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# Structure–Activity Relationships of Human Urotensin II and Related Analogues on Rat Aortic Ring Contraction*

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**The sequence of human urotensin II (UII) has been recently established as H-Glu-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH, and it has been reported that UII is the most potent mammalian vasoconstrictor peptide identified so far. A series of UII analogues was synthesized, and the contractile activity of each compound was studied *in vitro* using de-endothelialised rat aortic rings. Replacement of each amino acid by an L-alanine or by a D-isomer showed that the N- and C-terminal residues flanking the cyclic region of the amidated peptide were relatively tolerant to substitution. Conversely, replacement of any residue of the cyclic region significantly reduced the contractile activity of the molecule. The octapeptide $UII_{(4-11)}$ was 4 times more potent than UII, indicating that the C-terminal region of the molecule possesses full biological activity. Alanine or D-isomer substitutions in $UII_{(4-11)}$ or in $UII_{(4-11)}$-$NH_2$, respectively, showed a good correlation with the results obtained for UII-$NH_2$. Disulfide bridge disruption or replacement of the cysteine residues by their D-enantiomers markedly reduced the vasoconstrictor effect of UII and its analogues. In contrast, acetylation of the N-terminal residue of UII and UII-$NH_2$ enhanced the potency of the peptide. Finally, monoiodination of the $Tyr^6$ residue in $UII_{(4-11)}$ increased by 5 fold the potency of the peptide in the aortic ring bioassay. This structure–activity relationship study should provide useful information for the rational design of selective and potent UII receptor agonists and antagonists.**

*Keywords*: Urotensin II, Solid-phase synthesis, Vasoconstriction, Structure–activity relationships, Aortic rings

## INTRODUCTION

Urotensin II (UII) is a cyclic peptide initially isolated from the urophysis of the teleost fish *Gillichthys mirabilis* on the basis of its ability to contract the trout hindgut.[1] Subsequently, UII has been characterized in the brain and spinal cord of various vertebrate species including dogfish,[2] trout,[3] frog,[4] mouse,[5] rat,[5] pig[6] and human.[7,8] All isoforms of UII characterized so far possess a C-terminal cyclic hexapeptide that has been strongly conserved while the sequence of the N-terminal region of the peptide is highly variable (Figure 1). The cyclic core sequence of UII (Cys-Phe-Trp-Lys-Tyr-Cys) exhibits structural similarity with the biologically important sequence of somatostatin *i.e.* Phe-Trp-Lys-Thr.[14] Indeed, early studies have shown that disruption of the disulfide bridge suppresses the spasmogenic activity of UII[15,16] and that the cyclic hexapeptide possesses full biological activity.[17]

While in fish UII is primarily secreted by the caudal neurosecretory system,[18] in tetrapods and particularly in mammals the highest expression of pre-proUII mRNA occurs in motoneurons located in discrete brainstem nuclei and in the ventral horn of the spinal cord.[5,7,19,20] The UII gene is also expressed, albeit at a much lower level, in various peripheral tissues including the kidney, spleen, small intestine,

*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 of A Short Guide to Abbreviations and their Use in Peptide Science. *J. Peptide Sci.* **1999**, *5*, 465–471.

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| species | origin | sequence (disulfide bridge) | ref |
|---|---|---|---|
| Goby | urophysis | H-Ala-Gly-Thr-Ala-Asp-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **1** |
| Sucker A | urophysis | H-Gly-Ser-Gly-Ala-Asp-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **9** |
| Sucker B | urophysis | H-Gly-Ser-Asn-Thr-Glu-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **9** |
| Carp α | urophysis | H-Gly-Gly-Gly-Ala-Asp-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **10** |
| Carp β1 | urophysis | H-Gly-Gly-Asn-Thr-Glu-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **10** |
| Carp β2 | urophysis | H-Gly-Ser-Asn-Thr-Glu-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **10** |
| Carp γ | urophysis | H-Gly-Gly-Gly-Ala-Asp-**Cys-Phe-Trp-Lys-Tyr-Cys**-Ile-OH | **10** |
| Flounder | urophysis | H-Ala-Gly-Thr-Thr-Glu-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **11** |
| Dogfish | spinal cord | H-Asn-Asn-Phe-Ser-Asp-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **2** |
| Sturgeon | spinal cord | H-Gly-Ser-Thr-Ser-Glu-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **12** |
| Frog | brain | H-Ala-Gly-Asn-Leu-Ser-Glu-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **4** |
| Trout | brain | H-Gly-Gly-Asn-Ser-Glu-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **3** |
| Skate | brain | H-Asn-Asn-Phe-Ser-Asp-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **3** |
| SW lamprey | brain | H-Asn-Asn-Phe-Ser-Asp-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **13** |
| FW lamprey | brain | H-Asn-Asn-Phe-Ser-Asp-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **13** |
| Paddlefish | spinal cord | H-Gly-Ser-Thr-Ser-Glu-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **13** |
| Human | cDNA | H-Glu-Thr-Pro-Asp-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **7** |
| Mouse | cDNA | <Gln-His-Lys-Gln-His-Gly-Ala-Ala-Pro-Glu-**Cys-Phe-Trp-Lys-Tyr-Cys**-Ile-OH | **5** |
| Rat | cDNA | <Gln-His-Gly-Thr-Ala-Pro-Glu-**Cys-Phe-Trp-Lys-Tyr-Cys**-Ile-OH | **5** |
| Porcine A | spinal cord | H-Gly-Pro-Thr-Ser-Glu-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **6** |
| Porcine B | spinal cord | H-Gly-Pro-Pro-Ser-Glu-**Cys-Phe-Trp-Lys-Tyr-Cys**-Val-OH | **6** |

FIGURE 1 Comparison of the primary structure of urotensin II-related peptides from different species. The tissues from which UII peptides have been originally purified are indicated. The primary structures of human, rat and mouse UII were deduced from cDNA sequences. SW, sea water, FW, fresh water, <Gln, pyroglumatic acid.

thymus, prostate, pituitary, adrenal and pancreas.[7] The search for endogenous ligands of orphan receptors has recently led to the identification of GPR14 (an orphan receptor previously characterized in bovine[21] and rat[22]) as being the UII receptor.[6,8,23,24] The UII receptor is widely expressed in human tissues, notably in the cardiovascular system including the left atrium, ventricle, coronary artery and aorta.[8] GPR14 is also expressed in various regions of the central nervous system (olfactory epithelium, thalamus, substantia nigra, superior occipital gyrus, cerebellum, choroid plexus, motoneurons of the spinal cord), as well as in skeletal muscle and bladder.[8,21,23] Consistent with the widespread distribution of pre-proUII and GPR14 mRNAs, functional studies have shown that UII exerts a large array of biological activities. In particular, UII causes vasoconstriction in frog,[25] rat[26,27] and monkey,[8] provokes contraction of frog urinary bladder,[28] stimulates cortisol secretion in fish[29] and inhibits insulin release in rat.[30] It has been recently shown that intracerebroventricular administration of human UII in rat increases rearing, grooming and motor activity indicating that UII also exerts behavioral effects.[31]

In spite of the potential therapeutic value of GPR14 ligands for the treatment of cardiovascular, neuromuscular and/or endocrine diseases, only one structure–activity relationship study of UII-related peptides has been reported so far.[32] In the present work, we have synthesized a collection of 57 analogues of human UII (Table I) in order to investigate the minimal sequence for activity and to identify the position-related structural requirements for agonistic and antagonistic behavior. The contractile activity of each UII analogue has been studied by using a de-endothelialised rat aortic ring assay.

## MATERIALS AND METHODS

### General

All L-amino acid residues, preloaded polyethylene glycol-polystyrene resins (Fmoc-Val-PEG-PS, Fmoc-Ala-PEG-PS, Fmoc-Cys(Acm)-PEG-PS), the peptide amide linker polyethylene glycol-polystyrene resin (Fmoc-PAL-PEG-PS), *O*-(7-azabenzotriazolyl-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), dichloromethane (DCM) and trifluoroacetic acid (TFA) were purchased from Applera France (Courtaboeuf, France). All Fmoc-D-amino acids were from Advanced ChemTech (Aulnoy-lez-Valenciennes, France). Fmoc-3-iodo-Tyr-OH was from Bachem (Voisins-le-Bretonneux, France). 2-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) was from Neosystem (Strasbourg, France). 1-Hydroxybenzotriazole (HOBt) was from France Biochem (Meudon, France). *N*,*N*-dimethylformamide (DMF) was from SDS (Peypin, France) and isopropanol and acetonitrile were obtained from Carlo Erba (Val-de-Reuil, France). Diisopropylethylamine (DIEA), *t*-butylmethyl ether (TBME), 2-mercaptoethanol, acetic anhydride, anisole, thioanisole, 1,2-ethanedithiol, potassium ferricyanide and HEPES were from Sigma-Aldrich (St. Quentin Fallavier, France).

TABLE I Amino acid sequences of compounds **1–58**

| Compound number | Peptide | Primary structure* |
|---|---|---|
| **1** | UII | H-Glu-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH |
| **2** | UII-$NH_2$ | H-Glu-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **3** | [$Ala^1$]UII-$NH_2$ | H-Ala-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **4** | [$Ala^2$]UII-$NH_2$ | H-Glu-Ala-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **5** | [$Ala^3$]UII-$NH_2$ | H-Glu-Thr-Ala-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **6** | [$Ala^4$]UII-$NH_2$ | H-Glu-Thr-Pro-Ala-Cys-Phe-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **7** | [$Ala^6$]UII-$NH_2$ | H-Glu-Thr-Pro-Asp-Cys-Ala-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **8** | [$Ala^7$]UII-$NH_2$ | H-Glu-Thr-Pro-Asp-Cys-Phe-Ala-Lys-Tyr-Cys-Val-$NH_2$ |
| **9** | [$Ala^8$]UII-$NH_2$ | H-Glu-Thr-Pro-Asp-Cys-Phe-Trp-Ala-Tyr-Cys-Val-$NH_2$ |
| **10** | [$Ala^9$]UII-$NH_2$ | H-Glu-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Ala-Cys-Val-$NH_2$ |
| **11** | [$Ala^{11}$]UII-$NH_2$ | H-Glu-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Ala-$NH_2$ |
| **12** | [D-$Glu^1$]UII-$NH_2$ | H-***Glu***-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **13** | [D-$Thr^2$]UII-$NH_2$ | H-Glu-***Thr***-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **14** | [D-$Pro^3$]UII-$NH_2$ | H-Glu-Thr-***Pro***-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **15** | [D-$Asp^4$]UII-$NH_2$ | H-Glu-Thr-Pro-***Asp***-Cys-Phe-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **16** | [D-$Phe^6$]UII-$NH_2$ | H-Glu-Thr-Pro-Asp-Cys-***Phe***-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **17** | [D-$Trp^7$]UII-$NH_2$ | H-Glu-Thr-Pro-Asp-Cys-Phe-***Trp***-Lys-Tyr-Cys-Val-$NH_2$ |
| **18** | [D-$Lys^8$]UII-$NH_2$ | H-Glu-Thr-Pro-Asp-Cys-Phe-Trp-***Lys***-Tyr-Cys-Val-$NH_2$ |
| **19** | [D-$Tyr^9$]UII-$NH_2$ | H-Glu-Thr-Pro-Asp-Cys-Phe-Trp-Lys-***Tyr***-Cys-Val-$NH_2$ |
| **20** | [D-$Val^{11}$]UII-$NH_2$ | H-Glu-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-***Val***-$NH_2$ |
| **21** | $UII_{(2-11)}$ | H-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH |
| **22** | $UII_{(2-11)}$-$NH_2$ | H-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **23** | $UII_{(3-11)}$ | H-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH |
| **24** | $UII_{(3-11)}$-$NH_2$ | H-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **25** | $UII_{(4-11)}$ | H-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH |
| **26** | $UII_{(4-11)}$-$NH_2$ | H-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **27** | $UII_{(5-11)}$ | H-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH |
| **28** | $UII_{(5-11)}$-$NH_2$ | H-Cys-Phe-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **29** | $UII_{(5-10)}$ | H-Cys-Phe-Trp-Lys-Tyr-Cys-OH |
| **30** | [$Ala^1$]$UII_{(4-11)}$ | H-Ala-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH |
| **31** | [$Ala^3$]$UII_{(4-11)}$ | H-Asp-Cys-Ala-Trp-Lys-Tyr-Cys-Val-OH |
| **32** | [$Ala^4$]$UII_{(4-11)}$ | H-Asp-Cys-Phe-Ala-Lys-Tyr-Cys-Val-OH |
| **33** | [$Ala^5$]$UII_{(4-11)}$ | H-Asp-Cys-Phe-Trp-Ala-Tyr-Cys-Val-OH |
| **34** | [$Ala^6$]$UII_{(4-11)}$ | H-Asp-Cys-Phe-Trp-Lys-Ala-Cys-Val-OH |
| **35** | [$Ala^8$]$UII_{(4-11)}$ | H-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Ala-OH |
| **36** | [D-$Asp^1$]$UII_{(4-11)}$-$NH_2$ | H-***Asp***-Cys-Phe-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **37** | [D-$Phe^3$]$UII_{(4-11)}$-$NH_2$ | H-Asp-Cys-***Phe***-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **38** | [D-$Trp^4$]$UII_{(4-11)}$-$NH_2$ | H-Asp-Cys-Phe-***Trp***-Lys-Tyr-Cys-Val-$NH_2$ |
| **39** | [D-$Lys^5$]$UII_{(4-11)}$-$NH_2$ | H-Asp-Cys-Phe-Trp-***Lys***-Tyr-Cys-Val-$NH_2$ |
| **40** | [D-$Tyr^6$]$UII_{(4-11)}$-$NH_2$ | H-Asp-Cys-Phe-Trp-Lys-***Tyr***-Cys-Val-$NH_2$ |
| **41** | [D-$Val^8$]$UII_{(4-11)}$-$NH_2$ | H-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-***Val***-$NH_2$ |
| **42** | [$Ser^{5,10}$]UII | H-Glu-Thr-Pro-Asp-Ser-Phe-Trp-Lys-Tyr-Ser-Val-OH |
| **43** | [$Ala^{5,10}$]UII-$NH_2$ | H-Glu-Thr-Pro-Asp-Ala-Phe-Trp-Lys-Tyr-Ala-Val-$NH_2$ |
| **44** | [$Pen^{5,10}$]UII | H-Glu-Thr-Pro-Asp-Pen-Phe-Trp-Lys-Tyr-Pen-Val-OH |
| **45** | [D-$Cys^{5,10}$]UII-$NH_2$ | H-Glu-Thr-Pro-Asp-***Cys***-Phe-Trp-Lys-Tyr-***Cys***-Val-$NH_2$ |
| **46** | [$Ser^{2,7}$]$UII_{(4-11)}$ | H-Asp-Ser-Phe-Trp-Lys-Tyr-Ser-Val-OH |
| **47** | [$Cys(Acm)^{2,7}$]$UII_{(4-11)}$ | H-Asp-$Cys_{(Acm)}$-Phe-Trp-Lys-Tyr-$Cys_{(Acm)}$-Val-OH |
| **48** | [D-$Cys^{2,7}$]$UII_{(4-11)}$-$NH_2$ | H-Asp-***Cys***-Phe-Trp-Lys-Tyr-***Cys***-Val-$NH_2$ |
| **49** | Ac-$UII_{(3-11)}$ | Ac-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH |
| **50** | Ac-[D-$Glu^1$]UII | Ac-***Glu***-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH |
| **51** | Ac-$UII_{(4-11)}$ | Ac-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH |
| **52** | Ac-[D-$Glu^1$]UII-$NH_2$ | Ac-***Glu***-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **53** | Ac-$UII_{(3-11)}$-$NH_2$ | Ac-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **54** | Ac-UII | Ac-Glu-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH |
| **55** | Ac-UII-$NH_2$ | Ac-Glu-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-$NH_2$ |
| **56** | [D-$Trp^7$]UII | H-Glu-Thr-Pro-Asp-Cys-Phe-***Trp***-Lys-Tyr-Cys-Val-OH |
| **57** | [D-$Trp^4$]$UII_{(4-11)}$ | H-Asp-Cys-Phe-***Trp***-Lys-Tyr-Cys-Val-OH |
| **58** | [3-iodo-$Tyr^6$]$UII_{(4-11)}$ | H-Asp-Cys-Phe-Trp-Lys-$_{(iodo)}$Tyr-Cys-Val-OH |

*D-Amino acids are indicated in bold italic letters.

*N*-methylmorpholine (NMM) and piperidine were from Merck (Nogent-sur-Marne, France) and phenol was from Gibco Life Technologies (Cergy Pontoise, France).

## Peptide Synthesis and Cleavage

All C-terminal free analogues of human UII were synthesized (0.1 mmol scale) on a Fmoc-Val-PEG-PS, a Fmoc-Ala-PEG-PS or a Fmoc-Cys(Acm)-PEG-PS resin using a Pioneer PerSeptive Biosystems peptide synthesizer (Applera France) with the standard template and OH derivative procedures. All Fmoc-amino acids (0.4 mmol, 4 eq.) were coupled by *in situ* activation with TBTU/HOBt (0.5 mmol/0.5 mmol, 5 eq., 1:1) and DIEA (5 mmol, 5 eq.) in DMF. Reactive side chains were protected as follows: Glu and Asp,

*t*-butyl ester (O*t* Bu); Thr, Tyr and Ser, *t*-butyl ether (*t* Bu); Lys, Trp and D-Trp, *t*-butyloxycarbonyl carbamate (Boc); Cys, S-*t*-butyl disulfide (S*t* Bu) or acetamidomethyl (Acm). After completion of the chain assembly, cyclization of human UII analogues was performed on resin by removing the S*t* Bu ether protecting groups of the Cys residues with a mixture of DMF:2-mercaptoethanol (57 mmol, 57 eq. 1:1, v/v) for 5 h at room temperature (RT). After complete deprotection, the peptidyl-resin was filtered and washed three times with DMF, DCM, DMF, isopropanol and DMF, successively. Then, the peptidyl-resin was placed in an aqueous solution of potassium ferricyanide (0.1 mmol, 1 eq.) for 15 h at RT. The peptidyl-resin was washed 6 times with DMF:$H_2O$ (1:1) and then three times with DMF, DCM, DMF, iPrOH and DMF, successively.[33] Cyclic C-terminal free peptides were deprotected and cleaved from the resin by adding 10 mL of the mixture TFA/phenol/anisole/$H_2O$ (85:6.5:4.25:4.25, v/v/v/v) for 2 h at RT. After filtration, the crude peptides were precipitated by addition of TBME, centrifuged (4,500 rpm), washed twice with TBME and lyophilized.

All C-terminal amidated analogues of human UII were synthesized (0.05 mmol scale) on a Fmoc-PAL-PEG-PS resin using a Multiple Peptide Synthesizer (MPS) module coupled with a Pioneer PerSeptive Biosystems peptide synthesizer with the MPS template and OH derivative procedures. All Fmoc-amino acids (0.2 mmol, 4 eq.) were coupled by *in situ* activation with HATU (0.2 mmol, 4 eq.) and DIEA in DMF. Reactive side chains were protected as described above except for the indole nucleus of Trp which was not protected. After completion of the chain assembly, the resins were extensively washed with isopropanol and dried overnight *in vacuo*.

The linear free-carboxyl or C-terminal amidated peptides were deprotected and cleaved as previously described.[34] After a preliminary purification step (see below) and freeze-drying, the linear C-terminal amidated peptides were dissolved in HEPES pH 7.5–8 at a concentration of 0.1 mg/mL. The solution was vigorously stirred and the oxidation process was followed with Ellman reagent. The solutions were freeze-dried.

### Purification

All peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC) on a semi-preparative Vydac $C_{18}$ column (2 × 25 cm; Touzart et Matignon, Courtaboeuf, France) using a linear gradient (10–50% over 40 min) of acetonitrile/TFA (99.92:0.08, v/v) at a flow rate of 10 mL/min, or on a Deltapak $C_{18}$ column (1.9 × 30 cm; Waters, Saint-Quentin en Yvelines, France) using a linear gradient (15–40% over 30 min) of acetonitrile/TFA (99.92:0.08, v/v) at a flow rate of 12 mL/min. Analytical RP-HPLC (1 mL/min) was performed on a Vydac $C_{18}$ column (0.45 × 25 cm) using a linear gradient (10–40% over 30 min) of acetonitrile/TFA (99.92:0.08, v/v). The purified peptides were characterized by a MALDI-TOF-MS on a TOFSpecE (Micromass, Manchester, UK) or by ESI-MS on a Q-Star (Applera France).

### Measurement of Rat Aortic Ring Contraction

Male Wistar rats (200–250 g) were stunned and killed by cervical dislocation. The part of thoracic aorta corresponding to the 2-cm portion proximal to the carotid bifurcation of the aortic arch was collected in physiological saline solution (PSS, 130 mM NaCl, 5.6 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 11 mM glucose, 10 mM Tris, pH 7.4) cleaned of fat and adherent connective tissue, and cut in rings. The length of each arterial ring was 4 mm. The endothelium was carefully removed by gently rubbing the intimal surface with the tip of small forceps. Smooth muscle rings were then suspended under isometric conditions and connected to a force transducer (Pioden Controls Ltd, Canterbury, UK) in organ baths filled with Krebs-Henseleit solution (118.4 mM NaCl, 4.7 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 25 mM $NaHCO_3$, 11 mM glucose) maintained at 37°C, and equilibrated with $O_2/CO_2$ (95:5). The preparations were initially placed under a resting tension of 1500 mg, left to equilibrate for 1 h and washed at 20 min intervals. Contractile responses to 60 mM KCl were used as control responses at the beginning and at the end of each experiment. The absence of endothelium was confirmed in each ring by the inability of carbachol (10 μM) to relax the contraction induced by phenylephrine (PE, 1 μM). Cumulative concentration-response curves to UII and its analogues were obtained by increasing the agonist concentration in the organ chamber. To determine $EC_{50}$ values, concentration-response curves were fitted to a logistic equation using Origin software from Dipsi (Chatillon, France). The amplitude of the contraction induced in response to each concentration of peptide was measured and expressed as a percentage (± SEM) of the PE (1 μM)-induced tension.

### Statistical Analysis

Data are expressed as mean ± SEM. Differences between UII, UII-$NH_2$ and analogue potencies were analyzed by ANOVA followed by a Dunnett's multiple comparison test.

## RESULTS AND DISCUSSION

Exposure of de-endothelialised thoracic rat aortic rings to human UII (**1**) produced dose-dependent contraction with an $EC_{50}$ value of 11 nM (Figure 2A). In agreement with this observation, several studies have previously demonstrated that UII is a highly potent vasoconstrictor peptide.[8,25,35] A large proportion of regulatory peptides are α-amidated[36] and C-terminal amidation has been widely used to protect peptide analogues against proteolysis by carboxypeptidases.[37] In order to study the effect of amidation of the C-terminal Val residue on the activity of the peptide, we have compared the effects of human UII (**1**) and human UII-$NH_2$ (**2**) in the rat thoracic aortic ring assay. The dose-response curves showed that both peptides produced maximum contractile response at a concentration of $10^{-7}$ M (Figure 2) and that the $EC_{50}$ value of UII-$NH_2$ (**2**) was two times higher than that of UII (**1**) (Table II). Since α-amidation only produced a 2-fold reduction of the potency of UII, and since this modification is expected to enhance the stability of the peptide in biological fluids,[36,37] we have used the amidated form as a template to map the functionally important determinants of UII.

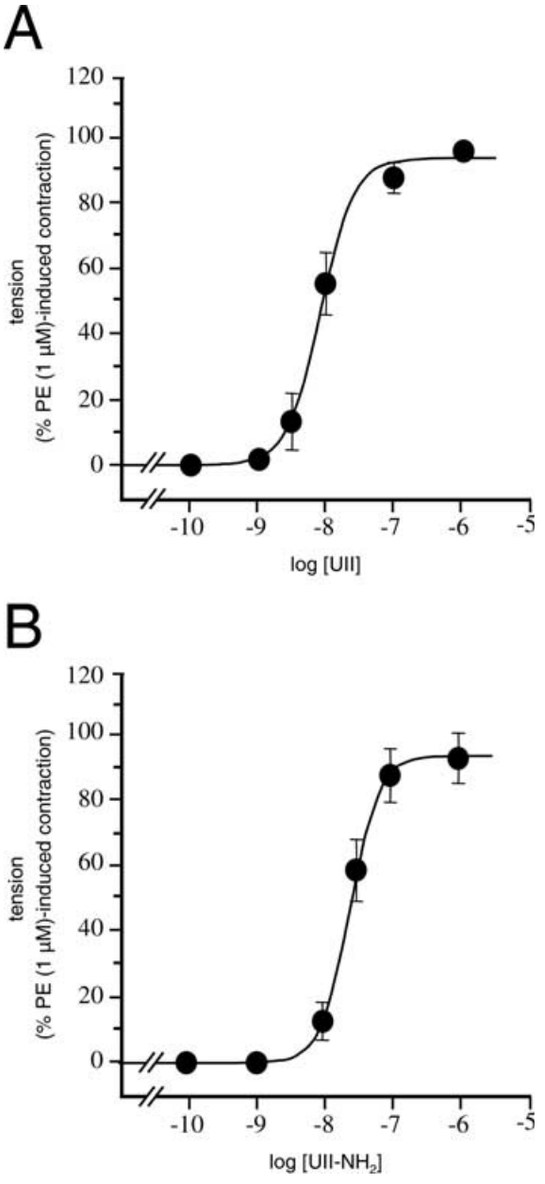


FIGURE 2 Typical dose-response curves showing the effect of human UII and UII-$NH_2$ on rat thoracic aortic ring contraction. Panel A shows the percentage of the contractile effect of human UII (**1**) related to the response to $10^{-6}$ M phenylephrine (PE). Panel B shows the percentage of the contractile effect of UII-$NH_2$ (**2**) related to the response to $10^{-6}$ M phenylephrine (PE).

The contribution of each side-chain motif in the biological activity of the peptide was investigated by systematic substitution of each amino acid residue of UII-$NH_2$ by an Ala moiety (Table II, **3–11**), except for the $Cys^5$ and $Cys^{10}$ residues to preserve the backbone conformation of the peptide. Replacement of individual amino acids in the extra-cyclic region of UII-$NH_2$ (**3–6** and **11**) did not substantially affect the contractile potency of the peptide (Figure 3A), indicating that the side-chains of $Glu^1$, $Thr^2$, $Pro^3$, $Asp^4$ and $Val^{11}$ are not essential for the biological activity of UII-$NH_2$. In particular, substitution of the $Pro^3$ residue (**5**) that is expected to confer flexibility to the N-terminal linear segment did not impair the vasocontractile effect of UII-$NH_2$. In all UII isoforms characterized so far (Figure 1), the cyclic region is invariably flanked by an acidic residue (Asp/Glu) on its N-terminal side and a voluminous hydrophobic residue (Val/Ile) on its C-terminal side.[7,38] The fact that the potency of [$Ala^4$]UII-$NH_2$ (**6**) was only reduced by 4 fold (Figure 3A) revealed that an acidic feature is not required for agonistic activity. Similarly, the potency of [$Ala^{11}$]UII-$NH_2$ (**11**) was only reduced by 6 fold (Figure 3A), indicating that a hydrophobic residue at the C-terminus is not essential for the contractile activity. In contrast, Ala substitution of individual amino acids in the cyclic region of the molecule (**7–10**) suppressed the activity of each analogue (Figure 3A). For instance, [$Ala^7$]UII-$NH_2$ and [$Ala^9$]UII-$NH_2$ (**8** and **10**), in which the $Trp^7$ and $Tyr^9$ residues have been substituted, were totally devoid of activity even at a concentration of $10^{-5}$ M. In addition, neither compound **8** nor compound **10** were able to antagonize human UII-evoked aortic contraction (data not shown), suggesting that the $Trp^7$ and $Tyr^9$ moieties may be involved in the binding of UII to its receptor. It is interesting to note that the tripeptide Phe-Trp-Lys which is present in the rings of both UII[1] and somatostatin[39] plays a pivotal role in the biological activity of both peptides.[40] Globally, the potency of the Ala-substituted amidated peptides reported herein, using the rat aortic ring assay, is similar to those of

TABLE II Analytical and Biological Data for Compounds **1–58**

| Compound number | Peptide | [MH]$^+$ | | HPLC | | Max efficacy[§] | n | $EC_{50}$ (nM) | $pEC_{50}$ |
|---|---|---|---|---|---|---|---|---|---|
| | | Calcd* | Obsd[†] | $t_R$ (min)[‡] | Purity (%)[¶] | | | | |
| **1** | UII | 1388.6 | 1388.6 | 16.2 | 99.9 | 94.5 | 7 | 11.9 ± 4.4 | 7.9 |
| **2** | UII-$NH_2$ | 1387.6 | 1387.6 | 20.8 | 99.9 | 96.0 | 4 | 25.8 ± 5.7 | 7.6 |
| **3** | [Ala$^1$]UII-$NH_2$ | 1329.6 | 1330.6 | 19.5 | 98.0 | 103.0 | 4 | 13.0 ± 5 | 7.9 |
| **4** | [Ala$^2$]UII-$NH_2$ | 1357.6 | 1356.5 | 20.9 | 99.5 | 97.3 | 5 | 13.0 ± 2 | 7.9 |
| **5** | [Ala$^3$]UII-$NH_2$ | 1361.6 | 1361.6 | 19.5 | 99.5 | 110.6 | 4 | 46.0 ± 20 | 7.3 |
| **6** | [Ala$^4$]UII-$NH_2$ | 1343.6 | 1343.6 | 21.0 | 99.8 | 104.9 | 4 | 46.0 ± 9 | 7.3 |
| **7** | [Ala$^6$]UII-$NH_2$ | 1311.5 | 1311.5 | 12.2 | 99.5 | 37.8 | 4 | 4447 ± 1333 | 5.4 |
| **8** | [Ala$^7$]UII-$NH_2$ | 1272.5 | 1272.4 | 11.5 | 99.9 | - | 4 | >10,000 | 5.0 |
| **9** | [Ala$^8$]UII-$NH_2$ | 1330.5 | 1330.5 | 15.4 | 99.9 | - | 4 | >10,000 | 5.0 |
| **10** | [Ala$^9$]UII-$NH_2$ | 1295.5 | 1295.4 | 15.2 | 99.8 | - | 4 | >10,000 | 5.0 |
| **11** | [Ala$^{11}$]UII-$NH_2$ | 1359.5 | 1359.3 | 16.5 | 99.8 | 94.5 | 10 | 75.0 ± 16 | 7.1 |
| **12** | [D-Glu$^1$]UII-$NH_2$ | 1388.6 | 1387.5 | 15.8 | 98.0 | 88.8 | 6 | 4.6 ± 0.8 | 8.3 |
| **13** | [D-Thr$^2$]UII-$NH_2$ | 1388.6 | 1387.4 | 15.6 | 98.0 | 96.4 | 4 | 24.4 ± 4.9 | 7.6 |
| **14** | [D-Pro$^3$]UII-$NH_2$ | 1388.6 | 1387.4 | 14.5 | 98.0 | 91.5 | 7 | 6.7 ± 1.7 | 8.2 |
| **15** | [D-Asp$^4$]UII-$NH_2$ | 1388.6 | 1387.5 | 14.7 | 99.9 | 99.2 | 5 | 3.6 ± 0.4 | 8.4 |
| **16** | [D-Phe$^6$]UII-$NH_2$ | 1388.6 | 1387.5 | 13.5 | 99.8 | - | 4 | >10,000 | 5.0 |
| **17** | [D-Trp$^7$]UII-$NH_2$ | 1388.6 | 1387.6 | 16.5 | 99.8 | 32.1 | 10 | 279 ± 97 | 6.6 |
| **18** | [D-Lys$^8$]UII-$NH_2$ | 1388.6 | 1387.5 | 15.2 | 99.0 | - | 4 | >10,000 | 5.0 |
| **19** | [D-Tyr$^9$]UII-$NH_2$ | 1388.6 | 1387.4 | 11.5 | 98.0 | - | 4 | >10,000 | 5.0 |
| **20** | [D-Val$^{11}$]UII-$NH_2$ | 1388.6 | 1387.6 | 18.6 | 99.8 | 86.0 | 5 | 157 ± 55 | 6.8 |
| **21** | $UII_{(2-11)}$ | 1259.5 | 1259.5 | 15.1 | 99.9 | 97.3 | 4 | 27.0 ± 7 | 7.6 |
| **22** | $UII_{(2-11)}$-$NH_2$ | 1258.5 | 1258.6 | 14.7 | 99.9 | 94.1 | 3 | 9.0 ± 2.4 | 8.0 |
| **23** | $UII_{(3-11)}$ | 1158.4 | 1157.0 | 13.1 | 99.9 | 104.0 | 6 | 6.7 ± 0.8 | 8.2 |
| **24** | $UII_{(3-11)}$-$NH_2$ | 1157.4 | 1157.4 | 21.8 | 99.5 | 87.9 | 4 | 19.0 ± 6 | 7.7 |
| **25** | $UII_{(4-11)}$ | 1061.2 | 1061.3 | 12.6 | 99.9 | 102.1 | 4 | 2.7 ± 1.1 | 8.6 |
| **26** | $UII_{(4-11)}$-$NH_2$ | 1060.3 | 1060.3 | 15.4 | 99.5 | 92.6 | 4 | 11.4 ± 3.5 | 7.9 |
| **27** | $UII_{(5-11)}$ | 946.1 | 946.3 | 14.8 | 99.9 | 88.8 | 4 | 1120 ± 404 | 6.0 |
| **28** | $UII_{(5-11)}$-$NH_2$ | 945.3 | 946.3 | 13.7 | 99.9 | 85.1 | 4 | 652 ± 213 | 6.2 |
| **29** | $UII_{(5-10)}$ | 847.0 | 847.0 | 11.2 | 99.9 | 90.7 | 4 | 2627 ± 256 | 5.6 |
| **30** | [Ala$^1$]$UII_{(4-11)}$ | 1017.2 | 1017.1 | 20.9 | 98.0 | 101.1 | 5 | 50.0 ± 27 | 7.3 |
| **31** | [Ala$^3$]$UII_{(4-11)}$ | 985.1 | 984.8 | 18.2 | 99.9 | 54.8 | 5 | 2414 ± 250 | 5.6 |
| **32** | [Ala$^4$]$UII_{(4-11)}$ | 946.1 | 945.7 | 17.4 | 99.9 | - | 4 | >10,000 | 5.0 |
| **33** | [Ala$^5$]$UII_{(4-11)}$ | 1004.1 | 1004.2 | 19.4 | 99.9 | 98.3 | 4 | 456 ± 185 | 6.3 |
| **34** | [Ala$^6$]$UII_{(4-11)}$ | 969.1 | 969.7 | 19.6 | 99.9 | 8.0 | 4 | >10,000 | 5.0 |
| **35** | [Ala$^8$]$UII_{(4-11)}$ | 1033.2 | 1034.5 | 19.4 | 99.9 | - | 4 | >10,000 | 5.0 |
| **36** | [D-Asp$^1$]$UII_{(4-11)}$-$NH_2$ | 1060.3 | 1060.3 | 15.7 | 99.9 | 97.3 | 4 | 10.3 ± 1.8 | 8.0 |
| **37** | [D-Phe$^3$]$UII_{(4-11)}$-$NH_2$ | 1060.3 | 1060.3 | 15.7 | 99.9 | - | 4 | >10,000 | 5.0 |
| **38** | [D-Trp$^4$]$UII_{(4-11)}$-$NH_2$ | 1060.3 | 1060.1 | 15.8 | 99.9 | 84.1 | 4 | 124 ± 46 | 6.9 |
| **39** | [D-Lys$^5$]$UII_{(4-11)}$-$NH_2$ | 1060.3 | 1060.1 | 15.2 | 99.9 | - | 4 | >10,000 | 5.0 |
| **40** | [D-Tyr$^6$]$UII_{(4-11)}$-$NH_2$ | 1060.3 | 1060.0 | 12.1 | 99.9 | - | 4 | >10,000 | 5.0 |
| **41** | [D-Val$^8$]$UII_{(4-11)}$-$NH_2$ | 1060.3 | 1060.4 | 15.4 | 99.5 | 96.4 | 4 | 1388 ± 544 | 5.9 |
| **42** | [Ser$^{5,10}$]UII | 1358.5 | 1358.7 | 19.2 | 99.9 | - | 4 | >10,000 | 5.0 |
| **43** | [Ala$^{5,10}$]UII-$NH_2$ | 1325.5 | 1325.5 | 23.8 | 99.9 | - | 4 | >10,000 | 5.0 |
| **44** | [Pen$^{5,10}$]UII | 1443.7 | 1444.1 | 22.0 | 97.5 | 84.1 | 4 | 97.0 ± 20 | 7.0 |
| **45** | [D-Cys$^{5,10}$]UII-$NH_2$ | 1387.6 | 1387.6 | 21.4 | 99.5 | - | 4 | >10,000 | 5.0 |
| **46** | [Ser$^{2,7}$]$UII_{(4-11)}$ | 1031.1 | 1031.1 | 18.9 | 99.9 | - | 4 | >10,000 | 5.0 |
| **47** | [Cys(Acm)$^{2,7}$]$UII_{(4-11)}$ | 1205.3 | 1205.3 | 19.6 | 99.9 | 52.0 | 6 | 1968 ± 700 | 5.7 |
| **48** | [D-Cys$^{2,7}$]$UII_{(4-11)}$-$NH_2$ | 1060.3 | 1060.2 | 20.8 | 99.9 | - | 4 | >10,000 | 5.0 |
| **49** | Ac-$UII_{(3-11)}$ | 1200.4 | 1200.6 | 22.5 | 99.9 | 94.5 | 4 | 3.8 ± 1.6 | 8.4 |
| **50** | Ac-[D-Glu$^1$]UII | 1430.7 | 1430.7 | 21.0 | 99.9 | 90.7 | 5 | 4.3 ± 2.8 | 8.4 |
| **51** | Ac-$UII_{(4-11)}$ | 1102.3 | 1103.5 | 21.5 | 99.9 | 102.1 | 4 | 1.7 ± 1 | 8.8 |
| **52** | Ac-[D-Glu$^1$]UII-$NH_2$ | 1429.7 | 1429.7 | 19.4 | 99.9 | 91.7 | 5 | 7.3 ± 1.1 | 8.1 |
| **53** | Ac-$UII_{(3-11)}$-$NH_2$ | 1199.4 | 1199.6 | 20.7 | 99.9 | 83.2 | 4 | 30.0 ± 23 | 7.5 |
| **54** | Ac-UII | 1430.7 | 1430.7 | 21.0 | 99.9 | 99.2 | 4 | 4.6 ± 1.9 | 8.3 |
| **55** | Ac-UII-$NH_2$ | 1429.7 | 1429.6 | 13.0 | 99.5 | 89.4 | 4 | 9.5 ± 1.9 | 8.0 |
| **56** | [D-Trp$^7$]UII | 1388.6 | 1388.2 | 21.3 | 99.9 | 23.6 | 4 | 180 ± 80 | 6.7 |
| **57** | [D-Trp$^4$]$UII_{(4-11)}$ | 1061.2 | 1061.2 | 21.0 | 99.9 | 64.7 | 4 | 71.0 ± 50 | 7.1 |
| **58** | [3-iodo-Tyr$^6$]$UII_{(4-11)}$ | 1188.2 | 1187.2 | 22.9 | 99.9 | 87.5 | 6 | 2.2 ± 1.9 | 8.7 |

*Theoretical molecular weight ([MH]$^+$, Da). †$m/z$ values by MALDI-TOF-MS or ESI-MS. ‡Retention time determined by RP-HPLC. ¶Assessed by RP-HPLC. §The maximum efficacy (contraction produced by $10^{-6}$ M peptide) is expressed as a percentage of the amplitude of the contraction induced by $10^{-6}$ M phenylephrine.

the C-terminal free counterparts recently determined on human GPR14-transfected CHO cells.[32]

In order to explore the importance of the orientation of the amino acid side-chains in the biological activity of the peptide, a series of D-isomer-substituted peptides was synthesized (**12–20**) and the ability of the analogues to contract rat thoracic aortic rings was measured (Table II). Replacement of each N-terminal amino acid by its D-enantiomer (**12–15**) did not affect the activity of

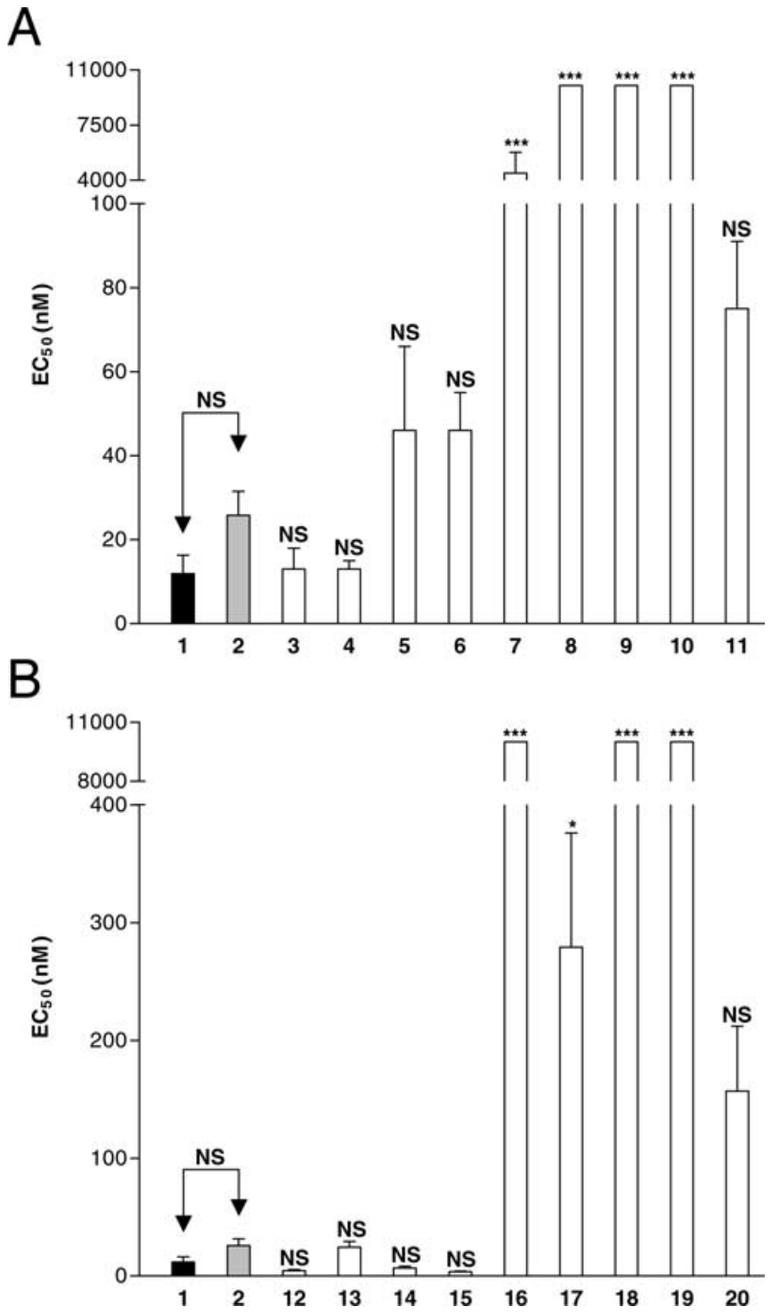


FIGURE 3 Comparison of the $EC_{50}$ values (nM) of L-alanine and D-isomer-substituted human UII-$NH_2$ analogues on rat thoracic aortic ring contraction. Panel A shows the mean (± SEM) $EC_{50}$ value of UII (**1**) (black bar), UII-$NH_2$ (**2**) (grey bar), L-alanine-substituted analogues (**3–11**) (open bars). Panel B shows the mean (± SEM) $EC_{50}$ value of UII (**1**) (black bar), UII-$NH_2$ (**2**) (grey bar), D-isomer-substituted analogues (**12–20**) (open bars). Each $EC_{50}$ value refers to the compound concentration yielding 50% of the maximal contractile response determined from each dose-response curve. $^*P < 0.05$, $^{***}P < 0.001$ *vs* UII-$NH_2$ by one-way ANOVA followed by *post hoc* Dunnett's test, NS, not statistically significant.

the peptide (Figure 3B). In contrast, [D-Val$^{11}$]UII-$NH_2$ (**20**) was 6 times less potent than UII-$NH_2$. Since [Ala$^{11}$]UII-$NH_2$ (**11**) was 2 times more potent than [D-Val$^{11}$]UII-$NH_2$, it appears that the orientation of the C-terminal side-chain residue is important for the activity of the peptide. Point-substitution of Phe$^6$, Lys$^8$ or Tyr$^9$ by their D-isomer (**16**, **18** or **19**) totally suppressed the activity of the UII-$NH_2$ analogues while [D-Trp$^7$] replacement (**17**) caused a 11-fold decrease in the potency (Figure 3B). Similar effects have been reported on calcium mobilization.[32] Altogether, these data demonstrate that not only the chemical structure but also the correct orientation of the side-chains of each residue of the cyclic region plays a critical role in the activity of the peptide.

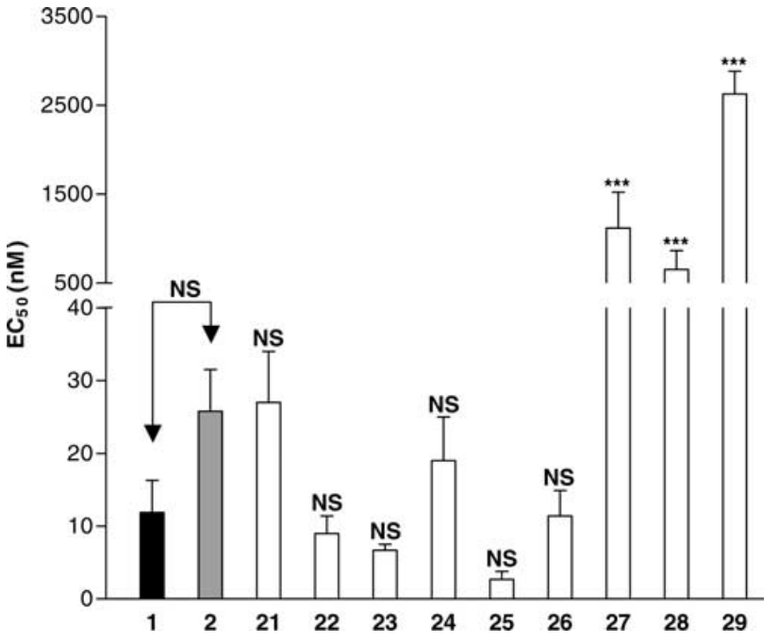


FIGURE 4 Comparison of the $EC_{50}$ values (nM) of human UII, UII-$NH_2$ and their N- and C-truncated fragments on rat thoracic aortic ring contraction. Each value represents the mean (± SEM) $EC_{50}$ of UII (**1**) (black bar), UII-$NH_2$ (**2**) (grey bar) and free C-terminal (**21**, **23**, **25**, **27** and **29**) and C-terminally amidated (**22**, **24**, **26** and **28**) peptides (open bars). See legend to Figure 3 for other designations. $^{***}P < 0.001$ *vs* UII (**21**, **23**, **25**, **27** and **29**, respectively) or UII-$NH_2$ (**22**, **24**, **26** and **28**, respectively) by one-way ANOVA followed by *post hoc* Dunnett's test, NS, not statistically significant.

To investigate the contribution of the N- and C-terminal linear sequences in the contractile potency of the peptide, we have synthesized truncated analogues of UII and UII-$NH_2$ (**21–29**). Figure 4 shows that deletion of $Glu^1$ (**21** and **22**), $Glu^1$ and $Thr^2$ (**23** and **24**) or $Glu^1$, $Thr^2$ and $Pro^3$ (**25** and **26**) did not affect the biological activity of the peptide. The potency of the amidated forms of truncated analogues (**24** and **26**) was generally lower than that of the corresponding C-terminal free counterparts (**23** and **25**, respectively) as already observed for the full-length peptides (**2** and **1**, respectively). Additional deletion of $Asp^4$ (**27** and **28**) markedly reduced the potency of the UII and UII-$NH_2$ analogues, while deletion of $Glu^1$, $Thr^2$, $Pro^3$, $Asp^4$ and $Val^{11}$ (**29**) suppressed the activity (Table II, Figure 4), indicating that the presence of a residue on the N- and C-termini of the ring structure is necessary for biological activity. In agreement with these observations, an early report had shown that the goby $UII_{(5-12)}$ fragment (homologous to human $UII_{(4-11)}$) was equipotent with the native peptide while the goby $UII_{(6-12)}$ and $UII_{(6-11)}$ fragments (homologous to human $UII_{(5-11)}$ and $UII_{(5-10)}$) were much less potent in contracting rat aortic strips and in displacing $^{125}$I-UII from rat aortic membranes.[17] Similarly, in a trout rectum assay, the goby $UII_{(5-12)}$ fragment was far more potent than the goby $UII_{(6-12)}$ or $UII_{(5-11)}$ fragments.[16] Taken together, these data

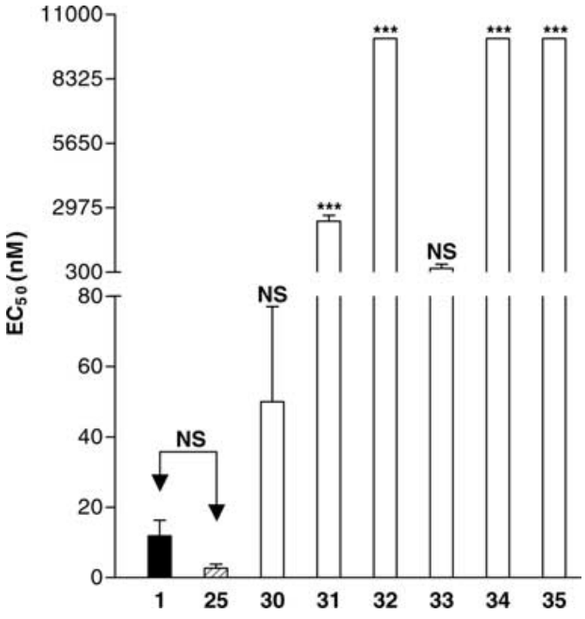


FIGURE 5 Comparison of the $EC_{50}$ values (nM) of L-alanine-substituted $UII_{(4-11)}$ analogues on rat thoracic aortic ring contraction. Each value represents the mean (± SEM) $EC_{50}$ of human UII (**1**) (black bar), $UII_{(4-11)}$ (**25**) (hatched bar) and L-alanine-substituted $UII_{(4-11)}$ analogues (**30–35**) (open bars). See legend to Figure 3 for other designations. $^{***}P < 0.001$ *vs* $UII_{(4-11)}$ by one-way ANOVA followed by *post hoc* Dunnett's test, NS, not statistically significant.

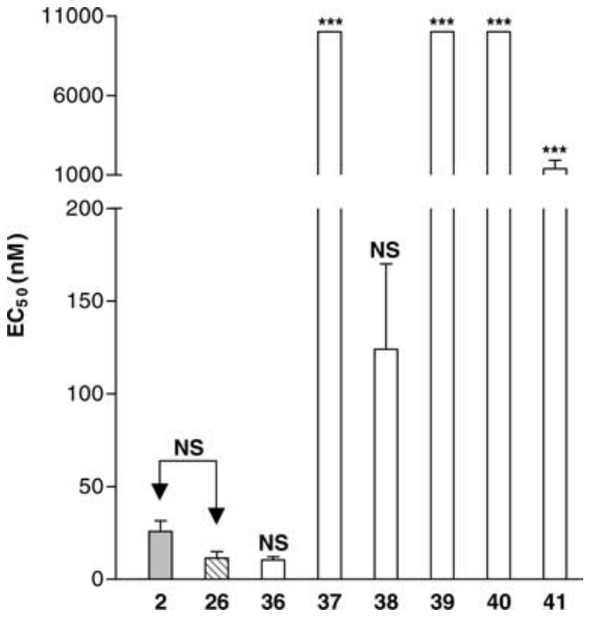


FIGURE 6 Comparison of the $EC_{50}$ values (nM) of D-isomer-substituted $UII_{(4-11)}$-$NH_2$ analogues on rat thoracic aortic ring contraction. Each value represents the mean (± SEM) $EC_{50}$ of UII-$NH_2$ (**2**) (grey bar), $UII_{(4-11)}$-$NH_2$ (**26**) (hatched bar) and D-isomer-substituted $UII_{(4-11)}$-$NH_2$ analogues (**36–41**) (open bars). See legend to Figure 3 for other designations. $^{***}P < 0.001$ *vs* $UII_{(4-11)}$-$NH_2$ by one-way ANOVA followed by *post hoc* Dunnett's test, NS, not statistically significant.

indicate that the minimal sequence for strong activity of UII requires 8 amino acids *i.e.* the cyclic region flanked by an amino acid at both its N- and C-terminal extremities. Interestingly, this domain corresponds precisely to the sequence which has been almost completely conserved across vertebrate UIIs (Figure 1), thus confirming that evolutionary pressure has acted to preserve the functionally important region of the UII molecule.[14] It should be mentioned however that, in human GPR14-transfected CHO cells, $UII_{(5-10)}$ has recently been reported to be equipotent to native UII in stimulating calcium mobilization,[32] suggesting that the Asp and Val residues flanking the ring structure are not essential for the biological activity of UII on the human receptor.[32]

The influence of modifications of $UII_{(4-11)}$ (**25**) and $UII_{(4-11)}$-$NH_2$ (**26**) was further evaluated by subjecting them to an Ala-scan and a D-scan, respectively (Table II, Figures 5 and 6). A good correlation was generally observed between the relative activities of the Ala- and D-isomer-substituted $UII_{(4-11)}$ analogues (**30–41**) and the corresponding substituted UII-$NH_2$ homologues (**6–11** and **15–20**). In particular, substitution of the $Asp^1$ residue of $UII_{(4-11)}$ by Ala or D-Asp (**30** and **36**, respectively) did not markedly affect the vasocontractile activity, in very much the same way as for the UII-$NH_2$ homologues (**6** and **15**, respectively). Concurrently, replacement of the $Phe^3$ or $Tyr^6$ residues within the cyclic region by Ala (**31** and **34**, respectively) or by their D-isomer (**37** and **40**, respectively) led to inactive peptides (Figures 5 and 6) like their UII-$NH_2$ homologues (**7**, **10**, **16** and **19**, respectively). The [$Ala^4$]$UII_{(4-11)}$ analogue (**32**), like the [$Ala^7$]UII-$NH_2$ analogue (**8**), was devoid of activity, while the [D-$Trp^4$]$UII_{(4-11)}$-$NH_2$ analogue (**38**), like the [D-$Trp^7$]UII-$NH_2$ analogue (**17**), exhibited reduced efficacy. These data confirm the importance of the amino acids inside the ring for the biological activity of UII.

To determine the role of the disulfide bridge and the cysteines themselves in the biological activity of UII and $UII_{(4-11)}$, we have synthesized several analogues in which the Cys residues have been modified (**42–48**). Substitution of the two cysteines by Ser residues in the UII molecule (**42**), or by Ala residues in the UII-$NH_2$ molecule (**43**), totally suppressed the contractile activity (Table II, Figure 7), indicating that the integrity of the disulfide bridge is essential for the biological activity of the peptide. Consistent with this data, it has been previously reported that the reduced form of goby UII was totally devoid of contractile activity in a trout rectum assay.[16] The cyclic analogue [$Pen^{5,10}$]UII (**44**) was 8 times less potent than native UII whereas [D-$Cys^{5,10}$]UII-$NH_2$ (**45**) was totally inactive. Similarly, any modification of the cysteine moieties of $UII_{(4-11)}$ or $UII_{(4-11)}$-$NH_2$ (**46–48**), led to inactive

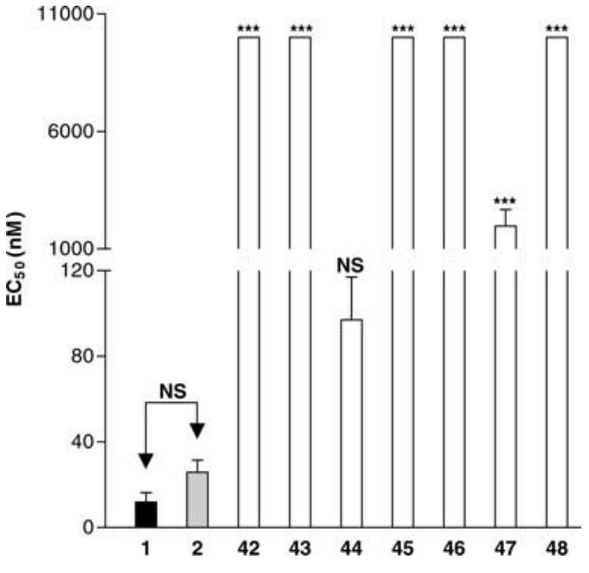


FIGURE 7 Comparison of the $EC_{50}$ values (nM) of human UII and UII-$NH_2$ analogues in which the cysteine residues have been replaced on rat thoracic aortic ring contraction. Each value represents the mean (± SEM) $EC_{50}$ of UII (**1**) (black bar), UII-$NH_2$ (**2**) (grey bar) and theirs analogues (**42–48**) (open bars). See legend to Figure 3 for other designations. $^{***}P < 0.001$ *vs* UII (**42**, **44**, **46** and **47**, respectively) or UII-$NH_2$ (**43**, **45** and **48**, respectively) by one-way ANOVA followed by *post hoc* Dunnett's test, NS, not statistically significant.

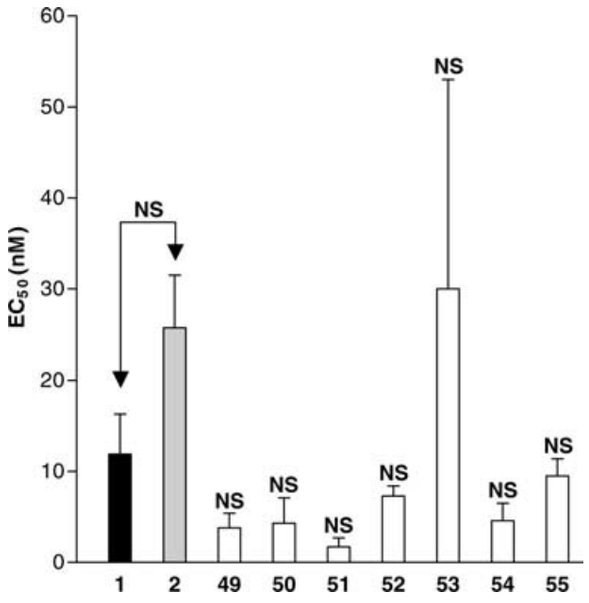


FIGURE 8 Comparison of the $EC_{50}$ values (nM) of acetylated human UII, UII-$NH_2$ and analogues on rat thoracic aortic ring contraction. Each value represents the mean (± SEM) $EC_{50}$ of UII (**1**) (black bar), UII-$NH_2$ (**2**) (grey bar) and their acetylated analogues (**49–55**) (open bars). See legend to Figure 3 for other designations. NS, not statistically significant *vs* UII (**49**, **50**, **51** and **54**, respectively) or UII-$NH_2$ (**52**, **53** and **55**, respectively).

compounds (Figure 7), indicating that the disulfide bridge and its natural configuration are crucial for the constrictor effect of UII on the rat thoracic aorta.

N-terminal acetylation is known to confer resistance to aminopeptidases and acetylated analogues usually exhibit longer half-lives in biological tissues.[41] However, acetylation may also affect the biological activity of peptides. For instance, acetylation of α-melanocyte-stimulating hormone enhances the melanotropic potency of the peptide[42] while acetylation of β-endorphin suppresses its opioid activity.[43] We have thus synthesized a series of 7 $N_\alpha$-acetylated UII analogues (Table I, **49–55**) and tested their contractile activity in the rat aortic ring assay (Table II, Figure 8). The acetylated forms of $UII_{(3-11)}$ (**49**), $UII_{(4-11)}$ (**51**), [D-Glu$^1$]UII-$NH_2$ (**52**) and $UII_{(3-11)}$-$NH_2$ (**53**) exhibited similar activity as their non-acetylated counterparts (**23**, **25**, **12** and **24**, respectively) while, acetylated UII and UII-$NH_2$ (**54** and **55**, respectively) were 2–3 fold more potent than the non-acetylated forms (**1** and **2**, respectively). These data indicate that acetylation, which may improve the stability of UII analogues against proteolysis, is not deleterious for the intrinsic activity of the peptides.

As previously noted, the tripeptide Phe-Trp-Lys is present in the cyclic regions of UII[1] and somatostatin[39] and this core sequence appears to play a pivotal role in the biological activity of both peptides. Since most peptidic somatostatin antagonists designed so far possess a D-Trp$^8$ residue[44] (somatostatin 14 numbering), it has been proposed that [D-Trp$^7$]UII analogues may behave as GPR14 antagonists.[45] To test this hypothesis, we have synthesized two D-Trp analogues, *i.e.* [D-Trp$^7$]UII (**56**) and [D-Trp$^4$]$UII_{(4-11)}$ (**57**). The data reported in Figure 9 revealed that, in fact, both compounds are rather moderately potent agonists. In agreement with this observation, it has been shown that Ac-[D-Trp$^3$]$UII_{(5-10)}$-$NH_2$ behaves as an agonist activating GPR14 in CHO-transfected cells.[24]

The Tyr residue at position 9 appears to be of paramount importance for the biological activity of UII inasmuch as all the Ala- or D-Tyr-substituted analogues tested in the present study (**10**, **19**, **34** and **40**) were totally inactive. However, previous studies have shown that radioiodinated UII has a rather high affinity for UII-binding sites in tissues[35] and GPR14-transfected cells.[24] To further explore the importance of the phenolic moiety of the Tyr residue in the biological activity, we have synthesized the monoiodinated analogue [3-iodo-Tyr$^6$]$UII_{(4-11)}$ (**58**). This analogue turned out to be 5 times more potent than natural UII in stimulating rat aortic ring contractions (Figure 9), suggesting that either a modification of the steric hindrance of the aromatic side-chain or an increase in the electronic density

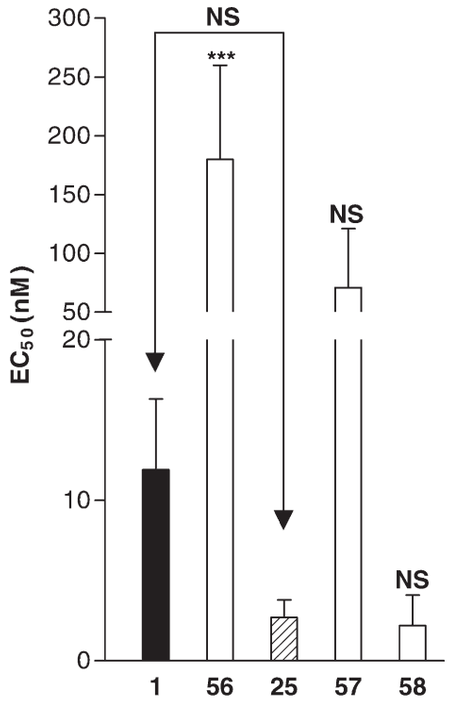


FIGURE 9 Comparison of the $EC_{50}$ values (nM) of D-Trp-substituted human UII and $UII_{(4-11)}$ analogues and of [3-iodo-Tyr$^6$]$UII_{(4-11)}$ on rat thoracic aortic ring contraction. Each value represents the mean (± SEM) $EC_{50}$ of UII (**1**) (black bar), $UII_{(4-11)}$ (**25**) (hatched bar) and D-Trp-substituted (**56** and **57**) and [3-iodo-Tyr$^6$]$UII_{(4-11)}$ (**58**) analogues (open bars). See legend to Figure 3 for other designations. ***$P < 0.001$ *vs* UII (**56**) or $UII_{(4-11)}$ (**57** and **58**) by one-way ANOVA followed by *post hoc* Dunnett's test, NS, not statistically significant.

enhances the affinity of the UII analogue for its vascular receptor.

## CONCLUSIONS

The importance of the various amino acid residues in the contractile activity of human UII on rat aortic rings can be summarized as follows: *(i)* amidated analogues substituted by an alanine or by their D-isomers in the extra-cyclic region of the molecule exhibit similar activity as UII-$NH_2$, *(ii)* alanine- or D-isomer-replacement in the cyclic region of the peptide markedly reduces the contractile response, *(iii)* the C-terminal octapeptide $UII_{(4-11)}$ retains full biological activity, *(iv)* point-substitution of residues in this core sequence by alanine or D-amino acid significantly reduces the contractile response, *(v)* the integrity of the disulfide bridge is crucial for biological activity, *(vi)* acetylation of the N-terminal residue does not impair the potency of UII and its C-terminal octapeptide analogues, and *(vii)* the potency of the mono-iodinated analogue [3-iodo-$Tyr^6$]$UII_{(4-11)}$ was 5 fold higher than that of native UII. Taken together, these results demonstrate the importance of the $Tyr^9$ residue of UII in determining the biological activity of the molecule. Our data also suggest that an increase in steric hindrance or in electronic density of this $Tyr^9$ residue may facilitate the interaction between the peptide and its receptor. This structure–activity relationship study, that provides crucial information regarding the chemical requirements for the conception of high affinity UII analogues, opens the way for the design of selective and potent UII receptor agonists and antagonists.

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