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Synthesis and Characterization of a Macrocyclic Near-Infrared Optical Scaffold

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Fluorescence-emitting molecules continue to play a major role in all facets of chemistry, materials science, and life sciences. With the advent of molecular medicine, the challenge for chemists is to develop new photoactive molecules that can probe specific molecular events and inhibit pathophysiological processes. Light in the near-infrared (NIR) wavelengths is particularly attractive to study chemical and biological processes in tissue because its absorption and scattering by endogenous biomolecules are minimal and, hence, can penetrate into deep tissues without the accompanying autofluorescence that occurs in the UV/visible wavelengths.[1,2]

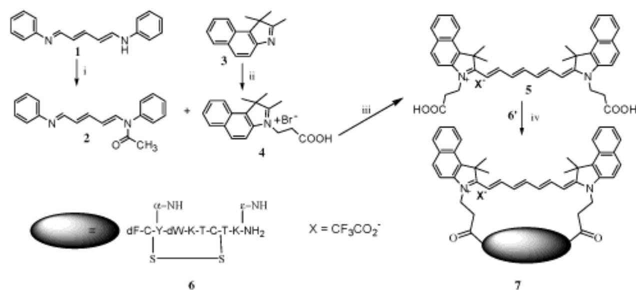
Recent studies have shown that conjugating NIR fluorescent compounds to bioactive peptides and proteins is a reliable method to selectively deliver the optical probes to tumors, resulting in the localization and treatment of the target pathologic tissue.[3-5] For small peptides, the fluorophore is typically attached to the N-terminus of the bioactive carrier. Unlike proteins, small peptides are highly susceptible to rapid degradation by proteolytic enzymes. One approach to improve metabolic stability of small peptides and to rigidify their low-energy bioactive conformations is to use head-to-tail cyclic peptide scaffolds.[6,7]

Accordingly, we report the first NIR macrocyclic fluorescent bioactive peptide scaffold that has great potential for a variety of applications. In a model system, we demonstrate that macrocyclization of a somatostatin receptor-avid nanopeptide with a bifunctional NIR carbocyanine optical probe gives a compound that retained the high receptor binding affinity of the peptide and the absorption and fluorescence emission properties of the fluorescent probe. NMR and computational modeling data show that the macrocyclization did not significantly perturb the established low-energy conformation of the peptide.

A major shortcoming to using NIR carbocyanine fluorescent probes in research is the difficulty in synthesizing high purity reactive intermediates in high yields by conventional methods. A review of the literature[8] shows that *N*-acetylation of glutaconaldehyde is an important step in the synthesis of NIR dyes. This acetylation is traditionally performed in situ and purification of the final product is very demanding. We observed that pre-acetylating glutaconaldehyde (**1**) prior to reaction with activated benzoindole (**4**) gives a good yield (>60%) of the desired bifunctional NIR dye (cypate, **5**) without the need for HPLC purification (Scheme 1).

To prepare the macrocyclic molecule from **5**, lysine was incorporated at the C-terminus of a cyclic somatostatin (sst) receptor-avid octapeptide [dF-cyclo(C2-Y3-dW4-K5-T6-C7)-T8; octreotate; **8**]. While it is possible to use the ϵ-amino group of K5 for the cyclization, this amino acid is part of the octapeptide's pharmacophore and, hence, we chose to introduce a second amino group at the C-terminus by using the readily available orthogonally

Scheme 1. Synthesis of NIR Macrocyclic Optical Scaffold[a]



[a] (i) Ac_2O/TEA, (ii) $BrC_2H_4CO_2H$, (iii) CH_3COONa, (iv) (a) PyBOP, (b) TFA. Compound **6′** is the protected peptide on resin.

protected lysine. The C-terminal lysine residue provides the second amino group for intramolecular cyclization on solid support. The choice of **8** has a practical implication because the sst receptor is up-regulated in a variety of human tumors.[9] Thus, starting with a preloaded Fmoc-Lys(Dde)-OH on Rink amide resin, the peptide was synthesized on a solid support by a standard procedure. While the peptide was still on the solid support, simultaneous removal of the orthogonal Dde and Fmoc protecting groups of α-dF1 and ϵ-K9 amino groups and subsequent reaction with the PyBOP preactivated carboxyl groups of **5** gave the desired macrocyclic compound (**7**) after cleavage from the resin with TFA (Scheme 1). The spectral properties of **7** ($\lambda_{max,abs}$ 792; $\lambda_{max,em}$ 811 nm) were similar to those of **5** ($\lambda_{max,abs}$ 786; $\lambda_{max,em}$ 811 nm) in 25% aqueous DMSO. Expectedly, the molar absorptivity of the dye (**5**) decreased from 2.97×10^5 to 1.1×10^5 cm^{-1} M^{-1} in **7** due to a combination of macrocyclization and "dye dilution" effects resulting from the addition of the peptide moiety.

To evaluate the effect of incorporating lysine at the C-terminus of **8** and subsequent macrocyclization of the peptide, we performed the receptor-binding assays of these compounds. The IC_{50} values of **8**, K9-octreotate [dF1-cyclo(C2-Y3-dW4-K5-T6-C7)-T8-K9-NH_2; **6**], and the macrocyclic analogue [cyclo cypate-(dF1-cyclo-(C2-Y3-dW4-K5-T6-C7)-T8-K9)-NH_2; **7**], which were obtained by competition with ^{111}In-DTPA-octreotide, were 0.12, 21.68, and 8.17 nM, respectively. This result shows that macrocyclization retained the receptor binding affinity of the precursor peptide **6** and that the IC_{50} value of **7** is comparable to those of previously reported linear analogues.[5] A preliminary stability study by HPLC of **7** and the linear analogue (cypate-octreotate)[5] in serum after a 24 h incubation at 37 °C shows a decrease of <10% of the macrocyclic compound and >25% of the linear analogue. This result suggests that macrocyclization enhanced the stability of the new construct.

Assuming that the bound conformation is selected by a receptor from the set of low-energy conformers of the peptide in solution, one can conclude that peptide **7** retained receptor binding affinity because its low-energy conformations are not significantly affected by macrocyclization. This conclusion was confirmed by NMR and

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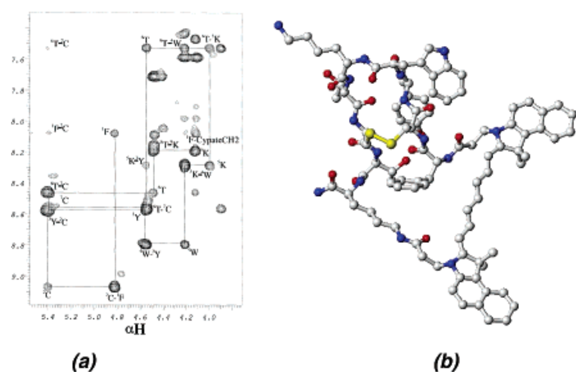

Figure 1. (a) Expansion of NH-αH region of 600 MHz NOESY spectra and (b) sketch of a representative structure of **7**.

computational modeling data. Proton chemical shifts of **7** were compared with those of **6** and **8**. The proton chemical shifts were assigned by analysis of TOCSY and NOESY spectra. Sequential assignments were obtained by using the NH-αH fingerprint region of the NOESY spectra. All the $d_{\alpha N}$ connections between adjacent residues were observed and a continuous path indicates the segment from dF1 to T8 or K9 C-terminal residue. Cyclization through disulfide S−S bond was evidenced by several observed NH-αH NOEs between dF1 and C2 as well as between C7 and T8. Strong NOE between dF1NH and cypate CH_2, and between K9εNH and cypate CH_2 and cypate-aromatic protons, confirm the dF1-cypate-K9 linkage in compound **7**. The presence of several αH_i-NH_{i+2} contacts such as C7-K5, T6-W4, and K5-Y3 suggests a tight β-reversal conformation of the 20-membered cyclic disulfide peptide. Furthermore, the observation of αH-αH NOE for the residues from C2 to C7 indicates the backbone at the cyclic moiety may deviate from the trans-amide conformation. Of particular interest is the observation of T6NH-W4αH, T6NH-Y3αH, T6NH-C2αH, and T8NH-T6αH NOE cross-peaks, suggesting the disulfide ring closure causes a β-reversal of the peptide in vicinity of T6 bringing T8, W4,Y3, and C2 in close proximity. All three peptides have similar sequential and αH_i-NH_{i+2} NOEs except that the T8NH-T6αH and C7NH-K5αH NOEs were not observed in compound **7** (Figure 1a). This indicates that an additional K9 residue and the cypate linkage may cause the 20-membered ring to adopt a less compact topology with a conformational change appearing only at the residues near the C-terminus of **7**.

Since the NMR data did not show a strong NOE between the peptide and the fluorescent probe, the computational modeling was restricted to the key common element for compounds **6** and **7**, namely, Ac-dF1-cyclo(C2-Y3-dW4-K5-T6-C7)-T8-K9-NH_2 (**9**). Energy calculations were performed in general accordance with a published procedure[10] (see Supporting Information for detailed description). The most characteristic feature of the obtained ensemble of low-energy conformers (206 structures of peptide backbone) is the distinct β-reversal centered on the dW4-K5 fragment. About 79% of low-energy structures possess that feature as compared to the six-residue β-reversal template (rms less than 2 Å, C^α atoms only). None of the individual structures satisfied all the 13 nonsequential interproton restraints suggested by NMR measurements. However, the ensemble of the found low-energy structures as a whole fully satisfies the NMR restraints, since for every experimentally observed NOE it is possible to find the corresponding interproton distance in the range under 4.5 Å in a number of low-energy conformers, which comprise from 6% to 52% of the entire ensemble depending on the particular NOE.

The dye itself is conformationally rigid, since its polyethylene chain most likely is fixed in all-*trans* configuration with the distance between nitrogen atoms of 12.3 Å (according to model building employing the readily available SYBYL computational package). Consequently, we do not expect that all low-energy conformers of **9** are compatible with attachment of the dye, but only those possessing the suitable distance between the α-amino group of dF1 and the ε-amino group of K9. Our calculations did not consider multiple low-energy conformations for the side chain of K9, but it is quite reasonable to expect that the option selected by the optimization procedure[11] and energy minimization is, at least, one of the lowest energy options possessing the highest statistical weight in solution. Therefore, selection of low-energy conformations with the $\alpha N_1-\epsilon N_9$ distance in the range of 12 ± 2 Å will rather accurately define the conformers of **9** most suitable for attachment of the dye. Such a selection yielded 66 conformations satisfying the above requirement. This new limited ensemble retains most of the features of the extended one: it has almost the same percentage of the β-reversal-like structures (ca. 77%), and again, all close interproton distances observed in the NMR experiments can be reproduced by this limited ensemble as a whole. This is in agreement with experimental finding that the same close interproton contacts are characteristic of both **8** and compounds with the dye attached (**7**). Several conformers possessing the most (but not all) individual interproton distances satisfying the NMR data belong to the same cluster of geometrically similar structures (rms < 2 Å, C^α atoms only). Figure 1b shows a representative structure from that cluster.

One advantage of the macrocyclization approach is the possibility of constraining the bioactive conformations of linear peptides that may adopt a more flexible conformation. For example, bioactive linear bombesin peptide analogues that possess a random coil structure,[12] may be forced to adopt a partial β-sheet or helical conformation after cyclization. The robust nature of the structural framework of these macrocyclic compounds lends itself to a variety of applications, including optimizing and monitoring by NIR optical methods the bioactivity of putative drugs, improving the in vivo metabolic stability of fluorescent drugs, enhancing receptor recognition, modifying spectral properties by altering the macrocyclic ring size, and elucidating the low-energy conformations of small linear biomolecules that would otherwise be difficult to obtain.

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Supporting Information Available: Detailed descriptions of synthetic, NMR, and computational modeling procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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