ACS Combinatorial Science

Research Article

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Fluorescent Mu Selective Opioid Ligands from a Mixture Based Cyclic Peptide Library

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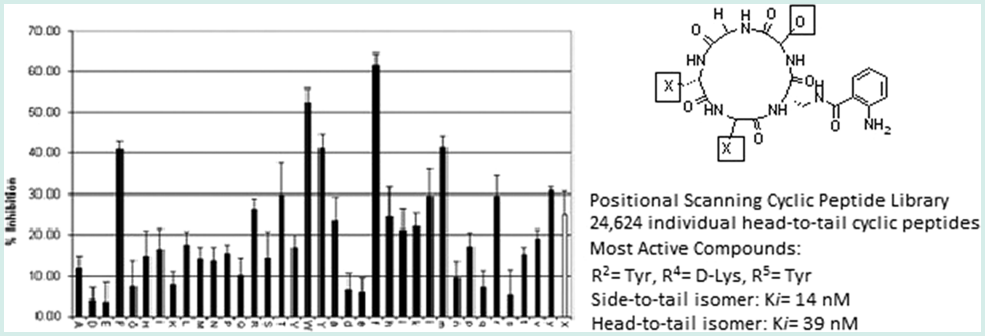
Supporting Information



ABSTRACT: A positional scanning cyclic peptide library was generated using a penta-peptide thioester scaffold. Glycine was fixed at position R^1. Diaminopropionic acid was fixed at position R^3, with its γ-amino attaching to an anthraniloyl group. Positions R^2 and R^4 contained 36 L- and D- amino acids and position R^5 contained 19 L- amino acids. Cyclization was performed in a mixture of acetonitrile and 1.5 M aqueous imidazole solution (7:1 v/v) at room temperature for 5 days. No significant cross-oligomerization was detected under the cyclization conditions. The library was screened in a binding assay for mu opioid receptor, identifying the active amino acid mixture at each position. A total of 40 individual cyclic peptides were identified and synthesized by the combinations of the most active amino acid mixtures found at three positions 5 × 4 × 2. Two cyclic peptides exhibited high binding affinities to opioid receptor. The most active cyclic peptide in the library was yielded to have Tyr at R^2, D-Lys at R^4, and Tyr at R^5. Further investigation on this compound revealed the side chain-to-tail isomer to have greater binding affinity (14 nM) than the head-to-tail isomer (39 nM). Both isomers were selective for the mu-opioid receptor.

KEYWORDS: *cyclic peptide, positional scanning library, synthetic cyclic peptide library, opioid ligand, mu selective ligand, fluorescent label*

INTRODUCTION

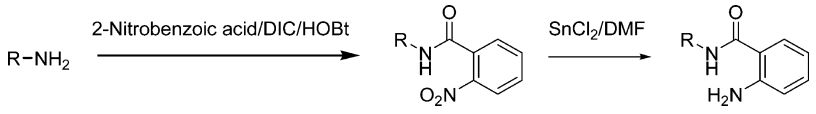
Cyclic peptides are widely recognized as potential therapeutic compounds. Compared to their linear counterparts, cyclic peptides have enhanced binding affinity and specificity to G-coupled receptors,[1] but more importantly they have increased stability in vivo owing to the constrained structure imposed by the cyclization. Therapeutic agents such as daptomycin, cyclosporine A, polymyxin, and octreotide are cyclic peptides.

A potential limitation of cyclic peptides is that they can be difficult to synthesize. The ground-state E geometry of the peptide bond prevents the peptides from attaining the ring-like conformation conducive to cyclization.[2] Furthermore, oligomerization is a common side reaction in macrocyclization. Such synthetic barriers have hindered the discovery of new therapeutically promising molecules and limited construction of cyclic peptide-based libraries. To date, there are only a handful of studies where cyclic peptide libraries have been applied to the identification of potent ligands in drug discovery.[3] Many of the cyclic peptide libraries reported were constructed by phage display strategies rather than de novo chemical synthesis.[4] The need for a facile synthetic strategy for the synthesis of cyclic peptide library is clear.

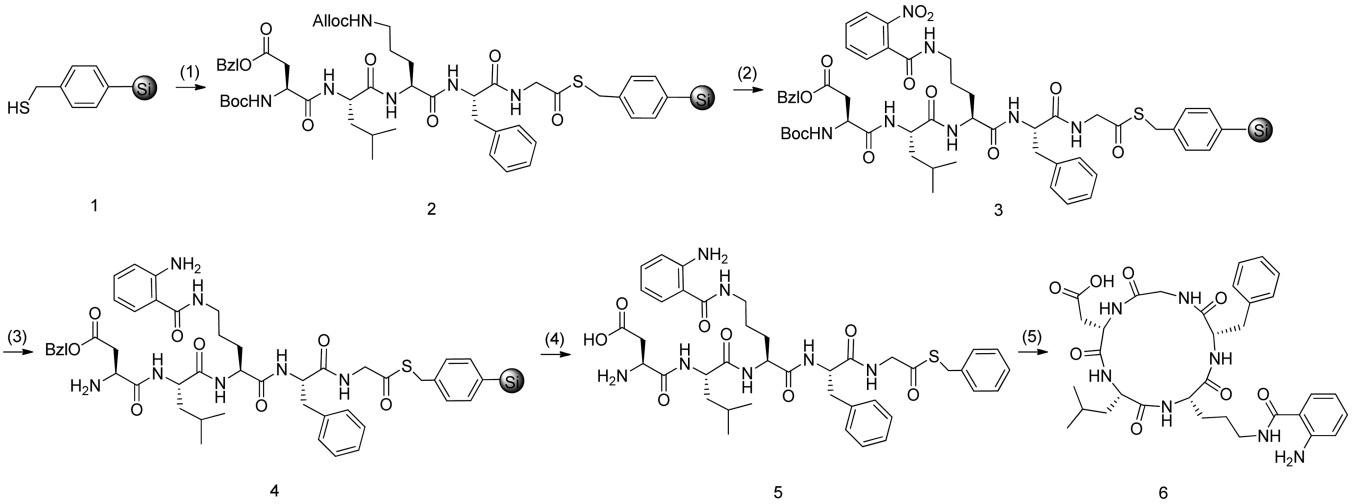
We have recently developed an imidazole-promoted cyclization approach to synthesize cyclic peptides from their fully unprotected linear peptide thioesters.[5] This method is highly efficient for the synthesis of cyclic peptides ranging from 5 to 11 amino acid residues. Our studies also demonstrate that oligomerization was minimal using the imidazole-promoted cyclization. In our continuing efforts to develop new potent ligand tools for opioid receptors, we designed, synthesized, and screened an anthraniloyl labeled cyclic peptide library in the positional scanning format using the imidazole-promoted cyclization method.

Received: September 12, 2012
Revised: October 18, 2012
Published: October 30, 2012

Scheme 1. Synthesis Approach for Anthraniloyl-Tagged Peptide



Scheme 2. Synthesis of Anthraniloyl-Labeled Cyclic Pentapeptide[a]



[a](1) Boc-AA−OH/PyBOP/DIEA; 55% TFA. (2) $Pd(PPh_3)_4/PhSiH_3$; 2-nitrobenzoic acid/DIC. (3) $SnCl_2$/DMF; 55% TFA. (4) HF (anhydrous)/anisole, 0 °C, 2 h. (5) 1.5 M imidazole (aq)/acetonitrile (1:7 v/v), r.t. 72 h.

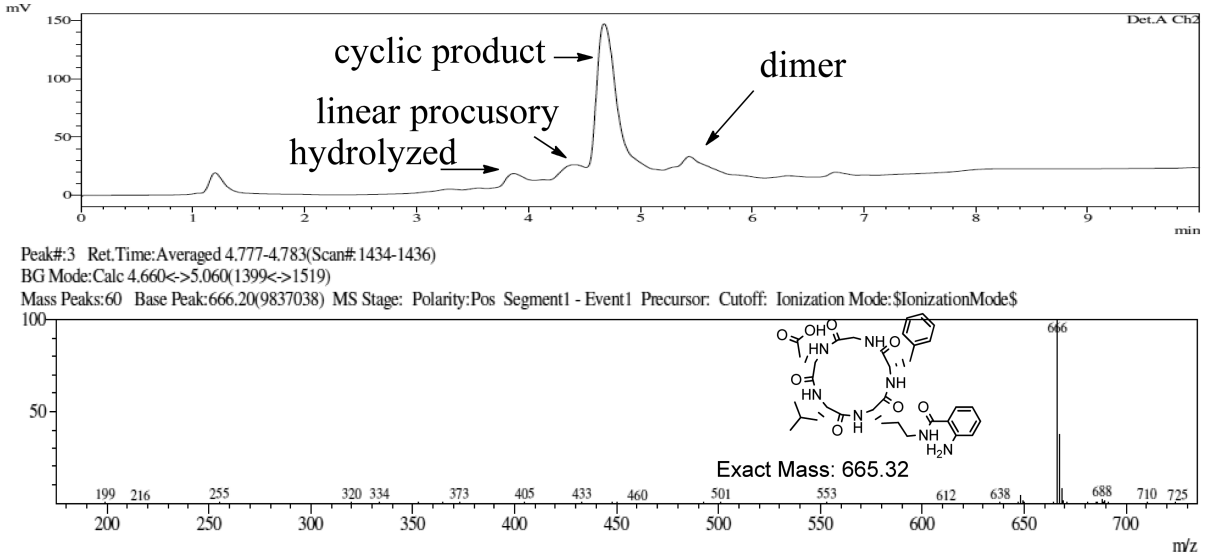


Figure 1. LC-MS of crude cyclic product cyclo[Asp-Leu-Orn(Ant)-Phe-Gly].

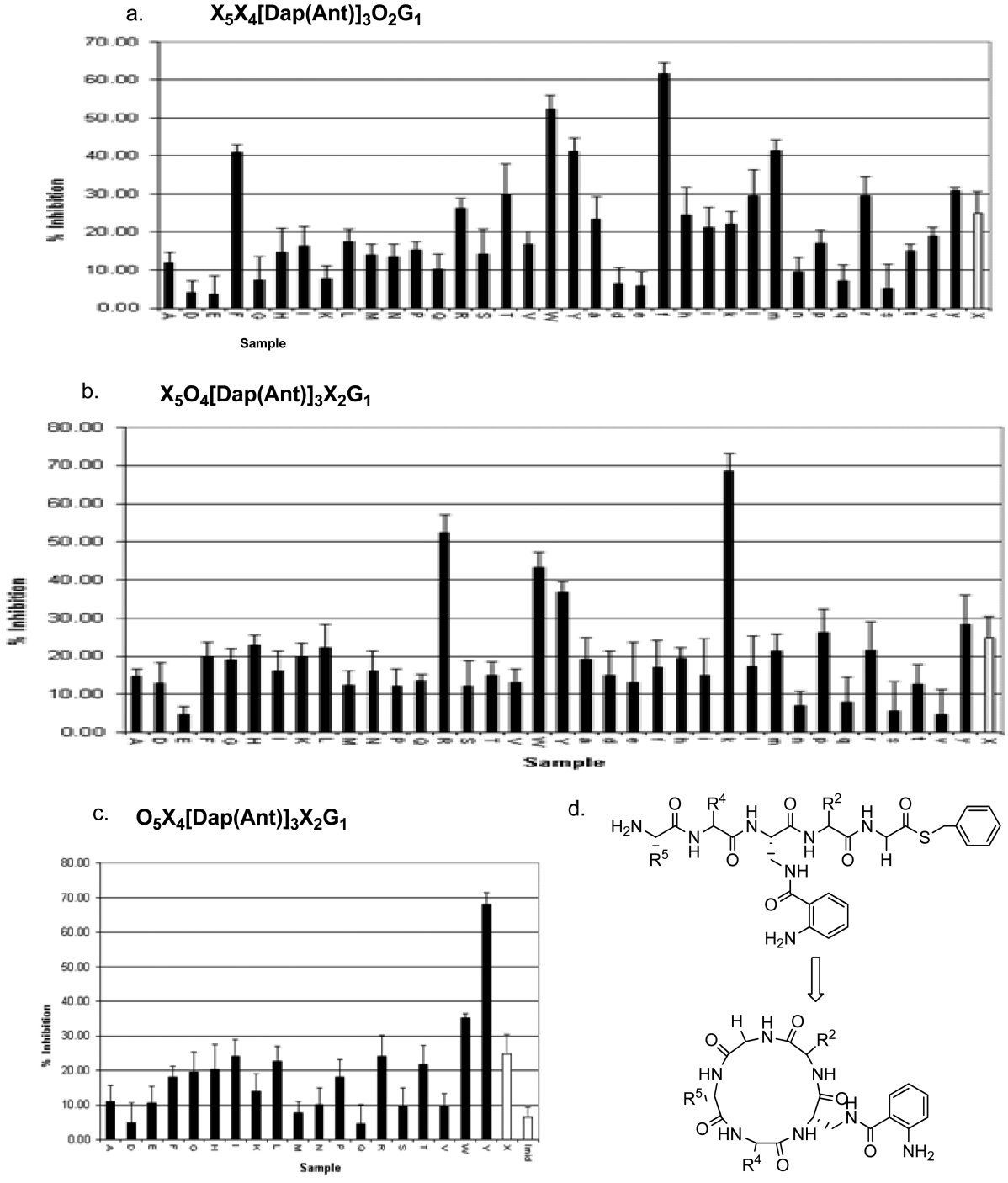
RESULTS AND DISCUSSION

Synthetic Approach to Anthraniloyl (Ant) Labeled Cyclic Penta-Peptide. The anthraniloyl group is a well characterized extrinsic fluorescent probe which has some important features such as a relatively high quantum yield at 415 nm (excitation at 330 nm) for its small size and hydrophilicity.[6] The anthraniloyl fluorophore is less likely to change the overall structural characteristics of a peptide and interfere with biological activity. Indeed in our previous studies we studied an N-terminal rhodamine-labeled tetrapeptide library. We found that the fluorescent moiety was an intrinsic component of the binding activity of the active compounds identified.[7] The synthetic strategy for the anthraniloyl-labeled peptide is illustrated in Scheme 1. The anthraniloyl group is generated by coupling of an *o*-nitrobenzoic acid to an amino group of a peptide, followed by reduction with tin chloride(II). The mild reduction in nearly neutral conditions is compatible with both Boc- and Fmoc- chemistry.[8]

A model cyclic peptide was used to optimize the synthetic approach. Synthesis of anthraniloyl-labeled *cyclo*[Asp-Leu-Orn(Ant)-Phe-Gly] is presented in Scheme 2. The anthraniloyl group was designed to attach to the third amino acid residue to limit its possible effect on cyclization. Boc-Gly, Phe, Orn-(Alloc), Leu, and Asp(OBzl) were subsequently coupled to the mercaptomethylphenyl-functionalized silica gel **1** developed in our lab as "volatilizable" support to obtain the resin bound

dx.doi.org/10.1021/co300110t | *ACS Comb. Sci.* 2012, 14, 673−679

Chart 1. Screening of the Cyclic Peptide PS-SCL for the Ability to Inhibit the Binding of Selective Radiolabeled [3H]DAMGO to the Mu Receptor[a]



[a]Each panel represents one of the three positional SCLs. Each bar within a panel represents percent inhibition by a cyclic peptide mixture defined in the O position. (a). Position 2; (b). Position 4; (c). Position 5 in a competitive radio receptor binding assay for the mu opioid receptor. (d). Cyclic peptide PS-SCL.

pentapeptide **2**.[9] After removal of the Alloc protecting group, 2-nitrobenzoic acid was coupled to the resin bound peptide **2** to yield the resin bound peptide **3**. The resin bound peptide **3** was then reduced in 2 M $SnCl_2$ to yield the resin bound anthraniloyl peptide **4**. The linear anthraniloyl peptide thioester **5** was obtained by the treatment of anhydrous HF at 0 °C for 1.5 h. Following removal of the HF with nitrogen stream and lypholization, the crude linear peptide was cyclized at a concentration of 1 mM in a mixed solution made up of acetonitrile and 1.5 M imidazole in water plus (7:1 v/v), forming the anthraniloyl-labeled fluorescent cyclic peptide **6**. Results from the LC-MS of the crude cyclization product are shown in Figure 1. The yield of the desired cyclic peptide was over 80%. There was less than 8% of the linear hydrolysis byproduct, 7% of the linear precursor, and 5% of the cyclic dimer.

dx.doi.org/10.1021/co300110t | ACS Comb. Sci. 2012, 14, 673−679

Table 1. Ki Values for Individual Compounds (Crude) from the Cyclic Peptide Library at Mu Receptor

#	R^2	R^4	R^5	Ki[a] /nM	Ki/26[b] nM	±STD[c] /26	#	R^2	R^4	R^5	Ki[a] /nM	Ki/26[b] /nM	±STD[c] /26
1	D-Phe	D-Lys	Tyr	1552	60	7	21	D-Met	Trp	Tyr	60535	2328	289
2	D-Phe	D-Lys	Trp	84860	3264	737	22	D-Met	Trp	Trp	34145	1313	94
3	D-Phe	Arg	Tyr	8803	339	43	23	D-Met	Tyr	Tyr	40500	1558	14
4	D-Phe	Arg	Trp	21910	843	217	24	D-Met	Tyr	Trp	168150	6467	2198
5	D-Phe	Trp	Tyr	30730	1182	195	25	Tyr	D-Lys	Tyr	463	18	1
6	D-Phe	Trp	Trp	n.a.	n.a.	n.a.	26	Tyr	D-Lys	Trp	5203	200	22
7	D-Phe	Tyr	Tyr	25360	975	477	27	Tyr	Arg	Tyr	24745	952	108
8	D-Phe	Tyr	Trp	n.a.	n.a.	n.a.	28	Tyr	Arg	Trp	n.a.	n.a.	n.a.
9	Trp	D-Lys	Tyr	673	26	2	29	Tyr	Trp	Tyr	n.a.	n.a.	n.a.
10	Trp	D-Lys	Trp	5525	212	14	30	Tyr	Trp	Trp	n.a.	n.a.	n.a.
11	Trp	Arg	Tyr	30995	807	36	31	Tyr	Tyr	Tyr	n.a.	n.a.	.a.
12	Trp	Arg	Trp	186500	7173	1887	32	Tyr	Tyr	Trp	n.a.	n.a.	n.a.
13	Trp	Trp	Tyr	54055	2079	130	33	Phe	D-Lys	Tyr	232	9	0.3
14	Trp	Trp	Trp	n.a.	n.a.	n.a.	34	Phe	D-Lys	Trp	7582	292	22
15	Trp	Tyr	Tyr	75970	2922	578	35	Phe	Arg	Tyr	89155	3429	1590
16	Trp	Tyr	Trp	46675	1795	253	36	Phe	Arg	Trp	27425	1055	195
17	D-Met	D-Lys	Tyr	4059	156	0.3	37	Phe	Trp	Tyr	n.a.	n.a.	n.a.
18	D-Met	D-Lys	Trp	49725	1912	260	38	Phe	Trp	Trp	n.a.	n.a.	n.a.
19	D-Met	Arg	Tyr	20755	798	108	39	Phe	Tyr	Tyr	6700	258	22
20	D-Met	Arg	Trp	35540	1367	231	40	Phe	Tyr	Trp	25120	966	224

[a]Measured Ki value of crude sample. [b]Adjusted Ki value of crude sample by using apparent concentration of peptide in the mixture. The apparent concentration of peptide was estimated using the mass ratio of peptide and imidazole in the mixture. [c]Adjusted standard error.

Synthesis and Deconvolution of a Positional Scanning Synthetic Combinatorial Library of Anthraniloyl-Labeled Cyclic Peptide. The synthetic strategy described above was applied to the construction of an anthraniloyl-labeled cyclic pentapeptide library in the positional scanning format.[10] Position 1 was fixed with glycine to ensure the efficient cyclization as determined in our previously study.[5] 2,3-Diaminopropionic acid was fixed at position 3. Its 3-amino was coupled with the fluorescent anthraniloyl-group. The library was composed of three sublibraries, in which each of the three positions 2, 4, or 5 were defined with either a single amino acid (O) or a mixture of amino acids (X). For each of the three subset mixtures the two remaining positions were made up of a mixture of amino acids (X). Position 2 and 4 contained 36 L- and D- amino acids; while position 5 contained 19 natural L- amino acids. Thus this positional scanning library was composed of 91 mixtures. Each mixture contained 684 head-to-tail penta-cyclic peptides at position 2 and 4 and 1296 penta-cyclic peptides at position 5. The library contains a total of 24,624 individual head-to-tail penta-cyclic peptides.

The anthraniloyl-labeled cyclic pentapeptide library was screened in a competitive radio receptor binding assay for the mu opioid receptor. Owing to the large excess of imidazole contained in each mixture, the effect of imidazole on the mu opioid receptor binding assay was tested before screening of the library. The result demonstrated that 1 mg/mL (14.7 mM) imidazole did not have a significant effect in the binding assay. Thus imidazole at this concentration exhibited negligible inhibition.

Each mixture in the library was then screened at a concentration of 1 mg/mL. The screening results for the mu selective binding assay are shown in Chart 1. Several mixtures at each position exhibited percent inhibitions above that of the all X mixture in which all cyclic peptides are present as a single mixture (all X, White bar labeled X). One mg/mL Imidazole was demonstrated not to have a significant effect in the binding assay, exhibiting negligible inhibition (White bar labeled Imid). When a cutoff value of 35% was used, the most active amino acids for the mixture making up position 2 were D-Phe, L-Trp, D-Met, L-Tyr, and L-Phe; at position 4 they were D-Lys, L-Arg, L-Trp, and L-Tyr; while L-Tyr and L-Trp were the two most active amino acids at position 5. Since the percent inhibitions observed were on the linear part of the competition curve (i.e., between 20 and 80% inhibition) IC_{50} values were not required in this instance to differentiate activities.

Individual Peptides from the Cyclic Library. A total of 40 individual cyclic peptides were synthesized in parallel by the combination of the most active amino acids identified at the three positions 5 × 4 × 2. The individual cyclic peptides were tested before purification in the binding assay for the μ-opioid receptor. The results of the 40 crude compounds are shown in Table 1. The molar ratio of imidazole and peptide in solution is 187:1 by comparing the concentration of imidazole (final 0.187 M) and peptide mixture (1 mM in total). A mass ratio of 25:1 is obtained by using an average molecular weight of 500 Da of the cyclic peptide. An adjusted Ki value by dividing by 26 is then calculated and listed to reflect the apparent concentration of cyclic peptide in the test samples.

Four individual cyclic peptides, #1, #9, #25, and # 33, exhibited significant mu receptor activity were selected for further purification. The four cyclic peptides have a similar sequence with D-Lys at position 4 and L-Tyr at position 5, differing only by the residue at position 2. Following removal of imidazole and other possible byproducts by HPLC, each purified cyclic peptide was tested in the binding assay for μ-opioid receptor. The results are shown in Table 2. The most active compound having L-Tyr at R^2, D-Lys at R^4, and Tyr at R^5 shows a Ki of 16 nM at the μ-opioid receptor.

Determination of the Structure of the Most Active Compound. The HPLC profile of the most active compounds actually showed two peaks having an identical mass weight that matched the desired cyclic product. This is caused by the head-to-tail and side chain-to-tail cyclization when R^4 is D-Lys (Scheme 3). Our previous study indicated that the imidazole-

Table 2. Binding Affinities of Active Compounds at Mu Receptor

peptide	R^1	R^2	R^3	R^4	R^5	K_i/ nM	STD
#1	Gly	D-Phe	Dap(Ant)	D-Lys	Tyr	144	37
#9	Gly	Trp	Dap(Ant)	D-Lys	Tyr	505	25
#25	Gly	Tyr	Dap(Ant)	D-Lys	Tyr	16	1
#33	Gly	Phe	Dap(Ant)	D-Lys	Tyr	20	1

promoted cyclization is not regioselective when Lys is an internal residue. Besides the cyclic isomers, the main product (impurity) was the linear peptide acid from hydrolytic side reaction. To determine the structure of the two cyclic isomers, they were isolated and reacted with 2,4-dinitro-1-fluorobenzene, separately. The two derivatives were hydrolyzed with 6 M HCl at 110 °C overnight. N^{ε}-(dinitrophenyl)-D-lysine was used as control to determine the cyclization pattern of the parent cyclic products. It was found that the side chain-to-tail cyclic peptide was eluted earlier than the head-to-tail cyclic peptide. These two isolated and structurally determined cyclic peptides were tested in binding assays for all three opioid receptors, mu, delta, and kappa. The results show that both cyclic isomers are selective for the μ-opioid receptor (Table 3); however, the side chain-to-tail cyclic isomer has a greater binding affinity (K*i* = 14 nM).

Cross-Oligomerization Control for Library Construction. Effective deconvolution of positional scanning synthetic combinatorial libraries relies on the precise construction of the library and the quality of the library components. For a cyclic peptide library, one of the major concerns is the possible side reactions of dimerization or oligomerization during cyclization. To check the possible extent of dimerization/oligomerization, a test sample containing two linear penta-peptide thioesters were mixed at a final concentration of 1 mM each (2 mM in total) to cyclize. Asp-Leu-Thr-Phe-Gly-SCH_2Ph and Asp-Leu-Val-Phe-Gly-SCH_2Ph were found to produce on intramolecular cyclization individually; no significant dimerization, oligomerization, and cross-oligomerization products could be identified by HPLC. Since the total concentration is 2-fold higher in the test sample than in each mixture in the library, we therefore assume that the oligomerization should not happen to each mixture in the library. Each linear peptide thioester in the mixture based library reacts individually to form cyclic monomer.

Even if dimers (or other side reaction) form to varying degrees, we test for activity in the mixtures and that information is used to make individual compounds. If a side reaction occurs, and it is fully responsible for the activity seen, then that side reaction becomes an interesting/desired product. While the goal is, of course, to cyclize 100% monomer, this is in realty highly unlikely. Since we are tracking the activity not the purity to make the individual compounds, as long as the side reaction is reproducible, mixture libraries should be deconvoluted successfully to identify the active compounds. It can be seen from this cyclic peptide library that the most active compound identified is the side-to-tail cyclic peptide, not the originally designed head-to-tail version.

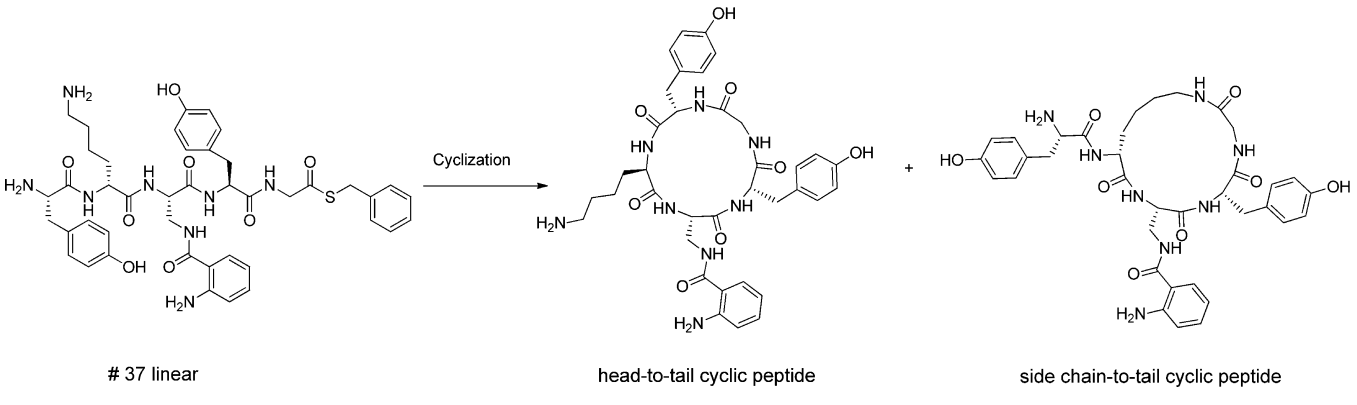
Table 3. Binding Affinities of cyclo[YkDap(Ant)YG] and Y-cyclo[kDap(Ant)YG] at Three Opioid Receptors

	K_i (nM)		
cyclization pattern	MOR	KOR	DOR
side chain-to-tail	14 ± 0.54	3231 ± 863	865 ± 424
head-to-tail	39 ± 1.06	3197 ± 129	1221 ± 278

Effect of Imidazole on Binding Assay and Removal of Imidazole. The cyclic peptide library contains a high ratio of imidazole to cyclizable starting material. The mole ratio of imidazole and peptide is 187:1 by comparing the concentration of imidazole and peptide mixture in the solution. Though imidazole is used as a biological buffer, imidazole at such high concentration may damage membranes and interfere with the binding assay. To explore the effect of high concentration of imidazole that may impose on the assay, imidazole at a concentration of 1 mg/mL has been tested in the binding assay. No significant effect was found on the binding of ^{3}H-DAMGO to the mu opioid receptor. Thus, the high ratio of imidazole in the mixtures will affect only the apparent concentration of each mixture, but not the affinity of the library components to the receptor.

Though imidazole did not appear to interfere with the screening results in this assay, the removal of imidazole has been investigated by sublimation under high vacuum (approximately 100 mTorr). Imidazole itself was removed quantitatively after sublimation for 3 days at 40 °C. A test sample containing a mixture of imidazole and a peptide, CH_3CO-His-Phe-Arg-Trp-Gly-NH_2, at a molar ratio of 80:1 was treated under the described sublimation conditions. Only 90% of imidazole in the mixture was readily removed (assuming that the loss of mass was resulted only by sublimation of imidazole). The remaining 10% of the imidazole could not be sublimed, leading to a mixture of imidazole and peptide at a molar ratio of 8:1. To check the structural consistency of the

Scheme 3. Two Possible Isomers of the Most Active Cyclic Peptide



dx.doi.org/10.1021/co300110t | ACS Comb. Sci. 2012, 14, 673−679

peptide during sublimation, LC-MS and ^{1}H NMR spectra of the samples before and after sublimation were recorded. LC-MS data showed the peptide in both samples had the same retention time and mass weight, indicating that the peptide kept unchanged during sublimation. Though the chemical shifts of the amide protons of the peptide in the sublimated sample slightly moved upfield in DMSO-d6 compared with pure peptide, the sublimated sample and a fresh-mixed sample containing a mixture of imidazole and the peptide at a molar ratio of 8:1 had nearly identical ^{1}H NMR spectra. Both LC-MS and ^{1}H NMR experiments supported that the peptide remained unchanged under the sublimation conditions.

CONCLUSION

A method has been developed to construct the positional scanning cyclic peptide combinatorial library by using our "volatilizable" resin and imidazole-promoted cyclization strategy. A positional scan formatted penta-cyclic peptide library has been synthesized and screened. Deconvolution of the library identified potent individual fluorescent ligands for the mu opioid receptor. The most active compound has been found to have Tyr at R^2, D-Lys at R^4, and Tyr at R^5. Further study on this compound revealed that the side chain-to-tail cyclic isomer had greater binding affinity than the head-to-tail isomer. Because of the minimal side reactions leading to oligomerization this method is promising for the construction of larger cyclic peptide libraries having greater structural diversities. The power to synthesize and screen libraries of cyclic peptides will have a dramatic impact on the identification of biologically active cyclic peptides for drug discovery.

EXPERIMENTAL PROCEDURES

Cyclic Peptide Synthesis. All linear peptide thioesters were synthesized by solid-phase synthetic method using the "tea-bag" approach. Functionalized mercaptomethylphenyl-silica gel was synthesized and used as "volatilizable" support as reported elsewhere.[9] A 100 mg portion of functionalized silica gel was sealed in each polypropylene tea bag. Boc amino acids activated with PyBOP/DIEA in dimethylformamide (DMF) were used in the peptide couplings. X-positions were coupled as mixtures of N^{α}-Boc protected amino acids using concertration ratios to compensate for the relative reaction rates in competitive couplings. Position 3 was coupled with a N^{α}-Boc-N^{β}-Alloc-Diaminoproponic acid. After peptide elongation on resin, the Boc-protected peptides were treated with $Pd(PPh_3)_4$ (0.1 equiv) in the presence of $PhSiH_3$ (20 equiv) in dichloromethane to remove the Alloc protection group. The resin bound peptides were coupled with 2-nitrobenzoic acid, following by the treatment of 2 M $SnCl_2$ in DMF overnight to reduce the nitro group to an amino group to generate the anthraniloyl-label. After removal of the Boc group with 55% TFA, the resin bound anthraniloyl-labeled peptides were treated with anhydrous HF in the presence of 5% anisole at 0 °C for 2 h. After removal of the HF with nitrogen stream and lypholization, the linear peptide thioesters were cyclized in a mixed solution of 1.5 M imidazole (aq.) and acetonitrile (1:7 v/v) at a concentration of 1 mM for 72 h, forming the anthraniloyl-labeled fluorescent cyclic peptides.

Determination of Structure of Most Active Compound—Preparation of N^{ε}-(dinitrophenyl)-D-lysine. Fmoc-D-Lys(Boc)−OH (47.5 mg, 0.1 mmol) was dissolved in 55% TFA in DCM for 30 min to remove the Boc protection group. After removal of the solvent in vacuo, 34 μL of DIEA in 1 mL of DCM was added. To the solution was added 2,4-dinitro-1-fluorobenzene (13 μL, 0.1 mmol). The mixture was reacted at room temperature for 10 min. After removal of the solvent in vacuo, the residue was treated with 2 mL of 20% piperidine in DMF for 10 min. The product was extracted with 25% acetonitrile in water with 10% TFA and purified by preparative HPLC and confirmed by ESI-MS (found 313.1, calculated for $[M + H]^+$ 313.1).

Determination of the Structure of the Most Active Compound. The two cyclization isomers of compound #25 were isolated by semipreparative RP-HPLC using H_2O (0.1% formic acid) and CH_3CN (0.1% formic acid) with a gradient ranging from 2 to 60% in 30 min. Two milligrams of each purified cyclic isomers were dissolved in 1 mL of DMF and then treated with 17 μL of DIEA and 13 μL of 2, 4-dinitro-1-fluorobenzene at room temperature for 10 min. After removal of the solvent in vacuo, the samples were hydrolyzed with 6 M HCl at 110 °C overnight. The hydrolysates were analyzed by LC-MS. N^{ε}-(dinitrophenyl)-D-lysine was used as control to determine its presence of the digestion product.

Opioid Binding Assay. Membrane suspensions were prepared and used on the same day. Rat brains, minus the cerebellum, were homogenized using 50 mM Tris-HCl, pH 7.4, centrifuged and rewashed. Each assay tube contained 0.5 mL of membrane suspension, 1.9 nM competitor [^{3}H]-DAMGO, 1 mg/mL mixture, and 50 mM Tris-HCl in a final volume of 0.65 mL. The assay was incubated for 1.5 h at room temperature, and the reactions were terminated by filtration through GF-B filters. Bound radioactivity was counted on a betaplate scintillation counter.

ASSOCIATED CONTENT

Supporting Information

Experimental detail, compound characterization, copies of ^{1}H NMR, ^{13}C NMR spectra and LC-MS for the most active compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by the State of Florida, Executive Officer of the Governor's Office of Tourism, Trade and Economic Development, National Science Foundation (R.A.H. CHE 0455072), the National Institute on Drug Abuse (DA031370), and NIH (R03DA025850).

Notes

The authors declare no competing financial interest.

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