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Solvatochromic Reagents for Multicomponent Reactions and their Utility in the Development of Cell-Permeable Macrocyclic Peptide Vectors

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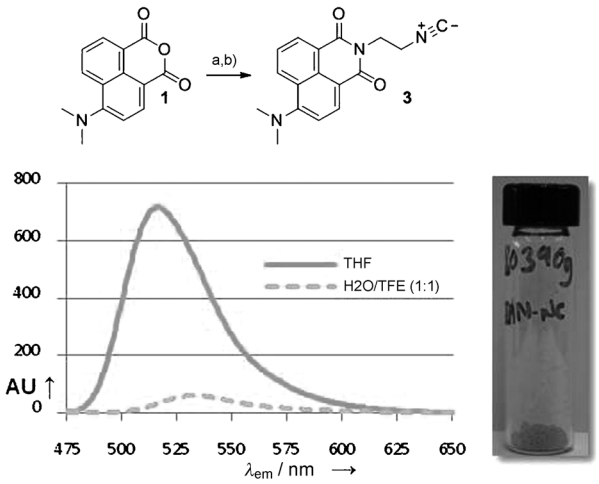
Peptides that are composed of proteinogenic amino acids have the capacity to engage in native interactions with protein targets, but usually lack the ability to effectively translocate membrane barriers.[1] Synthetic peptides have found numerous in cellulo applications, most commonly as fluorescent imaging agents and as activity-based probes.[2–4] The biochemical properties displayed by peptides would make them ideal scaffolds in the design of drugs and drug-delivery vectors, were it not for their limited stability against proteolysis in vivo. Cyclization of peptides can lead to enhanced specificity,[5] in vivo proteolytic stability,[6] and cellular permeability.[7] Computational studies have shown that internal hydrogen bonding in these compounds may facilitate passive membrane permeability.[8] Cyclic peptides based on the arginine–glycine–aspartic acid (RGD) sequence are a well-known class of probes. When constrained within a cyclic pentapeptide framework, RGD targets $\alpha_v\beta_3$ receptors on the surfaces of cancer cells. A change from a five- to a six-residue macrocycle switches the selectivity towards the $\alpha_{IIb}\beta_3$ receptor.[9] These findings highlight the tremendous opportunities available in making highly specific probes, therapeutics, and delivery vectors using peptide macrocycles.

The synthetic manipulation of peptide-based probes is complicated by numerous functional groups throughout the molecule. The placement of a fluorescent label must be both selective and carefully chosen to avoid interference in functional assays. Thus, simple, chemoselective, and site-specific strategies for the preparation of fluorescent peptide macrocycles can have a significant influence on a number of areas. We recently described the use of isocyanides and aziridine aldehydes in peptide macrocyclization.[10] Isocyanides are essential to many multicomponent reactions, and the wide functional group tolerance observed is evidence of their selectivity. Our current contribution describes a new generation of solvatochromic isocyanides for multicomponent reactions and documents their application in the synthesis of peptide macrocycles. Fluorescence cellular imaging and flow cytometry revealed that macrocyclization improves the cellular uptake of a mitochondria-penetrating peptide (MPP), in comparison to a closely related acyclic probe (Scheme 1).



Scheme 1. Synthesis and solvatochromic emission of fluorescent isocyanide **3**. a) 2-aminoethylformamide, abs. ethanol, 78 °C, 30 min; 82 % yield; b) $POCl_3$, NEt_3, CH_2Cl_2, 0 °C to RT, 1 h; 68 % yield.

Toward the goal of synthesizing macrocyclic molecular probes, we required a robust methodology for incorporation of a fluorophore into macrocycles. We desired to maintain the electrophilic aziridine "handle" within the macrocycles as a site for specific downstream conjugation. Our interest in expanding the repertoire of functional reagents for multicomponent condensations led us to consider solvatochromic isocyanides. These reagents could be of broad utility in the synthesis of environment-sensitive probes.[11] Isocyanides are 1,1-amphoteric functional groups; their reactivity is defined by α-additions. This has been exploited most famously by multicomponent reactions, such as the Passerini and Ugi reactions,[12] but their utilities as ligands for metal complexes,[13] coupling partners in transition metal catalysis,[14] and polymerization[15] are also notable. Novel reagents containing the isocyanide functionality could be applied widely (Scheme 2).

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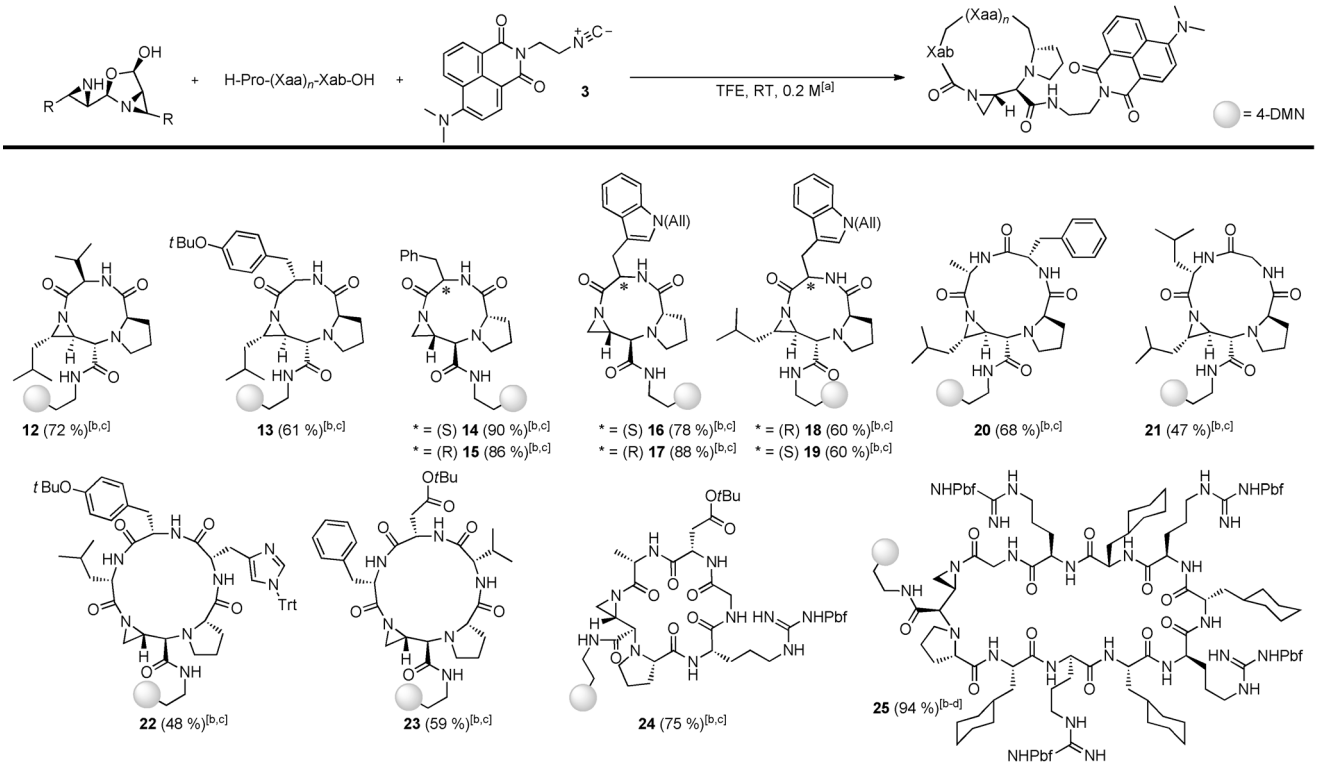
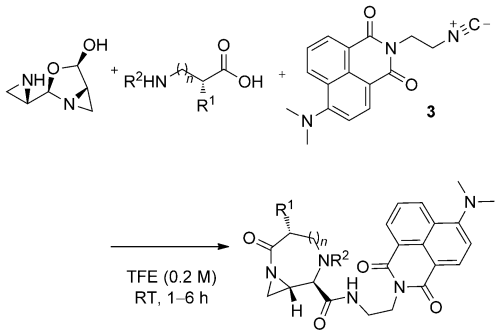


Scheme 2. Scope of the fluorescent peptide macrocycles synthesized. [a] Typical conditions: peptide (0.05 mmol), aziridine aldehyde (1 equiv), isocyanide (1 equiv), TFE (0.25 mL); [b] value in parentheses is the isolated product yield; [c] A diastereomeric ratio (d.r.) of >19:1 was confirmed in each case by analysis of ^{1}H NMR spectra of crude products [d] TFE (0.1 M), aziridine aldehyde (1.15 equiv), isocyanide (1.15 equiv); All = allyl, *t*Bu = *tert*-butyl, 4-DMN = 4-*N*,*N*-dimethylaminonaphthalimido, Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, Xaa, Xab = α-amino acid.

Our first goal was to develop novel isocyanide reagents equipped with solvatochromic groups. Naphthalimide-labelled molecules display solvent-sensitive quantum yield and, relative to many other commonly used fluorophores, are also distinguished by a fairly compact structure.[4c,d] Fluorescent naphthalic anhydride **1** can be easily converted to formamide **2** by treatment with 2-aminoethylformamide. We have found that dehydration gives the desired fluorescent isocyanide **3**, which is isolated after chromatography as a bench stable, crystalline, odorless, orange solid with a melting point of 164–165 °C (EtOH). Its photophysical properties have been investigated in aqueous and organic solvents. When dissolved in THF, the isocyanide exhibited a maximum emission wavelength (λ_{max}) of 516 nm, whereas in a 1:1 solvent mixture of H_2O:TFE λ_{max} was 532 nm. The excitation maxima were 335 and 474 nm in THF and 353 and 508 nm under aqueous conditions. The fluorescence intensity was uniformly increased by more than a factor of 10 in THF compared with the aqueous conditions. We were delighted that the fluorescence was not quenched by the isocyanide functionality and that the label was compatible with the macrocyclization chemistry. A series of fluorescent piperazinones was prepared from amino acids and an aziridine aldehyde dimer (Table 1). In addition to proteinogenic α-amino acids, β- and γ-amino acids were found to undergo cyclization to yield 7- and 8-membered rings. Given the utility of

Table 1. Synthesis of macrocycles from amino acids.

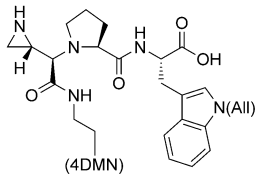


Entry[a]	Amino Acid	Time [h]	*n*	Product[b,c]
1[d]	L-Glu	6	0	**4** (76)
2	L-Phe	3	0	**5** (91)
3	L-His	1.5	0	**6** (93)
4	L-Leu	2	0	**7** (72)
5	L-Pro	1	0	**8** (88)
6	Gly	1	0	**9** (80)
7	β-Ala	2	1	**10** (71)
8	GABA	2.5	2	**11** (67)

[a] Typical conditions: amino acid (0.05 mmol), aziridine aldehyde (1 equiv), isocyanide (1 equiv), TFE (0.25 mL); [b] value in parentheses is the isolated product yield [c] >19:1 d.r. by NMR spectroscopy; see the Supporting Information for assignment of absolute stereochemistry [d] solvent: HFIP/H_2O (20:1); exclusively 6-membered ring product observed.

isocyanides in multicomponent reactions, our findings should be applicable to many different molecular scaffolds.[16]

Macrocyclization conditions were then applied to a series of short peptides to generate the macrocycles depicted in Scheme 2. Each linear peptide studied was constrained in good yield and with excellent diastereoselectivity. Using this methodology, peptide macrocycles equipped with a fluorescent tag as well as an accessible electrophile can be synthesized in a single step from a wide pool of linear peptides.[17] In cases where chromatographic purification was required, the brightly colored product was easily identified by visual inspection of collected fractions. In THF, the constrained di- and tripeptides were found to display a sequence-dependent λ_{max} in the 495–500 nm range. The diastereomeric pair Pro-L-Trp(allyl) and Pro-D-Trp(allyl) were also constrained and the fluorescence properties of the product macrocycles **16** and **17** were examined. Macrocycle **16** exhibited a significantly greater fluorescence excitation at 212 nm than **17** when studied in THF, indicating a possibility that fluorescence is responsive to macrocyclic configuration. This observation, as well as the observed shift in λ_{max} of the naphthalimide fluorophore upon macrocyclization, aroused our curiosity about a possible reporter for chemical transformations of the macrocycle. Linear control ***lin*-16** (Scheme 3) displayed λ_{max} of 507 nm (THF), outside of the observed range for the studied macrocycles. This trend was found to be general for ***lin*-15** and ***lin*-20** as well (Table 2).



Scheme 3. Linear control peptide ***lin*-16**.

Table 2. Fluorescence properties of macrocycles and derivatives.

Compound[a]	λ_{exc} [nm] THF	λ_{em} [nm] THF	λ_{exc} [nm] H_2O:TFE[b]	λ_{em} [nm] H_2O:TFE[b]
3	474	516	508	532
16	457	500	–	–
17	467	501	–	–
18	473	497	506	518
19	469	497	506	519
20	472	498	505	515
21	463	495	505	515
***lin*-15**	455	508	–	–
***lin*-16**	468	507	–	–
***lin*-20**	458	516	–	–

[a] Solutions were prepared to a concentration of 0.5 mM [b] 1:1 (v/v).

To demonstrate the potential of fluorescent macrocyclic peptide probes in cellular systems, we pursued organelle-selective macrocycles. Our previous investigations into linear MPPs had uncovered a series of sequences capable of transporting cargo across the mitochondrial membrane. By balancing positively charged and hydrophobic residues, synthetic hexa- and octapeptides exhibited localization in the mitochondria.[18] A representative peptide (Pro-(Cha-D-Arg)$_4$-Gly) (Cha = cyclohexylalanine) was chosen to study if a similar localization would be observed for a macrocyclic system. The peptide was constrained using an aziridine aldehyde and fluorescent isocyanide **3** to give **25** in 94% yield (Scheme 2). The aziridine ring was reduced by a two-step protocol involving ring-opening with thiobenzoic acid, followed by desulfurization in the presence of Raney nickel. This reaction "deleted" the aziridine ring by a formal ring-opening with "H^-". After deprotection of the arginine side-chains, an analysis of the cellular localization of **26** (Figure 1a) in HeLa cells, a cervical carcinoma cell line, was conducted. The probe exhibited excellent mitochondrial localization, which was confirmed by co-localization of the peptide with a mitochondrion-selective fluorescent probe, MitoTracker CMXRos (Figure 1b). As a control, fluorescent formamide **2** exhibited no cellular fluorescence.

To determine whether a cyclic MPP offered a significant advantage over a linear one, the side-chain deprotection and hydrolysis of the aziridine amide of macrocycle **25** was used to give a linear counterpart (***lin*-26**). Evaluation of localization of ***lin*-26** in HeLa cells revealed that the linear peptide produced less mitochondrial fluorescence than cycle **26** within the same concentration range (Figure 1). In the confocal microscopy images for both **26** and ***lin*-26**, bright spots are observed outside of the mitochondria, and in some cases outside of the cell. These arise from aggregation of the probes, as well as endosomal uptake. To better understand the localization results, we quantified the cellular uptake of **2**, **26**, and ***lin*-26** using flow cytometry. Importantly, whereas many fluorophores are known to increase the membrane permeability of probes, **2** showed minimal cellular uptake (See the Supporting Information, Figure S1). Furthermore, cyclic **26** consistently showed higher uptake than ***lin*-26**.

In conclusion, solvatochromic isocyanides are novel tools for the preparation of macrocycles using aziridine aldehyde-induced macrocyclization. We expect that these reagents will find a wide range of applications in other isocyanide-based multicomponent reactions[12] to generate libraries of fluorescently-tagged products. Our method should also be conducive to generating diverse libraries using recently designed microfluidic platforms.[19] A wide range of physiologically relevant linear peptide sequences are known and many of them avoid extended conformation upon binding to protein targets. These systems will provide excellent starting points for the one-step synthesis of solvatochromic macrocycles. The possibility of developing conformation-responsive macrocycles and ionophores[20] is equally exciting. Aziridine-containing fluorescent macrocycles may also find an application as irreversible probes of enzyme acitivity. Our current efforts are aimed at capitalizing on the increased amphipathicity of our macrocycles, conjugation of various types of molecular cargo using straightforward aziridine ring-opening transformations, and attenuation of the undesired aggregation and endosomal uptake observed with our current probes.

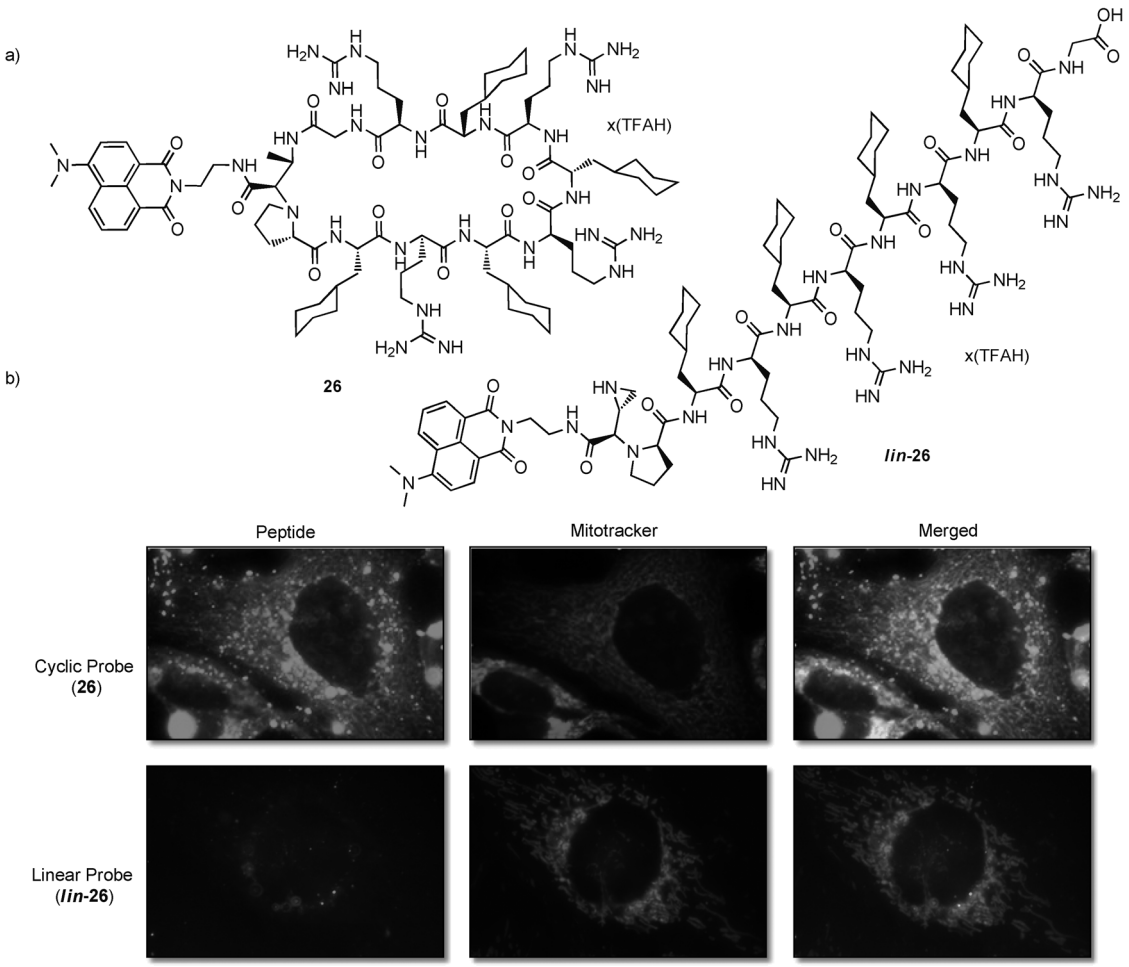


Figure 1. Localization of cyclic (**26**) and linear (***lin*-26**) MPP in live HeLa cells. Cells were incubated with cyclic/linear peptide (4 μM) for 60 min and then with MitoTracker CMXRos (25 nM) for 15 min before images were acquired. The merged images were acquired by adding the peptide images to the MitoTracker images in a cumulative manner to show the degree of co-localization.

Experimental Section

2-(4-dimethylamino-1,8-naphthalimido)ethylformamide (2): 4-Bromo-1,8-naphthalic anhydride (500 mg, 2.07 mmol) and absolute ethanol (25 mL) was added to a round-bottomed flask equipped with a reflux condenser and a magnetic stirring bar. 2-aminoethylformamide (925 mg, 10.5 mmol) was added to the mixture and the solution was brought to reflux. After refluxing for 30 min, the solution was cooled to room temperature and the solvent removed by rotary evaporation. The product was purified by flash chromatography (SiO_2, ethyl acetate, R_f 0.21) and isolated as an orange solid (530 mg, 1.7 mmol, 82%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.57 (dd, J = 7.2, 0.9 Hz, 1H), 8.49–8.43 (m, 2H), 8.12 (s, 1H), 7.66 (dd, J = 8.3, 7.2 Hz, 1H), 7.11 (d, J = 8.3 Hz, 1H), 6.45 (s, 1H), 4.46–4.38 (m, 2H), 3.71 (dd, J = 10.6, 5.1 Hz, 2H), 3.13 ppm (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 165.49, 164.97, 161.71, 157.60, 133.33, 131.94, 131.63, 130.65, 125.34, 125.07, 122.79, 114.34, 113.43, 44.97, 39.14, 38.79 ppm.

2-(4-dimethylamino-1,8-naphthalimido)ethyl isocyanide (3): Phosphorous oxychloride (0.23 mL, 2.56 mmol) was added dropwise to an ice-bath cooled, stirred solution of 2-(4-dimethylamino-1,8-naphthalimido)ethylformamide (500 mg, 1.6 mmol), triethylamine (0.93 mL, 6.72 mmol), and dichloromethane (80 mL) in a round-bottomed flask, After the bath was removed, the reaction was stirred at room temperature for 1 h. A 0.5 M solution of sodium carbonate (6.13 mL) was then added slowly to reaction mixture, which was then stirred for an additional 30 min. The organic phase was separated, washed once with brine, dried over anhydrous sodium sulphate, filtered and concentrated. The product was purified by flash chromatography (SiO_2, ethyl acetate/triethylamine 99:1, R_f = 0.79) and isolated as a crystalline orange solid (319 mg, 1.09 mmol, 68%). M.p. 164–165 °C (EtOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.59 (dd, J = 7.3, 1.1 Hz, 1H), 8.49 (d, J = 8.3 Hz, 1H), 8.46 (dd, J = 8.5, 1.1 Hz, 1H), 7.67 (dd, J = 8.5, 7.3 Hz, 1H), 7.12 (d, J = 8.3 Hz, 1H), 4.53 (t, J = 6.6 Hz, 2H), 3.80 (t, J = 6.6 Hz, 2H), 3.13 ppm (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 164.63, 163.94, 157.57, 133.33, 131.94, 131.65, 130.64, 125.34, 125.02, 122.60, 114.18, 113.41, 44.91, 39.48, 38.48 ppm. FTIR $\tilde{\nu}$ = 2855 (w), 2148 (m), 1683 (s), 1634 (s), 1580 (s), 1354 (s), 1318 (s).

General procedure for macrocyclization: a solution of aziridine aldehyde dimer in TFE (0.25 mL, 0.1 M) was added to a screw-cap vial containing a magnetic stirring bar and peptide (0.05 mmol) followed by isocyanide (0.05 mmol). The vial was sealed and the contents stirred at room temperature. Reactions were monitored by ESI-MS and/or TLC. When the reaction was determined to be complete, the mixture was concentrated under a stream of nitrogen gas. Diethyl ether and/or hexanes (≈2 mL total) were added to induce precipitation of the product. The solid was isolated after additional washes with diethyl ether and/or hexanes. In cases where impurities remained in the precipitate, the product was purified by flash chromatography or preparative reversed-phase HPLC.

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Keywords: fluorescent probes · isocyanides · macrocycles · multicomponent reactions · peptides · solvatochromism

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