Bioorganic & Medicinal Chemistry 17 (2009) 6742–6747

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Identification of new agonists of urotensin-II from a cyclic peptide library

Takenao Odagami [a], Yuko Tsuda [b,c,*], Yuji Kogami [a], Hiroyuki Kouji [a], Yoshio Okada [b]

[a] Research and Development, PRISM Bio. Lab. Corporation. 4259-3, Nagatsuta-cho, Midori-ku, Yokohama 226-8510, Japan
[b] Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Minatojima, 1-1-3, Chuo-ku, Kobe 650-8586, Japan
[c] Cooperative Research Center of Life Sciences, Kobe Gakuin University, Minatojima, 1-1-3, Chuo-ku, Kobe 650-8586, Japan

ARTICLE INFO

Article history:
Received 13 June 2009
Revised 17 July 2009
Accepted 21 July 2009
Available online 28 July 2009

Keywords:
Cyclic hexapeptide library
Lactam bridge
Agonist for urotensin-II receptor
GPR-14

ABSTRACT

Urotensin-II (UT-II) is thought to be involved in the regulation of cardiovascular homeostasis and pathology. A head-to-tail cyclic hexapeptide library based on UT-II sequence was designed, synthesized, and evaluated by the activity on the UT-II receptor (GPR-14). A new synthetic sequence, WK[Xaa] (Xaa: amino acid with aromatic side chain), was identified as a characteristic minimum fragment activating hUT-II receptor instead of the WK[Y] sequence. Compound **1** showed an agonistic activity with an EC_{50} value of 6.94 nM. The conformational investigation suggested that **1** did not have typical secondary structure in the message sequence. Structural analyses may enable us to investigate the active conformation of UT-II and lead to the identification of new ligands for GPR-14.

1. Introduction

Human urotensin-II (hUT-II: H-Glu-Thr-Pro-Asp-*c*[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH) is a cyclic undecapeptide having potent vasoactive effects.[1] Although UT-II had originally isolated from the fish neurosecretory system,[2] the cDNA encoding its precursor has been sequenced from many sources.[3,4] Ames et al.[5] showed that UT-II is the endogenous ligand of a new human G-protein-coupled receptor (GPCR), which possesses homologous sequence to the GPR-14, orphan receptor identified in rat[5] and currently referred to as the UT-II receptor. Following those observations, an increasing number of biological studies suggest that hUT-II is involved in the regulation of cardiovascular homeostasis and pathology,[1,6,7] and the UT-II system offers a great potential for novel therapeutic applications related to the treatment of cardiovascular diseases.

UT-II assumes various forms within the variation of its primary structure in the N-terminal domain;[8] however, the C-terminal cyclic hexapeptide (*c*[CFWKYC]) [UT-II(5–10)] is conserved across species. Flohr et al.[9] showed that UT-II(5–10) is the minimal sequence required to retain full agonist activity at the hUT-II receptor, although UT-II(5–10) exhibited about 1000-fold lower activity as compared to full-length UT-II in pharmacology studies. Further structure–activity relationship studies[9] suggested that the WKY [Trp^7-Lys^8-Tyr^9] motif is the most important sequence for full agonist activity of hUT-II.

With the aim to obtain a more stable moiety, the replacement of the disulfide bridge by a side chain to side chain lactam bridge has been reported in several biologically relevant peptides, such as endothelin-1[10,11] and somatostatin analogs.[12] The replacement of the disulfide bridge of UT-II analogs by the lactam bridge has been performed previously, but the most active peptide obtained, H-Asp-*c*[Orn-Phe-Trp-Lys-Tyr-Asp]-Val-OH,[13] was about 100-fold less potent than hUT-II itself. However, cyclic peptides are important targets for drug discovery because of their interesting biological properties. For example, constraining highly flexible linear peptides by cyclization is one of the most commonly used approaches to define the bioactive conformation of peptides. Furthermore, β-turn structures play an important role in the ligand–receptor interaction of many hormone peptides.[14] In many cases, cyclic peptides often show increased receptor affinity and metabolic stability compared to their linear counterparts. Based on UT-II and somatostatin sequence similarities, GlaxoSmithKline identified SB-710411,[15] GSK248451,[16] and BIM-23127[17] as UT receptor antagonists. It is interesting that diverse β-turn sequences from peptide hormones can be utilized for UT-II ligand identification. The design of UT-II lactam analogs might be a challenging method in order to develop more stable UT-II agonists or antagonists. In the present study, we describe an approach for designing and screening the biological activities of the cyclic hexapeptide library containing a 'head-to-tail' lactam bridge that is targeted to GPR-14. After identification of the peptides with agonist activity from the cyclic peptide library, the conformational properties of the agonists were investigated.

2. Rationale

The preparation of cyclic peptide library, being an important object in peptide chemistry due to its interesting biological proper-

* Corresponding author. Tel.: +81 78 974 1551; fax: +81 78 974 4661.
E-mail address: tsuda@pharm.kobegakuin.ac.jp (Y. Tsuda).

0968-0896/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.07.058

ties, we designed and synthesized a head-to-tail cyclic peptide library. Generally three procedures are applied for the head-to-tail cyclization. The first is a classical cyclization of a linear peptide released from a protected peptide resin. The second is cyclization on a resin,[18] in which a peptide anchored via side chain functional group such as acid,[19] amine,[20] alcohol,[21] or imidazol,[22] while the C-terminus of peptide is orthogonally protected by an ester. The protected peptide is elongated by ordinary Boc or Fmoc synthesis followed by saponification, cyclization, and cleavage. The third method is a cleavage–cyclization approach. An advantage of this procedure is that cyclization occurs as soon as cleavage of N-terminal protecting group is carried out. In the Boc/benzyl protocol, either the Kaiser oxime[23] resin or thioester[24] resin was employed. In those cases, the linker itself was so active that an unexpected cyclization often occurred during elongation of peptide backbone. Additionally, the cleavage required the treatment with strong acid such as hydrogen fluoride (HF) or trifluoromethanesulfonic acid (TFMSA). Instead of the Boc/benzyl procedure, Fmoc/Boc method is sufficiently more applicable to the synthesis of this peptide library using Kenner's 'safety catch' sulfonamide linker.[25] The sulfonamide linker is stable to nucleophilic attack in piperidine which was used in Fmoc deprotection and activated for nucleophilic displacement by treating with diazomethane or iodoacetonitrile. This results in the formation of *N*-alkyl-*N*-acylsulfonamide which can be cleaved with a primary amine to yield a cyclic peptide.

2.1. Design

Our cyclic peptide library has general sequence *c*[Gly-Xaa_1-Xaa_2-Xaa_3-Xaa_4-Pro] (Xaa_1-Xaa_4 represents the variable residues), where the cyclic peptides in place of Cys^5-Cys^{10} disulfide bridge have the structure Pro-Gly that generated head-to-tail *c*[GFWKYP] and some other analogs. Each of the variable amino acids was selected from their frequency in β turn sequences in peptide hormones: Xaa_1 contained three amino acids [Phe, Ser, Tyr], Xaa_2 was four amino acids [Phe, Ser, Trp, Tyr], Xaa_3 had five amino acids [Arg, Gln, Leu, Lys, Phe], and Xaa_4 revealed six amino acids [Arg, Asn, Asp, Phe, Trp, Tyr], thereby yielding 360 individual peptides. With the aim of elucidating the active conformation of hUT-II, our goal was to examine the sequence dependency and conformational effects on GPR-14 activation using our cyclic peptide scaffold, particularly the residue sequence where some combination of Phe^6, Trp^7, Lys^8 and Tyr^9 are present.

2.2. Synthesis

Cyclic peptide library was prepared on Sulfamylbutyryl AM resin (purchased from NOVA biochem, 1.06 mmol/g, 300 mg, 0.32 mmol) using standard solid-phase 9-fluorenylmethoxy carbonyl (Fmoc) based procedures. Initially, the Fmoc-glycine was loaded to the resin by using *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium haxafluorophosphate (HATU)/1-hydroxy-7-azabenzotriazole (HOAt) procedure. The Fmoc group was deprotected by piperidine, then the Fmoc-proline was coupled by the 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) procedure. Further extension of the peptide was accomplished using standard Fmoc strategy as described previously.[26] After elongation of the linear peptide, the N-terminal Fmoc was replaced by trityl (Trt) group. The safety-catch linker was activated by alkylation with iodoacetonitrile. Then Trt group was deprotected with diluted trifluoroacetic acid (TFA) and cyclization was immediately achieved. Finally, the side chain protecting group was deprotected and cyclic peptide was obtained by ether precipitation.

3. Results and discussion

3.1. Synthesis and evaluation of the peptide library

The protected linear peptides were prepared on the sulfamylbutyryl resin (the so-called safety-catch resin) using standard solid-phase procedures based on Fmoc chemistry (see Fig. 2). Fmoc-Gly-OH was coupled to the resin using HATU/ HOAt /*N*,*N*-diisopropyl-ethylamine (DIEPA) procedure. After attachment Fmoc group was removed with 20% piperidine in dimethylform-amide (DMF). Then Fmoc-Xaa-OH was coupled using HBTU/HOBt/DIEPA procedure to elongate the protected peptide chain on the resin. The amino acid side chain protection groups were as follows: *t*-butyl (*t*Bu) for Tyr, Ser, and Asp; *t*-butoxycarbonyl (Boc) for Trp and Lys; 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl (Pbf) for Arg; Trt for Gln and Asn. After elongation of the linear peptide, the N-terminal Fmoc group was replaced with Trt by following procedures: (1) 20% piperidine in DMF and (2) trityl chloride (Trt-Cl) and DIEPA in DMF. The safety-catch linker was activated with iodoacetonitrile. After activation, the N-terminal Trt group was removed by the treatment of TFA and triisopropylsilane (TIS) in dichloromethane (DCM) (1:5:94). The generating primary amine, cleaved the activated linker in the presence of DIEPA in DMF to provide the head-to-tail cyclic peptide. The side-chain protected cyclic peptide was dissolved in DCM, and the organic solution was washed with 10% citric acid followed by 5% sodium bicarbonate. The organic layer was concentrated in vacuo. The residue was then exposed to the cocktail of TIS, 1,2-ethanedithiol (EDT), H_2O and TFA (5:2.5:5:87.5) then precipitated with ether to yield the crude cyclic hexapeptide.

To assess the quality of the library, crude peptides were analyzed by LC/MS. Among them, 291 peptides were obtained with good purity (>80%), while 40 peptides were obtained with moderate purity (60–80%) and 29 peptides had low purity (<60%). The crude peptides were applied to preliminary screening without further purification. Eight crude peptides which had strong agonistic activity were purified by reverse phase HPLC using a Develosil C30 column (20 × 150 mm). The purity and physicochemical properties of the purified peptides were assessed by LC/MS and HRMS (see Table 1).

3.2. Calcium mobilizing activity

The biological activity of all the compounds was evaluated by calcium mobilization assay for the hUT-II receptor; the activity was calculated from the titration curve of calcium flux and the primary screening was performed at 1 μM concentration. Primary screening results are shown in Figure 1. The eight crude peptides with the strongest agonistic activities were purified by reversed phase HPLC to give compounds **1**–**8** and their EC_{50} values are summarized in Table 2. The cyclic peptides possess UT-II agonist properties not antagonism, as measured by their ability to block the response induced by UT-II (data not shown).

All endogenous UT-II peptides contain the hexapeptide sequence *c*[CFWKYC], which has been identified as the minimal peptide sequence crucial for hUT-II receptor (GPR-14) activation. In a previous study, side-chain lactam bridges of different lengths were introduced to replace the disulfide bridge in the active fragment of hUT-II, that is hUT-II (4–11).[13] The length of the lactam bridge was continuously modulated between 20 and 24 atoms by side-chain to side-chain cyclization and the length of the main-chain was changed to 18 atoms by insertion of Pro-Gly instead of Cys^5-Cys^{10}. Interestingly, the functional assay data shown in Table 2 indicated that compound **1** was five to six times less potent than hUT-II; EC_{50} value of compound **1** (*c*[GFWKYP]) is 6.9 nM. This sug-

Table 1
Analytical data for the purified cyclic peptides

Compound	Structure	Molecular formula	HPLC min[a]	MS (*m*/*z*)	HRMS (*m*/*z*)	
				Found	Found	Calcd
1	*c*(GFWKYP)	$C_{42}H_{51}N_8O_7$	3.27	779.93[M+1]$^+$	779.3889[M+1]$^+$	779.388[M+1]$^+$
2	*c*(GSWKYP)	$C_{36}H_{47}N_8O_8$	2.94	719.65[M+1]$^+$	719.3519[M+1]$^+$	719.3516[M+1]$^+$
3	*c*(GYWKYP)	$C_{42}H_{51}N_8O_8$	3.05	795.72[M+1]$^+$	795.3843[M+1]$^+$	795.3829[M+1]$^+$
4	*c*(GFWKFP)	$C_{42}H_{51}N_8O_6$	3.38	763.7[M+1]$^+$	763.3939[M+1]$^+$	763.3931[M+1]$^+$
5	*c*(GSWKFP)	$C_{36}H_{47}N_8O_7$	3.01	703.64[M+1]$^+$	703.3548[M+1]$^+$	703.3567[M+1]$^+$
6	*c*(GYWKFP)	$C_{42}H_{51}N_8O_7$	3.23	779.87[M+1]$^+$	779.3869[M+1]$^+$	779.388[M+1]$^+$
7	*c*(GFWKWP)	$C_{44}H_{52}N_9O_6$	3.35	802.78[M+1]$^+$	802.4021[M+1]$^+$	802.404[M+1]$^+$
8	*c*(GSWKWP)	$C_{38}H_{48}N_9O_7$	3.05	742.71[M+1]$^+$	742.3654[M+1]$^+$	742.3676[M+1]$^+$

[a] HPLC retention time.

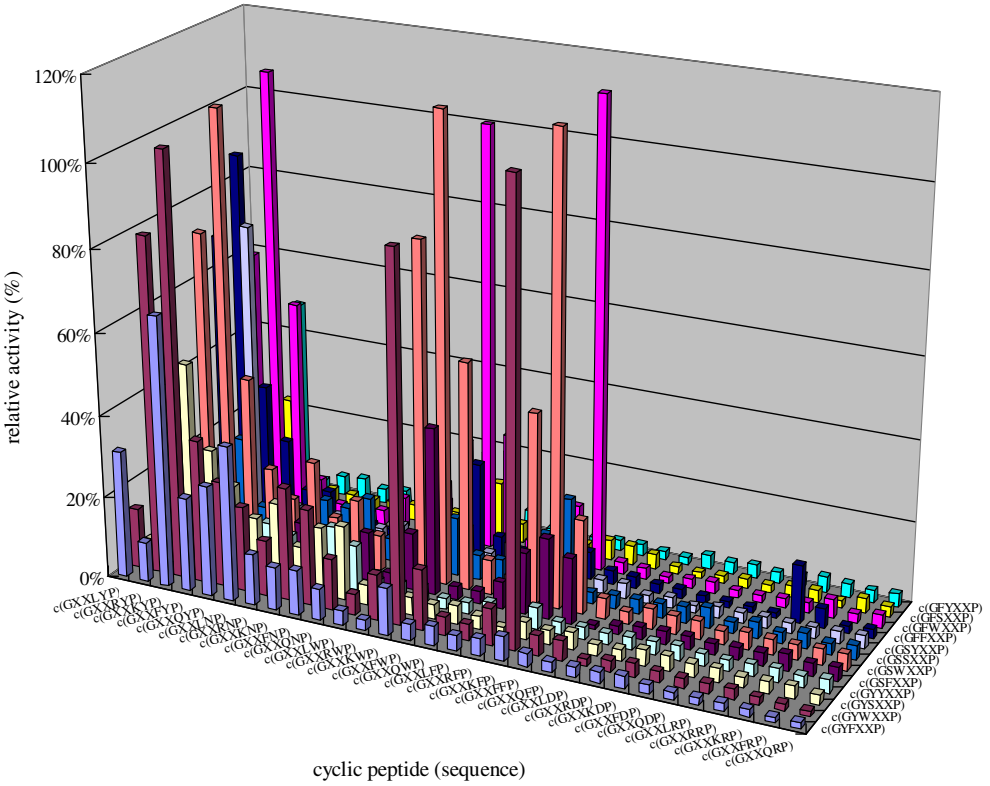

Figure 1. Primary screening result of cyclic peptide library. Relative activity was calculated from full agonistic activity of UTII.

gested that compound **1** still exhibited activity in the nM range, meaning that the insertion of a Pro-Gly sequence instead of Cys[5]-Cys[10] disulfide bridge is almost as tolerable as hUT-II for activation of the hUT-II receptor (GPR-14). Flohr et al.[9] reported a detailed structure–activity relationship study of hUT-II showing that the WKY sequence in the cyclic portion of the peptide is the most important for full agonist activity of hUT-II, whereas Phe[6] played only a minor role. In the present study, compounds **1**, **2**, and **3** gave full agonistic activity, although the potency was weaker than **1**. In agreement with these results, Phe[6] can be replaced with Tyr or Ser without losing considerable activity. These endocyclic modification results are similar to disulfide bridge analogs that were reported by Flor et al.[9] and Brkovic et al.[27] The EC_{50} values of **4** (*c*[GFWKFP]) and **7** (*c*[GFWKWP]) were 13.2 and 20.5 nM, respectively. The replacement of Tyr with Phe or Trp did not drastically decrease the hUT-II receptor activation, while that with Arg, Asp, or Asn completely diminished the agonistic activity as seen in the primary screening; namely, WK[Xaa] (Xaa: amino acid with aromatic side chain) is a characteristic minimum fragment of hUT-II necessary for activation instead of the WK[Y] motif, whereas the evolutionarily conserved hydroxy moiety of Tyr only plays a minor role. Furthermore, the aromatic ring of Tyr can replace with bicyclic portion of Trp. Kinney et al. [28] reported that the introduction of 1-naphthyl-L-alanine led to a potency comparable to that of the native Tyr residue based on the calcium flux assay. These results evidently suggested that the position of Tyr could accept a larger hydrophobic moiety.

3.3. Conformational study

Itoh et al. reported[29] UT-II SAR studies, which indicated that the C-terminal octapeptide UT-II (4–11) retained full agonist activity in rat aorta bioassays. These observations have been subsequently extended to hUT-II. The first structure–activity studies on human UT-II indicated the highly conserved cyclic portion of the molecule was essential for biological activity. Insertion of a penicillamine (Pen) residue in place of Cys[5,11] generated a 10 times more potent UT-II analog.[30] The NMR analyses suggested that the enhancement of the potency was ascribed to an increase in the population of the bioactive conformations which were induced by the replacement

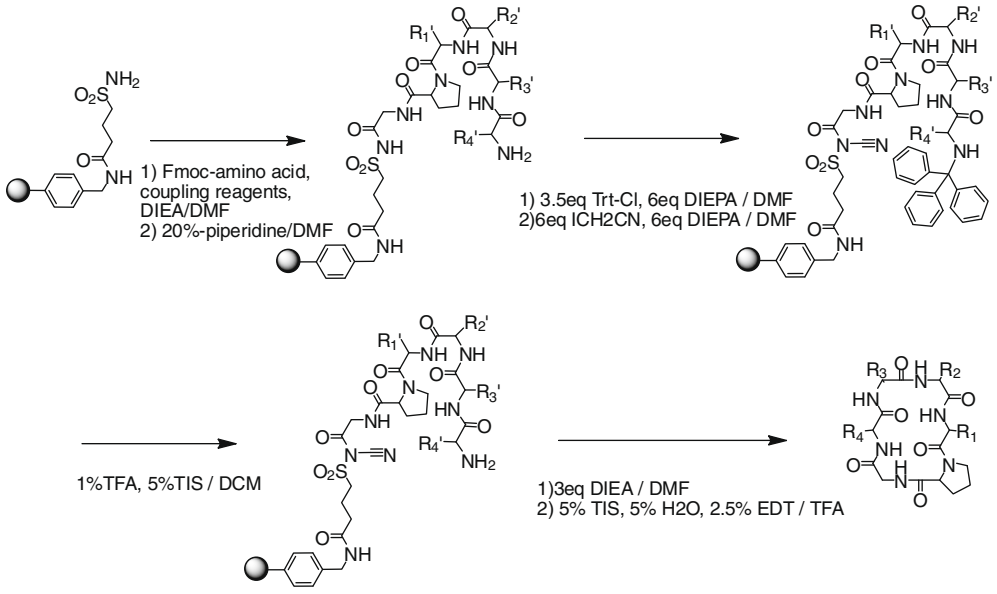

Figure 2. Synthetic scheme for cyclic peptides. R1′–R4′ represent protected amino acid side chain. R1–R4 represents amino acid side chain.

Table 2
Biological activity of purified cyclic peptides

Compounds	Structure	EC_{50}[a] (nm)	E_{max}[b] (%)
hUT-II	ETPD*c*(CFWKYC)V	1.09 ± 0.12	100
1	*c*(GFWKYP)	6.94 ± 0.18	98
2	*c*(GSWKYP)	8.51 ± 0.13	108
3	*c*(GYWKYP)	12.58 ± 0.14	86
4	*c*(GFWKFP)	13.2 ± 0.12	101
5	*c*(GSWKFP)	10.07 ± 0.14	96
6	*c*(GYWKFP)	75.66 ± 0.11	102
7	*c*(GFWKWP)	20.55 ± 0.1	92
8	*c*(GSWKWP)	16.72 ± 0.12	100

[a] Each value is the mean ± SD of at least four determinations.
[b] % of maximal agonistic effect.

of Pen.[30] Additionally, the changes of the distance between the primary amine function present at the side chain of Lys^8 and the peptide backbone might modulate both the efficacy and the potency of UT-II.[31] In particular the substitution of Lys^8 by Orn generated the first UT receptor partial agonist, [Orn^8]UT-II,[32] whose potency was later increased by the replacement of Cys^5 with Pen.[33] The insertion of D-Trp in position 7 as in [Pen^5, D-Trp^7, Orn^8]UT-II(4–11) (urantide) produced an increase in potency associated with elimination of efficacy in the rat aorta bioassay,[33] while in a calcium mobilization assay using cells expressing the human recombinant UT-II receptor, urantide behaved as a partial agonist.[34,35] The substitution of Orn with Dab led to the identification of the compound [Pen^5, D-Trp^7, Dab^8]UT-II(4–11), named UFP-803, which behaved as a UT-II receptor antagonist with negligible residual agonist activity even in cells expressing the recombinant rat and human UT-II receptor.[35] Recently, it has been demonstrated that it is possible to reduce peptide efficacy by replacing Phe^6 with cyclohexylalanine (Cha),[36] however, that compound, [Cha^6]UT-II(4–11), was only evaluated in the rat aorta bioassay.

3.3.1. NMR study

To explore the importance of the conformation of the message sequence, we examined the structure of **1** (*c*[GFWKYP]) using NMR spectroscopy and Monte-Carlo simulation. A qualitative analysis of short- and medium-range NOEs, was used to characterize the secondary structure of **1**. From the NOESY spectra a total of 37 NOEs were collected (14 intra residual, 17 sequential, and 6 medium range). From a qualitative evaluation of the NOE connectivities (Fig. 3), the presence of a turn encompassing residues GFWK is suggested by a weak αH_i-NH_{i+2} connectivity between Gly and Trp, and between Phe and Lys. Unfortunately, MD simulations based on NMR-derived constraints did not yield sufficient results (data not shown).

3.3.2. Monte-Carlo calculations

Therefore, we examined **1** by Monte-Carlo simulations using the BATCHMIN program with MMFF94 force field. Energy minimizations were performed in vacuum. A 100,000 step Monte-Carlo search was performed with rmsd of 0.25 Å for all heavy atoms and energy distance within 25 kJ from the global minimum. Total number of conformational families as result of the clustering run was 525. Number of conformational families with populations above 0.20% (#*F* 0.20%) was 33. Sum of the percent relative population of #*F* 0.20% was 91.20%. The number of conformers with the energy distance within 10 kJ from the global minimum was 15. Sum of the low energy of 15 conformers with accumulated population was 85.09%. The results of the calculations are shown in Figure 5. Figure 4 shows the Ramachandran plots obtained from the Monte-Carlo simulation of **1**. Gly with a torsion angle

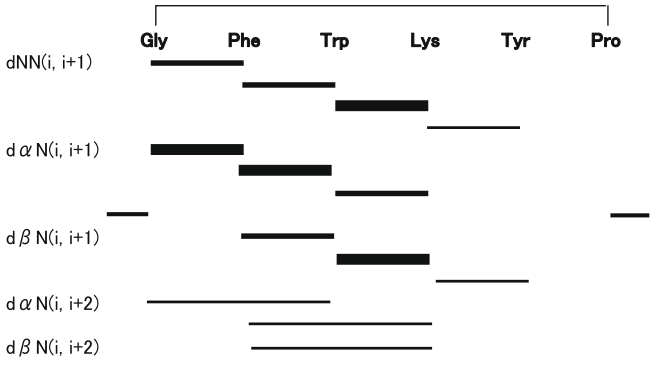

Figure 3. Summary of NMR derived data of compound **1**. Schematic bar diagrams show the NOE connectivities observed in the NOESY spectra. Thickness of the bars is related to the NOE intensities.

$93 < \varphi < 165$, $-34 < \psi < 9$ was considered to be in the β-turn conformation: Phe with a torsion angle $-162 < \varphi < -158$, $49 < \psi < 67$ is in the β-strand conformation; Trp with a torsion angle $66 < \varphi < 76$, $-81 < \psi < -59$ is in the γ-turn conformation; Lys with a torsion angle $-89 < \varphi < -77$, $-45 < \psi < 0$ is in the α-helical conformation; Tyr with torsion angle $-153 < \varphi < -92$, $159 < \psi < 165$ is in the β-strand conformation; and proline with a torsion angle $-75 < \varphi < -65$, $82 < \psi < 111$ is in the β-strand conformation. These results suggested that Phe-Trp-Lys-Tyr forms a distorted turn structure. Distance between Hα of Gly and NH of Trp Hα of Phe and NH of Lys were $4.4 < d < 5.1$and $4.6 < d < 4.8$, respectively. These results were in accordance with NMR study. In the case of head-to-tail cyclized analog of UT-II (**1**), no standard pattern of secondary structure was observed in message sequence (FWKY). This is in line with previous investigation on hUT-II.[9,30] The aromatic residues region (Phe, Trp, and Tyr) are aligned toward one side of the molecule to form a hydrophobic cluster. The calculated distances among the Trp-, Lys-, Tyr-residues of **1** are shown in Figure 6; the distances between Trp and Lys, Lys and Tyr, and Lys and Tyr are 12.57, 4.34, and 10.39 Å, respectively. On the other hand, Flohr et al.[9] demonstrated the distances between Trp and Lys, Lys and Tyr, and Lys and Tyr were 11.3, 6.4, and 12.2 Å, respectively. These differences in the distance between the pharmacophoric centers may arise from the replacement of the disulfide bridge by the head-to-tail lactam bridge, and well be responsible for the lower activity of **1** relative to UT-II. However, the fact that **1** still had low nM level activity is significant.

4. Conclusion

We reported on the design and synthesis of a cyclic peptide library and SARs of a new series of head to tail cyclized UT-II analogs that act as GPR-14 receptor agonists. Compound **1**, identified as replacing a disulfide bridge of UT-II(5–10) with Pro-Gly head to tail cyclization, had a low nM agonistic activity for the GPR-14 receptor. Structural conversion led to the identification of the SAR for the FWKY sequence. In accordance with previous studies, WKY was identified to most important sequence for full agonistic activity; however, WK(AR) still maintained moderate activity. We also investigated the conformation of **1** by NMR and Monte-Carlo simulation that suggested side chain conformation was slightly different from that previously reported.[9] This difference may well responsible for the lower activity determined for **1**. Moreover the identification of new chemo-type head-to-tail cyclized analog **1** and its structural analysis may well enable us to investigate the active conformation of UT-II that could lead to the identification of new non-peptide ligands for GPR-14.

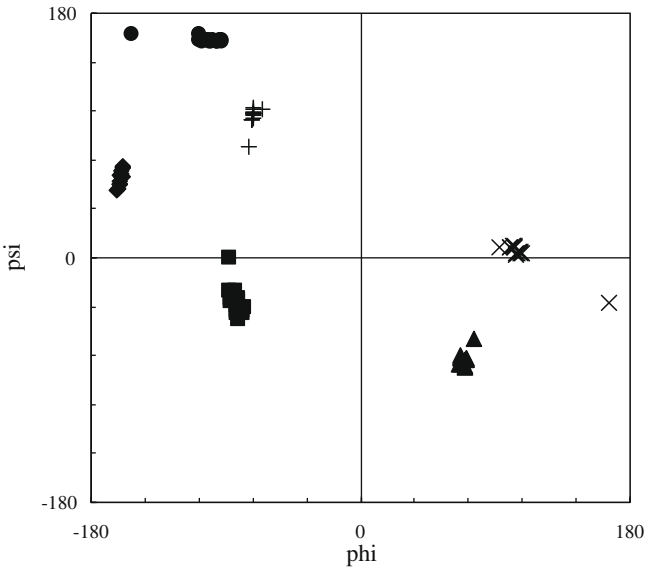

Figure 4. Ramachandran plots for lowest energy 15 conformer of **1**. Lowest energy15 conformers have relative calculated energy distance within 10 kJ from global minimum and accumulated population is more than 85%. φ and ψ angles plots of amino acids in **1**: ×, Gly; ♦, Phe; ▲, Trp; ■, Lys; ●, Tyr; +, Pro.

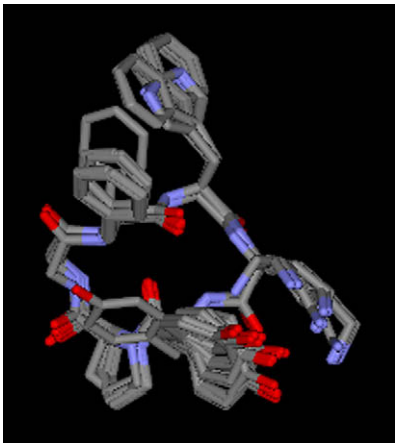

Figure 5. Superimposition of the lowest energy 15 conformers. Lowest energy for 15 conformers have relative calculated energy distance within 10 kJ from global minimum and accumulated population is more than 85%. Heavy atoms are shown with different colors (carbon, gray; nitrogen, blue; oxygen, red).

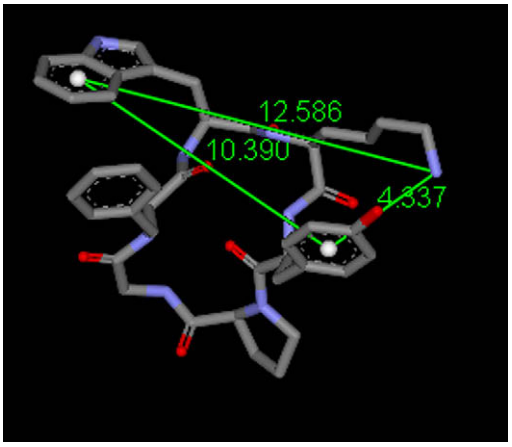

Figure 6. Lowest energy conformer of **1**. Heavy atoms are shown with different colors (carbon, gray; nitrogen, blue; oxygen, red). Distances between the pharmacophoric points are shown in Å.

5. Experimental section

5.1. Synthetic methods

LC/MS were obtained on Micromass ZMD (ESI) mass spectrometers and a Waters 600 HPLC System (Develosil C30-UG-5, 4.6 × 50 mm) with a linear gradient of 5% acetonitrile containing 0.1% acetic acid to 98% acetonitrile containing 0.1% acetic acid/water and 0.1% acetic acid over 4 min at a1.0 mL/min flow rate. Peak areas were integrated with SEDEX 75 evaporative light scattering detector (ELSD). The high resolution MS (HRMS) spectra of compounds were obtained by ESI (ABI, QSTAR pulsar-i). Semi-preparative HPLC was performed using Develosil C30-UG-5, 20 × 150 mm with a linear gradient of 5% acetonitrile containing

0.1% acetic acid to 98% acetonitrile containing 0.1% acetic acid over 20 min at a 10.0 mL/min flow rate.

Cyclic peptide library was prepared on Sulfamylbutyryl AM resin (purchased from NOVA Biochem, 1.06 mmol/g, 300 mg, 0.32 mmol) using standard solid-phase Fmoc based procedures. In the first place, the resin was shaken for 2 × 16 h with a mixture of Fmoc-glycine (4 equiv, 1.27 mmol)/HATU (4 equiv, 1.27 mmol)/HOAt (4 equiv, 1.28 mmol)/DIEPA (8 equiv, 2.56 mmol) in DMF. The coupling solution was drained and the resin was washed (5 × DCM, 5 × DMF, 5 × DCM). Fmoc group was removed with 20%-piperidine in DMF. Then the resin was shaken for 16 h with a mixture of Fmoc-proline (4 equiv, 1.27 mmol)/HBTU (4 equiv, 1.27 mmol)/HOBt (4 equiv, 1.28 mmol)/DIEPA (8 equiv, 2.56 mmol) in DMF. Further extension of the peptide was accomplished using standard Fmoc strategy as described. After elongation of the linear peptide, the N-terminal Fmoc group was replaced by Trt following treatment with 20% piperidine in DMF and shaken with Trt-Cl (3.5 equiv, 1.1 mmol), DIEPA (6 equiv, 1.9 mmol) in DMF for 24 h. The cyclization was achieved by shaking with iodoacetonitrile (10 equiv, 3.18 mmol), DIEPA (10 equiv, 3.18 mmol) in DMF for 24 h, treatment with TFA and TIS in DCM (1:5:94) for 3 × 3 min, and then treatment with DIEPA (3 equiv, 0.95 mmol) in DMF for 5 days. The side-chain protected cyclic peptides were dissolved in DCM, and the organic layer was washed with 10% citric acid followed by 5% sodium bicarbonate. The organic layer was concentrated in vacuo. The residue was then mixed with TIS, EDT, and H_2O in TFA (5:2.5:5:87.5) for 2 h. The crude peptide solution was concentrated in vacuo and a crude peptide was obtained by precipitation from ether.

5.2. UT-II agonistic activity assay

Calcium-mobilization assay was used for the estimation of UT-II agonistic activity of cyclic peptides in HEK293 cells stably transfected with human GPR-14. The cells were seeded into 96 well plates pre-coated with poly-D-lysine and incubated at 37 °C overnight under 5% CO_2/95% oxygen atmosphere. The growth medium was aspirated and replaced with 50 μL of assay buffer (20 mM HEPES, pH 7.4, 115 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 0.8 mM $CaCl_2$, 13.8 mM D-glucose, and 0.1% BSA) with 5 μM Fura-2 AM and 0.2% Pluronic F-127 (Molecular Probes, Inc.) in each well and incubated at 37 °C for 30 min. The cells were subsequently washed twice with same assay buffer and 80 μL left in each well. The washed cells were placed in an FDSS 4000 (Hamamatsu Photonics K.K.) and changes in cellular fluorescence using double excitation wavelength (340 and 380 nm) after the addition of 20 μL test compounds in assay buffer and recorded immediately. The peak height of the ratio of fluorescence (340/380 nm) was evaluated as relative agonistic activity.

5.3. NMR analysis

One- and two-dimensional NMR experiments were performed at 400 MHz in a JEOL LA-400 spectrometer at 25 °C. The NMR samples were prepared by dissolving 1 mg of the compound in DMSO-d_6 (0.5 ml). COSY and NOESY were collected by the methods of States et al.[37] Resonance assignments were determined by COSY spectrum using PFG technique.

5.4. Calculations

The three-dimensional structure of **1** was investigated by conformational analysis (MMFF94) using the BATCHMIN program included in the MacroModel v6.5 package.[38] A 100,000 step Monte-Carlo search was performed with rmsd of 0.25 Å for all heavy atoms. Energy minimizations were performed in vacuum.

Acknowledgments

The authors acknowledge Dr. Lawrence H. Lazarus, NIEHS, NC, USA for his advice during preparation of the manuscript. This work was supported in part by NEXT 'Academic Frontier' Project (2006).

References and notes

1. Maguire, J. J.; Davenport, A. P. *Br. J. Pharmacol.* **2002**, *137*, 579.
2. Pearson, D.; Shively, J. E.; Clark, B. R.; Geschwind, I. I.; Barkley, M.; Nishioka, R. S.; Bern, H. A. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 5021.
3. Coulouarn, Y.; Jegou, S.; Tostivint, H.; Vaudry, H.; Lihrmann, I. *FEBS Lett.* **1999**, *457*, 28.
4. Ong, K. L.; Lam, K. S.; Cheung, B. M. *Cardiovasc. Drugs Therap.* **2005**, *19*, 65.
5. Ames, R. S.; Sarau, H. M.; Chambers, J. K.; Willette, R. N.; Aiyar, R. V.; Romanic, A. M.; Louden, C. S.; Foley, J. J.; Sauermelch, C. F.; Coatney, R. W.; Ao, Z.; Disa, J.; Holmes, S. D.; Stadel, J. M.; Martin, J. D.; Liu, W. S.; Glover, G. I.; Wilson, S.; McNutty, D. E.; Ellis, C. E.; Eishourbagy, N. A.; Shabon, U.; Trill, J. J.; Hay, D. V. P.; Ohlstein, E. H.; Bergsma, D. J.; Douglas, S. A. *Nature* **1999**, *401*, 282.
6. Douglas, S. A.; Ohlstein, E. H. *Trends. Cardiovasc. Med.* **2000**, *10*, 229.
7. Douglas, S. A. *Curr. Opin. Pharmacol.* **2003**, *3*, 159.
8. Mori, M.; Sugo, T.; Abe, M.; Shimomura, Y.; Kurihara, M.; Kitada, C.; Kikuchi, K.; Shintani, Y.; Kurokawa, T.; Onda, H.; Nishimura, O.; Fujino, M. *Biochem. Biophys. Res. Commun.* **1999**, *265*, 123.
9. Flohr, S.; Kurz, M.; Kostenis, E.; Brkovich, A.; Fournier, A.; Klabunde, T. *J. Med. Chem.* **2002**, *45*, 1799.
10. Spinella, M. J.; Malik, A. B.; Everitt, J.; Andersen, T. T. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7443.
11. Abraham, W. M.; Ahmed, A.; Cortes, A.; Spinella, M. J.; Malik, A. B.; Andersen, T. T. *J. Appl. Physiol.* **1993**, *74*, 2537.
12. Mierke, D. F.; Pattaroni, C.; Delaet, N.; Toy, A.; Goodman, M.; Tancredi, T.; Motta, A.; Temussi, P. A.; Moroder, L.; Boverman, G.; Wunsch, E. *Int. J. Peptide Protein Res.* **1990**, *36*, 418.
13. Grieco, P.; Carotenuto, A.; Patacchini, R.; Maggi, C. A.; Novellino, E.; Rovero, P. *Bioorg. Med. Chem.* **2002**, *10*, 3731.
14. Hruby, V. J. *Nat. Rev.* **2002**, *1*, 847.
15. Behm, D. J.; Herold, C. L.; Ohlstein, E. H.; Knight, S. D.; Dhanak, D.; Douglas, S. A. *Br. J. Pharmacol.* **2002**, *137*, 449.
16. Behm, D. J.; Stankus, G.; Doe, C. P.; Willette, R. N.; Sarau, H. M.; Foley, J. J.; Schmidt, D. B.; Nuthulaganti, P.; Fornwald, J. A.; Ames, R. S.; Lambert, D. G.; Calo, G.; Camarda, V.; Aiyar, N. V.; Douglas, S. A. *Br. J. Pharmacol.* **2006**, *148*, 173.
17. Herold, C. L.; Behm, D. J.; Buckley, P. T.; Foley, J. J.; Wixted, W. E.; Sarau, H. M.; Douglas, S. A. *Br. J. Pharmacol.* **2003**, *139*, 203.
18. Kates, S. A.; Sole, N. A.; Albericio, F.; Barany, G. In *Peptides: Design, Synthesis and Biological Activity Solid Phase Synthesis of Cyclic Peptides*; Basava, C., Anamtharamaiah, G. M., Eds.; Brikhäuser: Boston, 1994; pp 39–58.
19. Spatola, A. F.; Darlark, D.; Romanovkis, P. *Tetrahedron Lett.* **1996**, *37*, 591.
20. Alsina, J.; Rabana, F.; Giralt, E.; Albericio, F. *Tetrahedron Lett.* **1994**, *35*, 9633.
21. Alsina, J.; Chiva, C.; Ortiz, M.; Rabana, F.; Giralt, E.; Albericio, F. *Tetrahedron Lett.* **1997**, *38*, 883.
22. Isied, S.; Kauehn, C. G.; Lyon, J. M.; Merrifield, R. B. *J. Am. Chem. Soc.* **1980**, *104*, 2632.
23. Mihara, H.; Yamabe, S.; Niidome, T.; Aoyagi, H.; Kumagai, H. *Tetrahedron Lett.* **1995**, *36*, 4837.
24. Richter, L. S.; Tom, J. Y. K.; Brunier, J. P. *Tetrahedron Lett.* **1994**, *35*, 5547.
25. Kenner, G. W.; McDermott, J. R.; Sheppard, R. C. *J. Chem. Soc., Chem. Commun.* **1971**, 636.
26. Amblard, M.; Fehrentz, J.-A.; Martinez, J.; Subra, G. *Mol. Biotechnol.* **2006**, *33*, 239.
27. Brkovic, A.; Hattenberger, A.; Kostenis, E.; Klabunde, T.; Flohr, S.; Kurz, M.; Bourgault, S.; Fournier, A. *J. Pharm. Exp. Ther.* **2003**, *306*, 1200.
28. Kinney, W. A.; Almond, H. A., Jr.; Qi, J.; Smith, C. E.; Santulli, R. J.; Garavilla, L.; Andrade-Gordon, P.; Cho, D. S.; Everson, A. M.; Feinstein, M. A.; Leung, P. A.; Maryanoff, B. E. *Angrew. Chem., Int. Ed.* **2002**, *41*, 2940.
29. Itoh, H.; Itoh, Y.; Rivier, J.; Lederis, K. *Am. J. Physiol.* **1987**, *252*, R361.
30. Grieco, P.; Carotenuto, A.; Campiglia, P.; Zampelli, E.; Patacchini, R.; Maggi, C. A.; Novellino, E.; Rovero, P. *J. Med. Chem.* **2002**, *45*, 4391.
31. Guerrini, R.; Camarda, V.; Marzola, E.; Arduin, M.; Calo, G.; Spagnol, M.; Rizzi, A.; Salvadori, S.; Regoli, D. *J. Pept. Sci.* **2005**, *11*, 85.
32. Camarda, V.; Guerrini, R.; Kostenis, E.; Rizzi, A.; Calo, G.; Hattenberger, A.; Zucchini, M.; Salvadori, D. *Br. J. Pharmacol.* **2002**, *137*, 311.
33. Patacchini, R.; Santicioli, P.; Giuliani, S.; Grieco, P.; Novellino, E.; Rovero, P.; Maggi, A. C. *Br. J. Pharmacol.* **2003**, *140*, 1155.
34. Camarda, V.; Song, W.; Marzola, E.; Spagnol, M.; Guerrini, R.; Salvadori, S.; Regoli, D.; Tompson, J. P.; Rowbotham, D. J.; Behm, D. J.; Douglas, S. A.; Calo, G.; Lambert, D. G. *Eur. J. Pharmacol.* **2004**, *498*, 83.
35. Camarda, V.; Spagnol, M.; Song, W.; Vergura, R.; Roth, A. L.; Thompson, J. P.; Rowbothman, D. J.; Gurerrini, R.; Marzola, E.; Salvadori, S.; Cavanni, P.; Regoli, D.; Douglas, S. A.; Lambert, D. G.; Calo, G. *Br. J. Pharmacol.* **2006**, *147*, 92.
36. Chatenet, D.; Dubessy, C.; Boularan, C.; Scalbert, E.; Pfeiffer, B.; Renard, P.; Lihrmann, I.; Pacaud, P.; Tonon, M.-C.; Vaudry, H.; Leprince, J. *J. Med. Chem.* **2006**, *49*, 7234.
37. States, D. J.; Haberkorn, R. A.; Ruben, D. J. *J. Magn. Reson.* **1982**, *48*, 286.
38. http://www.schrodinger.com.