Structure−Activity Relationships of a Novel Series of Urotensin II Analogues: Identification of a Urotensin II Antagonist

David Chatenet,[†,||] Christophe Dubessy,[†,||] Cédric Boularan,[‡] Elizabeth Scalbert,[§] Bruno Pfeiffer,[§] Pierre Renard,[§] Isabelle Lihrmann,[†] Pierre Pacaud,[‡] Marie-Christine Tonon,[†] Hubert Vaudry,[†,*] and Jérôme Leprince[†]

INSERM U413, Laboratory of Cellular & Molecular Neuroendocrinology, European Institute for Peptide Research (IFRMP 23), University of Rouen, 76821 Mont-Saint-Aignan, France; INSERM U533, Laboratory of Cellular & Molecular Physiopathology & Pharmacology, University of Nantes, 44322 Nantes, France; and IdRS, 11 rue des Moulineaux, 92150 Suresnes, France

Received February 23, 2006

Urotensin II (U-II) is a potent vasoconstrictor peptide which has been identified as the endogenous ligand for the orphan G protein-coupled receptor GPR14 now renamed UT receptor. As the C-terminal cyclic hexapeptide of U-II (U-II$_{(4-11)}$, H-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH) possesses full biological activity, we have synthesized a series of U-II$_{(4-11)}$ analogues and measured their binding affinity on *h*GPR14-transfected CHO cells and their contractile activity on de-endothelialized rat aortic rings. The data indicate that a free amino group and a functionalized side-chain at the N-terminal extremity of the peptide are not required for biological activity. In addition, the minimal chemical requirement at position 9 of U-II$_{(4-11)}$ is the presence of an aromatic moiety. Most importantly, replacement of the Phe^6 residue by cyclohexyl-Ala (Cha) led to an analogue, [Cha^6]U-II$_{(4-11)}$, that was devoid of agonistic activity but was able to dose-dependently suppress the vasoconstrictor effect of U-II on rat aortic rings. These new pharmacological data, by providing further information regarding the structure−activity relationships of U-II analogues, should prove useful for the rational design of potent and nonpeptidic UT receptor agonists and antagonists.

Introduction

Urotensin II (U-II)[a] is a cyclic peptide initially isolated from the urophysis of the teleost fish *Gillichthys mirabilis* on the basis of its spasmogenic action on the trout hindgut.[1] Subsequently, it has been found that U-II is also present in tetrapods and that its gene is expressed in the CNS.[2] The U-II precursor has now been cloned in various vertebrate species including frog,[3] rat and mouse,[4] pig,[5] monkey,[6] and human.[3] The mature forms of U-II have been characterized in frog,[2] pig,[5] and human.[7] Recently, a paralogue of U-II, called urotensin-related peptide (URP), has been identified in mammals.[8] In all U-II and URP isoforms known so far, the sequence of the cyclic C-terminal hexapeptide has been fully conserved across species.[9] The U-II and URP genes are primarily expressed in motoneurons located in discrete brainstem nuclei and in the ventral horn of the spinal cord.[3,4,8,10−13] U-II and URP mRNAs have also been detected, although at a much lower level, in various peripheral tissues including the pituitary, heart, spleen, thymus, pancreas, kidney, small intestine, adrenal, and prostate.[3,8,14]

U-II exhibits a wide range of biological activities in mammals. In particular, U-II can induce both contraction of vascular smooth muscles and endothelium-dependent vasorelaxation, bronchospactic actions in airway smooth muscles and inotropic/arrhytmogenic effects in isolated cardiac tissues.[15] In the isolated rat pancreas, U-II inhibits glucose-induced insulin secretion.[16] In addition, intracerebroventricular or intracerebral administration of U-II elicits various cardiovascular,[17] neuroendocrine,[18] and behavioral effects.[19]

Several groups have shown that U-II is the endogenous ligand for a G protein-coupled receptor, originally termed GPR14 and now renamed UT receptor.[5,20−22] The UT receptor is widely expressed in the central nervous system as well as in various peripheral organs including the cardiovascular system, kidney, bladder, pancreas, and adrenal gland.[14,18,20,21,23−27] The multiple effects of U-II and the broad expression pattern of its receptor indicate that U-II may be involved in physiopathological processes. Indeed, recent studies suggest that U-II may play a role in smooth muscle cell proliferation,[28] cardiac fibrosis and hypertrophy,[29] heart failure,[30] cardiac remodeling,[31] atherosclerosis,[32] and renal dysfunction.[33] The recently discovered U-II variant, URP, in very much the same as U-II itself, exhibits a high binding affinity for the human GPR14 (*h*GPR14) in transfected cell lines[8,9] and a high contractile potency in the rat aortic ring assay,[9] suggesting that some of the effects previously attributed to U-II may actually be exerted by URP.

It has been previously demonstrated that the C-terminal octapeptide of U-II retains full biological activity and binding properties.[34−38] To identify new leads for the development of both agonists and antagonists of UT receptor, we have studied the structure−activity relationships of a series of novel human U-II$_{(4-11)}$ analogues based on the chemical substitution of the particularly sensitive Asp[1], Phe[3], and Tyr[6] residues,[35−41] using a radioligand binding assay with GPR14-transfected cells and a rat aortic ring contraction assay.

* To whom correspondence should be addressed. Tel.: +33 235 14 6624. Fax: +33 235 14 6946. E-mail: hubert.vaudry@univ-rouen.fr.
[†] University of Rouen.
[‡] University of Nantes.
[§] IdRS.
[||] Authors who equally contributed to this work.

[a] Abbreviations: Symbols and abbreviations are in accord with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature: Nomenclature and Symbolism for Amino Acids and Peptides. *Biochem. J.* **1984**, *219*, 345−373, and with A Short Guide to Abbreviations and Their Use in Peptide Science. *J. Pept. Sci*. **2003**, *9*, 1−8. Additional abbreviations are as follows: Aib, 2-aminoisobutyric acid; BzThi, (3-benzothienyl)-alanine; Cha, cyclohexyl-alanine; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; Oic, octahydroindole-2-carboxylic acid; Pen, β,β-dimethyl-cysteine; RP-HPLC, reversed-phase high-performance liquid chromatography; Thi, (2-thienyl)-alanine. All optically active amino acids are of the L-configuration unless otherwise stated.

10.1021/jm0602110 CCC: $33.50 © 2006 American Chemical Society
Published on Web 10/27/2006

Table 1. Chemical Data for Compounds **1**−**19**

compd	peptide	HPLC t_R (min)[a]	HPLC purity (%)	MS calcd[b]	MS obsd[c]
1	U-II$_{(4-11)}$	21.1	99.9	1060.41	1061.41
2	[Glu^4]U-II$_{(4-11)}$	21.2	98.6	1074.43	1075.54
3	[Aib^4]U-II$_{(4-11)}$	21.0	98.9	1030.44	1031.47
4	[pGlu^4]U-II$_{(4-11)}$	22.0	99.7	1056.42	1057.53
5	[N-Me-Asp^4]U-II$_{(4-11)}$	21.2	99.8	1074.43	1075.44
6	[3-nitro-Tyr^9]U-II$_{(4-11)}$	23.0	99.9	1105.40	1106.27
7	[4-carboxy-Phe^9]U-II$_{(4-11)}$	20.7	99.9	1088.41	1089.49
8	[4-amino-Phe^9]U-II$_{(4-11)}$	22.6	98.2	1059.43	1060.33
9	[4-*tert*-butyl-Phe^9]U-II$_{(4-11)}$	26.4	99.8	1100.48	1101.55
10	[Thi^6]U-II$_{(4-11)}$	20.1	98.4	1066.37	1067.28
11	[BzThi^6]U-II$_{(4-11)}$	22.2	99.3	1116.39	1117.44
12	[4-*tert*-butyl-Phe^6]U-II$_{(4-11)}$	24.0	98.1	1116.47	1117.58
13	[Oic^6]U-II$_{(4-11)}$	20.4	99.6	1064.44	1065.28
14	[Leu^6]U-II$_{(4-11)}$	19.8	98.9	1026.43	1027.54
15	[Cha^6]U-II$_{(4-11)}$	22.0	98.9	1066.46	1067.51
16	[Cha^6]URP	22.4	98.2	1022.47	1023.61
17	[D-Cha^6]U-II$_{(4-11)}$	21.3	99.8	1066.46	1067.67
18	[Pen^5, Cha^6]U-II$_{(4-11)}$	21.1	98.3	1094.49	1095.67
19	[Cha^6, 3-iodo-Tyr^9] U-II$_{(4-11)}$	23.8	98.5	1192.36	1193.48

[a] Retention time determined by RP-HPLC. [b] Theoretical monoisotopic molecular weight. [c] m/z value assessed by MALDI-TOF-MS.

Results and Discussion

Previous studies have demonstrated that the C-terminal octapeptide of *h*U-II (U-II$_{(4-11)}$, H-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH, compound **1**) mimicked the effects of U-II on intracellular calcium concentration in GPR14-transfected cells and contraction of rat aortic rings.[35,37,38] To further explore the structural requirements of this core sequence, 18 U-II$_{(4-11)}$ analogues (Table 1) were synthesized and tested for their binding affinity on *h*GPR14-transfected CHO cells and for their contractile activity on de-endothelialized rat aortic rings.

To investigate the contribution of the N-terminal Asp residue in the biological activity of U-II$_{(4-11)}$, we have synthesized four analogues substituted at this position. Substitution of the native Asp^4 residue (compound **1**, Table 1) by a Glu moiety (compound **2**, Table 1), which preserves the acidic feature of the N-terminal amino acid, did not modify the binding affinity for *h*GPR14 (pK_i = 8.91 ± 0.04 and 8.53 ± 0.27, respectively) nor the contractile potency of the peptide (pEC$_{50}$ = 8.30 ± 0.11 and 8.32 ± 0.15, respectively) (Table 2). Similarly, replacement of Asp^4 by the nonchiral and nonpolar α-aminoisobutyric acid residue ([Aib^4]U-II$_{(4-11)}$, compound **3**, Table 1) did not affect the binding affinity and the contractile activity of the analogue (Table 2) suggesting that this position was fairly tolerant to the chemical nature and stereochemistry of the side-chain. These results are in agreement with previous data, indicating that URP, which corresponds to an Ala^4-substituted analogue of U-II$_{(4-11)}$, retains full binding affinity[9] and the same contractile activity as U-II.[9,38] For the U-II$_{(4-11)}$ amidated counterpart, a hydrogen bond acceptor CO group is mandatory within the N-terminal amino acid side-chain but not the negative charge.[39] It has been previously demonstrated that acetylation of the N-terminal Asp residue does not affect the contractile property of U-II$_{(4-11)}$ and even enhances the potency of U-II.[36,38] Here, we observed that the N-blocked analogues [pGlu^4]U-II$_{(4-11)}$ (compound **4**, Table 1) and [N-Me-Asp^4]U-II$_{(4-11)}$ (compound **5**, Table 1) exhibited pEC$_{50}$ values that were very similar to those of U-II$_{(4-11)}$ (Table 2). These data confirm that a free N-terminal function is not mandatory for the docking of the ligand and the activation of UT receptor. Since the presence of a blocked residue at the N-terminal position of U-II is likely to confer resistance to aminopeptidases,[42] the [pGlu^4]U-II$_{(4-11)}$ and [N-Me-Asp^4]U-II$_{(4-11)}$ analogues may possess longer half-lives than U-II and URP and thus may exhibit prolonged effects when administered in vivo.

We have previously found that [3-iodo-Tyr^9]U-II$_{(4-11)}$ is 5-fold more potent than *h*U-II in the aortic ring bioassay, suggesting that an increase in the steric hindrance or in the electronic density of the phenol moiety might be responsible for the enhancement of the agonistic property.[38] To further investigate the contribution of the Tyr^9 residue in the biological activity of U-II$_{(4-11)}$, this amino acid was substituted with various amino acid derivatives (compounds **6**−**9**, Table 1). The [3-nitro-Tyr^9]U-II$_{(4-11)}$ analogue (compound **6**) showed a moderate decrease in binding affinity and in contractile activity (Table 2). Replacement of the hydroxyl group of the tyrosine moiety by a carboxyl function led to the [4-carboxy-Phe^9]U-II$_{(4-11)}$ analogue (compound **7**) which exhibited a significant reduction of both affinity and potency (Table 2), whereas the amino counterpart, the [4-amino-Phe^9]U-II$_{(4-11)}$ analogue (compound **8**), showed only a marked decrease in contractile activity (Table 2). While this study was in progress, Guerrini et al. reported that the [4-amino-Phe^9]*h*U-II analogue, the full-length homologue of compound **8**, has a pEC$_{50}$ value of 7.56 in the rat aortic ring assay,[40] i.e. one order in magnitude higher than that measured herein for [4-amino-Phe^9]U-II$_{(4-11)}$. These authors also found that different chemical groups can be added to the phenyl part without significant modification of the biological activity of the peptide.[40] Introduction of the hindered *t*-butyl group in place of the hydroxyl moiety of Tyr^9 ([4-*t*butyl-Phe^9]U-II$_{(4-11)}$, compound **9**) did not significantly modify the binding affinity nor the contractile activity of the peptide. Indeed, it has been shown that the substitution of tyrosine by bulky aromatic amino acids such as (2-naphthyl)-L-alanine, L-biphenylalanine[36] or 3-iodo-tyrosine[38] may even increase the binding affinity and the biological activity possibly through an enhancement of the hydrophobic interactions within a putative Tyr binding pocket in the UT receptor. These observations, together with the present data, suggest that the minimal chemical requirement at position 9 of U-II$_{(4-11)}$ is the presence of an aromatic moiety.

Several studies have shown that the Phe moiety is the most tolerant intracyclic residue.[35,37,38] We have thus investigated the effects of replacement of this residue by nonpolar aromatic or aliphatic amino acids in U-II$_{(4-11)}$ (compounds **10**−**17**, Table 1). As shown in Table 2, substitution of the Phe^6 residue with Thi or BzThi yielded to compounds ([Thi^6]U-II$_{(4-11)}$, compound **10**, and [BzThi^6]U-II$_{(4-11)}$, compound **11**) that retained high binding affinity but were 3 and 107 times less potent than U-II$_{(4-11)}$ in the rat aortic ring assay, respectively. In contrast, replacement of the Phe^6 residue by 4-*t*-butyl-Phe (compound **12**) reduced by 48-fold the binding affinity but did not modify the contractile activity. Consistent with this latter observation, Boucard et al. have shown that substitution of the Phe^9 residue of *h*U-II by the sterically bulky 4-benzoyl-L-phenylalanine increases the binding affinity of the peptide without affecting inositol phosphate production in COS-7 cells transfected with rat UT receptor.[43] Replacement of the Phe^6 moiety with the constrained aliphatic residue Oic led to an analogue ([Oic^6]U-II$_{(4-11)}$, compound **13**) that was totally devoid of binding affinity for UT receptor as well as contractile activity. The Oic residue might induce a turn, like a prolyl moiety,[44] that would destabilize at this point the correct folding of the peptide backbone in the GPR14 binding pocket. In agreement with this hypothesis, we found that the introduction of a more flexible aliphatic side-chain amino acid through substitution with a leucine or a cyclohexylalanine residue yielded compounds ([Leu^6]U-II$_{(4-11)}$,

Table 2. Biological Data for Compounds **1**−**19**

		binding *h* GPR14		rat aortic ring contraction					
				agonist			antagonist		
compd	peptide	*n*	pK_i	*n*	max effect[a] (%)	pEC$_{50}$	*n*	max effect[b] (%)	pA$_2$
1	U-II$_{(4-11)}$	3	8.91 ± 0.04	4	102.1	8.30 ± 0.11	2	inactive	
2	[Glu^4]U-II$_{(4-11)}$	4	8.53 ± 0.27	4	82.1	8.32 ± 0.15	4	inactive	
3	[Aib^4]U-II$_{(4-11)}$	4	8.65 ± 0.09	4	83.9	8.25 ± 0.07	2	inactive	
4	[pGlu^4]U-II$_{(4-11)}$	4	9.04 ± 0.31	4	98.6	8.23 ± 0.16	3	inactive	
5	[N-Me-Asp^4]U-II$_{(4-11)}$	4	8.79 ± 0.37	3	93.1	8.22 ± 0.11	2	inactive	
6	[3-nitro-Tyr^9]U-II$_{(4-11)}$	3	8.62 ± 0.15	3	95.0	8.01 ± 0.08	3	inactive	
7	[4-carboxy-Phe^9]U-II$_{(4-11)}$	5	7.13 ± 0.13**	2	79.3	5.77 ± 0.80**	2	inactive	
8	[4-amino-Phe^9]U-II$_{(4-11)}$	5	8.00 ± 0.12	3	97.7	6.37 ± 0.14**	2	inactive	
9	[4-*tert*-butyl-Phe^9]U-II$_{(4-11)}$	5	8.04 ± 0.27	3	88.5	7.63 ± 0.14	2	inactive	
10	[Thi^6]U-II$_{(4-11)}$	5	8.54 ± 0.11	7	94.0	8.05 ± 0.08	2	inactive	
11	[BzThi^6]U-II$_{(4-11)}$	2	8.95 ± 0.41	5	73.8	6.54 ± 0.18**	2	inactive	
12	[4-*tert*-butyl-Phe^6]U-II$_{(4-11)}$	3	7.63 ± 0.37	4	76.7	8.39 ± 0.11	4	inactive	
13	[Oic^6]U-II$_{(4-11)}$	3	<5***	4	inactive	<5**	2	inactive	
14	[Leu^6]U-II$_{(4-11)}$	3	6.88 ± 0.05**	4	38.7[c]	<5***	3	inactive	
15	[Cha^6]U-II$_{(4-11)}$	4	6.39 ± 0.07**	2	inactive	<5***	7	95.7[d]	6.09
16	[Cha^6]URP	3	6.58 ± 0.16**	3	36.9	6.03 ± 0.23**	4	inactive	
17	[D-Cha^6]U-II$_{(4-11)}$	3	<5***	4	inactive	<5***	4	inactive	
18	[Pen^5, Cha^6]U-II$_{(4-11)}$	3	6.81 ± 0.09**	3	14.8	5.72 ± 1.93**	2	inactive	
19	[Cha^6, 3-iodo-Tyr^9]U-II$_{(4-11)}$	3	7.71 ± 0.05	5	82.1	6.45 ± 0.08**	3	inactive	

[a] The maximum effect is expressed as a percentage of the amplitude of the contraction induced by 10^{-6} M phenylephrine. [b] The maximal effect is expressed as a percentage of the amplitude of the contraction induced by 10^{-6} M hU-II. [c] Effect at 10^{-5} M. [d] Effect at $10^{-4.5}$ M. Statistically different vs control (hU-II(4-11)), $**p < 0.01$ and $***p < 0.001$ as assessed by analysis of variance followed by Dunnett's post-test.

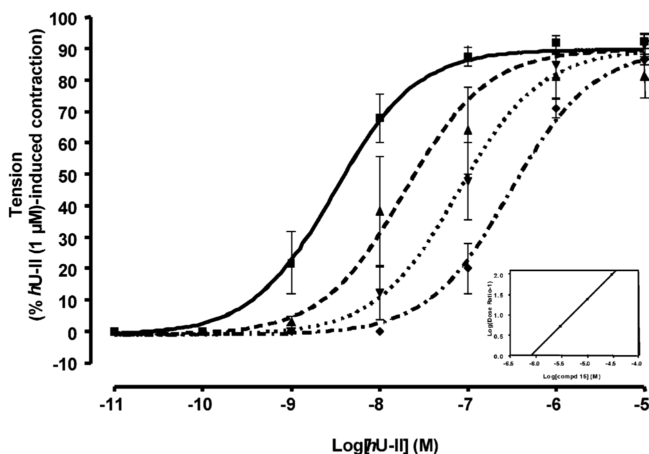

Figure 1. Effect of graded concentrations of [Cha^6]U-II$_{(4-11)}$ on *h*U-II-induced contractile response of rat aortic rings. The concentration−response curves show the tensive effect of *h*U-II in the absence (■-■) or presence of 3×10^{-6} M (▲- -▲), 10^{-5} M (▼- -▼) or 3×10^{-5} M (◆- -◆) [Cha^6]U-II$_{(4-11)}$. Each curve represents the mean ± SEM of four independent experiments. Inset: Schild plot revealed a slope of the Schild regression of 1.26. The *x*-axis intercept represents the pA$_2$ value according to Arunlakshana and Shild.[48]

compound **14** and [Cha^6]U-II$_{(4-11)}$, compound **15**) that exhibited residual affinity but were unable to induce the contraction of rat aortic rings (Table 2). Furthermore, [Cha^6]U-II$_{(4-11)}$ shifted the *h*U-II dose−response curve to the right in a concentration-dependent manner with a pA$_2$ value of 6.09, yielding a Schild slope of 1.26 and a Hill coefficient of 0.92 (Figure 1, Table 2). These data indicate that the simple substitution of the phenyl moiety of the Phe^6 residue with a cyclohexyl yields a pure competitive antagonist of UT receptor. Previous studies, however, have shown that UT ligands that behave as pure UT antagonists in the rat aorta ring bioassay exhibit residual activity in other tests such as calcium mobilization assay in cells expressing the recombinant UT receptor.[45−47] Therefore, the present data should be taken with some caution since [Cha^6]U-II$_{(4-11)}$ may not act as a pure antagonist on UT receptor-transfected cells and/or on native human UT receptors. Surprisingly, [Cha^6]URP (compound **16**, Table 1) did not exhibit substantial antagonistic activity but behaved as a weak agonist (Table 2). To further investigate the importance of the cyclohexylmethyl side-chain of this residue in the antagonistic activity, Cha^6 was substituted by its D-enantiomer (compound **17**, Table 1). [D-Cha^6]U-II$_{(4-11)}$ was totally devoid of binding affinity in very much the same as [D-Phe^6]U-II$_{(4-11)}$ (data not shown) and [D-Phe^6]URP.[9] Taken together, these data imply that the native phenylalanine residue plays a dual role: its chemical feature (aromatic vs aliphatic, Phe vs Cha) determines the pharmacological property (agonistic vs antagonistic, respectively) whereas its configuration (L- vs D-isomer) conditions the affinity (high vs low binding).

It has been found that [Pen^5]U-II$_{(4-11)}$[48] and [3-iodo-Tyr^9]U-II$_{(4-11)}$ (data not shown) exhibit higher affinity for GPR14 than *h*U-II. To increase the binding affinity of [Cha^6]U-II$_{(4-11)}$ (compound **15**), two analogues with dual substitutions [Pen^5, Cha^6]U-II$_{(4-11)}$ (compound **18**, Table 1) and [Cha^6, 3-iodo-Tyr^9]U-II$_{(4-11)}$ (compound **19**, Table 1) were synthesized. Both [Pen^5, Cha^6]U-II$_{(4-11)}$ and [Cha^6, 3-iodo-Tyr^9]U-II$_{(4-11)}$ bound *h*GPR14 with higher pK_i (6.81 ± 0.09 and 7.71 ± 0.05, respectively) than compound **15** ([Cha^6]U-II$_{(4-11)}$, pK_i = 6.39 ± 0.07). However, neither compounds **18** nor **19** affected *h*U-II-induced contraction of rat aortic rings (Table 2), indicating that the double substitutions Cys^5/Pen^5 and Tyr^9/3-iodo-Tyr^9 suppress the antagonistic activity of the Cha^6-substituted analogue.

Conclusion

In summary, this study reveals that a free N-terminal amino group or a functionalized side-chain are not required on the N-terminal position of short *h*U-II analogues to exert a full biological activity. Our data also demonstrate that replacement of the Phe^3 residue by a Cha group yields a weak antagonist that is devoid of agonistic activity. This structure−activity relationship study opens up new vistas for the design of potent U-II receptor antagonists.

Experimental Section

Materials. All L-amino acid residues, the preloaded poly(ethylene glycol)-polystyrene-resin (Fmoc-Val-PEG-PS), dichloromethane (DCM), 1-hydroxy-benzotriazole (HOBt), and trifluoroacetic acid

(TFA) were purchased from Applera-France (Courtaboeuf, France). All Fmoc-amino acids were purchased from Bachem Biochimie (Weil am Rhein, Germany) except Fmoc-Pen(Acm)-OH and Fmoc-D-Cha-OH which were from VWR (Fontenay-sous-Bois, France). *N*,*N*-Dimethylformamide (DMF), 2-propanol (^{i}PrOH), and acetonitrile were from Carlo Erba (Val-de-Reuil, France). Diisopropylethylamine (DIEA), *tert*-butyl methyl ether (TBME), acetic anhydride, thallium(III) trifluoroacetate ($Tl(OCOCF_3)_3$), *N*-methylmorpholine (NMM), lactoperoxidase, hydrogen peroxide, bovine serum albumin (BSA), Tris-HCl buffer, and piperidine were from Sigma-Aldrich (Saint-Quentin-Fallavier, France). 2-(1*H*-Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) was from NeoMPS (Strasbourg, France). Phenol and $MnCl_2$ were from Gibco Life Technologies (Cergy-Pontoise, France).

Peptide Synthesis. All analogues of U-II$_{(4-11)}$ and URP were synthesized (0.25 mmol scale) on a Fmoc-Val-PEG-PS resin on a Pioneer PerSeptive Biosystems peptide synthesizer (Applera-France) using the standard manufacturer's procedures. All Fmoc-amino acids (1 mmol, 4 equiv) were coupled by in situ activation with TBTU/HOBt (1.25 mmol:1.25 mmol, 5 equiv) and DIEA (2.5 mmol, 10 equiv) in DMF. Reactive side-chains were protected as follows: Tyr, *tert*-butyl (*t*Bu) ether; Lys and Trp, *tert*-butyloxycarbonyl (Boc) carbamate; Cys and Pen, acetamidomethyl (Acm) thioether; Glu, Asp, NMe-Asp and 4-carboxy-Phe, O-*tert*-butyl (O*t*Bu) ester. After completion of the chain assembly, cyclization of U-II and URP analogues was performed by $Tl(OCOCF_3)_3$ oxidation as previously described.[9]

Cleavage and Purification. All analogues of U-II$_{(4-11)}$ and URP were cleaved as previously reported.[38] Crude peptides were purified by semipreparative reversed-phase HPLC (RP-HPLC) on a Vydac 218TP1022 C_{18} column (2.2 × 25 cm; Alltech, Templemars, France) using a linear gradient (10−50% over 50 min) of acetonitrile/TFA (99.9:0.1, v/v) at a flow rate of 10 mL/min. Analytical RP-HPLC analysis, performed on a Vydac 218TP54 C_{18} column (0.46 × 25 cm; Alltech), revealed that the purity of all peptides was higher than 98.1% (Table 1). All peptides were characterized by MALDI-TOF-MS on a Voyager DE-PRO (Applera-France) in the reflector mode with a-cyano-4-hydroxycinnamic acid as a matrix.

Binding Experiments. Synthetic *h*U-II (3 μg) was radiolabeled with 0.5 mCi $Na^{125}I$ (Amersham Biosciences, Saclay, France) by the lactoperoxidase technique as previously described.[9] The radioiodinated peptides were purified by RP-HPLC on an Adsorbosphere C_{18} column (0.46 × 25 cm; Alltech) using a linear gradient (25−65% over 40 min) of acetonitrile/TFA (99.9:0.1, v/v) at a flow rate of 1 mL/min. Mono-iodinated ^{125}I-*h*U-II labeled on the Tyr^9 residue was collected and stored at 4 °C.

Binding assay was performed using CHO cells stably transfected with *h*GPR14 as previously described.[9] For competition studies 0.2 nM ^{125}I-*h*U-II was incubated with various concentrations of unlabeled U-II$_{(4-11)}$ and URP analogues. After a 3-h incubation, the cells were washed and lysed, and the radioactivity was counted on a LKB Wallac 1277 Gammamaster counter. Nonspecific binding, determined in the presence of 1 μM unlabeled *h*U-II, ranged between 10 and 15% of total binding.

Measurement of Rat Aortic Ring Contraction. De-endothelialized rat aortic rings were used to measure the contractile activity of the U-II-analogues as previously described.[9,38] Briefly, the proximal portion of the aortic arch was collected, and the endothelium was removed by gently rubbing the intimal surface with fine forceps. Aortic rings were then suspended under isometric conditions and connected to a force transducer (Pioden Controls, Canterbury, UK) in organ baths filled with Krebs−Henseleit solution, maintained at 37 °C and equilibrated with O_2/CO_2 (95:5). Contractile responses to 60 mM KCl were used as control at the beginning and at the end of each experiment. The absence of endothelium was verified by testing the inability of carbachol (10^{-5} M) to relax phenylephrine-evoked contraction (10^{-6} M). Cumulative concentration−response curves to synthetic peptides were set up by increasing the concentration of each peptide in the organ chamber. The amplitude of the contraction induced by each concentration of peptide was expressed as a percentage of the phenylephrine-induced contraction. The antagonistic activity of U-II$_{(4-11)}$ and URP analogues was determined by analyzing their ability to relax the contractile response induced by 10^{-6} M *h*U-II. For [Cha^6]U-II$_{(4-11)}$, that exhibited antagonistic properties, the effects of graded concentrations of the compound on U-II-evoked aortic ring contraction were tested and Schild analysis was performed in order to determine pA2 value.[49]

Statistical Analysis. Binding and functional experiments were performed at least in duplicate and data, expressed as mean ± SEM, were analyzed with the Prism software (Graphpad Software, San Diego, CA). The pK_i and pEC$_{50}$ values were determined from the concentration−response curves using a sigmoidal dose−response fit with variable slope. Statistical comparisons of the binding affinities and contractile potencies of U-II$_{(4-11)}$ and URP analogues were analyzed by ANOVA followed by a Dunnett's multiple comparison test, and differences were considered significant where $P < 0.05$.

Acknowledgment. This study was supported by INSERM (U413 and U533), the Ministère de la Jeunesse, de l'Education Nationale et de la Recherche, and the Conseil Régional de Haute-Normandie. D.C. and C.D. were recipients of fellowships from INSERM-SERVIER Laboratories.

References

(1) Pearson, D.; Shively, J. E.; Clark, B. R.; Geschwind, I. I.; Barkley, M.; Nishioka, R. S.; Bern, H. A. Urotensin II: a somatostatin-like peptide in the caudal neurosecretory system of fishes. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 5021−5024.

(2) Conlon, J. M.; O'Harte, F.; Smith, D. D.; Tonon, M. C.; Vaudry, H. Isolation and primary structure of urotensin II from the brain of a tetrapod, the frog *Rana ridibunda*. *Biochem. Biophys. Res. Commun.* **1992**, *188*, 578−583.

(3) Coulouarn, Y.; Lihrmann, I.; Jégou, S.; Anouar, Y.; Tostivint, H.; Beauvillain, J. C.; Conlon, J. M.; Bern, H. A.; Vaudry, H. Cloning of the cDNA encoding the urotensin II precursor in frog and human reveals intense expression of the urotensin II gene in motoneurons of the spinal cord. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 15803−15808.

(4) Coulouarn, Y.; Jégou, S.; Tostivint, H.; Vaudry, H.; Lihrmann, I. Cloning, sequence analysis and tissue distribution of the mouse and rat urotensin II precursors. *FEBS Lett.* **1999**, *457*, 28−32.

(5) Mori, M.; Sugo, T.; Abe, M.; Shimomura, Y.; Kurihara, M.; Kitada, C.; Kikuchi, K.; Shintani, Y.; Kurokawa, T.; Onda, H.; Nishimura, O.; Fujino, M. Urotensin II is the endogenous ligand of a G-protein-coupled orphan receptor, SENR (GPR14). *Biochem. Biophys. Res. Commun.* **1999**, *265*, 123−129.

(6) Elshourbagy, N. A.; Douglas, S. A.; Shabon, U.; Harrison, S.; Duddy, G.; Sechler, J. L.; Ao, Z.; Maleef, B. E.; Naselsky, D.; Disa, J.; Aiyar, N. V. Molecular and pharmacological characterization of genes encoding urotensin II-related peptides and their cognate G-protein-coupled receptors from the mouse and monkey. *Br. J. Pharmacol.* **2002**, *136*, 9−22.

(7) Chartrel, N.; Leprince, J.; Dujardin, C.; Chatenet, D.; Tollemer, H.; Baroncini, M.; Balment, R. J.; Beauvillain, J. C.; Vaudry, H. Biochemical characterization and immunohistochemical localization of urotensin II in the human brainstem and spinal cord. *J. Neurochem.* **2004**, *91*, 110−118.

(8) Sugo, T.; Murakami, Y.; Shimomura, Y.; Harada, M.; Abe, M.; Ishibashi, Y.; Kitada, C.; Miyajima, N.; Suzuki, N.; Mori, M.; Fujino, M. Identification of urotensin II-related peptide as the urotensin II-immunoreactive molecule in the rat brain. *Biochem. Biophys. Res. Commun.* **2003**, *310*, 860−868.

(9) Chatenet, D.; Dubessy, C.; Leprince, J.; Boularan, C.; Carlier, L.; Ségalas-Milazzo, I.; Guilhaudis, L.; Oulyadi, H.; Davoust, D.; Scalbert, E.; Pfeiffer, B.; Renard, P.; Tonon, M. C.; Lihrmann, I.; Pacaud, P.; Vaudry, H. Structure−activity relationships and structural conformation of a novel urotensin II-related peptide. *Peptides* **2004**, *25*, 1819−1830.

(10) Chartrel, N.; Conlon J. M.; Collin, F.; Braun, B.; Waugh, D.; Vallarino, M.; Lahrichi, S. L.; Rivier, J. E.; Vaudry, H. Urotensin II in the central nervous system of the frog *Rana ridibunda*: immunohistochemical localization and biochemical characterization. *J. Comput. Neurol.* **1996**, *364*, 324−339.

(11) Coulouarn, Y.; Fernex, C.; Jégou, S.; Henderson, C. E.; Vaudry, H.; Lihrmann, I. Specific expression of the urotensin II gene in sacral motoneurons of developing rat spinal cord. *Mech. Dev.* **2001**, *101*, 187−190.

(12) Pelletier, G.; Lihrmann, I.; Vaudry, H. Role of androgens in the regulation of urotensin II precursor mRNA expression in the rat brainstem and spinal cord. *Neuroscience* **2002**, *115*, 525−532.
(13) Pelletier, G.; Lihrmann, I.; Dubessy, C.; Luu-The, V., Vaudry, H.; Labrie, F. Androgenic down-regulation of urotensin II precursor, urotensin II-related peptide precursor and androgen receptor mRNA in the mouse spinal cord. *Neuroscience* **2005**, *132*, 689−696.
(14) Onan, D.; Hannan, R. D.; Thomas, W. G. Urotensin II: the old kid in town. *Trends Endocrinol. Metab.* **2004**, *15*, 175−182.
(15) Russell, F. D. Emerging roles of urotensin II in cardiovascular disease. *Pharmacol. Ther.* **2004**, *103*, 223−243.
(16) Silvestre, R. A.; Egido, E. M.; Hernandez, R.; Leprince, J.; Chatenet, D.; Tollemer, H.; Chartrel, N.; Vaudry, H.; Marco, J. Urotensin-II is present in pancreatic extracts and inhibits insulin release in the perfused rat pancreas. *Eur. J. Endocrinol.* **2004**, *151*, 803−809.
(17) Watson, A. M.; Lambert, G. W.; Smith, K. J.; May, C. N. Urotensin II acts centrally to increase epinephrine and ACTH release and cause potent inotropic and chronotropic actions. *Hypertension* **2003**, *42*, 373−379.
(18) Gartlon, J.; Parker, F.; Harrison, D. C.; Douglas, S. A.; Ashmeade, T. E.; Riley, G. J.; Hughes, Z. A.; Taylor, S. G.; Munton, R. P.; Hagan, J. J.; Hunter, J. A.; Jones, D. N. C. Central effects of urotensin-II following ICV administration in rats. *Psychopharmacology* **2001**, *155*, 426−433.
(19) Do Régo, J. C.; Chatenet, D.; Orta, M. H.; Naudin, B.; Le Cudennec, C.; Leprince, J.; Scalbert, E.; Vaudry, H.; Costentin, J. Behavioral effects of urotensin II centrally administered in mice. *Neuropharmacology* **2005**, *183*, 103−117.
(20) Ames, R. S.; Sarau, H. M.; Chambers, J. K.; Willette, R. N.; Aiyar, N. V.; Romanic, A. M.; Louden, C. S.; Foley, J. J.; Sauermeich, C. F.; Coatney, R. W.; Ao, Z.; Disa, J.; Holmes, S. D.; Stadel, J. M.; Martin, J. D.; Liu, W.; Glover, G. I.; Wilson, S.; McNulty, D. E.; Ellis, C. E.; Elshourbagy, N. A.; Shabon, U.; Trill, J. J.; Hay, D. W. P.; Ohlstein, E. H.; Bergsma, D. J.; Douglas, S. A. Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature (London)* **1999**, *401*, 282−286.
(21) Liu, Q.; Pong, S. S.; Zeng, Z.; Zhang, Q.; Howard, A. D.; Williams, D. L.; Davidoff, M.; Wang, R.; Austin, C. P.; McDonald, T. P.; Bai, C.; George, S. R.; Evans, J. F.; Caskey, C. T. Identification of urotensin II as the endogenous ligand for the orphan G-protein-coupled receptor GPR14. *Biochem. Biophys. Res. Commun.* **1999**, *266*, 174−178.
(22) Nothacker, H. P.; Wang, Z.; McNeill, A. M.; Saito, Y.; Merten, S.; O'Dowd, B.; Duckles, S. P.; Civelli, O. Identification of the natural ligand of an orphan G-protein-coupled receptor involved in the regulation of vasoconstriction. *Nat. Cell Biol.* **1999**, *1*, 383−385.
(23) Tal, M.; Ammar, D. A.; Karpuj, M.; Krizhanovsky, V.; Naim, M.; Thompson, D. A. A novel putative neuropeptide receptor expressed in neural tissue; including sensory epithelia. *Biochem. Biophys. Res. Commun.* **1995**, *209*, 752−759.
(24) Clark, S. D.; Nothacker, H. P.; Wang, Z.; Saito, Y.; Leslie, F. M.; Civelli, O. The urotensin II receptor is expressed in the cholinergic mesopontine tegmentum of the rat. *Brain Res.* **2001**, *923*, 120−127.
(25) Matsushita, M.; Shichiri, M.; Imai, T.; Iwashina, M.; Tanaka, H.; Takasu, N.; Hirata, Y. Co-expression of urotensin II and its receptor (GPR14) in human cardiovascular and renal tissues. *J. Hypertens.* **2001**, *19*, 2185−2190.
(26) Totsune, K.; Takahashi, K.; Arihara, Z.; Sone, M.; Satoh, F.; Ito, S.; Kimura, Y.; Sasano, H.; Murakami, O. Role of urotensin II in patients on dialysis. *Lancet* **2001**, *358*, 810−811.
(27) Jégou, S.; Cartier, D.; Dubessy, C.; Gonzalez, B.; Chatenet, D.; Tostivint, H.; Scalbert, E.; Leprince, J.; Vaudry, H.; Lihrmann, I. Localization of the urotensin II receptor in the rat central nervous system. *J. Comp. Neurol.* **2006**, *495*, 21−36.
(28) Sauzeau, V.; Le Mellionnec, E.; Bertoglio, J.; Scalbert, E.; Pacaud, P.; Loirand, G. Human urotensin II-induced contraction and arterial smooth muscle cell proliferation are mediated by RhoA and Rho-kinase. *Circ. Res.* **2001**, *88*, 1102−1104.
(29) Tzanidis, A.; Hannan, R. D.; Thomas, W. G.; Onan, D.; Autelitano, D. J.; See, F.; Kelly, D. J.; Gilbert, R. E.; Krum, H. Direct actions of urotensin II on the heart: implications for cardiac fibrosis and hypertrophy. *Circ. Res.* **2003**, *93*, 246−253.
(30) Watanabe, T.; Pakala, R.; Katagiri, T.; Benedict, C. R. Synergistic effect of urotensin II with mildly oxidized LDL on DNA synthesis in vascular smooth muscle cells. *Circulation* **2001**, *104*, 16−18.
(31) Behm, D. J.; Harrison, S. M.; Ao, Z.; Maniscalco, K.; Pickering, S. J.; Grau, E. V.; Woods, T. N.; Coatney, R. W.; Doe, C. P. A.; Willette, R. N.; Johns, D. G.; Douglas, S. A. Deletion of the UT receptor gene results in the selective loss of urotensin-II contractile activity in aortae isolated from UT receptor knockout mice. *Br. J. Pharmacol.* **2003**, *139*, 464−472.
(32) Bousette, N.; Patel, L.; Douglas, S. A.; Ohlstein, E. H.; Giaid, A. Increased expression of urotensin II and its cognate receptor GPR14 in atherosclerotic lesions of the human aorta. *Atherosclerosis* **2004**, *176*, 117−123.
(33) Totsune, K.; Takahashi, K.; Arihara, Z.; Sone, M.; Murakami, O.; Ito, S.; Kikuya, M.; Ohkubo, T.; Hashimoto, J.; Imai, Y. Elevated plasma level of immunoreactive urotensin II and its increased urinary excretion in patients with type 2 diabetes mellitus: association with progress of diabetic nephropathy. *Peptides* **2004**, *25*, 1809−1814.
(34) Itoh, H.; McMaster, D.; Lederis, K. Functional receptors for fish neuropeptide urotensin II in major rat arteries. *Eur. J. Pharmacol.* **1988**, *149*, 61−66.
(35) Flohr, S.; Kurz, M.; Kostenis, E.; Brkovich, A.; Fournier, A.; Klabunde, T. Identification of nonpeptidic urotensin II receptor antagonists by virtual screening based on a pharmacophore model derived from structure−activity relationships and nuclear magnetic resonance studies on urotensin II. *J. Med. Chem.* **2002**, *45*, 1799−1805.
(36) Kinney, W. A.; Almond, H. R.; Qi, J.; Smith, C. E.; Santulli, R. J.; de Garavilla, L.; Andrade-Gordon, P.; Cho D. S.; Everson, A. M.; Feinstein, M. A.; Leung, P. A.; Maryanoff, B. E. Structure−function analysis of urotensin II and its use in the construction of a ligand−receptor working model. *Angew. Chem., Int. Ed.* **2002**, *41*, 2940−2944.
(37) Brkovic, A.; Hattenberger, A.; Kostenis, E.; Klabunde, T.; Flohr, S.; Kurz, M.; Bourgault, S.; Fournier, A. Functional and binding characterizations of urotensin II-related peptides in human and rat urotensin II-receptor assay. *J. Pharmacol. Exp. Ther.* **2003**, *306*, 1200−1209.
(38) Labarrère, P.; Chatenet, D.; Leprince, J.; Marionneau, C.; Loirand, G.; Tonon, M. C.; Dubessy, C.; Scalbert, E.; Pfeiffer, B.; Renard, P.; Calas, B.; Pacaud, P.; Vaudry, H. Structure−activity relationships of human urotensin II and related analogues on rat aortic ring contraction. *J. Enz. Inhib. Med. Chem.* **2003**, *18*, 77−88.
(39) Coy, D. H.; Rossowski, W. J.; Cheng, B. L.; Taylor, J. E. Structural requirements at the N-terminus of urotensin II octapeptides. *Peptides* **2002**, *23*, 2259−2264.
(40) Guerrini, R.; Camarda, V.; Marzola, E.; Arduin, M.; Girolamo, C.; Spagnol, M.; Rizzi, A.; Salvadori, S.; Regoli, D. Structure−activity relationship study on human urotensin II. *J. Pept. Sci.* **2005**, *11*, 85−90.
(41) Camarda, V.; Guerrini, R.; Kostenis, E.; Rizzi, A.; Calo, G.; Hattenberger, A.; Zucchini, M.; Salvadori, S.; Regoli D. A new ligand for the urotensin II receptor. *Br. J. Pharmacol.* **2002**, *137*, 311−314.
(42) Praissman, M.; Fara, J.; Praissman, L. A.; Berkowitz, J. M. Preparation of an *N*-acetyl-octapeptide of cholecystokinin. The role of *N*-acetylation in protecting the octapeptide from degradation by smooth muscle tissues. *Biochim. Biophys. Acta* **1982**, *716*, 240−248.
(43) Boucard, A. A.; Sauve, S. S.; Guillemette, G.; Escher, E.; Leduc, R. Photo-labeling the rat urotensin II/GPR14 receptor identifies a ligand-binding site in the fourth transmembrane domain. *Biochem. J.* **2003**, *370*, 829−838.
(44) Fauchère, J. L.; Thurieau C. Evaluation of the stability of peptides and pseudopeptides as a tool in peptide drug design. *Adv. Drug Res.* **1992**, *23*, 127−159.
(45) Camarda, V.; Song, W.; Marzola, E.; Spagnol, M.; Guerrini, R.; Salvadori, S.; Regoli, D.; Thompson, J. P.; Rowbotham, D. J.; Behm, D. J.; Douglas, S. A.; Calo, G.; Lambert, D. G. Urantide mimics urotensin-II induced calcium release in cells expressing recombinant UT receptors. *Eur. J. Pharmacol.* **2004**, *498*, 83−86.
(46) Camarda, V.; Spagnol, M.; Song, W.; Vergura, R.; Roth, A. L.; Thompson, J. P.; Rowbotham, D. J.; Guerrini, R.; Marzola, E.; Salvadori, S.; Cavanni, P.; Regoli, D.; Douglas, S. A.; Lambert, D. G.; Calo, G. In vitro and in vivo pharmacological characterization of the novel UT receptor ligand [Pen^5, DTrp^7, Dab^8]urotensin II$_{(4-11)}$ (UFP-803). *Br. J. Pharmacol.* **2006**, *147*, 92−100.
(47) 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. The peptidic urotensin-II receptor ligand GSK248451 possesses less intrinsic activity than the low-efficacy partial agonists SB-710411 and urantide in native mammalian tissues and recombinant cell systems. *Br. J. Pharmacol.* **2006**, *148*, 173−190.
(48) Grieco, P.; Carotenuto, A.; Campiglia, P.; Zampelli, E.; Patacchini, R.; Maggi, C. A.; Novellino, E.; Rovero, P. A new, potent urotensin II receptor peptide agonist containing a Pen residue at the disulfide bridge. *J. Med. Chem.* **2002**, *45*, 4391−4384.
(49) Arunlakshana, O.; Schild, H. O. Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.* **1959**, *14*, 48−58.

JM0602110