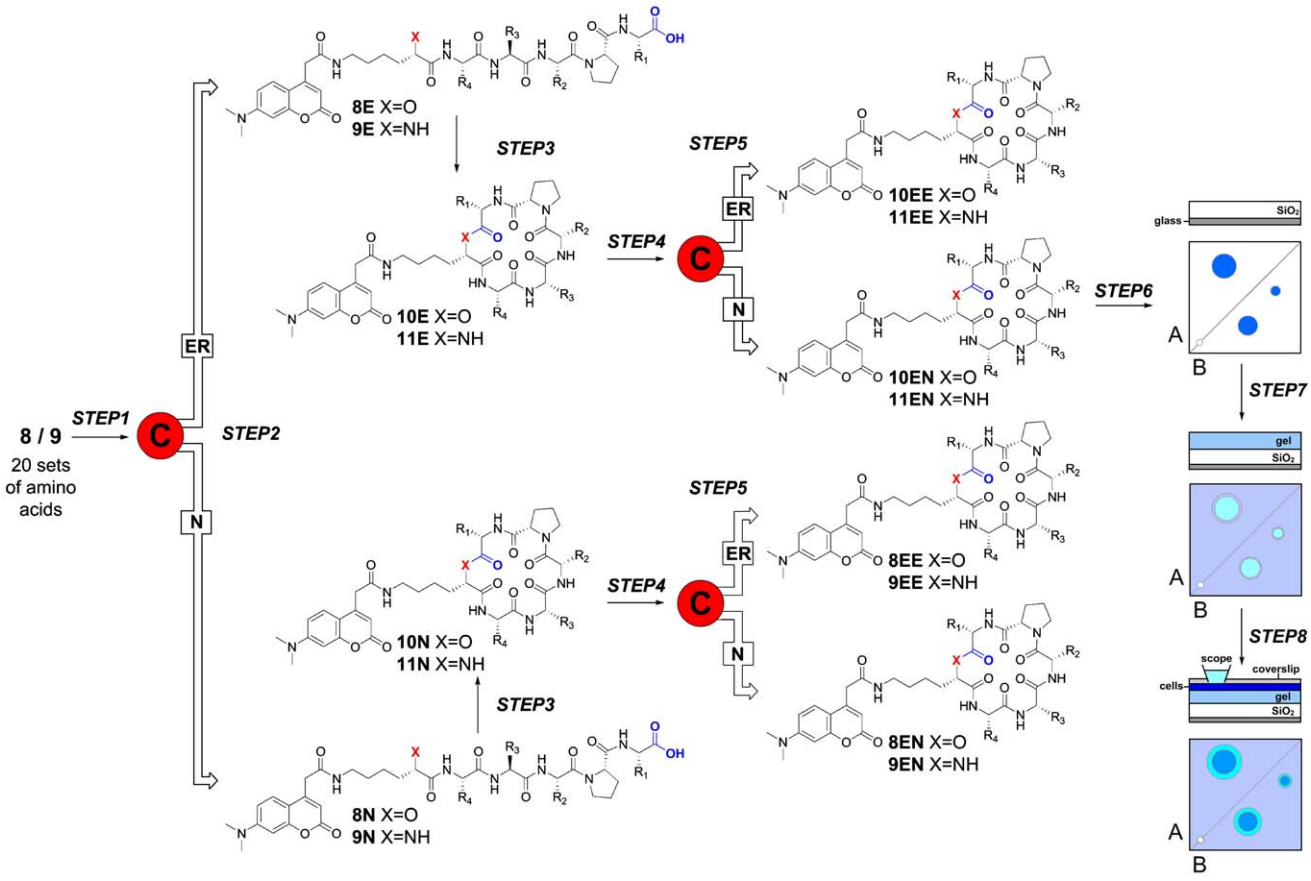
Cells depicting fluorescence in the ER or nucleus were then fractionated into nuclear and endoplasmic reticulum components (step 2). The fluorescent peptides were then extracted from these components to provide the corresponding sets of nuclear-localizing linear peptides **8N** and **9N** and ER-localizing peptides **8E** and **9E**. Samples from each reaction mixture were purified by pTLC K18-reverse phase silica gel or C(18)-reverse phase HPLC to provide pure peptides. The uptake and localization of each purified peptide was determined microscopically.

At this point, the crude sets of peptides were cyclized (step 3). Sets **8E**, **8N**, **9E**, and **9N** at 250±10 μM/peptide (0.05 μmol) in DMF were treated with HATU (0.95 mg, 2.5 μmol), DTT (3.8 μg, 0.025 μmol), and *sym*-collidine (0.2 μL, 2.5 μmol) in DMF. The reaction mixture was stored at rt under argon in the absence of light for 24 h, and the resulting material was filtered through a plug of K18-reverse phase silica gel (300 mg).

Samples of the cyclized reaction mixtures **10E**, **10N**, **11E**, and **11N** in DMSO at 10.0±0.5 μM were presented to HeLa cells and imaged (step 4). Cells were again fractionated and the peptides were extracted from the corresponding cell lysates (step 5). The conditions used for this process were identical to that used to screen for linear peptides in step 2. Samples of the resulting products were purified by 2D-pTLC on Partisil KC18 plates to provide peptide sets **10EE** and **10EN** from **10E** and **11EE** and **11EN** from **11E** (step 6). The corresponding analysis could also be conducted on **10N** and **11N**.



Scheme 1. Reagents and conditions: (a) 20% piperidine, DMF; (b) Fmoc-AA-OH, HATU, *sym*-collidine, DMF; (c) HATU, *sym*-collidine, DMF; (d) 20% piperidine, DMF; (e) 95% TFA, 2.5% triisopropylsilane (TIS), 2.5% water; and (f) HATU, *sym*-collidine, DTT, DMF.



Scheme 2. Cell-directed synthesis. An eight-step process is used. (step 1) Cell uptake and localization; (step 2) nuclear and ER subcellular fractionation; (step 3) macrocyclization as given by reagents (f) in Scheme 1; (step 4) cell uptake and localization; (step 5) nuclear and ER subcellular fractionation; (step 6) 2D-pTLC isolation; (step 7) transfer fluorescent peptides from TLC plate to HeLa cells is conducted through an agarose film by incubation at 37 °C; (step 8) the localization of the cyclic peptides was imaged on an LED-fluorescence microscope. For the pTLC analysis (A) denotes acid elution and (B) basic elution.

The uptake of the probes was imaged directly using a TLC plate. The silica gel was scraped from the plate in regions lacking blue fluorescence. The plate was then coated with a 0.10±0.02 mm layer of low-melt agarose and covered with cover slip containing live HeLa cells (steps 7 and 8). Cells were positioned such that at least ~10^4 cells were placed facing down over a single fluorescent band on the TLC plate. The agarose film served to transfer the peptides from silica gel on plate to the upper layer of the cells. Alternately, the peptide components could be extracted from the pTLC plate and added in a DMSO solution and transferred into the HeLa cell cultures. The uptake and localization of the peptides obtained at this stage were imaged on an LED microscope (step 8). The structures of the peptides identified in sets **10EN** and **11EN** were determined by a combination of TLC–MS[18] and FABMS peptide-sequencing.[19] These assignments were confirmed by synthesizing the peptides individually.

2.5. Discussion

The aim of this study was to develop peptide materials that could be trafficked between the ER and the nucleus.

As indicated in Scheme 2, this transition is depicted by the preparation of probes in sets **10EN** and **11EN**. To simplify matters for this study, sets **10EE**, **10NE**, and **10NN** as well as **11EE**, **11NE**, and **11NN** were collected but not analyzed. The analysis of sets **11EN** and **10EN** is provided in Figures 1 and 2, respectively.

When examined over the entire pool, **9E** contained a total of 121 linear peptides. Images depicting five of these materials **13**–**17** are presented in Figure 1. Upon macrocyclization, only cyclic peptides **18** and **19** localized in the nucleus as given by the requirements of set **11EN**. The remaining 119 peptides either failed to cyclize, as illustrated by **15** or **16**, or failed to provide a cyclic peptide that localized in the nucleus, such as **17**. Interestingly, the nuclear localization of cyclic peptide **19** degraded with time resulting in an indistinguishable signal after 10 h in HeLa cells. A new linear peptide **20** was isolated and its structure was determined by MS peptide-sequencing.

A comparable set of depsipeptides **10EN** was also obtained (Fig. 2). A total of 93 peptides in pool **8** localized in the ER, designated as set **8E**. Only three materials were identified as displaying the conversion of **8E** to **10EN** (Scheme 1). Upon cyclization, peptides **21**–**23**, which were observed in the ER were converted to nuclear-localizing depsipeptides **24**–**26**, respectively. This localization was reverted after 2–8 h in HeLa cells. Cyclic peptide **26** was the fastest, retreating with the ER in 2 h witnessed the formation of **23** by TLC analysis of the corresponding cell lysates. The other two cyclic peptides **24** and **25** were more stable as given by the loss

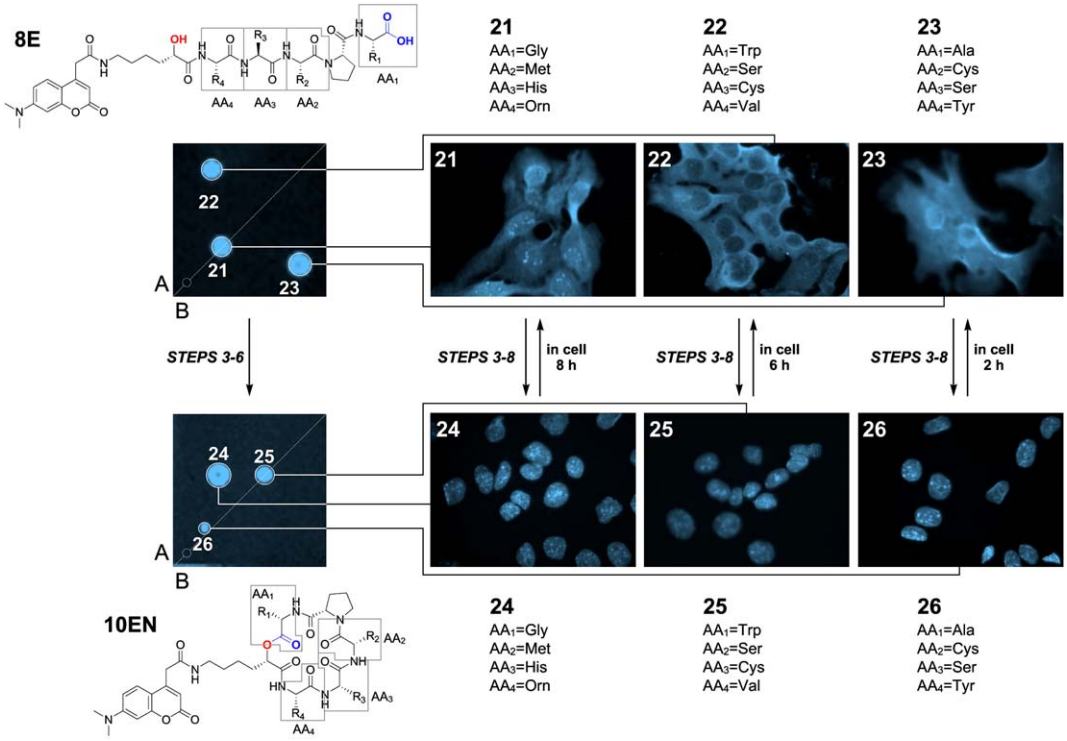


Figure 2. Cell-directed synthesis of depsipeptide probes. Images depicting the cellular localization of three linear peptides **21**–**23** from sets **9E** denotes the uptake in the ER. Each of these probes was purified by pTLC purification (left) and the images shown were developed from individual TLC spots containing single materials. These peptides were cyclized according to steps 3–6 in Scheme 2 to provide three new cyclic peptides **24**–**26**. The uptake and localization of these peptides was developed using the methods in steps 7–8 in Scheme 2. After incubation at 37 °C, depsipeptides **24**–**26** were hydrolyzed to **21**–**23**, respectively, in HeLa cells to afford **14** and **20**. Note that the AA sequence of **21/24** are identical to **13/18** and **22/25** differ from **14/19** by methyl group. For the pTLC analysis (A) denotes acid elution and (B) basic elution.

of nuclear localization after 6 h. Interestingly, the interplay between **21** and **24**, **22** and **25**, and **23** and **26** now provides a set of probes whose localization can be modified over a given time interval.

3. Conclusion

A method was developed that employed cells as a means of selection through the combination of subcellular fractionation and extraction. Through this routine, cyclic peptides **18**, **19** and depsipeptides **24**, **26** were synthesized to provide an effective correlation between nuclear and ER localization.

Advantageously, the proteolysis of probes **18**, **19**, and **24**–**26** provided a signal whose trafficking within the cell could be addressed in a logical fashion. It is within this conduit that methods such as that described herein can be used to expedite the design and synthesis of probes whose activity within a cell is not only regulated in thermodynamic terms but also can act as multidimensional kinetically-regulated signal.

The intracellular trafficking of fluorescent peptide probes was engineered so that the subcellular localization can be guided by in vivo proteolysis. This protocol offers a favorable means to prepare probes whose intermediates and metabolic products can be engineered to target specific organelles. While the molecular target of the peptides described herein was not examined, this manuscript develops syntheses whose function mimics biological selection at the cellular level. It is within this mimetic that new avenues in the development of biologically-inspired synthesis are unveiled.

4. Experimental

4.1. General methods

7-Dimethylaminocoumarin-4-acetic acid was obtained from GFS Chemicals Inc. Fluorescent lysine analogs **1** and **2** were prepared from 7-dimethylaminocoumarin-4-acetic acid using established procedures.[21] Wang resin was obtained from Calbiochem Inc. Fmoc-amino acids were prepared in-house and coupled with Wang resin to provide **3** with a substitution level of 0.40–0.55 mmol/g. Resins **3** could also be purchased from Anaspec Inc. or Polymer Labs Inc. The amine substitution level of each resin was determined by a spectrophotometric Fmoc-quantization.[22] Anhydrous solvents, *N*,*N*-dimethylformamide (DMF), tetrahydrofuran (THF), and methanol (MeOH), from EMD Chemicals were used. EtN^iPr_2, *sym*-collidine, 1-acetylimidazole, dimethylaminopyridine (DMAP), triisopropylsilane (TIS), and DL-Dithiothreitol (DTT) were obtained from TCI Inc. HATU was obtained from Anaspec Inc. Analytical samples of the reagents used to screen the macrocyclization process were obtained from number of laboratories including generous donations from Profs M. M. Joullie and M. Goodman.

HeLa cells (ATCC CCL-2) were cultured in phenol red-free Dulbecco's modification of Eagle's medium (DMEM) with 4.5 g/L glucose, 4.5 g/L L-glutamine, and 5% heat

inactivated fetal calf serum (FCS). Cells were cultured in 250 mL or 1 L culture flasks and as needed were grown on 24×60 mm glass Superslips (VWR Scientific).

4.2. Peptide synthesis

The reaction conditions were corrected for the level of substitution.[22] Fmoc-Ala-resin **3** (1 g, 0.45 mmol, 0.45 mmol/g of resin) was added to a reaction vessel of a self-built peptide synthesizer and solvated with CH_2Cl_2 (10 mL) for 10 min. The resin was filtered and 20% piperidine in DMF (10 mL) was added. After agitation for 30 min, the resin was filtered, and washed three times with DMF (10 mL). The resin was treated twice for 20 h with a solution of Fmoc-proline (379.6 mg, 1.125 mmol), HATU (427.7 mg, 1.125 mmol), and *sym*-collidine (364 μL, 4.5 mmol) in DMF (5 mL). After the second treatment, the resin was washed three times with DMF (5 mL) and capped by treatment with 1-acetylimidazole (743.2 mg, 6.75 mmol) and DMAP (27.5 mg, 0.225) in DMF (5 mL). After 20 h, the resin was washed three times with DMF (5 mL), three times with MeOH (5 mL), and three times with THF (5 mL). The amine substitution level of the resulting resin **4** within three repetitions was determined to be 0.41±0.02 mmol/g by Fmoc-quantization.[22]

An aliquot of resin **4** AA_1=Ala (200 mg, 0.08 mmol, 0.40 mmol/g of resin) was loaded in a self-built peptide synthesizer and solvated with CH_2Cl_2 (3 mL). The resin was filtered and treated with 20% piperidine in DMF (3 mL) for 30 min, filtered, and washed three times with DMF (3 mL). Amino acids AA_2, AA_3, and AA_4 were introduced in series using an isokinetic mixture[23] composed of: Fmoc-Ala, 3.4%; Fmoc-Arg(Pmc), 6.1%; Fmoc-Asn(Trt), 5.6%; Fmoc-Asp(O^{t}Bu), 3.5%; Fmoc-Cys(Trt), 3.6%; Fmoc-D-Orn(Boc)-OH, 3.9%; Fmoc-Gln(Trt), 5.4%; Fmoc-Glu(O^{t}Bu), 2.9%; Fmoc-Gly, 3.3%; Fmoc-His(Trt), 3.5%; Fmoc-Ile, 15.5%; Fmoc-Leu, 4.9%; Fmoc-Lys(Boc), 5.5%; Fmoc-Met, 2.6%; Fmoc-Phe, 2.6%; Fmoc-Pro, 4.3%; Fmoc-Ser(^{t}Bu), 4.2%; Fmoc-Thr(^{t}Bu), 4.1%; Fmoc-Tyr(^{t}Bu), 3.9%; and Fmoc-Val, 11.2%. A sample of this mixture (182 mg, 0.40 mmol) in DMF (4 mL) was activated for 15 min by incubation with HATU (304.2 mg, 0.80 mmol) and *sym*-collidine (162 μL, 2.0 mmol) in DMF (1 mL) and presented to the resin. After agitation for 5 h, the resin was filtered, washed twice with DMF (5 mL), and treated with a second aliquot of Fmoc-AA mixture (182 mg, 0.40 mmol), HATU (304.2 mg, 0.80 mmol), and collidine (162 μL, 2.0 mmol) in DMF (5 mL) and presented to the resin. After agitation for 10 h, the resin was filtered, washed three times with DMF (5 mL), treated with 20% piperidine in DMF (3 mL) for 30 min, filtered, and washed three times with DMF (3 mL). This procedure was repeated twice to incorporate AA_3 and AA_4. Resin **5** was always stored prior to removal of the N-terminal Fmoc protecting group. The amine substitution level of resin **5** AA_1=Ala within three repetitions was determined to be within 0.32±0.04 mmol/g by Fmoc-quantization.[22]

Resin **6** was prepared by solvating Fmoc-protected resin **5** AA_1=Ala (100 mg, 0.03 mmol, 0.3 mmol/g) in 20% piperidine in DMF (3 mL) for 30 min followed by filtering and washing three times with DMF (2 mL). Fluorescent hydroxyacid **1** or amino acid **2** (56.4 mg, 0.15 mmol), HATU (114 mg, 0.30 mmol), and *sym*-collidine (60.5 μL, 0.75 mmol) in DMF (2 mL) was incubated for 10 min and then added to the resin. After 3 h, the resin was filtered, washed three times with DMF (2 mL), and treated with a second aliquot of the fluorescent hydroxyacid **1** or amino acid **2** (56.4 mg, 0.15 mmol), HATU (114 mg, 0.30 mmol), and *sym*-collidine (60.5 μL, 0.75 mmol) in DMF (2 mL). After 6 h of agitation, the resin was filtered, and washed three times with DMF (2 mL), twice with MeOH (2 mL), three times with THF (2 mL), and air dried to provide resin **6** or **7**. The amine substitution level within three repetitions was found to be 0.21±0.03 and 0.26±0.02 mmol/g for resin **6** and **7**, respectively.[22]

Peptides **8** and **9** were cleaved from the resin using the same protocol. Resin **6** AA_1=Ala or **7** AA_1=Ala (100 mg, 0.025 mmol, 0.25 mmol/g) was treated with 20% piperidine in DMF (1 mL) for 30 min, filtered, washed three times with DMF (1 mL), and washed twice with CH_2Cl_2 (2 mL). After drying in vacuo, the resin was incubated with a cleavage solution (2 mL) containing 95:2.5:2.5 TFA/TIS/H_2O for 1 h. The solution was collected and the resin was washed twice with the cleavage solution (0.5 mL). The solution and washes were combined and concentrated in vacuo. The resulting materials were dissolved in 0.2 mL DMSO and filtered through a plug of K18-reverse phase silica gel (300 mg) using a gradient of 10–80% aq CH_3CN. The fluorescent fractions were collected and lyophilized. The amount of peptide obtained from three repetitions of this procedure was determined to contain 0.22±0.03 mmol of **8** or 0.26±0.03 mmol of **9** (see Section 4.3). This procedure provides ~20 mg of net peptide or ~2.5 μg of each peptides at the theoretical production of 8000 peptides per set.

4.3. Fluorescence imaging and quantification

The fluorescence from the 7-dimethylaminocoumarin-4-acetamide provided an effective handle to quantify the concentration of peptide on resins **6** and **7** and the concentration of solutions of **8–11**. Fluorescence was quantified in solution using a spectrophotometer or plate reader with λ_{ex}^{max}=375±6 nm, λ_{em}^{max}=462±5 nm, and ε=23,500±2500 M^{-1} cm^{-1}. The concentration of dye on resin or in cell was determined using quantitative fluorescence microscopy on an LED-fluorescence microscope (Xenobe Research Institute) using fluorescent microspheres as standards (Molecular Probes Inc.).

LED-fluorescence images were collected using the excitation from a 100 mW, 370 nM GaN UV-LED (Roithner Lasertechnik), filtration through a dichroic filter set FF400 (Semrock Inc.) with an excitation at 377±50 nm, a dichroic with >98% reflection at 344–404 nm and >98% transmission at 415–570 nm, an emission filter at 447±60 nm. Images were collected using a 60X Oil Immersion Epiplan Neofluar objective (Zeiss), and image collection on an Electron Bombardment CCD Camera EBCCD (Hamamatsu Corp.).

4.4. Cell uptake

Samples of the peptide sets **8** or **9** were dissolved in DMSO. The concentrations were adjusted to 10 μM/peptide-based on the expected fluorescence from 8000 peptides per set. A 200 μL aliquot of this solution was added to 20 mL of

DMEM in 250 mL culture flask containing ~106 cells/cm^2. After incubation at 37 °C for 30 min, the cells were washed three times with 50 mL of DMEM containing 10% ethanol and the resulting cells were imaged on an LED-fluorescence microscope (see Section 4.3). The cells were then harvested by brief digestion with trypsin, isolated by centrifugation at 1000 rpm, and washed five times with DMEM (10 mL).

Samples of sets of depsipeptides **10** and cyclic peptides **11** were imaged as crude mixtures or directly after TLC analysis. 2D-pTLC was conducted on 2×2 cm Partisil KC18 plates using a 1–2 mm diameter spot. The plates were eluted with 10:80:5:2.5:2.5 CH_2Cl_2/MeOH/H_2O/pyridine/Et_3N in one direction, turned 90°, and run in the other direction with 10:80:5:5 CH_2Cl_2/MeOH/H_2O/acetic acid. The nonfluorescent regions were scraped from the plate and the plate was gently washed with 2:1 mixture of hexane and ethyl acetate to remove silica particles. The plate was wet with 50% aq CH_3CN using an aerosol spray and covered with 0.10±0.02 mm layer of low-melt agarose.[24] A cover slip containing ~10^6 cells/cm^2 was placed on top of the agarose layer, cells facing down into the agarose layer. The agarose layer served to transport the fluorescent peptide to the cells. This sandwich was incubated at 37 °C for 1 h and then imaged. Though effective, this technique required a resolution of at least 2 mm between each spot.

Alternatively, samples of the depsipeptides **10** and cyclic peptides **11** were extracted from a 10×10 cm Partisil KC18 plates using a 1:1 mixture of MeOH and DMF. The resulting aliquots were dried and dissolved at 10 μM/peptide in DMSO. This solution was added to 2 μL/mL of DMEM and placed on a microscope slide containing ~10^6 cells/cm^2 and incubated at 37 °C for 30 min. The cells were washed with 3×50 mL of DMEM containing 10% ethanol and imaged on an LED-fluorescence microscope (see Section 4.3). The cells were harvested by release, by brief digestion with trypsin, isolated by centrifugation at 1000 rpm, and were washed five times with DMEM (10 mL).

4.5. Cell fractionation

Isolation of nuclei was conducted using a procedure developed by Roeder.[25] HeLa cell pellets were suspended in five volumes of 4 °C phosphate buffered saline pH 7.4 and collected by centrifugation for 10 min at 2000 rpm at 4 °C. The subsequent steps were performed at 4 °C. The cells were diluted five-fold with 10 mM HEPES (pH 7.9 at 4 °C) containing 1.5 mM $MgCl_2$, 10 mM KC1, and 0.5 mM DTT; and allowed to stand for 10 min. The cells were collected by centrifugation as before and resuspended by two-fold dilution in 10 mM HEPES (pH 7.9 at 4 °C) containing 1.5 mM $MgCl_2$, 10 mM KC1, and 0.5 mM DTT and lysed by 10 strokes of Dounce all-glass homogenizer with a type B pestle. The homogenate was checked microscopically for cell lysis and centrifuged for 10 min at 2000 rpm (470 g) and 20 min at 25,000 g to provide the crude nuclei. Materials were extracted from these nuclei using the method in Section 4.6.

Subcellular fractionation of the ER was conducted using Endoplasmic Reticulum isolation kit (ER0100) from Sigma Aldrich. Materials were extracted from these ER using the method in Section 4.6.

4.6. Peptide extraction

Nuclear and ER fractions were lysed in four volumes of PBS 7.2 by sonication at 4 °C, extracted twice with cyclohexane (10 mL) and ethyl acetate (10 mL) to remove lipids, and lyophilized. The resulting dried material was extracted twice with THF (1 mL), MeOH (1 mL), CH_3CN (1 mL), DMF (1 mL), and the combined extracts were dried in vacuo. The residue was dissolved in DMF and standardized to concentration of 250±10 μM per peptide using the fluorescence from 7-dimethylaminocoumarin-4-acetamide (see Section 4.3). The number of peptides present was determined by 2D-pTLC on 2×2 cm Partisil KC18 plates using a 1–2 mm diameter spot. The plates were eluted with 10:80:5:2.5:2.5 CH_2Cl_2/MeOH/H_2O/pyridine/Et_3N in one direction, turned 90°, and run in the other direction with 10:80:5:5 CH_2Cl_2/MeOH/H_2O/acetic acid. Preparatory purification was possible by repeating the same purification on a 10×10 cm Partisil KC18 plates using an 8–9 mm diameter spot.

4.7. Macrocyclization

A 200 μL aliquot of solution containing peptides **8** or **9** standardized to 250±10 μM/peptide (0.05 μmol) in DMF was diluted to 1:1 by addition of HATU (0.95 mg, 2.5 μmol), DTT (3.8 μg, 0.025 μmol), and *sym*-collidine (0.2 μL, 2.5 μmol) in DMF. The reaction mixture was stored at rt under argon in the absence of light. After 24 h, the reaction mixture was passed through a plug of K18-reverse phase silica gel (300 mg) using 1 mL of 50% MeOH in DMF and concentrated. The number of peptides present was determined by 2D-pTLC on 10×10 cm Partisil KC18 plates using an 8–9 mm diameter spot. The plates were eluted with 10:80:5:2.5:2.5 CH_2Cl_2/MeOH/H_2O/pyridine/Et_3N in one direction, turned 90°, and run in the other direction with 10:80:5:5 CH_2Cl_2/MeOH/H_2O/acetic acid.

The mass of peptides **13–26** was determined by pTLC-MALDI-MS.[18] Their sequence was determined using established methods for MS based peptide-sequencing[19] after isolating individual peptides from a pTLC plate. The identity of each peptide was established by repeating its synthesis in a single substrate manner at a 20 mg scale. Cell uptake and localization data for each peptide were confirmed by repeating the cellular imaging experiments with this material.

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