UNIVERSITA' DEGLI STUDI DI NAPOLI FEDERICO II

DIPARTIMENTO DI FARMACIA

Dottorato di ricerca in Scienza del Farmaco

XXXII ciclo

"DESIGN, SYNTHESIS AND BIOLOGICAL ACTIVITY OF PEPTIDES AND PEPTIDOMIMETICS AS MODULATORS THE UROTENSINERGIC SYSTEM"

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To my mammy and my sister,

For always support me

Abbreviations

Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, 247, 977- 983. Amino acid symbols denote L-configuration unless indicated otherwise.

The following additional abbreviations are used:

ANP atrial natriuretic peptide
Bip Biphenylalanine
BNP brain natriuretic peptide
Btz Benzothiazolylalanine
Cha Cyclohexylalanine
CNS Central Nervous System
CNSS caudal neurosecretory system
DBU 1,8-Diazabicycloundec-7-ene
DCM Dichloromethane
DIC N,N' -Diisopropylcarbodiimide
DIEA N,N-Diisopropylethylamine
DMAP 4-Dimethylaminopyridine
DMF Dimethylformamide
DMSO Dimethyl sulfoxide
ESI Electrospray ionization
Fpa 4-Fluorophenylalanine
HATU 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOAt 1-Hydroxy-7-azabenzotriazole
HOBt 1-Hhydroxybenzotriazole
HPLC High-Performance Liquid Chromatography
***h*U-II** Human Urotensin-II
IBC Isobutylchloroformate
LC Liquid Chromatography
MeCN Acetonitrile
MeOH Methanol
MS Mass Spectrometry
Nal Naphthylalanine
NBS 2-Nitrobenzenesulfonyl
NMP N-Methyl-2-pyrrolidone

NMR Nuclear Magnetic Resonance
ORF open reading frame
Orn Ornithine
Pal 3-Pyridylalanine
Pen Penicillamine
Phg Phenylglycine
RP-HPLC Reversed-Phase High-Performance Liquid Chromatography
SAR Structure-activity relationship
SDS Sodium-dodecyl-sulfate
SMC Smooth Muscle Cell
TFA Trifluoroacetic acid
THF Tetrahydrofuran
Tic Tetrahydro-isoquinoline-3-carboxylic acid
TIS Triisopropylsilane
U-II Urotensin-II peptide
URP Urotensin Related Peptide
UT Urotensin receptor

Abstract

In the humane, the urotensinergical system is involved in several pathological conditions, from cardiovascular disease at the neoplastic condition. The urotensinergical system is mediated by two cyclic peptides U-II and URP, these conserve the same cyclic region but differ the N-terminal domain. Previous studies have individuated two lead compounds P5U and Urantide, respectively superagonist and antagonist for the UT receptor. To improve the antagonistic activity of Urantide, the research activity of my PhD has been focused on Design, synthesis and characterization of analogues of this peptide. In particular, I've performed the synthesis of three different libraries of compounds that they have characterized the introduction of analogues of Tyrosine, Lysine, in respective position and the modification of the N-terminal domain. Another goal has been characterized at structural studies of URP through the use of peptoids and N-methylation, to better define the structural elements that allow maintaining the peptide bioactive structure. These modifications have been carried out to improve the knowledge of elements that improve the antagonistic activity so that these peptides could be used at the therapeutic scope.

1.0 Urotensin-II and the Urotensinergic System

1.1 Discovery of U-II

1.2 To invertebrate at mammalian

1.3 Urotensin Related Peptide

1.4 The Urotensin-II Receptor (UTR)

1.5 Features of UT receptor activation

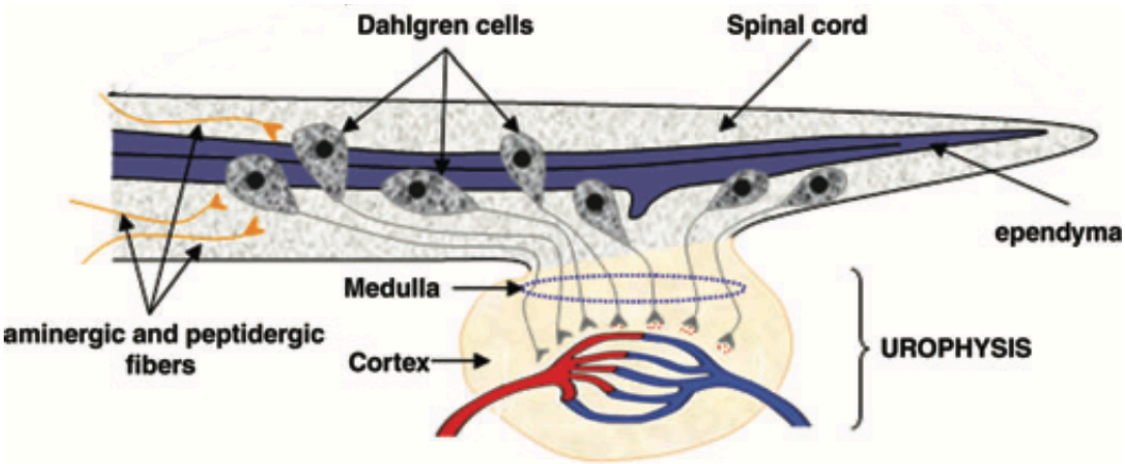
1.6 Signaling mechanism

1.7 Different role of URP/U-II in UT activation

1.1 Discovery of U-II

Urotensin II is a cyclic hormone peptide belonging to a series of regulatory neuropeptides that was isolated for the first time in caudal neurosecretory system of the teleost, *Gillichthys mirabilis*. The caudal neurosecretory system (CNSS) is a singular neurosecretory system located in the caudal region of the spinal cord defined only in teleosts as a complex of secretory neurons (Dahlgren cells) (Figure 1) The CNSS in teleosts receives extensive and diverse innervation from the higher central nervous system, with evidence for the presence of cholinergic, noradrenergic, serotonergic, and peptidergic descending inputs. The CNSS of teleosts is thus anatomically and functionally similar to the hypothalamo-neurohypophysial neurosecretory system [(1)].

Figure 1. Schematic representation of the organization of the caudal neurosecretory system of vascular connections in the urophysis of teleost fish.



Different studies have shown that urophysial extracts contain molecules that could have important pharmacological activities both in fish and in mammals. From an extract of the urophysis of fish *Gillichthys mirabilis* have been isolated two peptides, Urotensin-I, a 41amino acid peptide that was found to be an analogue of corticotropin-releasing factor (CRF) and Urotensin-II, a 12-amino acid peptide, that was found to exhibit some structural similarity to somatostatin. The most convincing evidence as to a physiological role for the CNSS in fish has stemmed from the direct and indirect influence of the urotensins on osmoregulatory function. The sequence of U-II has been relatively well

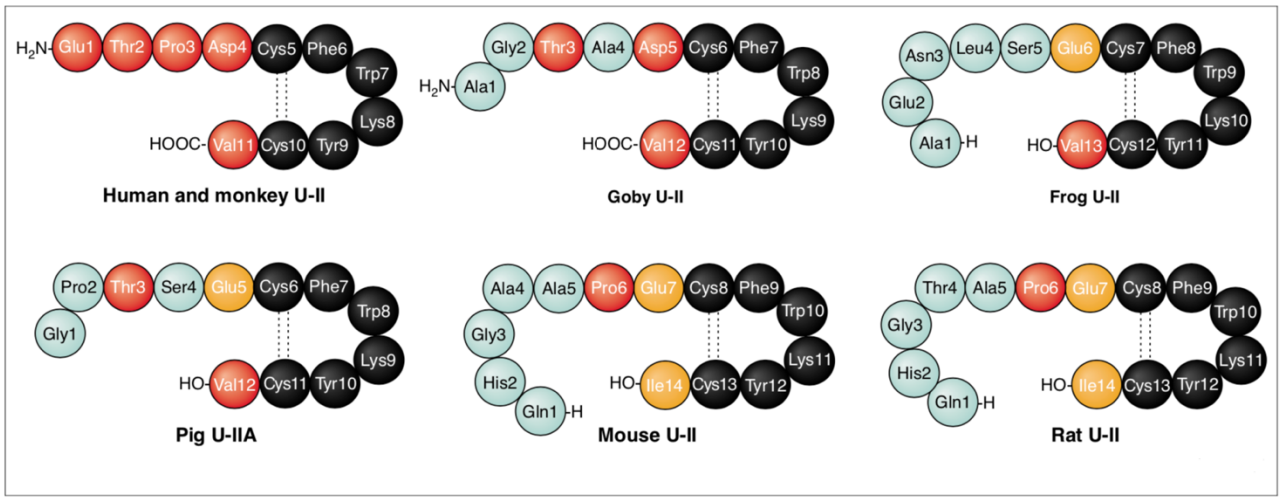
preserved in all fish, suggesting that the peptide exerts important biologic functions. As a matter of fact, in fish, U-II induces a general spasmogenic activity that has been documented in various tissue as preparations including the trout (*Salmo gairdneri*) urinary bladder, the trout posterior intestine, the guppy (*Poecilia reticulata*) oviduct, the goby sperm duct, the eel (Anguilla rostrata) caudal lymph heart, and the dogfish (*Scyliorhinus canicula*) vascular ring. Also, in fish U-II participates in the control of hydromineral balance through a direct action on ion transport across the skin, gill, intestine, and urinary bladder. Finally, U-II participates in the neuroendocrine regulation of prolactin secretion in tilapia (*Oreochromics mossambicus*) and cortisol secretion in the trout and the European flounder (*Platichtys flesus*). Because the urophysis is a neurosecretory organ that is exclusively found in teleosts fish, it was thought that U-II was present only in fish and not in others vertebrate.

1.2 From invertebrate to mammalians

Several observations, however, suggested that this view might not be correct. U-II-immunoreactive neurons are localized in the anterior (extraurophyseal) region of the spinal cord and the brain of fish and U-II-like immunoreactivity had even been described in the cerebral ganglia of gastropods; U-II has been characterized in brain extracts from the long-nose skate and rainbow trout; also binding studies using radio iodinated goby U-II had revealed the existence of functional U-II receptors in rat vascular tissue. Pharmacological studies have shown that fish U-II provoked relaxation of the mouse anococcygeal muscle, caused endothelium-dependent relaxation of rat aorta strips and lowered blood pressure in the anaesthetized rat. These observations provided many clues for the existence of a U-II-like peptide in extra-uropygial organs, including in mammalian tissues[(2)]. However, the confirmation of an implication of U-II in physiological processes in other species and not only in teleost, was demonstrated through the discovery of cDNA pre-pro U-II encoding in European green frog *Rana ridibunda*. Subsequently, prepro U-II cDNAs or genomic sequences were characterized in humans [(2)], chimpanzee (XP 001157678), cynomolgus monkey[(3)], pig[(4)], mouse[(5)], rat[(5)] , and chicken [(6)], and more in zebra finch (XP 002195510.1) (Figure 2). In mammalian, the open reading frame

(ORF) sequence of cDNA of pre-pro of encoding U-II is organized like the frog and carp preprohormones but with a different partial peptide sequence[7]. Notwithstanding the peptide sequence of U-II is expressed by invertebrates up to more complex organisms with mammals preserved her chemical structure. The primary structure of the cyclic hexapeptide, at Cys^5 to Cys^2, is unchanged in all species, this has made us speculate and subsequently confirm her importance in biological activity. However, the length of the mature peptide is very variable, from 11 amino acids in humans to 17 amino acids in the mouse.

Figure 2: example of different isoforms of U-II in mammalians and non-mammalian species. In black the hexapeptide preserved in all species.



1.3 Urotensin Related Peptide.

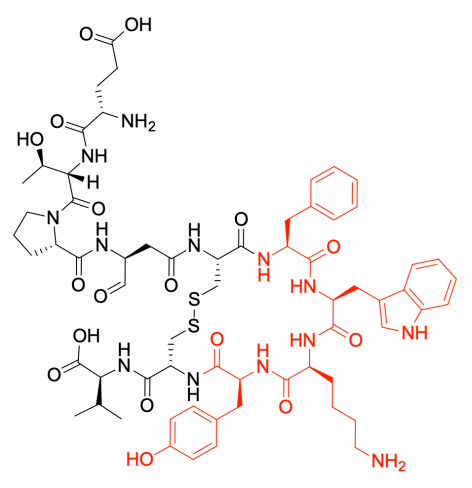
The URP (*Urotensin Related Peptide*) is a cyclic peptide that with the U-II belongs to the urotensinegic system. URP was isolated for the first time from an extract of the rat brain using an anti-U-II monoclonal antibody prepared using goby U-II as an antigen. However, the discovery of this peptidic analogue has been quite lucky. In fact, the research group, with the aim to isolate Urotensin-II in rat brain, isolated URP that have lower molecular weight respect U-II. In this work was been used two types of antibody. One antibody (AU-II5-6-10a) showed equal reactivity to all of the U-II molecules examined indicating that it might recognize the common structure to U-IIs, that

is, the Cys–Cys ring moiety (CFWKYC). Another antibody (AU-II103-5-41a) reacted with higher specificity to human and goby U-II than to other U-II peptides indicating it might recognize DCFWKYC which is the structure that is shared by human and goby U-II. These two antibodies did not cross-react with somatostatin and cortistatin, which have the same partial sequence, Phe–Trp–Lys, in the Cys–Cys ring as U-II, or other bioactive peptides such as endothelin, angiotensin II[9]. However, URP differs from U-II only for the extra cyclic portion in the N-terminus part of U-II, remaining unchanged the cyclic portion Cys5-Cys10 conserved the amino acids indispensable for the biological activity. URP exhibits a high binding affinity for the human UT receptor in transfected cell lines and high contractile potency in the rat aortic ring assay, suggesting that some physiological effects could be not completely attributed to U-II.

1.4 The Urotensin-II Receptor (UTR)

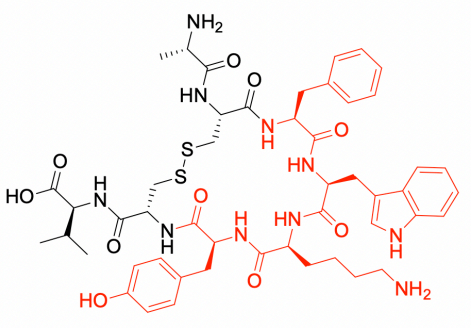
The presence of an FWK tripeptide motif within the cyclized region of all U-II isoforms originally led to this peptide family being described as 'somatostatin or cortistatin-like' (Figure 3). However, despite the apparent structural similarity, U-II and somatostatin are not homologous. Indeed, although it was originally proposed that U-II and somatostatin shared a common binding site[10] , both binding ([^{125}I]-radiolabeled goby urotensin-II in rat aortic membranes) and functional (aortic contraction) studies demonstrated that U-II mediated its biological action(s) via unique 'U-II-specific receptor(s)'**.**

Figure 3. Urotensin-II, URP, Somatostatin-I structural analogies



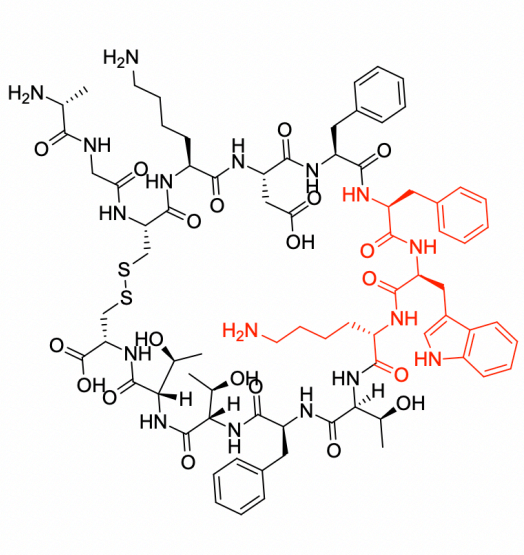
Urotensin-II

H-Glu-Thr-Pro-Asp-[Cys-**Phe-Trp-Lys-Tyr**-Cys]-Val-OH



Urotensin-Related-Peptide (URP)

H-Ala-[Cys-**Phe-Trp-Lys-Tyr**-Cys]-Val-OH



Somatostatin-I

H-Ala-Gly-[Cys-Lys-Asp-Phe-**Phe-Trp-Lys**-Thr-Phe-Thr-Thr-Cys]-OH

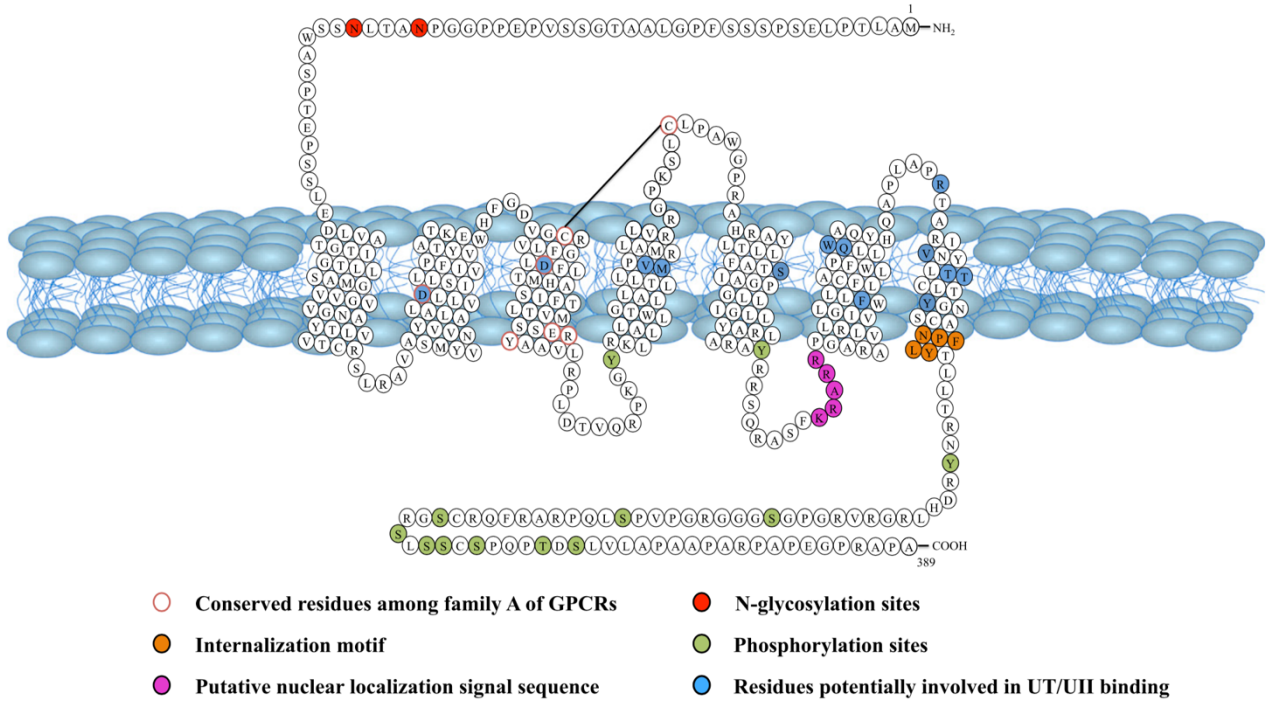
However, U-II is a poor agonist of the somatostatin receptor. This hypothesis was confirmed thought the work of four independent laboratories that using a reverse pharmacological strategy, reported that Urotensin II have great affinity with orphan receptor GPR14[11] This receptor also called SENR (sensory epithelial neuropeptide-like receptor) because Transcript for the receptor was identified predominantly in sensory and neural previously characterized in bovine, and indicated some sequence similarities with the rat somatostatin sst4 and δ-opioid receptors and a human galanin receptor[12].

Both groups reported that the novel gene was intronless and encoded a 386 amino acid receptor being indeed the U-II receptor[13] the Humane isoform of UT receptor was isolated for the first time when a research group searching for novel human GPCRs, used rat GPR14 to probe a human genomic library and isolated a clone encoding a 389 amino acid human GPCR and possesses 75% identity with rat GPR14. Messenger RNA encoding this human receptor was abundantly expressed in heart and pancreas and also detected in brain, human atria, ventricle, aorta, and endothelial and smooth muscle cell lines, but not in venous tissue[13]. The interaction of rat GPR14 and humane homologous with U-II has been obtained through use of reverse pharmacological approach. Through the screening of hundreds of potential ligands only one goby U-II elicited a potent biological response. In contrast to most neuropeptides that usually possess several receptor isoforms, GPR14/SENR, now renamed U-II receptor (UT)[14], is the only high affinity receptor for U-II/URP known so far, at least in mammals. UT-II belongs to family of rhodopsin an G protein–coupled receptor exhibits the highest degree of identity with the somatostatin receptors SST2 (26%) and SST4 (27%) and the mu, delta and kappa opioid receptors[11] (25–27%).

1.5 Features of UT receptor activation

As all class A GPCRs, UT is characterized by a short N-terminal segment, an Asp residue in trans-membrane domain 2 (TMD2) that is essential for ligand binding as in somatostatin and opioid receptors , a D/ERY motif at the junction between TMD3 and the second intracellular loop 2 (ICL2), a NP(XX)Y motif in TMD7 that is required for receptor internalization and 12 potential Ser/Thr phosphorylation sites in the intracellular loop 3 and the cytoplasmic tail (Figure 4). Conserved Cys123/Cys199 residues, which likely form a disulfide bridge, are present in the first and second extracellular loops (ECL1 and ECL2), respectively. Two putative N-glycosylation sites are also observed in the N-terminal extracellular domain (Figure 4). A putative palmitoylation site (Cys339) is present in rat UT [11] but absent in human UT [13].

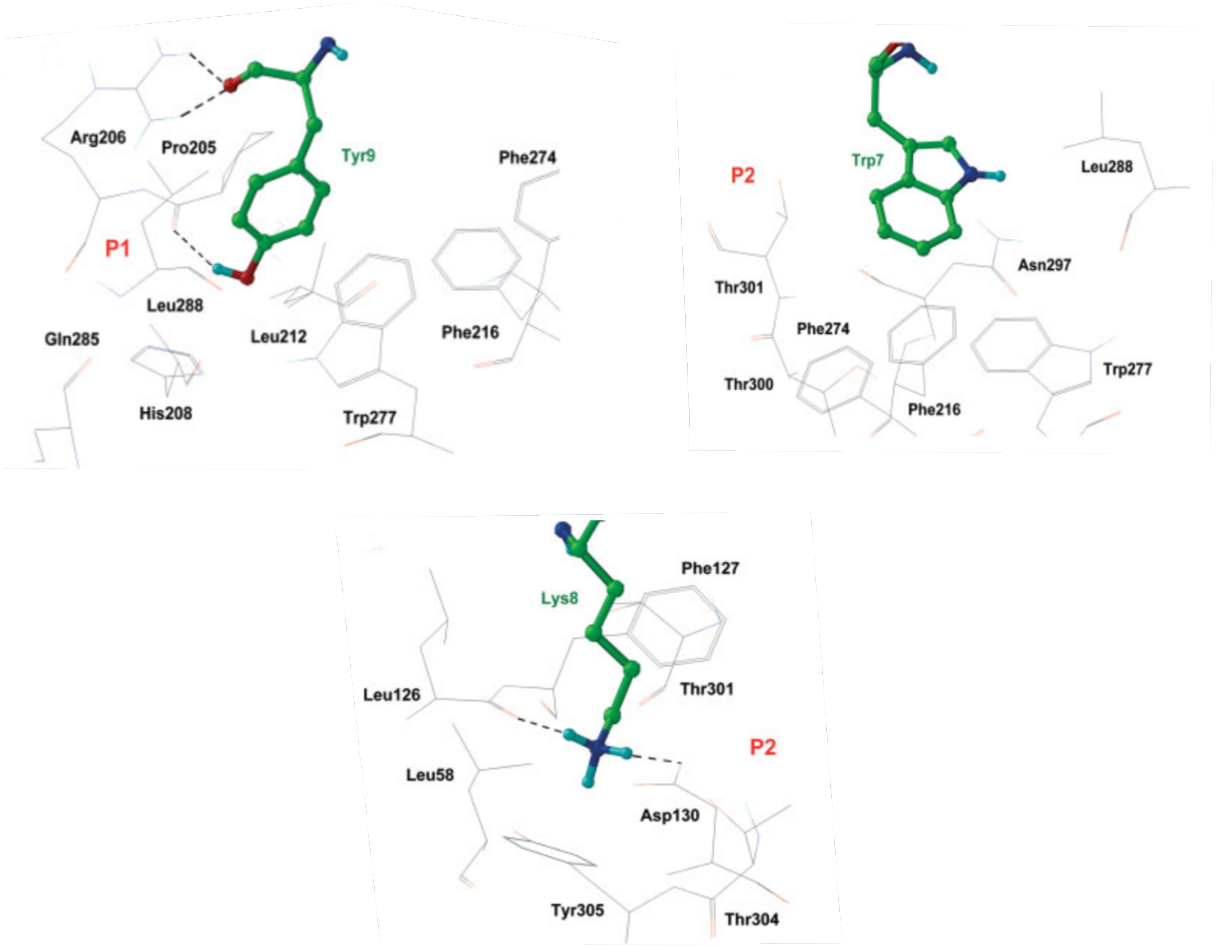
Figure 4. Human-UT receptor



Through the use of docking studies, it was possible theorize the 3D conformation of UT-II receptor. As model was taken the bovine rhodopsin sequence whose crystallographic structure is known[15] (PDB ID, 1F88) at 2.6 A resolution. The binding site of *h*U-II, situated in the entrance of the TM bundle on the extracellular side, is formed by TM-III, TM-IV, TM-VI and TM-VII and the EC-II and EC-III loops. The active site cavity has taken of global volume 1162 A˚, adopts a T-shaped form composed of 3 pockets which we named P1, P2 and P3. The entrance of the active site is located between the P1 and P2 pockets, and the P3 pocket is buried in the interior of the TM bundle. The largest pocket, P1, is delimited by the TM- IV/TM-VI helices and the EC-II/EC-III loops in the upper part of the cavity. It is mainly lined by hydrophobic residues [Phe274, Phe216, Leu212, Ala281, Leu288, Trp277] with a polar extremity [Arg206, Pro205, His208 and Gln285]. The P2 pocket is delimited by the TM-III and TM-VII helices and lined with hydrophobic residues [Phe127, Phe274 and Leu126] in the upper part and with polar residues [Thr301, Thr304, Tyr305 and Asp130] in the

lower part. The P3 pocket is embedded between the TM-III, TM-IV and TM-VI helices and lined with hydrophobic residues [Phe274, Phe216, Phe271, and Phe131], forming the bottom of the cavity [16]. *h*U-II only binds in the P1 and P2 pockets of the active site, leaving the P3 pocket empty. The highly conserved residue of Asp 120 in P2 pocket of the *h*UT-II receptor was assumed to participate in the binding. In fact, like in the somatostatin where lysine residue was essential for receptor activation so as the residue of lysine 8 in core sequence of U-II in could be necessary for the interaction with this residue. Since the positive charge of the hU-II lysine nitrogen was thus positioned close to the Asp130 carboxylate group where interact through one H-bond between the carboxylate group oxygen end the nitrogen of Lysine. The Phe^6, Trp^7 and Tyr^9 residues of hU-II form an aromatic cluster, with their rings in an off-centered parallel orientation[17]. Trp^7, localized between the P1 and P2 pockets close to the surface of the receptor (Figure 5), is close to Phe274 and Trp 277, and Tyr^9 is close to Phe216 and His208, that establish with their van der Waals interaction, the indole NH of Trp7 is close to the Asn297 side chain establishing an electrostatic interaction. Moreover, Trp^7 is surrounded by other hydrophobic amino acids [Phe216 and Leu288 (EC-III)] and polar residues [Thr301, Thr300, Asn297] that also they take a part during receptor binding. Tyr^9 is embedded within the large P1 pocket where interacts with His208 and Phe216. It is observed that two H-bonds involving the phenol group of Tyr^9 with the backbone carbonyl of Pro205 and the carbonyl of Tyr^9 with the side chain of Arg206. For the residue of Tyr^9, van der Waals interactions seem to be more crucial than electrostatic interactions. In fact, like confirmed in experimental results the substitution of Tyr^9 with the related amino acid Phe maintained potency in both the functional and binding assays [18]. The Phe^6 is located in the external region of the binding site, above the P2 pocket with no particular interactions except an H- bond between the backbone O and OH group of the Tyr111 phenol.

Figure 5 Tryptophan[7], Lysine[8] and Tyrosine[8] binding sites.

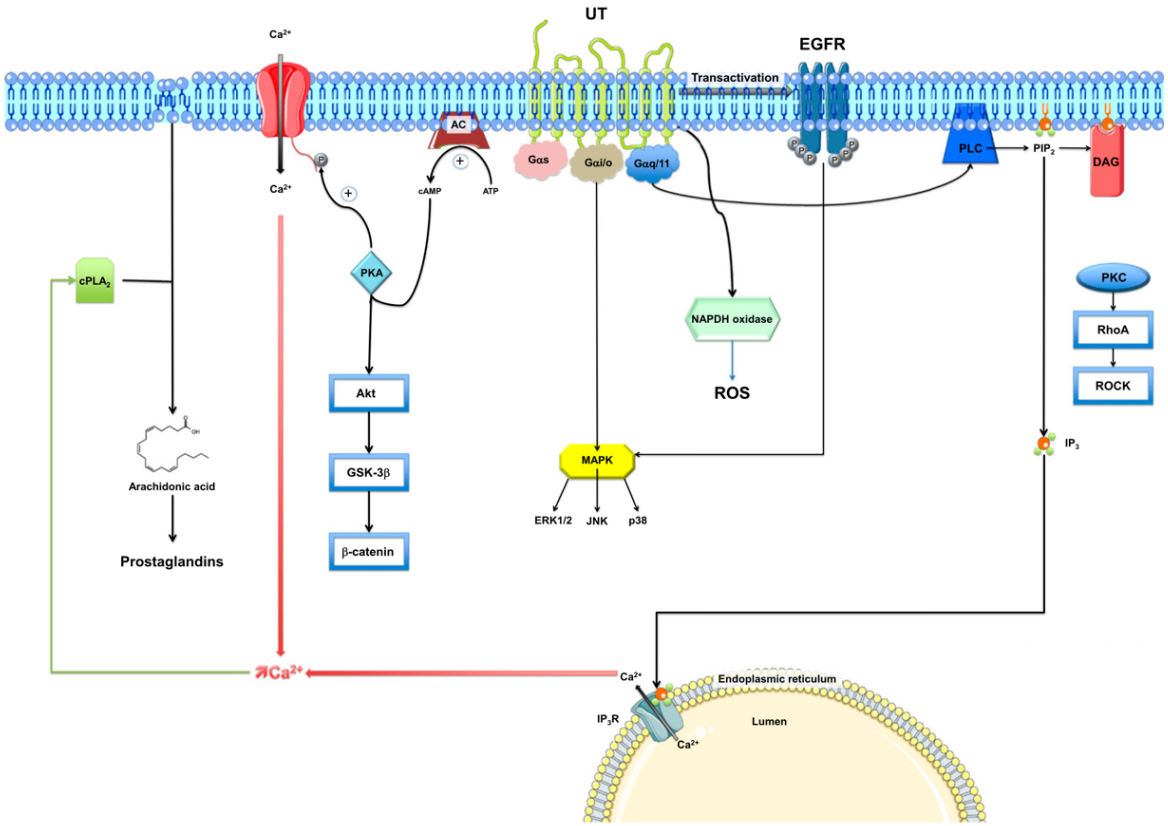


An important electrostatic interaction with the receptor site occurs for Val[1], because of three H-bonds between the carboxylate C-terminal fragment and the guanidinium group of Arg206 (EC-II loop) in the upper part of the active site. This suggests that the presence of the C-terminal fragment is more important than the residue type. The N-terminal sequence Glu1-Thr2-Pro3-Asp4 lies above the P1 and P2 pockets with a strong electrostatic interaction between Asp4 and the receptor. An important electrostatic interaction was also calculated for Glu1, but this residue is situated far from the receptor and its closest contact is observed with the guanidinium group of Arg291.

1.6 Signaling mechanism

The Urotensin II and URP bind with high affinity UT receptor there belong to class of metabolomic receptor G protein $Ga_{q/11}$. This class of G protein-coupled receptors upon U-II binding to UT, activation of PLC causes hydrolysis of phosphatidylinositol-4-5 bisphosphate (PIP2) to inositol-1-4-5 triphosphate (IP3) and diacyl- glycerol. With the release of IP3 there are the activation of IP3 receptor, a calcium channel on the membrane of the endoplasmic reticulum, resulting in an increase in cytoplasmic calcium levels. The increased level of cytoplasmatic calcium depending by U-II-UT binding was documented in different cell type, porcine renal epithelial cell line LLCPK1[19], human aorta endothelial cells[20], and rat aorta vascular smooth muscle cells[21]. U-II-induced Ca^{2+} entry occurs through various types of voltage-operated Ca^{2+} channels in fact in rat spinal cord cholinergic neurons, U-II causes calcium influx from the extracellular space via N-type Ca^{2+} channels through the protein kinase A pathway[22], instead in arterial smooth muscle cells, the Ca^{2+} influx U-II determined occur through L-type Ca^{2+} channels.

Figure 6. UT receptor pathways



UT is also coupled to Gai/o, leading to activation of the mitogen-activated protein kinase (MAPK) pathway (Figure 6). Thus, U-II stimulates P38 MAPK and extracellular signal-regulated kinase 1/2 in UT-transfected cell lines[23] this evidence was reported in different types of cells as cardiac myocytes [24][25], vascular smooth muscle cells[26][27], airway smooth muscle cells [28], endothelial cells [29][30], and endothelium-denuded rat aorta[31]. The activation of pathway p38 and p44/42 MAPK stimulates proliferation of endothelial progenitor cell, this evidence confirmed that U-II play a role in different physio-pathological disease. The stimulatory effect of U-II on P38MAPK and extracellular signal-regulated kinase 1/2 in neonatal rat cardiomyocytes and cardiac fibroblasts depends on transactivation of epidermal growth factor. The activation of UT by U-II significantly increased the phosphorylation level of JNK in cardiac side population cells in dose-dependent manner. JNK phosphorylates AP-1 (activator protein 1) transcription factor complex proteins, such as the conserved N-terminal Ser63 and Ser73 residues of c-Jun, and this increases AP-1 transcriptional activity, which can regulate both cell death and cell proliferation. U-II inhibits the cardiac side population cells proliferation through up-regulation of JNK phosphorylation and inhibits proliferation of these stem/ progenitor cells, but it induces proliferation of cardiac fibroblasts through phosphorylation of ERKs[32]. U-II-induced activation of the small GTPase RhoA, induced contraction activity on rat vascular rings, actin cytoskeleton organization, and proliferation of Arterial Smooth Muscle Cell and its chemo-attractant activity on human monocytes[33]. U-II increases phosphorylation of both Akt and its downstream target glycogen synthase kinase-3 in rat cardiomyocytes. In these cells, U-II also phosphorylates b-catenin a protein regulated downstream to GSK-3 and which is potentially involved in the control of the gene program related to the cellular growth/hypertrophy. Because the Akt/glycogen synthase kinase-3 signaling pathway plays a pivotal role in cardiomyocytes hypertrophy, these observations suggest that UT antagonists may prove useful for the treatment of cardiac hypertrophy[34]. A possible implication of phospholipase A2 in the contractile effect of U-II has long been postulated[35], in fact the contraction activity of U-II, in hamster ovary (CHO) cells and in CHO of human embryonic kidney has been attenuated by a phospholipase A2 inhibitor. In addition,

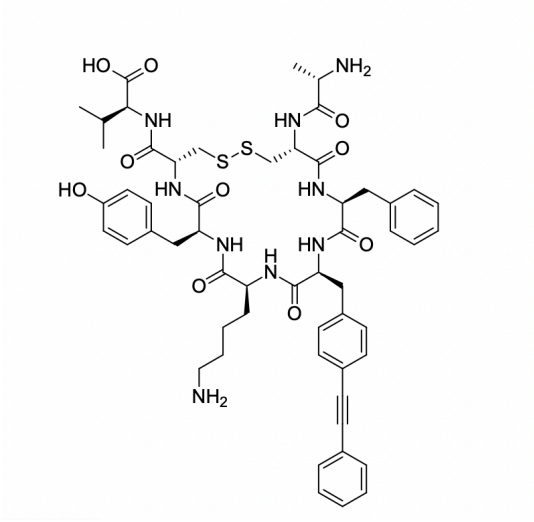
it was observed that U-II-induced contractions in guinea pig ileum, bladder and ileum was blocked by indomethacin, the cyclooxygenase inhibitor. Similarly, U-II-and URP-induced vasodilation in the rat heart is significantly attenuated by indomethacin[36], suggesting that the biologic actions of U-II are mediated, at least in part, through stimulation of prostaglandin synthesis. However, an implication of prostaglandins during the activation of UT receptor aren't evident in other species as dogfish[37], rat[35], and rabbit aorta[38]. Furthermore, U-II stimulates the expression of the NADPH oxidase subunits P22phox and NOX4 and potently activates the production of reactive oxygen species (ROS) in human pulmonary artery smooth muscle cells. Evidence that suggest that the peptide may play a role in pulmonary hypertension through activation of NADPH oxidases. Generation of ROS also plays an important role in U-II signaling in cardiac fibroblasts[39]. In fact, U-II-mediated ROS generation inhibits Src homology 2–containing tyrosine phosphatase activity, thereby facilitating epidermal growth factor receptor transactivation[40].

1.7 Different role of URP/U-II in UT activation

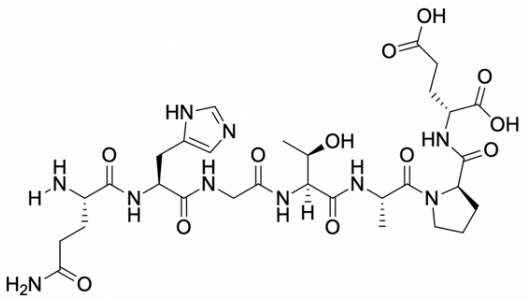
U-II-and URP- associated actions are mediated by the activation of a specific GPCR, UT-II receptor, which plays a seminal role in the physiological regulation of major mammalian organ systems. The presence of two biological ligands for the same receptor has made discuss the possibility of these two ligands could bind in a different way UT receptor or provoke a different biological activity. Different studies, in fact, have shown that Urotensin-II and URP can manifest different biological effects in different organs. In isolated ischemic heart experiments, both peptides were able to dilate the coronary arteries and reduce myocardial injury through creatine kinase reduction, but only U-II was able to reduce atrial natriuretic peptide (ANP) production[36]. Also, U-II, and, to a lesser extent, URP can activate a higher-affinity site coupled to a PTX-sensitive G-protein that would mediate a hypothetical PI3K activation, inducing astrocyte cellular proliferation. Finally, URP, but not U-II, may bind sst expressed in rat astrocytes whose activation would mimic the UT-Ca^{2+} coupling and/or counteract the

mitogenic role of UT[41]. Furthermore, the discovery of a specific nuclear/perinuclear expression of the U-II receptor in the rat and monkey heart, have made in evidence a possible role of U-II and URP in a modulatory effect on transcription. U-II and URP are able to reach the internal cellular compartment through independent receptor-mediated endocytosis in cell lines that do not express the UT receptor. Through use of FITC analogs of U-II and URP it was possible evaluate the major propensity of U-II to permeate the plasmatic membrane respect URP, and major capacity to escape from endosome–lysosome system. For this reason, U-II was associate with a specific and biologic role in nuclear UT activation. Also, U-II is able to specifically activate nuclear UT receptors, but not URP, acting as an intracrine factor[42]. Therefore, U-II and URP, despite the high sequence homologies should be identified as two chemically distinct entities. This affirmation was confirmed also by different pharmacological evidence. In fact, with the synthesis of a novel analogue of URP called UCA (Figure 7), it was observed a particular action of the latter. When UCA was used as the antagonist was able to selectively and significantly reduce the *h*U-II-induced contraction without altering URP-mediated vasoconstriction. This result has made thinking that on basis of UT activation there is a mechanism of allosteric modulation. Indeed, in contrast to an orthosteric ligand, that is *h*U-II, an excess concentration of UCA accelerated the ^{125}I-*h*U-II dissociation rate, thus suggesting that UCA can bind to ^{125}I-*h*U-II-occupied UT, and then change the receptor conformation in such a way that the radioligand is released from the receptor. Surprisingly, no difference in ^{125}I-URP dissociation kinetics was observed in similar conditions. Therefore, also URP, an equipotent U-II paralog, was able to accelerate the dissociation rate of membrane-bound 125I-hU-II, while *h*U-II had no noticeable effect on URP dissociation kinetics. Altogether, these results support to some extent the presence of specific pockets/interactions within UT, aimed at selecting a specific UT conformation that can differentiate U-II and URP biological activity.

Figure 7. Urocortin A and rU-II (1-7) as allosteric modulators of UT receptor



UROCORTIN A

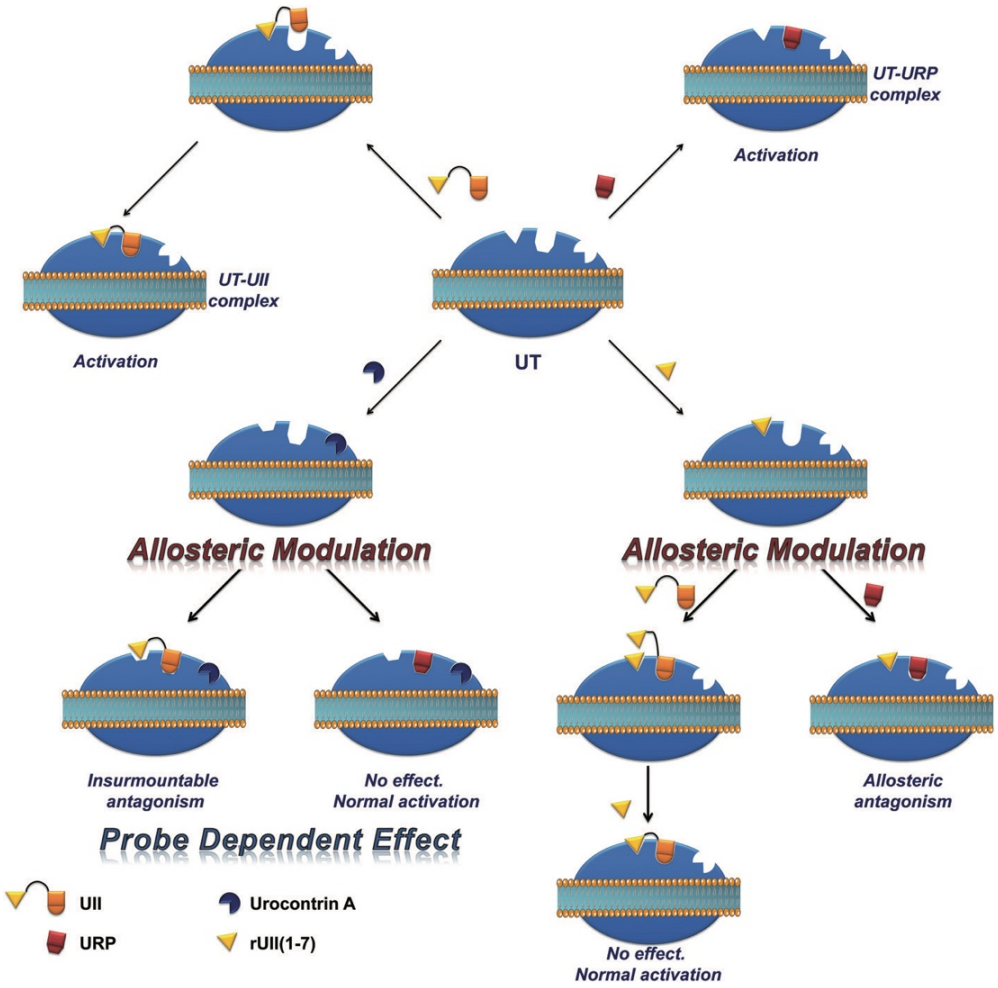


***r*U-II(1–7)**

Moreover, these observations seem to be by the concept of *h*U-II or URP being an endogenous biased agonist of the urotensinergic system. The concept of biased agonist has recently emerged from various studies, putting forward the notion that specific ligand-induced conformational changes can lead to precisely directed signaling. Then, based on this different way to binding UT by U-II and URP. However, URP, lacking this N-terminal portion is also able to bind UT but in a slightly different manner, probably characterized by the activation of a different subset of signaling pathways. By acting to an allosteric binding site, UCA is able to modify the receptor topography preventing the proper interaction of UT with the linear U-II N-terminal region ultimately leading to an inefficient activation characterized by a reduced efficacy. On the opposite, such receptor conformational change has no effect on URP-mediated action. Conversely, binding of the rU-II (1–7) N-terminal segment initiates a topographical change that antagonizes the effect of URP, but not U-II. Further experiments revealed that an interaction between the glutamic residue at position 1 of *h*U-II and the UT receptor seems to be critical to induce specific conformational changes associated with *h*U-II agonistic

activation. Indeed, replacement of this residue by an alanine moiety, that is [Ala1] hU-II, provoked an increase of the dissociation rate of *h*U-II. Therefore, this evidence was confirmed examining the role of rU-II (1–7), N-terminus portion of *h*U-II, on rU-II- or URP-induced contraction on rat aortic ring. Striking results demonstrated the propensity of such analogue to selectively block URP- but not rU-II-associated vasoconstriction. Confirmed the hypothesis that N-terminus domain of U-II, rU-II (1–7), act as an allosteric modulator, on rU-II and URP-mediated vasoconstriction (Figure 8).

Figure 8. Schematic representation of the proposed binding mode of hU-II, URP, UCA, rU-II (1-7).



2.0 Biologic and Pharmacologic Effects of Urotensin II and Urotensin II–Related Peptide

2.1 U-II in Central nervous system

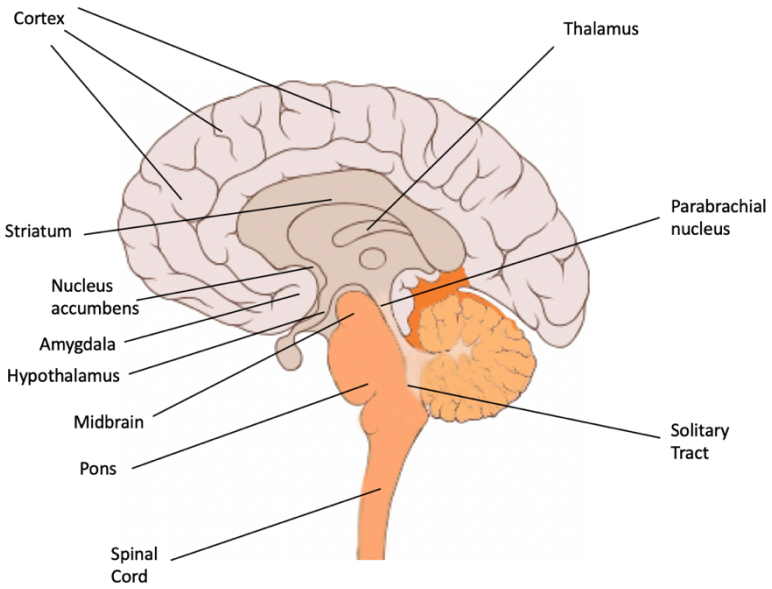
2.2 U-II in the regulation of cardiovascular system

2.3 U-II in the regulation of renal function

2.1 U-II in Central nervous system

The UT receptor is present in different organs, evidence of its expression has reported in CNS, cardiovascular system, gastro ethical and urogenital tract. In CNS the UT receptor is widespread in different regions, these indicate that U-II and URP may regulate various neurophysiological and behavioral activities. The expression of UT receptor in the pons-midbrain transition area, zone involved in the control of rapid eye movement (REM) sleep, suggests that U-II may be implicated in the regulation of the sleep-wake cycle. In accord with this hypothesis, the intra-cerebroventricular injection of U-II into in this area increases the number of REM sleep episodes in the rat. In the hypothalamus the expression of UT receptor, in the arcuate nucleus, and the ventromedial hypothalamic nucleus, and in the brainstem, the nucleus of the solitary tract and the parabrachial nucleus, indicate that U-II could have the control feeding and behavior[(43)]. Following this evidence intracerebroventricular injections of U-II in food-restricted mice it has been observed an increase of food consumption but in normally fed rats U-II induces a modest decrease of food intake[(44)]. Furthermore, U-II stimulates the expression of the genes encoding the uncoupling proteins UCP1 and UCP3 (mitochondrial proteins having a role in thermogenesis) in brown adipose tissue and causing an increase in sympathetic nerve activity. These observations indicate that U-II may act centrally to regulate food intake and energy expenditure. The UT receptor is also coded in the region of CNS designate at the locomotory activity, in fact, its expression has been found in motoneurons like brainstem and spinal cord regions, arousals, such as the cortex, thalamus, amygdala, striatum, nucleus accumbens, motor nuclei of the brainstem, and spinal cord [(45)(46)(47)(48) (49)(50)] . This evidence suggests that U-II and URP could have an important role in the control of motor activity. Confirmed through different in vivo test, in fact, intracerebroventricular injection of U-II in mouse, rat and frog induces a dose-dependent increase of ambulatory movements, indeed U-II induces a concentration-dependent stimulation of acetylcholine release[(51)(52)(53)].

Figure 9. UT distribution in CNS



The U-II and URP have also a neuroendocrinal activity; in fact, the presence of UT mRNA is identified in hypothalamic region depurated at the release of the hormonal mediators, notably in the arcuate nucleus, the supraoptic nucleus, the ventromedial hypothalamic nucleus, the magnocellular aspect of the paraventricular nucleus, and the median preoptic nucleus[54]. These hypotheses were confirmed when intracerebroventricular administration of U-II in unanesthetized ewes provokes a marked increase in plasma adrenocorticotropin (ACTH) and adrenaline levels[55]; but, when U-II is injected trough intravenous way there aren't observed the effect on ACTH secretion, this indicates that U-II acts a role on ACTH only when it was released in CNS[56]. The expression of the UT receptor in the cholinergic and/or adrenergic neuronal pathways and in the regions of pons and medulla oblongata indicates that U-II and URP could regulate the in CSN the cardiovascular system. In fact, in rat treated with an antagonist of the nicotinic receptor, it was observed a conspicuous reduction of the pressure and tachycardic responses. Furthermore, intracerebroventricular administration of U-II in normotensive rats and sheep provoke a prolonged increase in heart rate, cardiac output, and blood pressure[57]. (Figure 9)

2.2 U-II in the regulation of cardiovascular system

Another compartment where the UT receptor is widespread is the cardiovascular system, in this area, U-II and URP perform an important role both in the control of the homeostasis of the cardiovascular system than the onset pathological condition. The huge action of U-II on the cardiovascular system has been one of the major reasons whereby the scientific community has hired a great interest in this peptide. Whit the characterization of U-II and URP in the human we noted that these peptides have a vasoconstriction action 1-2 time greater than the endothelin-1, since the most powerful vasoconstrictor known in the humane. Moreover, in the cardiovascular system in physiologic conditions, U-II and URP regulate the control of vascular tone, blood pressure, and blood glucose levels, mediating the release of endothelial-derived vasodilators, such as NO, thus controlling the contraction and relaxation of vascular smooth muscle cells [(58)(59)]. In the vascular tissue, the activation of the UT receptor is responsible for a large number of actions that could have both physiological and pathological action on humane. The only activation of the UT receptor in vascular stimulates proliferation of vascular smooth muscle cells via epidermal growth factor (EGF) receptor transactivation[(60)]. Therefore, the effect of U-II on smooth cells growth is abrogated by the Rho-kinase inhibitor, indicating that the growth-stimulating effect of U-II is mediated through activation of the small GTPase RhoA and its downstream effector Rho-kinase. In fact, both in humane and rat endothelial cells, U-II in vitro have proangiogenic action of angiogenic cytokine fibroblast growth factor-2, also the U-II increases mRNA and protein expression of the proangiogenic factors vascular endothelial growth factor (VEGF), endothelin-1, and adrenomedullin. The U-II and URP play a cardinal role in tissue remodeling associated with cardiovascular diseases. These observations have been demonstrating U-II increases the expression of mRNAs for procollagens type I and III and fibronectin, also U-II promoting expression of growth factor-b1[(61)] (TGF-b1). The chief actions of U-II and URP in the cardiovascular system is characterized by a regulation of the vascular tone. In fact, initial study demonstrated that U-II and URP, from invertebrate to mammalian, have a potent

vasoconstriction action much more of endothelin-1in human coronary artery and radial arteries when they are denuded of their endothelium, in fact, the potency of hU-II, in the isolated rat aorta, (-log[EC50] of 9.09) was found to be greater than that of endothelin-1[13] (-log[EC50] of 7.9). The vasoactive effects of U-II could be related to the blood vessel calibre: in small vessels, there is endothelium-mediated vasodilation, whereas in large vessels the predominant response is a vascular smooth muscle cells-mediated vasoconstriction. In fact, this vasoconstrictive is mediated via UT receptors present on smooth muscle of large blood vessels and this vasoconstriction activity is primarily relayed by cells mobilization of cytosolic calcium[13]. Calcium recruited by UT is derived partly from an intracellular pool via the activation of channel receptors sensitive to inositol triphosphate (IP3) and partly from the extracellular pool via L-type calcium channels. Calcium activates Calmodulin, in turn, activates myosin light-chain kinase, responsible for the phosphorylation of MLC-2 and the contraction of actomyosin. In the sidelines of this principal intracellular signaling pathway, other pathways involved in the contractile activity of U-II, such as the PKC/ERK and the RhoA/ROCK pathways, have also been identified[62][65]. Regarding the vasorelaxant activity property that manifests in small caliber blood vessels are mediated via endothelium. In fact, the activation of UT receptor present on endothelial tissue determine, as in the smooth cells, mobilization of intracellular Ca^{2+} in the same way, but induce endothelium-dependent relaxation through release of relaxing factors such as nitric oxide and endothelium-derived hyperpolarizing factor (EDHF), causing vasodilation[66]. Moreover, considering the pseudo-irreversible binding properties of U-II with to the UT receptor has led to the suggestion that U-II may function as a chronic regulator of basal vascular tone, rather than a short-term regulator of vascular resistance. Concerning the cardiovascular system, the UT receptor is also present in myocardial tissue where it is involved in different function. The interaction both hU-II and UT receptor in the right atrium and right ventricle at the concentration of 20 nM cause an increase in contractile force of myositis, also, in the same tissues, *h*U-II has a potent concentration-dependent positive inotropic

effects. Therefore, U-II in myocardial tissue has been demonstrated to be the most potent inotropic agent to date, surpassing in activity endothelin-1, serotonin, and noradrenaline[67].

2.3 U-II in the regulation of renal function

The excretory system plays a pivotal role in the maintenance of homeostasis of the vascular tone of blood vessels, influencing through natriuresis and diuresis the cardiac preload (plasma volume) and afterload (peripheral resistance). In fact, in this compartment, the UT receptor lies in expressed having an active action in the control of blood pressure. In primates, including humans, the U-II mRNA is expressed highly in the kidney relative to other tissues. U-II immunoreactivity is associated with the epithelia of the distal convoluted tubule and collecting duct, with lower expression in capillaries and glomerular endothelium, and specific, high-affinity [^{125}I]hU-II- binding sites are located in human renal cortex[68]. Indeed, U-II infusion causes a marked reduction in glomerular filtration rate, urine flow, and sodium excretion rate, having, therefore a great impact in the control of cardiovascular tone. Different study also has demonstrated that U-II and URP could have a role in the diseases that affect the kidney, in fact plasma levels of U-II are elevated significantly in hemodialysis[69] patients and in diabetics with proteinuria. The U-II gene is also expressed in human corpus cavernous and in endothelial cells of these tissues. In the rat, in fact, intracavernous injection of U-II causes an increase in intracavernous pressure without affecting systemic blood pressure[70], and vessel blood relaxation through involving the NO pathway. This evidence, therefore, could give an indication of the use of U-II in erectile dysfunction[71].

3 Pathological roles of U-II

3.1 Heart failure

3.2 Hypertension

3.3 Arteriosclerosis

3.4 Chronic renal disease

3.5 Effects of U-II on the Immune System

3.6 UT receptor in neoplasia

3.7 Prostate cancer

3.8 The bladder cancer

3.9 Colorectal carcinoma

3.1 Heart failure

In patients, with cardiovascular disease, have been observed both elevated plasma levels of the U-II that on overexpression of UT receptor in cardiomyocytes endothelial cells and vascular smooth cells. This has made conceivable that U-II could play an important role in the mechanism of cardiac remodeling. Different evidence support this hypothesis, the expression UT receptor is significantly increased in end-stage cardiovascular heart failure independent of the etiology of the disease[72], also the mRNA both in infarcted and non-infarcted sections of the left ventricle (LV) in a rat heart failure model, U-II and UT mRNA were elevated. Therefore, The UT-induced hypertrophic response was associated with up-regulation of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) as well as inflammatory cytokines, such as interleukin-6 known to be a potent contributor to the remodeling process via activation of the JAK/STAT pathway[73]. Besides its hypertrophic effects, U-II acts as a fibrotic factor, inducing the mRNA synthesis of pro-collagen type I and III and fibronectin mRNA in rat neonatal cardiac fibroblasts. In fact, with the examination of U-II's effects on cardiac fibroblasts in vivo, procollagens alfa1(I), alfa1(III), and fibronectin mRNA transcripts were significantly increased: 139%, 15%, 59%, 5%, and 141%, 14%, respectively. This was further supported from demonstrating that U-II has high potency and low efficacy in its mitogenic effects on cardiac fibroblasts. When it was administered in cardiac fibroblasts at 1 nM and 100 nM U-II was observed cells proliferation after 48h incubation, but this doesn't happen when SB-611812 an antagonist for UT receptor was co-administered at a concentration of 1 μM at 100 nM U-II the proliferation activity in neonatal fibroblast cells was completely inhibited[74]. Therefore, these evidences confirm the role of the UT receptor in the pathological condition of the cardiovascular system that could lead to heart failure.

3.2 Hypertension

U-II and URP, as observed previously have an important action on vascular tone being the endogenous most powerful vasoconstrictors in humane. As described the action of U-II and URP on vessel blood is different depending on their caliber. In fact, in a smalls blood vessel where the endothelial component is much more than the smooth cells, there is vasodilatation, instead in vessels with greater caliber prevail vasoconstriction induced by smooth cells. Therefore, in hypertension, which is characterized by endothelial dysfunction, the endothelium-independent vasoconstriction induced by U-II could be enhanced. Studies have shown that elevated U-II plasma levels are positively correlated with blood hypertension, systolic and diastolic, although there was no association between U-II and nitric oxide (NO) metabolite levels, which were measured to evaluate endothelial dysfunction[(75)]. Also, examining the gene expression of U-II, URP, and UT in the heart and aorta of hypertensive rats there are increased expression of the entire urotensinergic system[(76)]. Within of pathologies that interested the mechanisms of control vascular tension, U-II and URP could have a role also in pulmonary hypertension. As in the hypertension of the cardiovascular system also in the pulmonary artery hypertension (PAH), the principal cause is attributed to dysfunction of vascular endothelium. Among the principal factor that involves in this pathologic condition, there are inflammation, fibrosis, vasoconstriction, medial hypertrophy and intimal hyperplasia mediated by cytokines. U-II and URP relate to vascular constriction and inhibits the secretion of atrial natriuretic peptide (ANP) secretion, which is a vasodilator of the pulmonary circulation[(77)]. The administration of palorusan and urantide, two of principal UT antagonist, we can observe positive outcomes in PAH treatment[(78)(79)(80)(81)].

3.3 Arteriosclerosis

The arteriosclerosis among cardiovascular pathologies is one of the first cause of death in western society. Trough the study of a possible role of U-II in the genesis of the atherosclerotic plate, in the carotid arteria and aorta, have increased expression of UT receptor compared with healthy vessels, in presence of atherosclerotic lesions[82]. The U-II imply in the genesis of atherosclerotic plaques, in fact, U-II upregulates the expression of cellular adhesion molecules (CAMs) in endothelial cells, namely ICAM-1 and VCAM-1, enabling leukocyte adhesion and infiltration into the vascular wall [83], also U-II promotes the proliferation of vascular smooth cells activating fibroblasts and accelerating macrophage-derived foam cell formation owing to the upregulation of acetyl- coenzyme A acetyltransferase 1 (ACAT-1) expression[84][85]. This is important evidence ACAT-1 is a key enzyme in cholesterol homeostasis that functions to convert intracellular free cholesterol into cholesterol ester for storage in lipid droplets and formation of foam cells.

3.4 Chronic renal disease

The UT receptor, in a healthy condition of the kidney, contributes to control of renal homeostasis. In patients with renal dysfunction, in particular, in patients with Mellitus diabetes and glomerulonephritis, is observed an increased U-II plasma concentration respect healthy subjects. In different renal pathological condition, diabetic nephropathy and in renal chronical disease has reported an increase of U-II plasmatic concentration, urinary U-II concentration, and in particular, an increase of 64-fold increase in renal U-II and expression and a 2000-fold increase in UT expression [86]. This data focus attention on how, also in kidney, alteration of normal concentration of U-II and its receptor is strictly related to the pathological condition both renal than systemic. In fact, in cardiovascular disease is associate the reduction of renal function and glomerular flow rate (GFR), inducing a marked increase in renal blood flow and GFR. This effect is nitric oxide-dependent,

inducing vasodilatation in renal vascular, this hypothesis was confirmed using fluorescent NO indicator 4,5-diamino-fluorescein diacetate, which showed that *h*U-II induced NO production in renal artery endothelium[87]. Instead, particular evidence it has been shown when it in patients with end-stage renal disease, the plasma U-II concentration is inversely related to sympathetic activity and brain natriuretic peptide implying that high plasma U-II is associated with relative cardiovascular protection in end-stage renal disease.

3.5 Effects of U-II on the Immune System

The UT receptor is also expressed on the surface of immunity cells, natural killer (NK) lymphocytes, monocytes, and macrophages, which infiltrate zones displaying high levels of immunoreactivity for U-II[88]. This evidence has highlighted that the U-II and URP not only play a role in physiological and pathological condition of the cardiovascular system but also there find himself involved in the control of homeostasis of the immune system. Pro-inflammatory signals, such as tumor necrosis factor-α (TNF-α), lipopolysaccharide (LPS), or interferon-γ (IFN-γ), promote the expression of UT receptor[89], while U-II induces the secretion of cytokines, such as interleukine-6 (IL-6), in UT transfected human cardiomyocytes and lung adenocarcinoma cells, also U-II has a chemotactic action toward for human monocytes, inducing the extravasation of plasma in mice[90] and rat[91]. When instead, is stimulate the inflammatory process trough injection of LPS, in leukocytes, especially monocytes and NK cells, the expression of the UT receptor is strongly stimulated through the NF-κB pathway[92]. Instead, in liver endothelial cells, pretreated UT receptor with the antagonist Urantide, show decrease NF-κB activation and inflammatory cytokine (TNF-α, IL-1β, IFN-γ) expression[93]. In vascular tissue over to have action on control of the tone, U-II, particularly in coronary smooth muscle cells or endothelial cells in culture, increase the synthesis of inflammatory and pro-thrombotic markers like the plasminogen activator inhibitor-1, the intercellular adhesion molecule-1, and the

tissue factor through activation of the necrosis factor NF-κB, a pro-inflammatory transcription factor [94]. Therefore, in inflammation process, pathogens factors stimulate the expression and secretion of U-II from innate immune cells (including vascular endothelial cells and macrophages) in local tissues. The release of U-II, through autocrine/paracrine effects, stimulates the further upregulation of U-II expression and induces cascading release of pro-inflammatory cytokines. At the same time, U-II serves as an inflammatory chemotactic molecule to recruit circulating UT-expressing inflammatory cells, such as monocytes and macrophages, to local lesions, thereby aggravating inflammatory damage to tissues[95]. In addition, U-II can be secreted into the blood from the inflamed organs, which may produce inflammatory hormone-like effects on distant organs and even induce systemic inflammatory responses.

3.6 UT receptor in neoplasia

Among the principal characters in the genesis of cancers is inflammation, angiogenesis, motility, invasion, uncontrolled growth. Different studies have shown an important correlation between the UT receptor and the genesis of cancers. As previously described, the UT receptor is overexpressed in different pathological conditions joined together by the inflammatory condition. In fact, in cells that promote the inflammatory process, the UT receptor not only is overexpressed but perform different actions. The activation of the UT receptor, therefore, beyond the vascular know effect, induces the secretion of pro-inflammatory mediators like Chemokine. These promote in cancer the metastasis, sustaining cancer cell proliferation and survival, stimulating cancer cell migration and invasion, and inducing chronic inflammation and angiogenesis. Different study has reported overexpression of the UT receptor in different types of cancer[96].

3.7 Prostate cancer

The first evidence of relationships among the UT receptor and cancer has been shown in prostatic adenocarcinoma, in fact in benign hypertrophic prostate tissues this protein is moderately expressed. In cancer, instead, the expression of the UT receptor is variable on the basis of disease grading. In well-differentiated prostate adenocarcinoma cells, the UT receptor is expressed with elevate intensity, but it is less expressed or absent when the pathologies grade advance. This data suggests, in fact, the use of the UT receptor as a prognostic marker independent of the Gleason score, able to reveal the presence of prostatic adenocarcinoma in advanced stages. The treatment of these cells with peptide antagonist shows a dose-dependent decrease of the area occupied by migrating and invading cells. Urantide also has effects on adhesion factors and many of these regulate processes of extracellular matrix/cytoskeleton interaction responsible for cell-proliferation, migration, and invasion[97].

3.8 The bladder cancer

Another evidence of a relationship among UT receptor and cancer has been reported evaluating the expression of this receptor in the bladder neoplasia. Bladder cancer is divided on a histologic basis in non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC). This tissue has reported that the overexpression of the UT receptor is related to the pathological condition of this organ and in particular, U-II/UTR-related pathways are able to modulate proliferation, migration, and invasion. In fact, the expression of the UT receptor is related to cancer grading, being higher in low-grade neoplasia than in high-grade cancers. Therefore, also in this type of cancers, the UT receptor could have a prognostic role. When the bladder cell line has been treated with *h*U-II for 72h, it hasn't been observed growth stimulation, instead, when have been treated with super-agonist for this receptor it has been reported growth inhibition caused by the internalization of the receptor.

Instead, when these cells have been treated with an antagonist like urantide, an antagonist, causes an inhibition of about 50% of migratory and invasive capacity[98].

3.9 Colorectal carcinoma

It has been demonstrated that the UT receptor is overexpressed in the different cancer cell lines of the colon. Trough technique Western-Blot it has shown the expression of the UT receptor went up in different neoplastic stages. In fact, in healthy colon tissue, it is observed a normal expression of the UT receptor, but this increases exponentially both in adenomatous polyps than colon adenocarcinomas. Test to evaluate the biological activity of U-II has shown an increase of U-II expression in cells line of colon adenocarcinoma, instead in the presence of antagonist, urantide is reported a cell growth inhibition. Other studies report that in initial steps of cancerogenesis process the interaction U-II/UTR carries out an important role in the progression of neoplastic disease, moreover, Tumor tissue could produce a higher amount of U- II, and at the same time, it could become more susceptible to U-II, acting as autocrine and paracrine factor of tumour expression[99].

4 State of art

The peptides of urotensinergical system have obtained on the part of scientific community great interest pursuant by biological, pharmacological and pathological evidence. The activation of the UT receptor can regulate the contractility and growth of cardiac and peripheral vascular vessels, having a role in cardiovascular failure. Moreover, U-II can induce an inflammation status trough release of inflammatory cytokines that in the chronic condition they culminate in cancers. It was found a relation between overexpression of U-II in chronic inflammatory and tumors in cancers of bladder, colon, and prostate. Since U-II it involved in physiological and pathological condition, the scientific community taking by reference hU-II, has focused his studies at the optimization of the chemical structure of this peptide. The aim of various studies it has been to understand the elements key that involve in the recognition, activation or inhibition of the UT receptor. The first steps, therefore, to develop new peptide-based drugs, was reducing the peptide sequence maintain same biological activity respect the *h*U-II. These it has been obtained by sequential deletion of exocyclic residues from the N- and C-terminal in *h*U-II, made up by GLU-THR-PRO, didn't significantly affect the [Ca^{2+}]-mobilizing potency and efficacy of the analogues in hGPR14-transfected CHO cells respect the wild type. In contrast, any residue removal within the cycle domain causes a marked reduction or total suppression of biological activity. Therefore, this shortest analogues of *h*U-II, H-Asp-c[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH, preserved the full potency respect *h*U-II[(100)]. Subsequentially, this evaluation has been confirmed by the discovery of a second endogenous peptide belonging at Urotensin-II system, called URP, (*urotensin related peptide*, H-Ala-c[Cys-Phe-Trp-Lys-Tyr-Cys]-Val- OH). Then, in order to define which amino acids of U-II and URP are required for binding and receptor activation, has been performed point mutation of each amino acids. Therefore, for this purpose, has been synthesized L-alanine-substituted *h*U-II analogues. From this study emerged that the intra-cyclic core -*Phe-Trp-Lys-Tyr*- sequence plays an essential role in peptide-receptor interactions; in fact, their substitutions cause a marked decrease of Ca^{2+}-mobilizing activity on UT receptor[(101)]. Another important steps to better understand the key elements in the receptor activation, has been to explore the orientation of the amino acid side chain, replacing each amino acid with its corresponding stereoisomer[(102)]. The

replacement of each amino acid with their D-enantiomers in N-terminal portion doesn't alter the activity of the peptide. Instead, when this change was made in the cyclic portion of U-II, U-II(4-11) and URP, this modification abrogated the binding affinity and, markedly, reduced the contractile activity except for Trp^{9}[103]. With the replacement of L-Trp9 with D-Trp in U-II and URP sequences, the resulting peptides retain substantial binding affinity, acting as a partial agonist with moderate potency and a full antagonist with low potency, indicating that the substitution of Trp residue could determine a shifting from agonist to antagonist activity[104] The cyclic structure is essential for the activity of this peptide; in fact in the linear sequence has been observed a total lack in activity, therefore the cyclic structure is determinant for the binding at UT receptor[105]. Additionally, using analogues peptides with a different ring-closing method, different from disulfide bond, like triazole and lactam, has been observed a reduced affinity for the receptor respect to the U-II. Therefore, it has been supposed that the U-II bind the UT receptor through a pseudo-irreversible manner, and that the disulfide bond should play an important role[106][107]. In fact, previous docking studies have demonstrated that U-II binds UT receptor in way that the disulfide bond is near to the TM helix (TM-III) and the second extracellular loop (EC-II) (Cys123 and Cys199). This binding mode could settle, through a redox mechanism, a typical pseudo-irreversible binding.

The subsequent replacement of the Cys^{5} with penicillamine (Pen), generated the most potent agonist known to data, named P5U [Pen5]hU-II (4-11)[108]. NMR studies performed on this peptide showed that the Pen^{5} residue stabilizes the active conformation of the peptide[109]. The additional Ala-scan study has shown that the core sequence -Phe-Trp-Lys-Tyr- is essential for the recognition and activation of UT receptor, so all the studies regarding the Structure-activity-relationships (S.A.R.) have been focused mainly on these residues. Some of these studies reported in literature have demonstrated that the Phe^{6} is the most tolerated residue within the cyclic region; in fact, its replacement with nonpolar aromatic or aliphatic residues doesn't improve the potency and the binding affinity respect to U-II (4-11). In particular, its replacement with constrained amino acids, like octa-hydro indole-2-carboxylic (Oic) residue, cause a devoid both binding affinity and contractive activity

with the receptor, probably caused by an unfavorable conformation of the structure, that could destabilize the correct folding of the peptide. Differently, introducing a residue characterized by a flexible aliphatic side chain, like as Leu and Cha (cyclohexyl alanine) residues, the peptides generated showed to exhibit an affinity with the receptor but are unable to induce contraction, resulting weak antagonists. These results seem show that replacing the aromatic group of Phe with a residue characterized by an aliphatic or alicyclic side chain, could determine the shifting from agonist to antagonist activity. The Trp^7 is the first of three amino acids of the "core sequence" important for the U-II activity; its substitution with Ala determines a total loss of activity. Surprisingly, the use of its D-isomer determines the shifting from agonist to antagonist activity. The role of indolic group has been deeply investigated and its replacement with a 4-benzoyl-L-phenylalanine, generating the [Bpa7]U-II derivative, which has a reduced binding affinity and lower potency on inositol phosphate production in UT-transfected COS-7 cells[110], suggesting that the indolic NH may establish a hydrogen bond with UT receptor important for its activation. Moreover, by NMR studies has been demonstrated that there is a type II b-hairpin backbone conformation between Phe6 and Trp7, and to stabilize this conformation the Trp residue in P5U and Urantide, has been replaced with Tpi (Fmoc-L-1,2,3,4-tetrahydronorharman-3-carboxylic acid). The corresponding analogues have been shown that the beta-turn conformation is essential to have biological activity because allow the maintained the pharmacophoric proprieties[111]. Anyway, Lysine residue appears to be the most important residues of the core sequence of U-II and URP. To evaluate the importance of a basic residue in this position, Lys has been replaced with amino acids with different sidechain and the presence of an aliphatic structure in the side chain give compounds with a total loss of activity. Moreover, the replacement of Lys^8 with residues with different basicity, has demonstrated that the presence of a primary aliphatic amine at position 8 is an essential requirement for U-II activity. The importance of Lys side chain has been investigated using ornithine (Orn), 2,4-diaminobutyric acid (Dab) and 2,3-diaminopropionic acid (Dap), showing that there is a reduction of potency and efficacy in these analogues. Actually, [Orn8]U-II behaves as a full agonist on calcium mobilization assay in HEK293

hUT- and rUT-transfected cells, inducing maximal effects similar to those of U-II, but exhibits weak contractile activity on rat aorta strips[112]. Gradual concentrations of [Orn8] U-II induce a rightward shift of the dose-response curves of hU-II indicating that this compound serves as a competitive antagonist. By docking studies, it has been observed that the Lys side chain is essential for the interaction with the receptor because interacts with Asp130 in TM3 of UT receptor. Reducing the length of the side chain of Lys, like in Orn, maintain the affinity with the receptor but doesn't able to activate it. Finally, the Tyr^9 residue has been deeply investigated as the fourth important element of the "core sequence" of U-II, indispensable for the biological activity. Like the other residues, Tyr^9 has been deeply investigated and its replacement with Ala, provokes an inactive analogue.

Among the most important modifications performed in this position, deserve to be mentioned the modification on phenolic group of Phe residue. In particular, the substitution of the hydroxyl group of tyrosine with methoxy (–OMe), nitro ($–NO_2$), amino ($–NH_2$), methyl ($–CH_3$), fluoride (–F), or by a hydrogen atom does not affect in the potency and the efficacy of the U-II analogues in the rat aorta bioassay[113]. The mono-iodinated analogue [3-iodo-Tyr] U-II (4–11) was found to be five times more potent than the parent peptide in producing rat aortic ring contraction[114]. The [3-nitro-Tyr] U-II (4–11) analogue showed a moderate decrease in binding affinity and in contractile activity. The [4-carboxy-Phe]U-II(4–11) analogue, in which the hydroxyl group of the tyrosine moiety is replaced by a carboxyl radical, exhibited a significant reduction of both affinity and potency, whereas the amino counterpart, the [4-amino-Phe]U-II(4–11) analogue, retained affinity but showed a marked decrease in contractile activity. Also, the replacement of Tyr^9 in urantide, with (pCN)Phe or (pNO_2) Phe improved the antagonistic activity of these analogues. More interestingly, the replacement of the Tyr with the Btz 2-amino-3-(benzo[d]thiazol-2-yl)propanoic acid) or the (3,4-Cl)Phe residues led to superagonist with pEC_{50} values at least 1.4log higher than that of 1, being one of the most potent UT receptor agonists discovered to date.

5 Design and Biologicals results

5.1 Mono N-Methylation of the "*core sequence*" of URP

5.2 Biological data of N-Methylated URP analogues and Discussion

5.3 Peptoids of URP

5.4 N-terminal modifications of Urantide and U-II sequences

5.5 Biological data of N-Terminal Urantide Analogues

5.6 Modifications at Tyr^{8} position of U-II (4-11) and Urantide

5.7 Biological results of U-II (4-11) and Urantide containing Tyrosine analogs

5.8 Incorporation of Lys constrained analogues in Position 7 of Urantide

5.9 Biological activity of Urantide analogues containing constrained Lysine

Prologue

The research activity performed during my PhD course has been focused on increasing the knowledge on structure-activity relationship on the urotensinergic ligands with aim to find more appropriate ligands as potential drugs.

In this context and starting from the previous results reported in literature, the research activity has been addressed in exploring different structural aspects of U-II and URP sequences.

In particular, following our previous studies on N-methylation performed on U-II (4-11), I extended this study on URP sequence to evaluate a possible discrepancy that could explain their unique biological activity (Section 5.1).

Subsequently, I have synthesized a small library of peptoids correlated to URP sequence (Section 5.3).

Additionally, modifications have involved the N-terminal sequence of hU-II to well understand the contribution of this portion in binding mode with the human receptor (Section 5.4).

Subsequently, starting from U-II (4-11) and Urantide sequences, I have synthesized a library of new compounds containing unconventional amino acids at Tyr position (Tyr^9). This study was performed to better evaluate the influence of this position in binding with the receptor and hoping to improve the antagonist activity (Section 5.6).

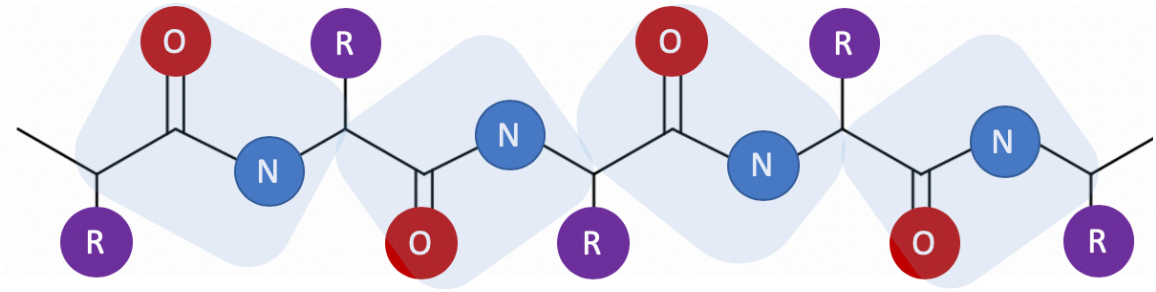
Finally, an additional small library of peptides has been synthesized starting from Urantide sequence by replacing the Lys^8 residue with unconventional amino acids, isosteres of this residue (Section 5.8)

The synthesis of these compounds has been performed using different synthetic strategies based on conventional approaches and new methodology, like as the Solid-Phase Peptide Synthesis by Ultrasonication developed in our laboratory, and Microwave-assisted solid-phase peptide synthesis.

5.1 Mono N-Methylation of the "*core sequence*" of URP

The potential of peptides as drug candidates is limited by their poor pharmacokinetics properties. In particular, two are the factors critical, that is, short half-life and poor absorption. Low serum stability is generally the cause of short half-life. In fact, the peptides, in vivo are metabolically unstable and susceptibly at proteolytic degradation. In order to improve these unfavorable pharmacokinetics profiles, the principal strategies are or use D-enantiomer or introduce N-methylation on backbone of amino acids recognized by proteases. In this regard, N-methylation of amide bonds in a peptide may overcome such limitations, improving stability and[114][115][116] bioavailability. Both factors are strictly linked with an increased activity and selectivity. A multiple N-methylation of peptide bonds may have complementary consequences to improve bioavailability and[117] target selectivity. Driven in part by the success of natural N-methylated peptides, such as the immunosuppressant drug cyclosporine A and the nematicide[118] omphalotin, in last two decades the N-methylation has been pursued to improve "drug-like" properties[11 9], and enhance a wide range of peptides possessing antibiotic, anticancer, as well as various other activities.

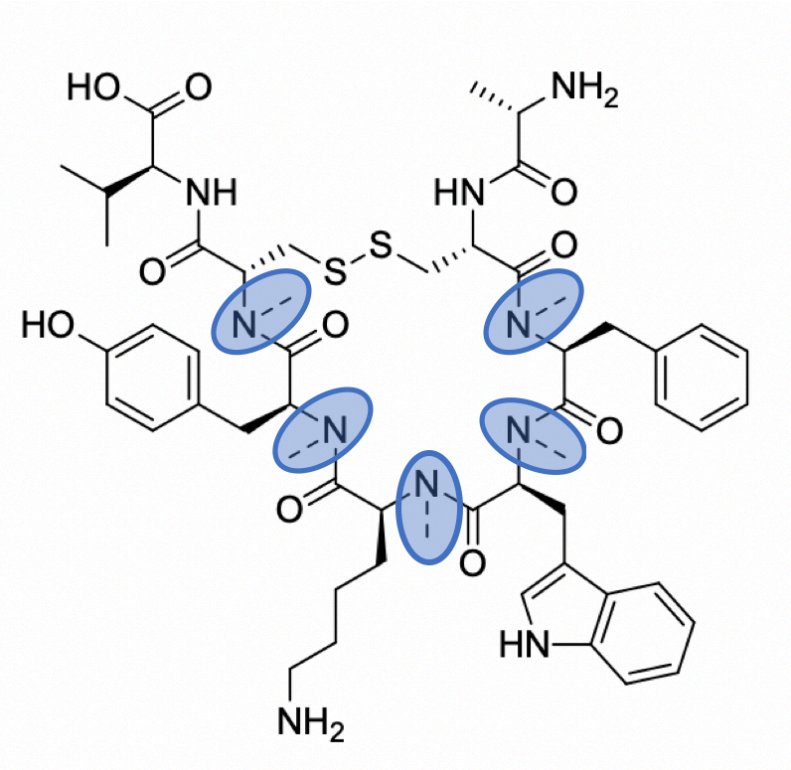
Figure 10. Peptide bond geometry.



The strategy to introduce the N-methylation of amide bond in a peptide, in addition to improve the pharmacokinetic property of peptide, could be a tool used to study the structural characteristics of the peptide. The backbone of peptides is in general flexible due to their low rotational barriers about the

bond between N and Cα, determined by the Φ dihedral angle, and the bond between Cα and CO, given by the ψ dihedral angle (Figure 10). Such flexibility hampers peptides as drugs in therapeutic applications, because it renders them susceptible to parasitic receptors such as proteolytic enzymes, leading to unfavorable pharmacokinetic properties such as poor oral and tissue absorption. N-Methylation can increase the steric hindrance between the N-methylated amide bond[120] and the adjacent amino acid side chain and modify the amide bond *cis-trans* isomer equilibrium by enhancing the population of the *cis* isomer[121]. Therefore, these modifications on peptide backbone could stabilize active peptidic structure improving the pharmacodynamic property of the peptide. In this context, cyclic peptides are more vulnerable at this type of modification. In fact, a specific N-methylation of amide bond may disrupt secondary structure due to the long-range steric interactions. Furthermore, the replacement of hydrogen by methyl on the amide may break-up intramolecular and intermolecular hydrogen-bonds, essential for peptide conformation. The replacement of hydrogen of amide bond improves the peptide lipophilic character, increasing the cellular permeability, that in the case of peptides that interact on a nuclear receptor, this could improve the pharmacodynamic profile. In view of these considerations, it is clear that the mono N-methylation of peptide amide bond represents an important tool in peptide chemistry to change/increase the pharmacological properties. Sequential introduction of N-Me amino acid in the peptide sequence is a modification that could give several information regarding the structural factors of a peptide responsible of the biological activity. In fact, it discriminates which hydrogen bond is essential for maintaining biological activity, stabilizing the peptide secondary structure and increasing its lipophilia. Recently, the research group where I have performed my PhD course, has reported in literature a structural study in which a systematic introduction of N-Methylated amino acids in the "core sequence" of U-II and U-II (4-11), has been performed. In order to complete the structural studies of peptides belonging at urotensinergic system, I designed and synthesized a short library of analogues of URP (Urotensin Related Peptide) containing N-Methyl amino acids in the core sequence. More specifically, the amino acids of the bioactive hexapeptide sequence of URP ([Cys^2-Phe^3-Trp^4-Lys^5-Tyr^6-Cys^7]), have been changed in a

systematic manner with their corresponding N-methylated analogues to provide URP derivatives (Table 1). The structural and biological effects of the modifications on URP sequence have been compared with the results already obtained on U-II and U-II (4-11) sequences. These peptides have been synthesized using standard SPPS (Section 6.1) while the N-Methyl amino acids have been synthesized directly on solid phase during the synthesis of the single peptide (Section 6.2)



URP

Table 1. N-Methyl analogues of URP

Name	Structure
URP	**H-Ala^1-[Cys^2-Phe^3-Trp^4-Lys^5-Tyr^6-Cys^7]-Val^8-OH**
1	H-Ala-c[(*N*-Me)Cys-Phe-Trp-Lys-Tyr-Cys]-Val
2	H-Ala-c[Cys-(*N*-Me)Phe-Trp-Lys-Tyr-Cys]-Val
3	H-Ala-c[Cys-Phe-(*N*-Me)Trp-Lys-Tyr-Cys]-Val
4	H-Ala-c[Cys-Phe-Trp-(*N*-Me)Lys-Tyr-Cys]-Val
5	H-Ala-c[Cys-Phe-Trp-Lys-(*N*-Me)Tyr-Cys]-Val
6	H-Ala-c[Cys-Phe-Trp-Lys-Tyr-(*N*-Me)Cys]-Val

5.2 Biological data of N-Methylated URP and Discussion

The N-methylation have allowed to investigate the conformational and biological aspects related to URP. The amino acids included into the cyclic exapeptide sequence Cys^2 -Cys^6, recognized as the pharmacophoric sequence due to the maintenance of biological activity, have been exchanged in a systematic manner giving mono-N-methylated peptides **1-6**.

Figure 13. Representative concentration-response curves obtained with rat thoracic aorta rings after adding cumulative concentrations of related *N*-methylated analogs **1-6**

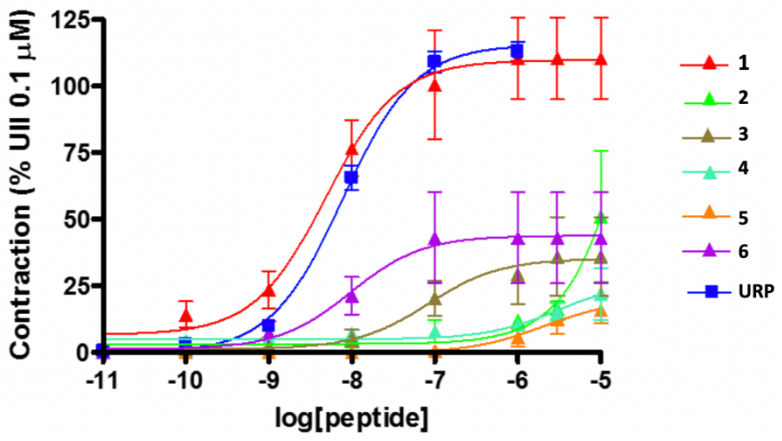


Table 2. Receptor affinity and functional assay of N-Me analogues of URP

Name	Bind affinity pK_i[a]	Aortic ring contraction	
		pEC_{50}[b]	Emax[c]
URP	8.20 ± 0.12	8.11 ± 0.06	116 ± 4
1	8.76 ± 0.07*	8.31 ± 0.19	110 ± 6
2	6.61 ± 0.06**	51% @ 10 μM	
3	8.66 ± 0.13*	7.08 ± 0.45***	35 ± 5***
4	5.82 ± 0.21***	22% @ 10 μM	
5	5.27 ± 0.84***	16% @ 10 μM	
6	8.45 ± 0.15	8.02 ± 0.49	44 ± 6***

[a]pK_i: $-\log K_i$; [b]pEC_{50}: $-\log EC_{50}$; [c]maximum efficacy is expressed as a percentage of the KCl- induced contraction (40 mM) divided by the tissue-response induced by **1** (10^{-7} M). All values are expressed as mean ± S.E.M. Statistical analysis were performed using unpaired Student's test, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, *versus* values obtained for **2**. Each replicate (n) was conducted on tissue from different animal or on different passage.

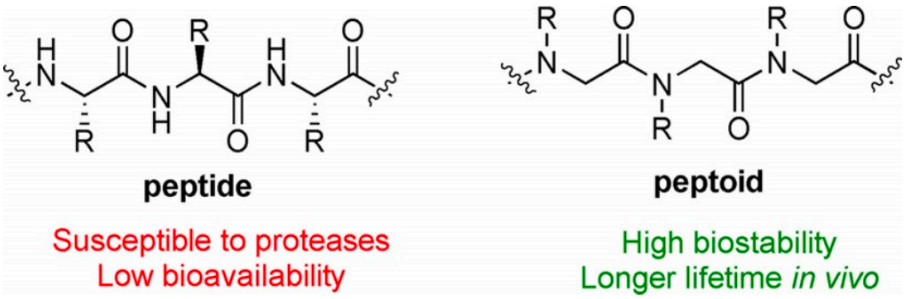
The propensity of each analog to bind UTR was initially assessed by a competitive binding assay. N-Methylated analogs 1-6 were examined on either HEK293 or CHO-K1 cells stably expressing the human UTR isoform (Figure.13). All peptides showed binding at the recombinant human UT receptor expressed on membranes obtained from stable CHO-K1 cell line of HEK293 or CHO-K1 (Table. 2). These peptides showed a different behavior, regarding the biological activity, depending on the site of N-methylation. Indeed, the introduction of N-Methylation has been tolerated only in (**1**) [(N-Me)-Cys2]-URP that showed increasing of binding affinity respect native peptide, maintaining complete

agonistic activity. The effect of N-Methylation of amide nitrogen in the compound (**1**) could be similar to that obtained by the introduction of Pen residue in position 2 of the URP sequence[122]. In fact, as happen in P5U (super agonist analog of urotensine-II) the presence of a methyl group in β in the side chain of penicillamine stabilizes the bioactive peptide conformation[123]. Regarding compounds (**3**) [(N-Me)-Trp3]-URP and (**6**) [(N-Me)-Cys7]-URP, the N-methylation causes a reduction of biological activity; in fact, these compounds act as partial agonist. Finally, the compounds (**2**), (**4**) and (**5**), respectively, [(N-Me)-Phe3] URP, [(N-Me)-Lys5] URP and, [(N-Me)-Tyr6] URP, showed a great reduction of binding affinity and lacking biological activity. The absence of biological activity for the compounds (**2**), (**4**), (**5**) has made thinking that N-methylation of the amide on peptide backbone of these peptides engraves negatively in peptide folding. The negatively affected due to the N-methylation of nitrogen amide of the compounds (**2**), (**4**), (**5**) could be due by destabilizing of peptide conformation. This may be due both by the impossibility to generate hydrogen-bonds aside amide nitrogen of peptide backbone or that the peptide assuming inactive conformation because of the methylene group. In case of N-Me analogues of Tyr^3 and Phe^6 the lack of activity is explained by previous an NMR study that has demonstrated that the amide NH and CO moieties of Phe^3 and Tyr^6 engaged in two hydrogen-bonds stabilizing the β-hairpin conformation in peptides[124]. The effect of N-Methylation on URP analogues has shown divergent results compared with previously obtaining on U-II and U-II (4-11). In fact, in case of compound (**6**), N-Methylation caused partial agonist (Emax = 44 ± 6%), while in [(N-Me)-Cys10]-U-II and in [(N-Me)-Cys10]U-II(4-11) the full agonist activity is preserved. The same happens in the compound (**3**) where the N-Methylation causes partial agonist (35±5***) in contrast with the compounds [(N-Me)Trp7]U-II and [(N-Me)Trp7]U-II(4-11), that preserve full agonist activity. Instead, the N-Methylation of lysine, tyrosine and phenylalanine in the core sequence, causes great reduction of agonistic activity in all peptides belongs at urotensinergic system.

5.3 Peptoids of URP

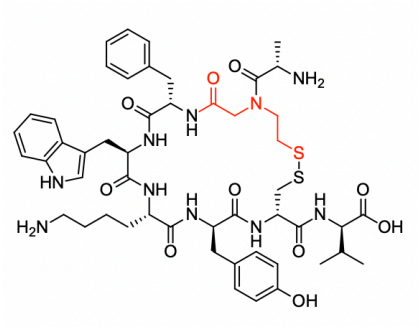
Peptoids, or poly-N-substituted glycines (NSG), are a class of peptidomimetics whose side chains are appended to the nitrogen atom of the peptide backbone, rather than to the α-carbons (Figure.11). In the field of Solid Phase Peptide Synthesis (SPPS), the use of peptoid is another tool to introduce variability in the peptidic sequence. Such as the introduction of D-amino acids, N-methylation or β amino acids, the use of peptoids in a peptide sequence is a strategy that allows having an increased resistance to proteolysis, improving consequentially the pharmacokinetic profile.

Figure 11. difference between peptoid and peptide



Their biological interest lies in their capability of forming a wide variety of secondary structures, including: α-helix, polyproline type I helix, and type I and type III β-turns. They can also mimic more complex secondary structures[125]. Zuckermann demonstrated that single amphiphilic peptoid helices could be assembled in bundles to form a hydrophobic core. Interestingly, peptoids exhibit good cellular permeability in comparison to their analogue peptides, therefore their potential as cellular transporters has also been studied. The backbone tertiary amides within peptoids are able to adopt cis- or trans-conformations and any stable secondary structures are derived purely from steric and/ or electronic interactions[126]. However, the backbone of the peptoids is intrinsically flexible; therefore, a peptoid that exhibits high affinity to biomolecules has not been reported except for rare exceptions. Previously has been reported a similar structural study on U-II (4-11) using N-alkyl-glycines, by a

systematic introduction of these structural entities in core sequence of this peptide. In order to perform similar study on URP sequence, I synthesized a small library of compounds containing URP-related peptoids modified in core sequence (Table 3). Regarding the side chain of $Cys^{2,7}$ and Trp^{4} have been protected manually using different synthetic strategies (Section 6.4). The library of compounds has been synthesized using a new SPPS approach using ultrasonication in different reaction steps. The ultrasonication allows to reduce considerably the time reaction and reduce the use of reactive, respect standard SPPS (section 6.3). In literature exist different synthetic strategies that allow the synthesis of peptoids both in solution and on solid phase. For the synthesis of URP peptoids, the Zuckermann methodology was used because it greatly increased the synthetic efficiency, synthesis yields, and available side-chain diversity, while dramatically reducing time and costs (section 6.4).



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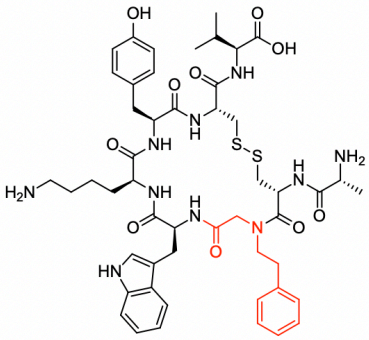

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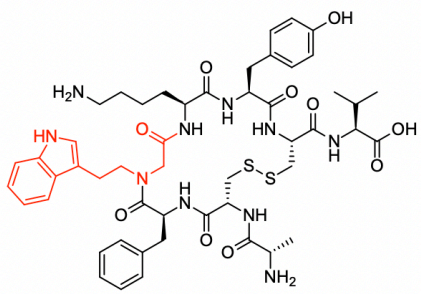

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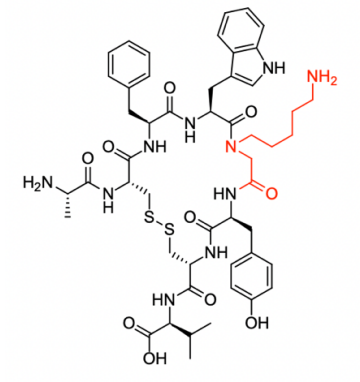

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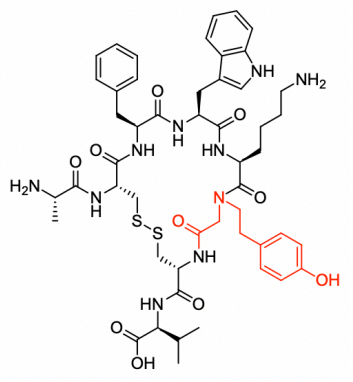

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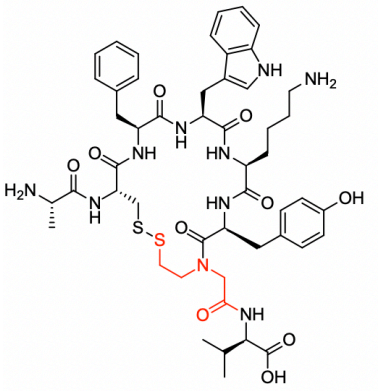

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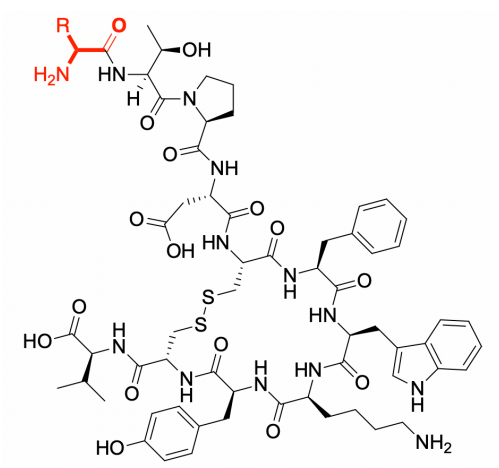
Table 3. Peptoids of URP

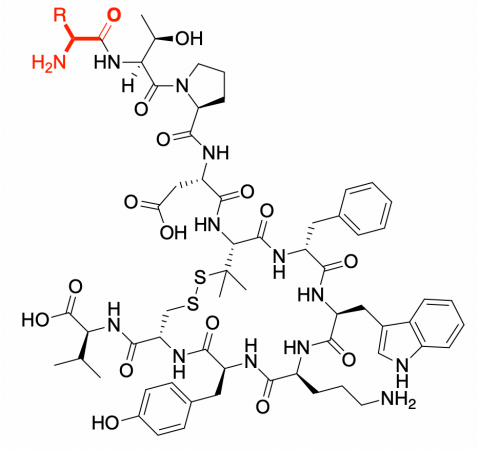
Name	Structure
Urp	**H-Ala[1]-[Cys[2]-Phe[3]-Trp[4]-Lys[5]-Tyr[6]-Cys[7]]-Val[8]-OH**
7	H-Ala[N-(2-Sulfanylethyl)Gly-Phe-Trp-Lys-Tyr-Cys]Val-OH
8	H-Ala[Cys-N-(2-Phenylethyl)Gly-Trp-Lys-Tyr-Cys]Val-OH
9	H-Ala[Cys-Phe-N-(2-indolethy)lGly-Lys-Tyr-Cys]Val-OH
10	H-Ala[Cys-Phe-Trp-N-(5-aminopentyl)Gly-Tyr-Cys]Val-OH
11	H-Ala[Cys-Phe-Trp-Lys-N-(4-hydroxyphenylehtyl)Gly-Cys]Val-OH
12	H-Ala[Cys-Phe-Trp-Lys-Tyr- N-(2-Sulfanylethyl)Gly]Val-OH

5.4 N-terminal modifications of Urantide and U-II sequences

Recent studies have highlighted that the peptides belonging at urotensinergic system, can modulate the UT receptor activation in a different manner. This is possible by the presence on UT receptor of allosteric sites specific for U-II and URP as reported by Chatenet[(127)]. This discovery has been possible thanks the synthesis of two new analogues, that is, rU-II (1-7) and UCA, respectively the N-terminal domain of the U-II in rat, and antagonist of urotensinergic system analog of URP.



***h*U-II**



***h*U-II with antagonist residue in core sequence**

Table 4. Urantide analogues containing changes in N-terminal domain.

Name	Structure
U-II	H-Glu^{1}-Thr^{2}-Pro^{3}-Asp^{4}-[Cys^{5}-Phe^{6}-Trp^{7}-Lys^{8}-Tyr^{9}-Cys^{10}]-Val^{11}-OH
U-II(4-11)	H-Asp-[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH
Urantide	H-Asp-[Pen-Phe-DTrp-Orn-Tyr-Cys]-Val-OH
13	H-**Ala**-Thr-Pro-Asp-[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH
14	H-**Glu**-Thr-Pro-Asp-[Pen-Phe-DTrp-Orn-Tyr-Cys]-Val-OH
15	H-**Ala**-Thr-Pro-Asp-[Pen-Phe-DTrp-Orn-Tyr-Cys]-Val-OH
16	H-**Gln**-Thr-Pro-Asp-[Pen-Phe-DTrp-Orn-Tyr-Cys]-Val-OH
17	H-**Dab**-Thr-Pro-Asp-[Pen-Phe-DTrp-Orn-Tyr-Cys]-Val-OH
18	H-**Dab**-Thr-Pro-Asp-[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH
19	H-**hSer**-Thr-Pro-Asp-[Pen-Phe-DTrp-Orn-Tyr-Cys]-Val-OH
20	H-**Ala**-Thr-Pro-**Ala**-[Pen-Phe-DTrp-Orn-Tyr-Cys]-Val-OH
21	H-**hSer** -Thr-Pro-Asp-[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH

When the UCA is used as an antagonist it can reduce *h*U-II-induced contraction without altering URP-mediated vasoconstriction; instead, rU-II(1-7) can block URP, but not the vasoconstriction associated at rU-II. This evidence has reevaluated the importance of N-terminal domain of U-II in the activation of the UT receptor, until now defined marginal. For this reason, an improves knowledge about this peptidic portion of U-II is necessary. Once identified the structural elements necessary to interactions with allosteric sites, it could be useful synthesize peptides capable to induce or block specifically the biological response inducted from U-II or URP. For this purpose, to increase our knowledge regarding the structural elements responsible for this additional interaction with the allosteric binding site, I synthesized a short library of compounds analogues of U-II and Urantide containing modification in N-terminal position (Table 4). More specifically, the first amino acid of U-II sequence, that is, Glu^{1} has been replaced with several natural amino acids with different physiochemical properties. In particular, the amino acids are characterized by a polar (**16**, **19**, **23**),

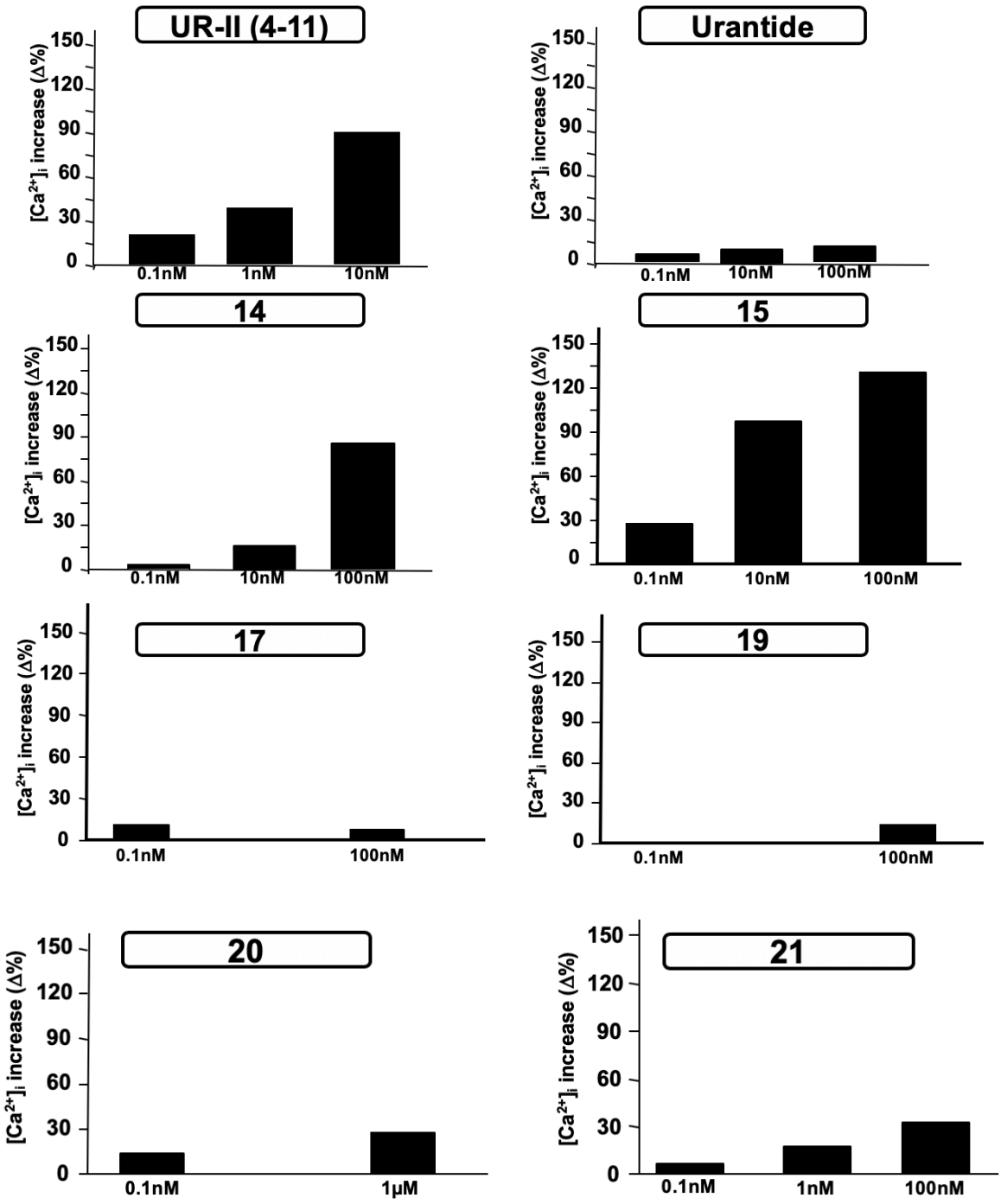
nonpolar (**13**, **15**, **20**) and basic (**17**, **18**) group in sidechain. Besides, the Urantide sequence has been included inside the whole sequence of U-II to well weight the contribute of the "*antagonist sequence*" in promote the cooperation with the allosteric binding site. The synthesis of these peptides has been carried out using an innovative SPPS approach, that through use of ultrasonication improves any synthetic steps (section 6.3).

5.5 Biological data of N-Terminal Urantide Analogues

The propensity of these compounds to activate UT receptor has been determinate through the ability of these compound to induce the release of intracellular calcium. The compounds (**13** to **21**) have been tested using Herk 293 cells transfected with *h*UT receptor. To avoid phenomena to receptor sensitization the concentration of these compounds has been tested in the range 0,1 nM to 100 nM. The compounds respond differently to the modifications carried out in the N-terminal peptide domain, providing important information about the element necessaries so that this portion interacts with the allosteric site. In particular, in the compound (**14**), U-II sequence containing in the core sequence the residues that confer antagonistic activity, shows an increase of the agonist activity to Urantide, in the 10 nM than 100nM. Surprisingly, replacing of Glu^1 with Ala, compound (**15**), the agonist activity is predominant already at 10nM. In fact, the compound (**15**) could be considered an agonist ligand at *h*UT receptor. This unexpected result demonstrates that Ala^1 has an opposite behavior on the allosteric binding site respect to Glu^1, although the peptide contains the core sequence typically of the antagonist Urantide. The biological result highlighted with the compound (**15**) is an evidence that this position could play an important role in discriminating between agonist and antagonist activity in the whole sequence of UII. Important results have been observed when in the compound (**17)** and (**19**), in which Glu^1 has been replaced respectively with basic residue as Dab (2,4-diaminobutyric acid) (**17**) and hSer (**19**). Both compounds restored the antagonist activity with an impressive loss of

agonist activity for the compound (**17**) containing Dab residue in position 1. The compound (**19**), characterized to have a hSer residue in same position, conserved a residual agonist activity at 100 nM. Instead, the replace of Glu1 with Dab and hSer, respectively (**17**) (**19**), cause important reduction of agonist activity, acting at concentration of 10 nM as full antagonist, better than Urantide that at this concentration conserve residual agonistic activity. In the case of (**17**), Dab could fold up as Glu^1 but it has different chemical-physical property; in fact, could interact with allosteric site of the UT receptor, but not be able to activate it. Instead, in the case of (**19**), hSer and Glu^1 have the side chain of the same length but different ability to realize hydrogen bonds; therefore, hSer interact with allosteric site but cannot fully activate it. As in the compound (**19**) in the compound (**21**), analogue of *h*U-II, the replacement of Glu^1 with hSer influence negatively the biological activity; in fact, in (**20**) there are a reduction of agonistic activity respect U-II (4-11) already at concentration of 100 nM. The compound (**20**) is an analogue of compound (**15**) in which the Asp^4, typically present in UII sequence, has been replaced with Ala residue of the URP sequence. This modification has reinforced the antagonist activity respect to the compound (**15**) highlighting that also this position could crucial for the binding of UII and related analogues with at UT receptor. This position could be an interesting point to be investigated in next future to get additional information about the its contribution in binding with the UT receptor. In view of these results it is clear that the Glu^1 plays an important role in receptor interaction, and the allosteric binding site could drive the discrimination between agonist and antagonist activity, depending the core sequence of the considered ligands (Figure 12).

Figure 12. Intracellular calcium release.

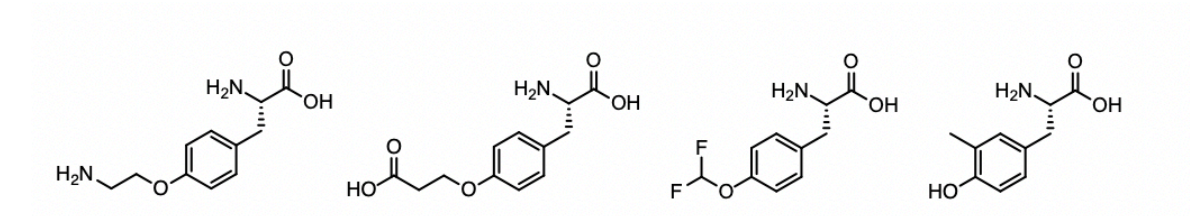


5.6 Exploring the Tyr^{8} position of U-II (4-11) and Urantide

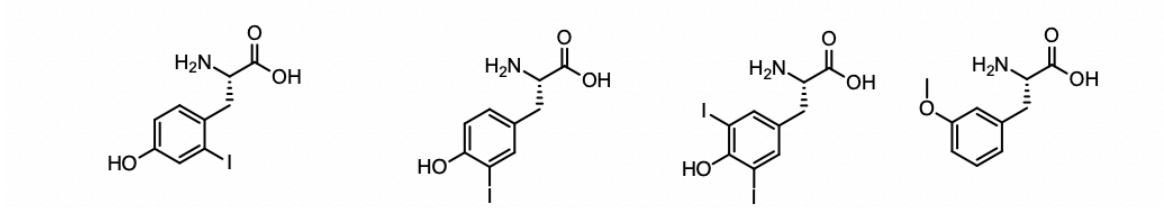
Tyrosine residue presents in the core sequence of hU-II is an indispensable key element for the biologic activity, in both UII and URP. In fact, its replacement with Alanine causes a complete reduction of activity in urotensinergic system ligands. Previously, several studies have highlighted the importance of the aromatic residue (**15**) in this position but are still missing some additional information regarding the conformational and structural elements responsible for interaction with the counterpart of receptor. With aim to explore deeply the contribute of the aromatic residue in this position it has been decided to use several unconventional tyrosine analogues differently substituted on aromatic ring (Figure 13).

Thus, a library of compounds analogues of Urantide and hU-II (4-11) containing sequential introduction of Tyrosine analogues in position 8 (Table 5). Taking in consideration the previous SAR studies already reported in literature, I have selected different non-coded Tyrosine analogues in attempt to improve antagonistic activity. Substitutions include bio-isosteres of phenolic group OH (**34**-**37**); introduction of electron-withdrawing groups on aromatic ring such us iodine (**25**-**26**, **31**-**33**) and NO_2 (**50**-**51**); groups sterically hindranced on aromatic ring, such as Methyl (**44**-**45**) and Tert-butyl (**46**-**47**); shifting of phenolic OH in meta-position on aromatic ring (**38**-**39**); replacement of OH phenolic with MeO (**52**-**53**) and Me groups (**24**-**30**); introduction of an electron-withdrawing group, such as Phenyl (**40**-**43**) and OH (**48**-**49**) in meta-position of the aromatic ring, regarding the unconventional Tyrosine analogs of (**40**-**43**), these have been synthesized manually using Suzuki reaction (Section 6.6). Finally, the introduction of groups that improve the ability to carry out a hydrogen bond (**22**-**23**, **28**-**29**). These peptides have been synthesized using the CEM Liberty Blu Synthesizer, that have allowed synthesize these compounds quickly ensuring excellent quality of synthesis (Section 6.5).

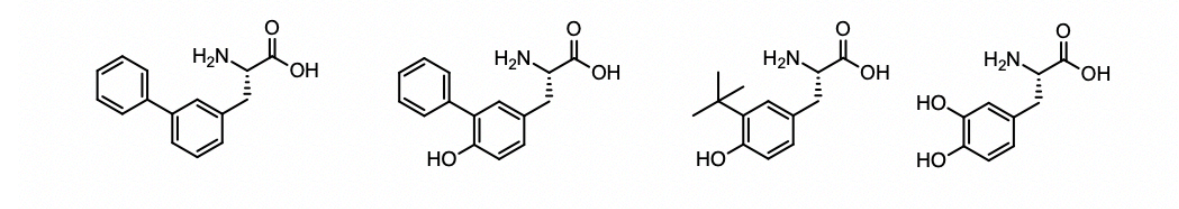
Figure 13. Tyrosine Analogue utilized for compounds **22-53**



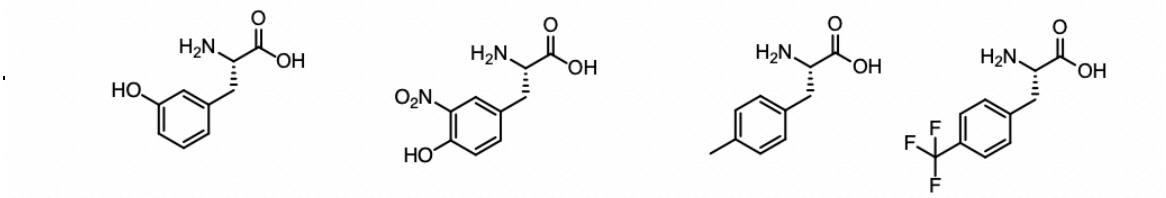
Aef **Cbf** **4OCHF2Phe** **3MeTyr**



2ITyr **3ITyr** **3,5ITyr** **3MeOPhe**



mBip **3PhTyr** **3tBuTyr** **Dopa**



3OHPhe **3NO₂Tyr** **4MePhe** **4CF3Phe**

Table 5. Urantide and U-II sequences containing unconventional Tyrosine analogues

Name	Structure
U-II (4-11)	H_2N-Asp-[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH
Urantide	H_2N-Asp-[Pen-Phe-DTrp-Orn-Tyr-Cys]-Val-OH
22	H-Asp-[Pen-Phe-DTrp-Orn-Cbf-Cys]-Val-OH
23	H-Asp-[Pen-Phe-DTrp-Orn-Aef-Cys]-Val-OH
24	H-Asp-[Pen-Phe-DTrp-Orn-4Mf-Cys]-Val-OH
25	H-Asp-[Pen-Phe-DTrp-Orn-3ITyr-Cys]-Val-OH
26	H-Asp-[Pen-Phe-DTrp-Orn-2ITyr-Cys]-Val-OH
27	H-Asp-[Pen-Phe-DTrp-Orn-3,5ITyr-Cys]-Val-OH
28	H-Asp-[Cys-Phe-Trp-Lys-Cbf-Cys]-Val-OH
29	H-Asp-[Cys-Phe-Trp-Lys-Aef-Cys]-Val-OH
30	H-Asp-[Cys-Phe-Trp-Lys-4Mf-Cys]-Val-OH
31	H-Asp-[Cys-Phe-Trp-Lys-3ITyr-Cys]-Val-OH
32	H-Asp-[Cys-Phe-Trp-Lys-2ITyr -Cys]-Val-OH
33	H-Asp-[Cys-Phe-Trp-Lys-3,5ITyr -Cys]-Val-OH
34	H-Asp-[Pen-Phe-DTrp-Orn-OCHF2Phe-Cys]-Val-OH
35	H-Asp-[Cys-Phe-Trp-Lys-OCHF2Phe-Cys]-Val-OH
36	H-Asp-[Pen-Phe-DTrp-Orn-CF3Phe-Cys]-Val-OH
37	H-Asp-[Cys-Phe-Trp-Lys-CF3Phe-Cys]-Val-OH
38	H-Asp-[Pen-Phe-DTrp-Orn-3OHPhe-Cys]-Val-OH
39	H-Asp-[Cys-Phe-Trp-Lys-3OHPhe-Cys]-Val-OH
40	H-Asp-[Pen-Phe-DTrp-Orn-mBip-Cys]-Val-OH
41	H-Asp-[Cys-Phe-Trp-Lys-mBip-Cys]-Val-OH
42	H-Asp-[Pen-Phe-DTrp-Orn-3PheTyr-Cys]-Val-OH
43	H-Asp-[Cys-Phe-Trp-Lys-3PheTyr-Cys]-Val-OH
44	H-Asp-[Pen-Phe-DTrp-Orn-3MeTyr-Cys]-Val-OH
45	H-Asp-[Cys-Phe-Trp-Lys-3MeTyr-Cys]-Val-OH
46	H-Asp-[Pen-Phe-DTrp-Orn-3tBuTyr-Cys]-Val-OH
47	H-Asp-[Cys-Phe-Trp-Lys-3tBuTyr-Cys]-Val-OH
48	H-Asp-[Pen-Phe-DTrp-Orn-Dopa-Cys]-Val-OH
49	H-Asp-[Cys-Phe-Trp-Lys-Dopa-Cys]-Val-OH
50	H-Asp-[Pen-Phe-DTrp-Orn-3NO2Tyr-Cys]-Val-OH
51	H-Asp-[Cys-Phe-Trp-Lys-3NO2Tyr-Cys]-Val-OH
52	H-Asp-[Pen-Phe-DTrp-Orn-3OCH3Phe-Cys]-Val-OH
53	H-Asp-[Cys-Phe-Trp-Lys-3OCH3Phe-Cys]-Val-OH

5.7 Biological results of U-II (4-11) and Urantide containing Tyrosine analogs.

A preliminary scan of these peptides has been carried out examining their propensity to activate UT receptor; in fact, this was performed measuring the ability to induce intracellular calcium release. These compounds have been tested on cells HEK293 stably transfected with UT receptor and the measurement of intracellular calcium, it was carried out through the use of calcium-sensitive indicator Fura-2. Since the studies of these compounds haven't completed, and are at the preliminary stadium, I will report the biological activity of compounds (**25** to **33**, **38** and **39**, **50** and **51**). The modifications carried out on U-II (4-11), agonistic sequence, and Urantide have influenced differently the activity biologic of these peptides. In particular, the introduction of electron-withdrawing groups on the aromatic ring of Tyrosine as $-NO_2$ (**50** and **51**) and iodine (in a different position) (**31** to **33**, **25** to **27**), cause a reduction of agonist activity in each peptidic analogue. In fact, in the case of U-II (4-11) analogues, the introduction of iodine in orto and meta in the aromatic ring cause a reduction of agonistic activity (Table 6). The same behavior is observed in (**51**), the introduction of nitro group in meta position, cause the reduction of agonistic activity. The negative influence on the biological activity of these modifications has been shown also in Urantide analogues containing same modifications (**25** to **27**, **50** and **51**), in fact, in these analogues the antagonistic activity was conserved, particularly in the compound (**50**) this has been improved respect Urantide (Table 7). These results indicate that the introduction of electron-withdrawing groups on the aromatic ring have a negative effect on biological activity of the agonist, and it allows to conserve (**25**, **26, 50**) and improve the antagonist activity (**27**) in Urantide analogues. This different behavior could be due a different steric hindrance caused about iodine and nitro group on aromatic ring of Tyrosine. An interesting result has been obtained with the compounds (**28**) and (**29**), in order to improve the ability to create hydrogen bond from Tyrosine with the receptor pocket, this has been replaced of Carboxy-Butoxy-Phenylalanine and Amino-Ethoxy-Phenylalanine. These modifications have reduced, on the agonistic sequence, the ability to release intracellular calcium, having an influence negative on the

biological activity respect UII (4-11). Finally, to obverse the importance of phenolic OH in agonist and antagonist sequence, the OH replaced with a methyl group (**24** and **30**). These modifications have caused a reduction of agonistic activity in U-II (4-11). Further studied are required in order to have a complete overview about the influence of these modified Tyrosine on Urantide sequence. (Figure 14)

Table 7. Tyrosine Scan on antagonist sequence

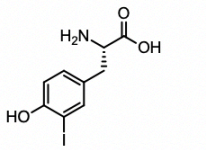
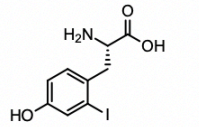
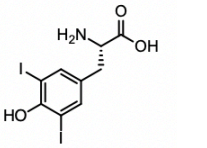
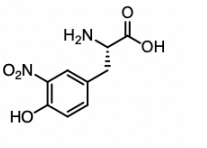
Name	Structure	Log IC_{50}	Emax
	H_2N-Asp-[Pen-Phe-DTrp-Orn-X-Cys]-Val-OH		
Urantide	H-Asp[4]-[Pen[5]-Phe[6]-DTrp[7]-Orn[8]-Tyr[9]-Cys[10]]-Val[11]-OH	9.16±0.14 nM	
25		9.38±0.17 nM	
26		9.38±0.27 nM	
27		9.53±0.39 nM	
50		9.06±0.13 nM	

Table 6. Tyrosine Scan on antagonist sequence

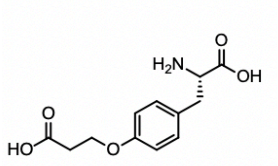
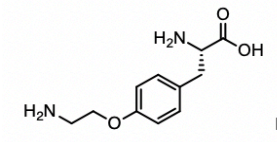
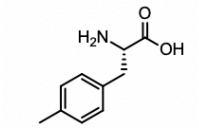
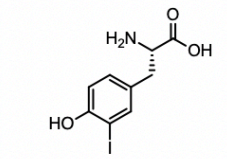
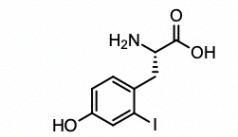
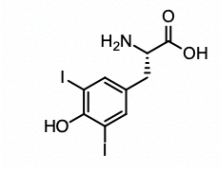
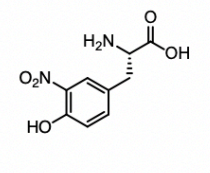
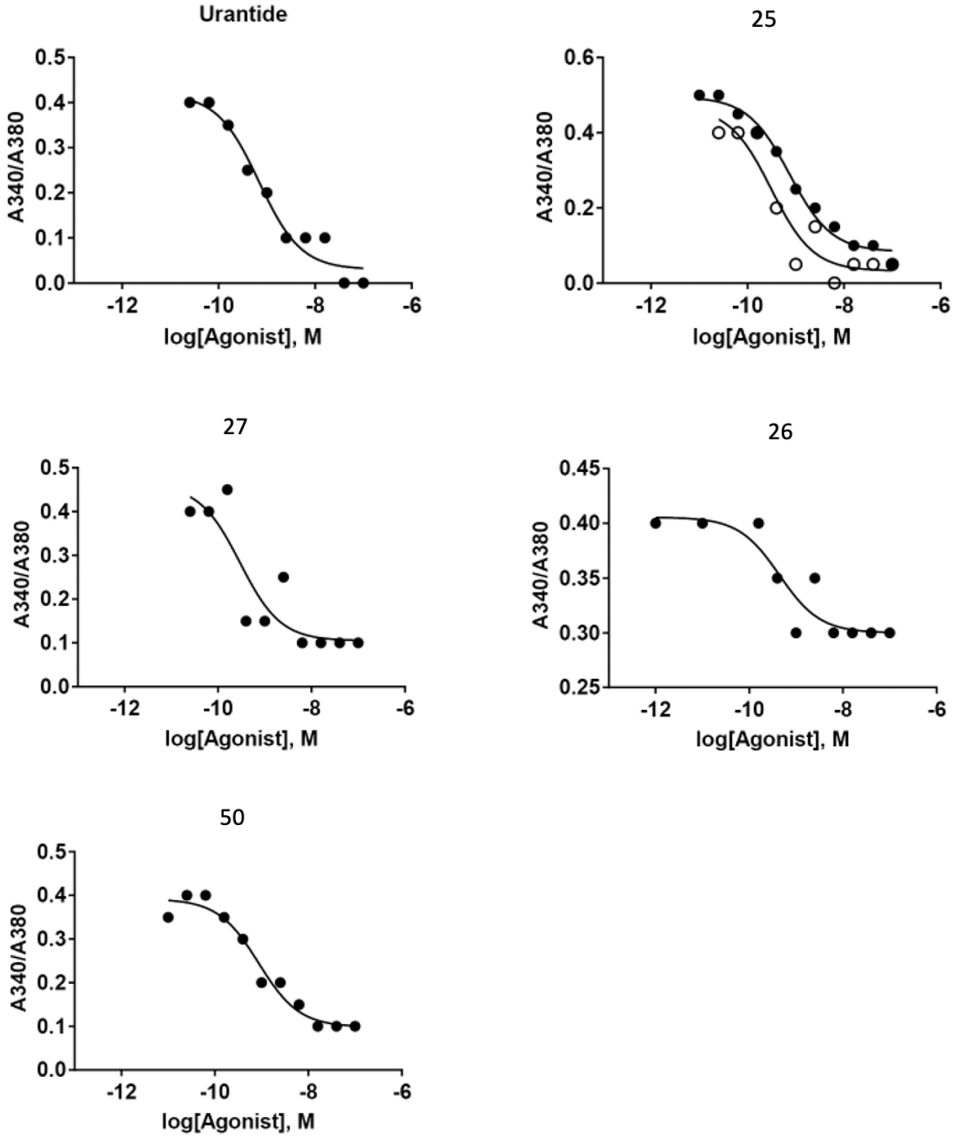
Name	Structure	Log IC50	Emax
U-II (4-11)	H_2N-Asp-Cys-Phe-Trp-Lys-X-Cys-Val-OH	0,8±0.1 nM	
28		6.90±0.3 nM	30%
29		6.90±0.4 nM	30%
30		8.15±0.4 nM	70%
31		8.30±0.3 nM	70%
32		7±0.5 nM	70%
33		7 ± 0,4 nM	70%
51		8.45± 0.3 nM	80 %

Figure 14. Curves concentrations-response compounds **25, 26, 27, 50**



5.8 Incorporation of Lys constrained analogues in Position 8 of Urantide

The Lysine in position 8 is an additional critical element responsible for interaction with UT receptor. To date, several studies have been reported in literature where has been demonstrated that the modifications of this residue can dramatically change the biological activity of U-II.

In fact, the simple replacement of Lys^{8} with alanine residue, produce a compound completely inactive. As it is well known, the importance of Lysine in this position is due to the interaction between the basic residue of Lysine with Asp130 in pocket P2, performing an important role in

recognition and activation of UT receptor[128]. The most important modification performed in this position it represented by the reduction of length of the side chain of lysine of one methyl group [Orn]; in fact, this modification is able reduce the potency and the intrinsic activity of the U-II, shifting the activity of this peptide from agonist to antagonist. Also, conformational NMR studies on urantide have demonstrated that the side chain of DTrp is orientated close to Orn residue[129]. For this reason, in order to stabilize this conformation, trough the formation of π-π interactions, it has been decided to replace Lys\Orn with analogues of Lysine contain an aromatic moiety (Table 8). These peptides have been synthetized using CEM Liberty Blue Synthesizer (Section 6.5).

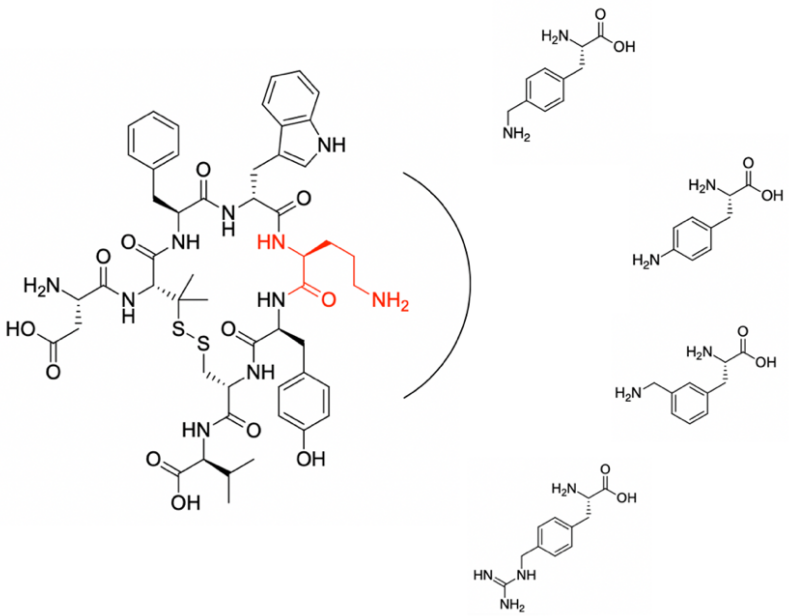


Table 8. Urantide analogues containing modified lysine

Name	Structure
Urantide	**H-Asp^4-[Pen^5-Phe^6-DTrp^7-Orn^8-Tyr^9-Cys^{10}]-Val^{11}-OH**
54	H-Asp-c[Pen-Phe-DTrp-**PAF**-Tyr-Cys]-Val-OH
55	H-Asp-c[Pen-Phe-DTrp-**GuF**-Tyr-Cys]-Val-OH
56	H-Asp-c[Pen-Phe-DTrp-**4AminoPhe**-Tyr-Cys]-Val-OH
57	H-Asp-c[Pen-Phe-DTrp-**MAF**-Tyr-Cys]-Val-OH

5.9 Biological activity of Urantide analogues containing constrained Lysine

The biological activity of analogues **54** to **57** reported in table 6, has been evaluated in base on their ability to induce intracellular calcium release. The cells utilized in this bioassay are Herk293 stably transfected with human UT receptor and the measurement of intracellular calcium was performed using calcium-sensitive indicator Fura-2. The preliminary data of these compounds shown that the introduction of a basic aromatic moiety, in core-sequence of antagonist sequence, increases the agonist activity compared to Urantide. Consequently, these preliminary results obtained on a short library of compounds seem to indicate that the constriction imparted by the aromatic moiety is not favorable for the agonist activity but also neither appropriate for the antagonist activity (Table 9).

Table 9. Biological data Lys scan on antagonist sequence

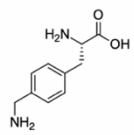
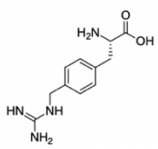
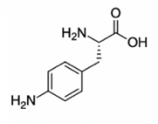
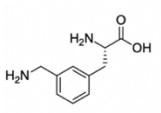
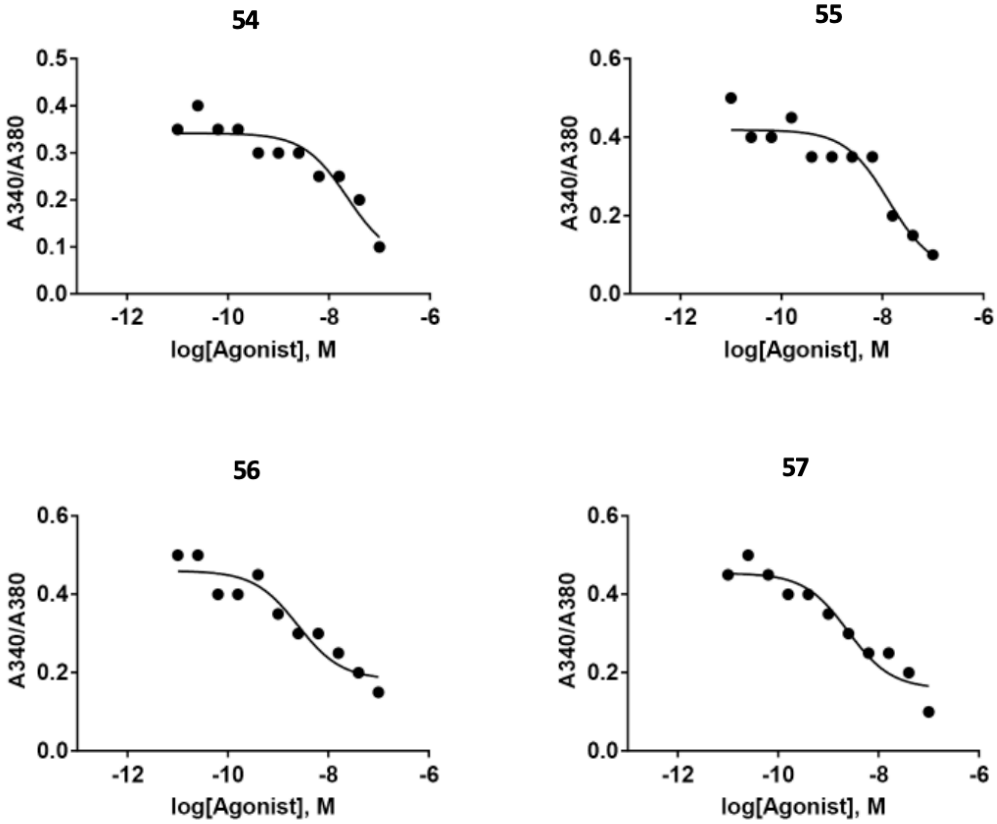
Name	Structure	Log IC50	Emax
	H-Asp[4]-[Pen[5]-Phe[6]-DTrp[7]-X[8]-Tyr[9]-Cys[10]]-Val[11]-OH		
Urantide	H-Asp[4]-[Pen[5]-Phe[6]-DTrp[7]-Orn-Tyr[9]-Cys[10]]-Val[11]-OH	9.16±0.14 nM	
54		7.64±0.30 nM	40%
55		7.87±0.26 nM	40%
56		8.61±0.24 nM	40%
57		8.61±0.21 nM	40%

Figure 15. Curves concentrations-response compounds **54, 55, 56, 57**



6 Synthetic strategies

6.1 General method for room temperature peptide synthesis

6.2 N-methylation on solid phase

6.3 General method for peptide synthesis with Ultrasonication boost

6.4 Synthesis of N-alkyl-glycine analogues

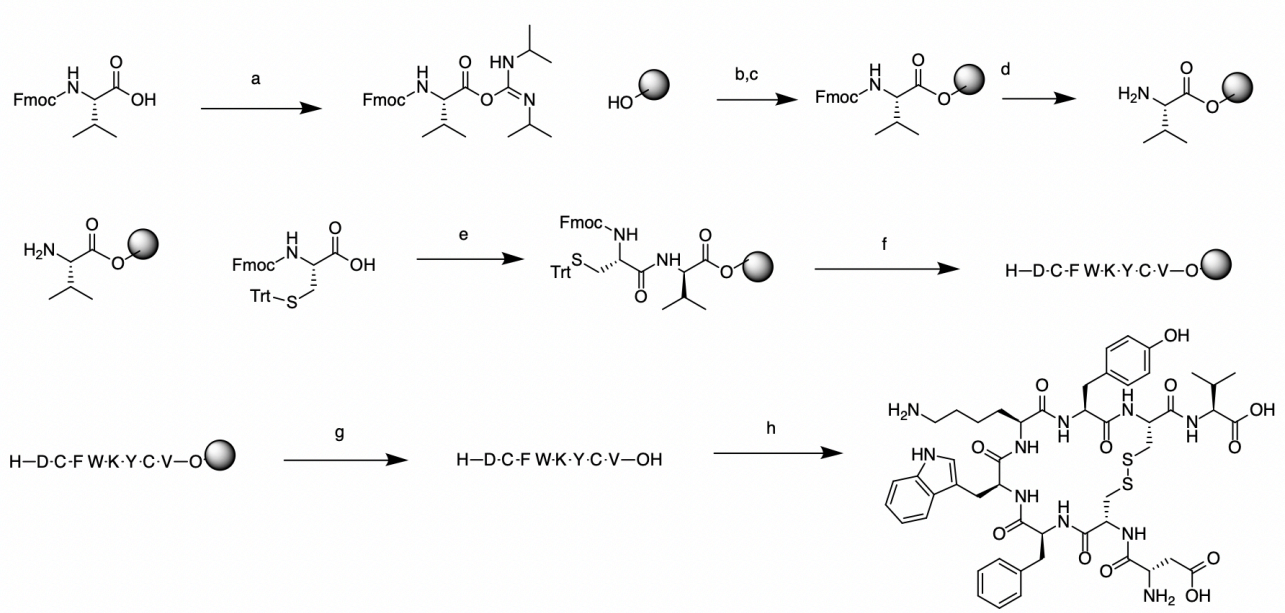
6.5 Peptide synthesis on microwaves

6.6 Amino acids derivatizations

6.1 General method for room temperature peptide synthesis.

The standard SPPS at room temperature method was performed in a stepwise fashion on solid phase using a Fmoc/tBu strategy and Wang linker resin as solid support. The coupling of first amino acids was performed using the pathway of asymmetric anhydride (5 eq.) Fmoc-Amino acid they have been reacted (2.5 eq.) of DIC (N, N'-Diisopropylcarbodiimide) for 30 min at 0°C. Then the mixture was dried in vacuo end added at Wang-OH resin and a catalytic amount of DMAP (4-Dimethylaminopyridine) to facilitate ester formation for 1 h. After the reaction went to completion, the resin was washed with DMF (3x) and DCM (3x) and was capped with acetic anhydride (1.2 eq.) and DIEA (2.4 eq.) in DMF for 30 min at rt to avoid the potential parallel synthesis of side products. The resin was washed with DMF (3x) and DCM (3x). The Nα-Fmoc protecting group was removed from the Val residue by the treatment with piperidine (20% in DMF; 1 x 5 min and 1 x 25 min). The resin was washed with DMF (3x). A positive Kaiser ninhydrin test was observed. The following protected amino acids (3 eq.) were added using as coupling reagent HBTU (3 eq.), HOBt (3 eq.), DIEA (6 eq.) for 2h. After the coupling was judged completed with Kaiser test the peptide resin was washed with DCM (3x) and DMF (3x). The Fmoc deprotection protocol described above was repeated and the next coupling step was initiated in a stepwise manner. The Kaiser test was used as colorimetric test to confirm every coupling/deprotection step occurred in the peptide sequence elongation. Analytical HPLC and MS spectrometry monitored the achievement of linear sequences for the compounds. When last Fmoc-protective of the linear sequence was removed as described above, the linear peptide was released from the solid support and all protective group was cleaved using a cocktail of TFA/TIS/H_2O (95:2.5:2.5, v/v/v) for 2h. The resin was removed by filtration. The crude peptide was recovered by precipitation from the filtrate using chilled ether and lyophilized to give a powder. The disulfide bonds were carried out solubilizing lyophilized peptide in a solution of H_2O/AcOH (50:50) in a concentration of 2 mg/ml and adding dropwise a solution of I_2 10% in MeOH until the reaction mixture turned to a persistent yellow coloration, for 15 minutes at room temperature

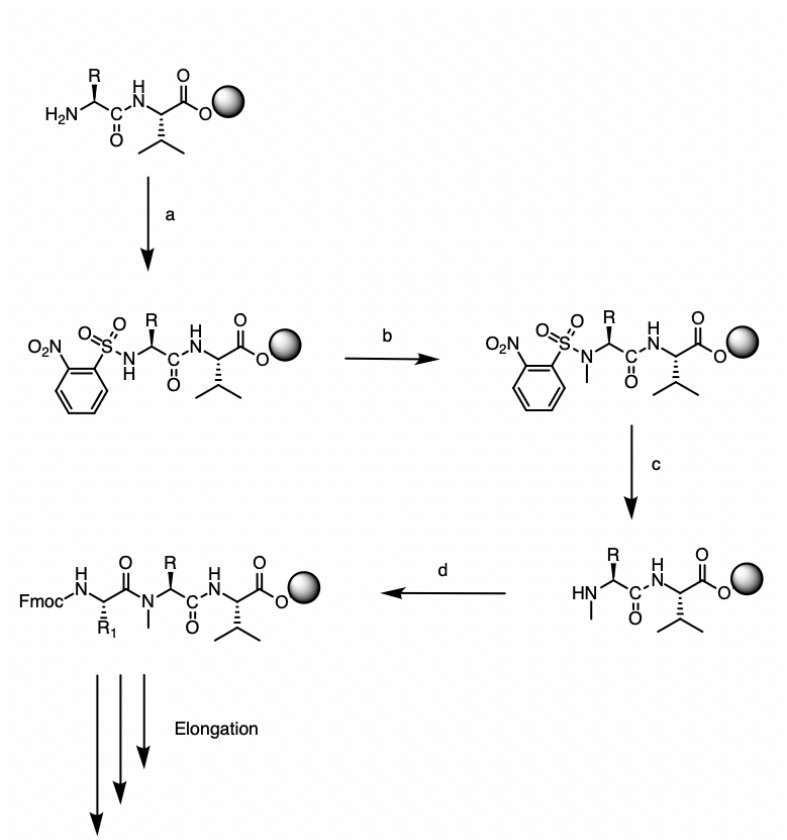
(Scheme 1). The reaction was monitored through HPLC, judged completed the iodine excess was quenched using a solution 1 M of Ascorbic acid in H_2O until the reaction mixture becomes colourless and lyophilized to have powder. The crudes peptides were purified by reverse-phase (RP) high-pressure liquid chromatography (HPLC) equipped with a Phenomenex Kinetex C18 column (150 mm × 4.6 mm, 5 µm, 100 Å), using as mobile phase a gradient of H_2O and MeCN containing 0.1% TFA (from 10 to 90% over 30 min) The product was obtained by lyophilization of the appropriate fractions after removal of the MeCN by rotary evaporation under reduced pressure. Analytical RP-HPLC indicated >98% purity and the correct molecular ions were confirmed by LC/ESI- MS.



Scheme. 1 a) Fmoc-Val-OH(5eq.); Dic (3eq.); b) DMAP (cat.) c) acetic anhydride (1.2 eq.), DIEA (2.4 eq.); d) 20% piperidine in DMF; 1 x 5 min and 1 x 25 e) HBTU (3 eq.), HOBt (3 eq.), DIEA (6 eq.) for 2 h; f) standard spps; g) TFA/TIS/H_2O (95:2.5:2.5, v/v/v) for 2 h; h) 10% in MeOH.

6.2 N-methylation on solid phase

N-methylated amino acids are commercially available, but expensive. N-Methyl amino acid building blocks have also been synthesized in solution and by solid-phase methods. The solid-phase protocol was based on reported methods that proved compatible with most amino[113][114][115] acids, and consisted of three fundamental steps: a) amine protection with the *o*-nitrobenzenesulfonyl (*o*-NBS) group; b) sulfonamide methylation; and c) selective removal of the *o*-NBS group. After Fmoc removal, the peptide amine terminal was reacted with *o*-NBS chloride and collidine in *N*-methyl-2-pyrrolidone (NMP). The methylation step was carried out through mitsunobu reaction, the sunfonamide was treated with (MeOH), triphenylphosphine (PPh_3) diisopropyl azodicarboxylate (DIAD) in THF for 10 min. Subsequently, resin treatment with mercaptoethanol and DBU removed the O-NBS group by way of a Meisenheimer intermediate. This reaction is reported to be selective for N-methylated derivatives and does not occur when the protected amine is not alkylated (Scheme 2). Couplings on Nα-methylamino acids are known to be more challenging due to steric hindrance. For this reason, these couplings were performed with HATU and HOAt instead of HBTU and HOBt using DIEA as base in NMP, which yielded complete couplings after reaction overnight at rt. The evolution of the procedure was monitored by the observation of cholorimetric tests such as Kaiser test for primary amine and p-chloranilin test for secondary amine, and by HPLC and ESI-MS systems. This method gave an efficient solution to synthesize libraries of N-methyl URP.



Scheme 2. General N-methylation strategy on solid phase during construction of peptides. (**a**) *o*-NBS-Cl (4 eq.), 2,4,6-Collidine (10 eq.), NMP, 15 min; (**b**)MeOH (10 eq.), DIAD(5 eq.),PPH_3(5 eq.), THF, 15 min;(**c**) 2-mercaptoethanol (10 eq.), DBU (5 equiv), NMP, 2 x 5 min (**d**) Fmoc-AA-OH (4 equiv), HATU (4 equiv), HOAt (4 equiv), DIEA (8 equiv), NMP, overnight.

6.3 General method for peptide synthesis with Ultrasonication boost.

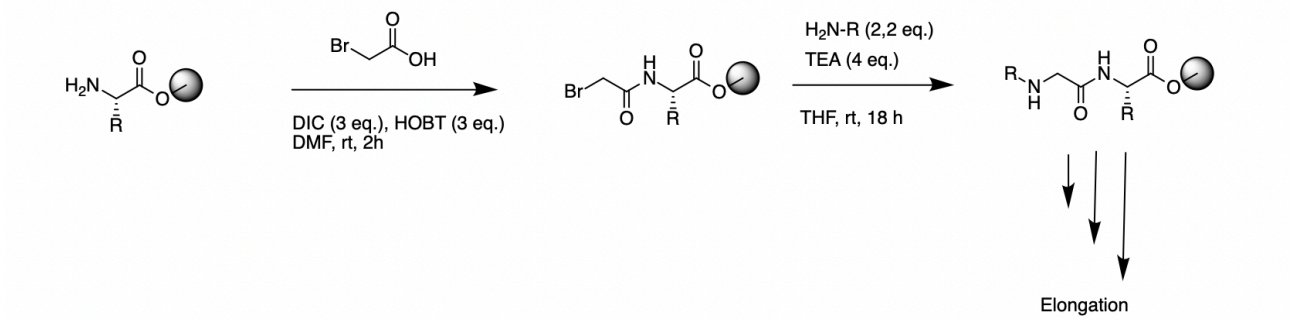
The use of sonication in peptide synthesis in solid phase is an innovative methodology that allows improves synthesis quality and reduces considerably reaction time[130]. In fact, the sonication through the phenomena of cavitation and the propagation of ultrasounds, allow increasing the kinetic energy into the reaction mixture, improving exponentially the molecular clash reducing consequently reaction time. Another vantage of this methodology is the reduction of equivalents of amino acids and activators necessary in the coupling reaction regard the standard SPPS, maintaining a high quality of synthesis. These peptides were synthesized using Wang resin that has been loaded using the same method descript before. The Nα-Fmoc protecting group was removed from the Val residue by the treatment with piperidine (20% in DMF; 0,1 x 5 min and 1 x 1min). The resin was washed with DMF

(3x). A positive Kaiser ninhydrin test was observed. The following protected amino acids were added using as coupling reagent HBTU (2 eq.), HOBt (2 eq.), DIEA (4 eq.) for 5 min. After the coupling reaction was judged completed the peptide resin was washed with DCM (3x) and DMF (3x). The Fmoc deprotection protocol described above was repeated and the next coupling step was initiated in a stepwise manner. the linear peptide was released from the solid support and all protective group was cleaved using a cocktail of TFA/TIS/H_2O (95:2.5:2.5, v/v/v) for 2h**.** The resin was removed by filtration. The crude peptide was recovered by precipitation from the filtrate using chilled ether and lyophilized to give a powder. The disulfide bond was performed using the same method described before. These crude peptides have been purified using reverse-phase (RP) high-pressure liquid chromatography (HPLC) equipped with a Phenomenex Kinetex C18 column (150 mm × 4.6 mm, 5 μm, 100 Å), using as mobile phase a gradient of H_2O and MeCN containing 0.1% TFA (from 10 to 90% over 30 min). The product was obtained by lyophilization of the appropriate fractions after removal of the MeCN by rotary evaporation under reduced pressure. Analytical RP-HPLC indicated >98% purity and the correct molecular ions were confirmed by LC/ESI- MS.

6.4 Synthesis of N-alkyl-glycine analogues

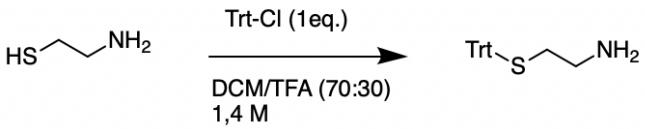
Peptoids provide major advantages as research and pharmaceutical tools include the economy of synthesis, highly variable backbone, and side-chain chemistry possibilities. The single introduction of peptoid in linear peptide sequence was performed using the methodology of Zauckermann that was as a major breakthrough because it greatly increased the synthetic efficiency, synthesis yields, and available side-chain diversity, while also dramatically reducing time and costs[(123)(124)] (Scheme 3). In the synthesis of N-alkyl-glycine derivate on Nα-Fmoc un-protected amino acid, was first acylated by a pre-activated bromoacetic acid, with N, N-diisopropyl carbodiimide (DIC) and HOBt for 2h. the reaction was judged completed through the Kaiser test.

Scheme 3. synthesis of N-alkyl-glycine analogs



Then Bromo was displaced by a primary amine, by SN2 reaction, in the presence of triethylamine (TEA) for 18hours at room temperature. The reaction was checked using the chloranil test that allows individuating the presence of secondary amine and through analytical HPLC. When the reaction is judged completed the synthesis of linear peptide continuing normally as before described. The removal of linear peptide from resin support, the cyclization reaction, and purification they have been done as descript before. Different amine used to mimic the amino acid side chain, in the synthesis of peptoid analogs, has been manually protected before to react with the Bromo acetic acid. The synthesis of side chain of (**7**) and (**12**) represented by Cys^2 and Cys^7 in original sequence of URP were synthesized, treating cysteamine with trityl chloride in a solution DCM/TFA (70:30) for 3h. (Scheme 4)

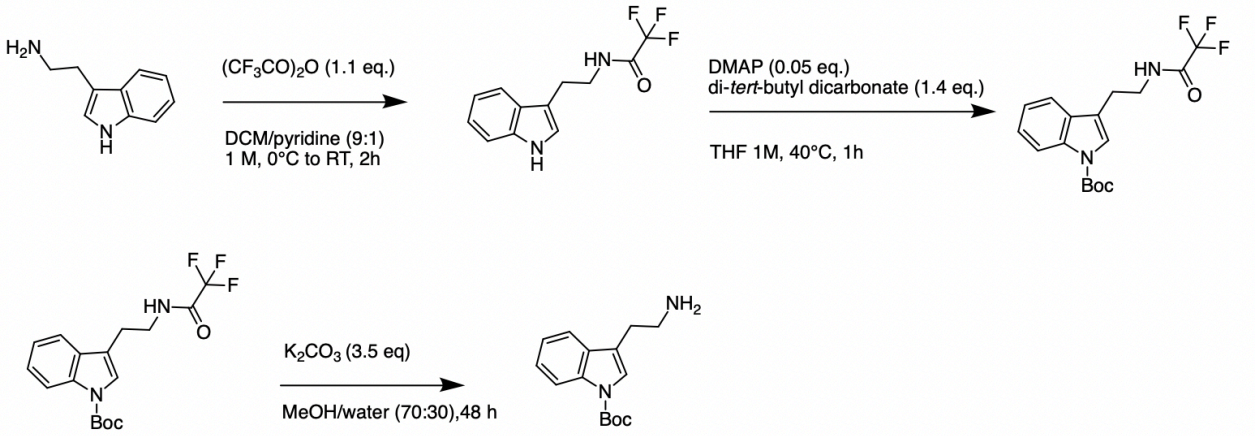
Scheme 4. Cysteamine protection



The amine of (**9**) that represent the Trp^4, was synthesized, dissolving Tryptamine in a cooled solution of DCM/Pyridine (90:10), then trifluoroacetic anhydride was added dropwise and the mixture was stirred for 5 min at 0 °C. The ice bath was removed, and the mixture was stirred for another 2h a RT. The reaction was judged completed through TLC. Then the reaction mixture was washed with

saturated $NaHCO_3$ (3x) the phases were separated, and the organic layer was washed with saturated aqueous NH_4Cl (3x), water(3x), brine solution (1x) and dried over Na_2SO_4. The solvent was removed under reduced pressure and the crude product was purified by chromatography (silica gel 60Å, 70-230 mash, 0% to 50% EtOAc in n-hexane) to give a yield of 90% of (**1**) as white solid. Then, in order to protect indoles amine, the product (**1**) has been reacted with DMAP (4-N, N-dimethyl aminopyridine), di-tert-butyl decarbonate, in THF for 1h at 40°C under magnetic stirring. When the reaction was completed, it was added DCM (30 ml) and washed with H_2O (3x), brine solution (1x). The phases were separated, and the organic layer dried over Na_2SO_4 and purified by flash chromatography (silica gel 60Å, 70-230 mash, 0% to 50% EtOAc in n-hexane) to give a yield of 70% of (**2**) as with solid. In order to remove the trifluoracetic protective group of γNH_2, the compound (**2**) was treated with K_2CO_3 in a solution of MeOH/water (70:30) under magnetic stirring for 48h. The reaction mixture was diluted with 100 mL water and extracted three times with 100 mL DCM. The combined organic layers were dried over Na_2SO_4. The solvent was removed under reduced pressure to give yield of 80% of a slightly yellow oil. (scheme 5)

Scheme 5 Tryptamine protection



6.5 Peptide synthesis on microwaves

The use of microwave technology for solid-phase peptide synthesis is one of the most significant breakthroughs of the past decade in the field of peptide chemistry. In fact, allowed the synthesis of complex peptide and to reduce considerably time of peptide synthesis. These types of vantages are due to the conductive heating caused by microwave irradiation. In fact, the energy of the microwave is transferred to the reaction mixture via convective currents or thermal conductivity, heating reaction mixture due to interactions of the material with the alternating electric field. Also, the common solvents used in peptide synthesis as DMF (N,N-Dimethylformamide) and NMP (N-methyl-2-pyrrolidione), have a very good ability to be heated by microwaves. Several reports speculate that microwaves to a significant extent may interact directly with amide dipoles of the dipole moments in peptides and that this effect causes direct heating of peptides. Therefore, the great amount of energy transferred by microwaves increases the kinetic energy into the reaction mixture increased exponentially the random clash between molecules, reducing reaction time. Also, the microwaves in peptide synthesis allowed the synthesis of complex peptide sequence obtaining high synthetic quality. This, it is due to the ability of microwave to the breaking of molecular interactions that there may create during the peptide grown, increasing the synthetic quality of complex peptides. Short time reactions and high synthetic quality have allowed the creation of automated peptidic synthesizer using microwaves.

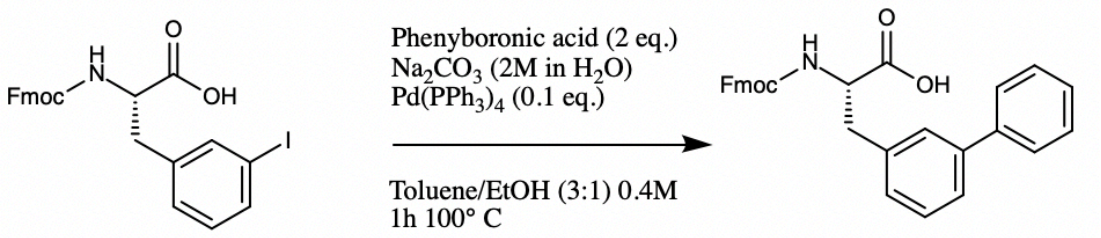
In the synthesis of analogs of U-II (4-11), Urantide containing modified Lysine and Tyrosine it was used the microwaves synthesizer CEM Liberty Blu. The scale of synthesis used for these peptides has been 0.2 mmol each peptide. The resin used in the synthesis of these peptides was Wang, that it was been loaded using method descript before. The Nα-Fmoc protecting group was removed from the Val residue using 10 ml of piperidine (20% in DMF) into vessel containing the resin-linked peptide and heat with MW irradiation (75°C at 220W for 15 s, then 90°C at 45W for 50 s). Then the reactor was drained, and the resin was washed 4 time using 7 ml of DMF. The following protected

amino acid (5 eq. 5ml of solution 0.2M in DMF) was added using as activator DIC (5 eq. 2 ml of a solution 1 M in DMF) and oxyma (5 eq. 2 ml of a solution 1M in DMF) and 2 mL of DMF to the resin-linked peptide in the reaction vessel. The reaction mixture was heated using microwaves irradiation (75°C at 235W for 15 s, then 90°C at 50W for 110 s). Then the vessels were dried, and resin washed 2 times with DMF. The Fmoc deprotection protocol described above was repeated and the next coupling step was initiated in a stepwise manner. After the last Fmoc-group was removed the peptide was released from resin support and cleaved all protective group using a cocktail of TFA/TIS/H_2O (95:2.5:2.5, v/v/v) for 2h under magnet stirring. The peptides were cyclized as described before. The crudes peptides were purified by reverse-phase (RP) high-pressure liquid chromatography (HPLC) equipped with Deltapack C18, (15µm, 100 Å, double cartridge 40x100mm). The mobile phase used in gradient has been H_2O[A] and MeCN [B] containing 0.1% TFA (from[%B] 10 to 40% over 20 min) at flow of 80 ml/min.

6.6 Amino acids derivatizations

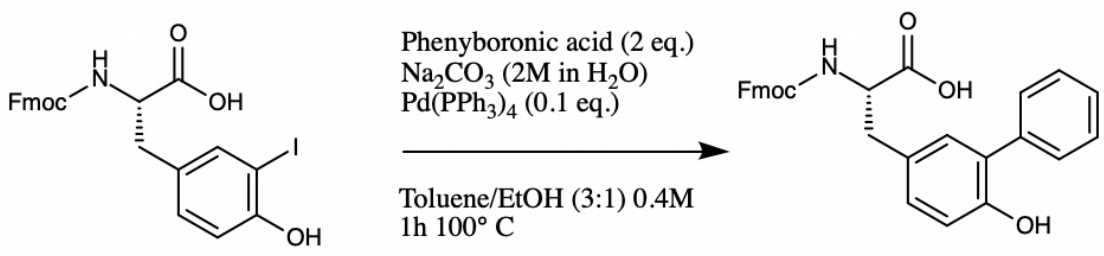
The tyrosine 9 of (**40**), (**41**) peptides was been synthesized using Suzuki reaction. The Fmoc-3-I-Phe-OH was dissolved in a solution of dry Toluene/EtOH (3:1), then was added phenylboronic acid, a solution Na_2CO_3 (2M in H_2O) and tetrakis (triphenylphosphine) palladium (0) under argon atmosphere. The reaction was stirred for 1 h at 100° C and checked through uPLC/MS. Then the reaction mixture was quenched with HCl 1N and extracted with AcOEt (x3). The combined organic extract was washed with brine solution and dried over with Na_2SO_4. The solvent was removed under reduced pressure and the reaction mixture was purified by *Biotage isolera* apparatus using column LUKNOVA 12g (10% to 60% EtOAc 0,5% AcOH in n-hexane 0,5% AcOH at flow rate 17ml/min) to give white powder with a yield of 77% (Scheme 6).

Scheme 6. Synthesis Fmoc-mBip-OH



The tyrosine 9 of (**42**) and (**43**) peptides was been synthesized using Suzuki reaction. The Fmoc-3-I-Tyr-OH was dissolved in a solution of dry Toluene/EtOH (3:1), then was added phenylboronic acid, a solution Na_2CO_3 (2M in H_2O) and tetrakis (triphenylphosphine) palladium (0) under argon atmosphere. The reaction was stirred for 1 h at 100° C and checked through uPLC/MS. Then the reaction mixture was quenched with HCl 1N and extracted with AcOEt (x3). The combined organic extract was washed with brine solution and dried over with Na_2SO_4. The solvent was removed under reduced pressure and the reaction mixture was purified by *Biotage isolera* apparatus using column LUKNOVA 12g (0% to 10% EtOAc 0.5% AcOH in DCM 0.5% AcOH at flow rate 17ml/min) to give white powder with a yield of 60%. (scheme 7)

Scheme 7. Synthesis Fmoc-3-phenyl-Tyr-OH



7 Conclusion

In summary, with the aim to get more insight about the structure-activity relationships on UII and URP, new analogues have been designed and synthesized. All compounds have been synthesized by conventional and innovative methodologies to modify peptide properties, incorporating non-natural amino acids or performing a N-methylation.

In this context, the peptide sequences of UII, U-II (4-11), URP and Urantide have been deeply explored the get more information regarding the conformational and structural elements responsible in interaction with the UT receptor. Regarding the N-methylation performed on the core sequence of URP, this modification has demonstrated to be deleterious for the biological activity with a general reduction of affinity with the UT receptor, except for the compound (**1**) which conserves full agonist activity. This result is the evidence that the hydrogens amidic of specific peptide bonds in the core sequence of URP are important for the interaction with the receptorial counterpart. This is coherent and in line with the results previously found for the N-methylation performed on UII(4-11) sequence.

The investigation performed on the first amino acid of the N-terminal domain of Urotensin-II has been highlighted the role of Glu^1 in interaction with an allosteric binding site of UT receptor. In fact, the replacement of this residue with different amino acids, shift the activity from agonist to antagonist. In particular, the replacement of Glu^1 with Ala (**15**), is able to improve the agonistic activity also in the peptide containing the core sequence typically of the well know antagonist Urantide. Instead, the replacement of Glu^1 with Dab (**17**) and hSer (**19**), produced ligands with an improved antagonist activity respect to the Urantide. As a matter of fact, the ligands (**17**) and (**19**), showed a behavior of full antagonist at UT receptor already at 10 nM, concentration to the which Urantide still conserve a residual agonist activity. These results reevaluate the role of the N-terminal domain of Urotensin-II until now defined as marginal. For this purpose, further studies will be necessary to better define the structural elements which participle in activation of UT receptor.

In addition, the introduction of Tyrosine analogues in the core sequence of U-II (4-11) and Urantide, has revealed important structural elements necessary in order to improve the antagonist activity. In fact, the introduction of electron-withdrawing groups on the aromatic ring of Tyrosine cause the reduction of agonist activity in U-II (4-11) sequence. Similar behavior has been observed in antagonist sequence of Urantide, where introduction on aromatic ring electron-withdrawing groups causes a further reduction of the residual agonist activity typic of the Urantide, promoting this kind of modification as a potentially key element to improve antagonist activity.

Finally, the latest study performed on Orn^8 of the core sequence of Urantide, confirmed the importance of the conformational freedom of sidechain of this residue. The preliminary data obtained replacing the Orn^8 with some analogues endowed of an aromatic residue in sidechain showed that the compounds are endowed of improved agonist activity. Therefore, these results suggest that the aromatic moiety of these Orn analogues stabilizes the active conformation of peptides for the agonist activity, also in peptide sequences that containing structural elements responsible for antagonist activity.

All these results will be extensively analyzed and on more representative compounds will be performed conformational studies using NMR spectroscopic techniques. The final results will be used in the design of further analogues with improved physicochemical properties and biological activities.

8 Materials and Methods

8.1 Materials

8.2 Aortic ring contraction experiments.

8.3 Ligand binding.

8.4 Intracellular Ca^{2+} release assay

8.5 Statistical analysis.

8.1 Materials

The *N*-Fmoc-protected amino acids, HBTU and HOBt were purchased from Inbios (Naples, Italy) and bachem, and used as received. *N*,*N*-Diisopropylcarbodiimide (DIC) was purchased from Aldrich and used as received. Wang resin was purchased from Advanced ChemTech. Protected Penicillamine HATU, HOAT was purchased from Bachem (Basel, Switzerland). The microwave synthesized was pursued from CEM (Matthews, NC). Tetrakis(triphenylphosphine)palladium and anhydrous solvents [tetrahydrofuran (THF), *N*,*N*-dimethylformamide (DMF), acetonitrile, and dichloromethane (DCM)] were pursued was purchased from Aldrich. Ethyl acetate (EtOAc) and hexanes were purchased from Fisher Chemical®. The N,N-Diisopropylethylamine, O-NBS, DBU, bromo acetic acid, collidine and triphenylphosphine was pursued from fluorochem and used as received.

The amine Tyramine, cysteamine, N-Boc-cadaverine, tryptamine was pursued from Aldhric and 2 feniletilamina from VWR. Flash chromatography was on 230-400 mesh silica gel. Flash chromatography apparatus was pursued by Biotage (Uppsala, Svezia). The trifluoroacetic anhydride anhydrous, DMAP, and di-tert-butil-dicarbonate, was pursued from abcr GmbH. The Hplc analisys was performed using Shimaszu and the LC\Ms analysis was carried out with Waters technology.

8.2 Aortic ring contraction experiments.

The ability of the compounds (**1** -**6**) to induce contraction of Aortic ring was carried out using Adult male Sprague-Dawley rats (Charles-Rivers, San Diego, CA) weighing 250-300 g were housed in group cages under controlled illumination (12:12h light-dark cycle), humidity, and temperature (21-23 °C) and given free access to tap water and rat chow. All experimental procedures were performed by David Chatenet, PhD of *Institut National de la Recherche Scientifique-Institut Armand-Frappier*. The thoracic aorta was cleared of surrounding tissue and then excised from the aortic arch to the diaphragm. Conjunctive tissues were next removed from the thoracic aorta and the vessels were

divided into 4 mm rings. The endothelium of each aortic ring was removed by gently rubbing the vessel intimal surface. Aortic rings were then placed in a 5 mL organ bath filled with oxygenated normal Krebs-Henselheit buffer. 80 µL of a 2.5 M KCl solution (40 mM final concentration in bath) was used to evaluate the contractile responses of each vessel. In one bath, U-II (10^{-7} M) was applied as a control, and the tissue-response was expressed as the ratio with the KCl-induced contraction. Cumulative concentration-response curves to synthetic peptides were obtained by increasing the concentration of each peptide in the organ chamber (10^{-11} to 10^{-5} M). The amplitude of the contraction induced by each concentration of peptide was expressed as a percentage of the KCl-induced contraction divided by the tissue-response induced by U-II. The median effective concentrations (EC_{50}) are expressed as the mean ± S.E.M., and the *n* values, representing the total number of animals from which the vessels were isolated, varied from 3-19 animals.

8.3 Ligand binding.

Synthetic U-II or URP was radiolabeled with $Na^{125}I$ using the chloramine T techniques previously reported. Iodinated ^{125}I-URP or ^{125}I-*h*UII were purified on a C_{18} cartridge, collected and stored at −20°C until use. Competitive binding experiments were performed using a stable HEK 293-UT cell line, expressing the human UTR isoform. Cells plated in 96-well plate at a density of 10,000 cells/well were incubated on ice and for 2 h with increasing concentration of various peptides (10^{-11} to 10^{-5} M) in the presence of ^{125}I-URP or ^{125}I-*h*U-II (0.05 nM). Non-specific binding was established by exposing cells to 10^{-5} M cold U-II or URP. Cells were washed twice with cold binding buffer then lysed with a solution of NaOH (1 M). Cell- bound radioactivity was quantified using a g-counter. Results were expressed as a percentage of the specific binding of ^{125}I-URP or ^{125}I-*h*U-II obtained in the absence of competitive ligands.

8.4 Intracellular Ca^{2+} release assay

The propensity to activate the UT receptor has been established through the measurement of intracellular calcium release. These compounds have been tested on cell HEK293 stably transfected with UT receptor. The measurement of intracellular calcium was carried out through the use of calcium-sensitive indicator Fura-2. This indicator once inside the cell is able to bind intracellular Ca^{2+}. In particular, Fura-2 have a different behavior if it is bind with Ca^{2+} or not. Ca^{2+}-bound Fura has its excitation maximum at 335 nm, Ca^{2+}-free fura-2 AM has its excitation maximum at 363 nm. In both states, the emission maximum is about 510 nm. The typical excitation wavelengths used are 340 nm and 380 nm for Ca^{2+}-bound and Ca^{2+}-free fura-2 AM, respectively. Therefore, the ratios 510 nm/340 nm and 510 nm/380 nm are directly related to the amount of intracellular Ca^{2+} . Due to fast kinetic of activation of GPCR and ion channel, the measurement of intracellular Ca^{2+} release must be carried out simultaneously at the administration of compound we intent to test. For this purpose, the bioassay was carried out using Multilabel Plate Readers Envision 2105 equipped with an injector. This instrument is equipped with a modular optical mirror allowing the selection the wavelength necessary for the assay. Due to fast kinetics of GPCR, the acquisition of data can start some seconds before the sub ministration of the compound.

In the execution of test the HEK293 cells have been treated with Fura-2 dyes for 45 minutes at 37° C. Subsequentially these compounds, treated as an antagonist, has been added in each well and the cells incubated at 37°C for prior to the measurement. Then, once loaded the agonist, in this case, U-II (4-11), was dispensed on-line using the EnVisionTM dispense unit. The instrument was set up to excite at two wavelengths 340 – and 380 nm while the emission was recorded at 510 nm. The data from the dual excitation per well minus the minimum ratio (max-min) after agonist addition, indicated the amount of cytoplasmatic Ca^{2+}.

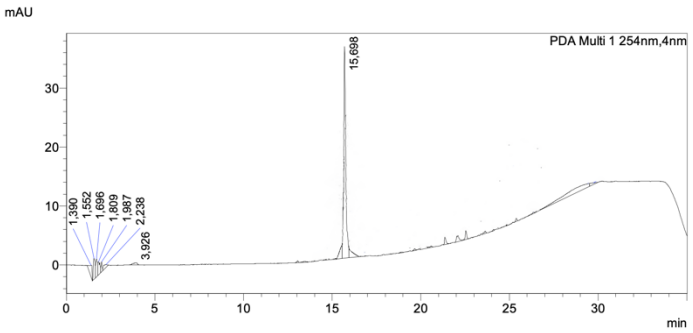
8.5 Statistical analysis.

Aortic contraction assays, binding experiments were performed at least in triplicate. Data, expressed as mean ± S.E.M., were analyzed with the Prism Software (Graphpad Software, San Diego, CA, USA). Sigmoidal dose-response fits with variable slope and one-site competition functions were used to determine EC_{50} and IC_{50}, respectively. K_i values were calculated from IC_{50} using the Cheng-Prusoff equation ($K_i = IC_{50} / (1 + [\text{radioligand}] / K_d)$, with [radioligand] = 0.05 nM and $K_d = 0.067 \pm 0.002$ nM for **1** or $K_d = 0.059 \pm 0.007$ nM for **2**. Statistical comparisons were analyzed by the Student's t-test, and differences were considered significant where $^*P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$.

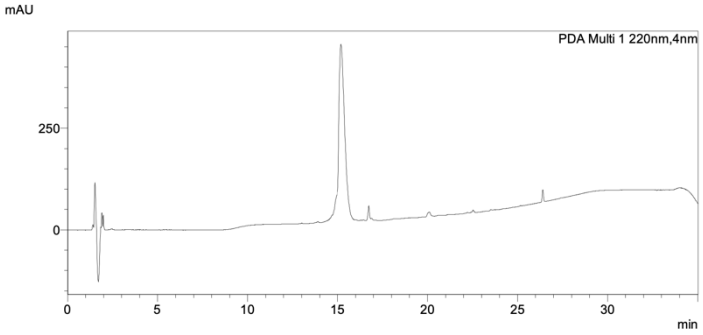
9 Characterization

Name	Structure
UII	H-Glu^{1}-Thr^{2}-Pro^{3}-Asp^{4}-[Cys^{5}-Phe^{6}-Trp^{7}-Lys^{8}-Tyr^{9}-Cys^{10}]-Val^{11}-OH
UII (4-11)	H_2N-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH
URP	Ala^{1}-[Cys^{2}-Phe^{3}-Trp^{4}-Lys^{5}-Tyr^{6}-Cys^{7}]-Val^{8}-OH
Urantide	H-Asp^{1}-[Pen^{2}-Phe^{3}-DTrp^{4}-Orn^{5}-Tyr^{6}-Cys^{7}]-Val^{8}-OH
1	H-Ala-[(*N*-Me) Cys-Phe-Trp-Lys-Tyr-Cys]-Val
2	H-Ala-[Cys-(*N*-Me) Phe-Trp-Lys-Tyr-Cys]-Val
3	H-Ala-[Cys-Phe-(*N*-Me) Trp-Lys-Tyr-Cys]-Val
4	H-Ala-[Cys-Phe-Trp-(*N*-Me)Lys-Tyr-Cys]-Val
5	H-Ala-[Cys-Phe-Trp-Lys-(*N*-Me)Tyr-Cys]-Val
6	H-Ala-[Cys-Phe-Trp-Lys-Tyr-(*N*-Me)Cys]-Val
7	H-Ala[N-(2-Sulfanylethyl)Gly -Phe-Trp-Lys-Tyr-Cys]-Val-OH
8	H-Ala-[Cys-N-(2-Phenylethyl)Gly -Trp-Lys-Tyr-Cys]-Val-OH
9	H-Ala-[Cys-Phe- N-(2-indolethy)lGly -Lys-Tyr-Cys]-Val-OH
10	H-Ala-[Cys-Phe-Trp- N-(5-aminopentyl)Gly -Tyr-Cys]Val-OH
11	H-Ala-[Cys-Phe-Trp-Lys-N(4-hydroxyphenylehtyl)Gly-Cys]-Val-OH
12	H-Ala-[Cys-Phe-Trp-Lys-Tyr- N-(2-Sulfanylethyl)Gly]Val-OH
13	H-Ala-Thr-Pro-Asp-[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH
14	H-Glu-Thr-Pro-Asp-[Pen-Phe-DTrp-Orn-Tyr-Cys]-Val-OH
15	H-Ala-Thr-Pro-Asp-[Pen-Phe-DTrp-Orn-Tyr-Cys]-Val-OH
16	H-Gln-Thr-Pro-Asp-[Pen-Phe-DTrp-Orn-Tyr-Cys]-Val-OH
17	H-Dab-Thr-Pro-Asp-[Pen-Phe-DTrp-Orn-Tyr-Cys]-Val-OH
18	H-Dab-Thr-Pro-Asp-[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH
19	H-hSer-Thr-Pro-Asp-[Pen-Phe-DTrp-Orn-Tyr-Cys]-Val-OH
20	H-Ala-Thr-Pro-Ala-[Pen-Phe-DTrp-Orn-Tyr-Cys]-Val-OH
23	H-hSer -Thr-Pro-Asp-[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH
22	H -Asp-[Pen-Phe-DTrp-Orn-Cbf-Cys]-Val-OH
23	H -Asp-[Pen-Phe-DTrp-Orn-Aef-Cys]-Val-OH
24	H -Asp-[Pen-Phe-DTrp-Orn-4MePhe-Cys]-Val-OH
25	H -Asp-[Pen-Phe-DTrp-Orn-3ITyr-Cys]-Val-OH
26	H -Asp-[Pen-Phe-DTrp-Orn-2ITyr-Cys]-Val-OH

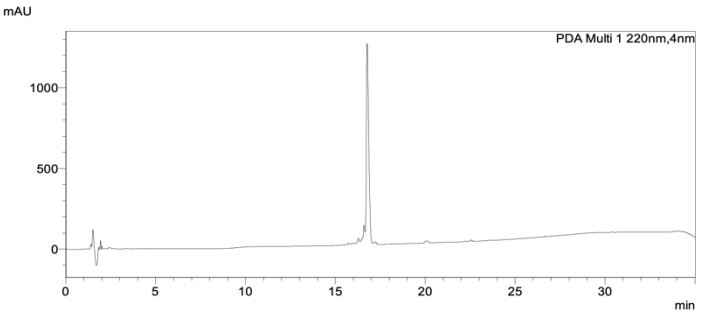
27	H -Asp-[Pen-Phe-DTrp-Orn-3,5ITyr-Cys]-Val-OH
28	H-Asp-[Cys-Phe-Trp-Lys- Cbf -Cys]-Val-OH
29	H-Asp-[Cys-Phe-Trp-Lys- Aef -Cys]-Val-OH
30	H-Asp-[Cys-Phe-Trp-Lys-4MePhe -Cys]-Val-OH
31	H-Asp-[Cys-Phe-Trp-Lys-3ITyr -Cys]-Val-OH
32	H-Asp-[Cys-Phe-Trp-Lys-2ITyr -Cys]-Val-OH
33	H_2N-Asp-[Cys-Phe-Trp-Lys-5ITyr-Cys]-Val-OH
34	H-Asp-[Pen-Phe-DTrp-Orn-OCHF2Phe-Cys]-Val-OH
35	H-Asp-[Cys-Phe-Trp-Lys-OCHF2Phe-Cys]-Val-OH
36	H-Asp-[Pen-Phe-DTrp-Orn-CF3Phe-Cys]-Val-OH
37	H-Asp-[Cys-Phe-Trp-Lys-CF3Phe-Cys]-Val-OH
38	H-Asp-[Pen-Phe-DTrp-Orn-3OHPhe-Cys]-Val-OH
39	H-Asp-[Cys-Phe-Trp-Lys-3OHPhe-Cys]-Val-OH
40	H-Asp-[Pen-Phe-DTrp-Orn-mBip-Cys]-Val-OH
41	H-Asp-[Cys-Phe-Trp-Lys-mBip-Cys]-Val-OH
42	H-Asp-[Pen-Phe-DTrp-Orn-3PhTyr-Cys]-Val-OH
43	H-Asp-[Cys-Phe-Trp-Lys-3PhTyr-Cys]-Val-OH
44	H-Asp-[Pen-Phe-DTrp-Orn-3MeTyr-Cys]-Val-OH
45	H-Asp-[Cys-Phe-Trp-Lys-3MeTyr-Cys]-Val-OH
46	H-Asp-[Pen-Phe-DTrp-Orn-3tBuTyr-Cys]-Val-OH
47	H-Asp-[Cys-Phe-Trp-Lys-3tBuTyr-Cys]-Val-OH
48	H-Asp-[Pen-Phe-DTrp-Orn-Dopa-Cys]-Val-OH
49	H-Asp-[Cys-Phe-Trp-Lys-Dopa-Cys]-Val-OH
50	H-Asp-[Pen-Phe-DTrp-Orn-3NO_2Tyr-Cys]-Val-OH
51	H-Asp-[Cys-Phe-Trp-Lys-3NO_2Tyr-Cys]-Val-OH
52	H-Asp-[Pen-Phe-DTrp-Orn-3MeOPhe-Cys]-Val-OH
53	H-Asp-[Cys-Phe-Trp-Lys-3MeOPhe-Cys]-Val-OH
54	H-Asp-[Pen-Phe-DTrp-PAF-Tyr-Cys]-Val-OH
55	H-Asp-[Pen-Phe-DTrp-GuF-Tyr-Cys]-Val-OH
56	H-Asp-[Pen-Phe-DTrp-4NH_2Phe-Tyr-Cys]-Val-OH
57	H-Asp-[Pen-Phe-DTrp-MAF-Tyr-Cys]-Val-OH



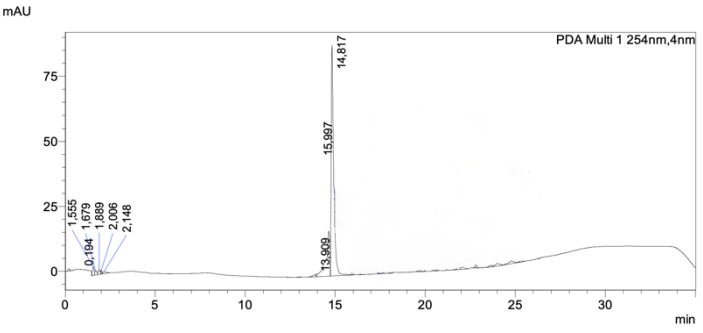
Peptide **1**: purity: 99%, t_R: 15.7min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 20 min, flow rate of 1 mL/min), molecular formula: $C_{50}H_{66}N_{10}O_{10}S_2$, calculated mass; 1031.2500, found: 1031.4493



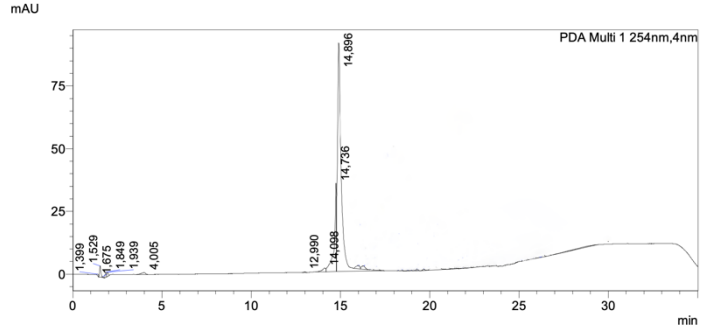
Peptide **2**: purity: 99%, t_R: 15.2 min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 20 min, flow rate of 1 mL/min), molecular formula: $C_{50}H_{66}N_{10}O_{10}S_2$, calculated mass; 1031.2500, found: 1031.4493



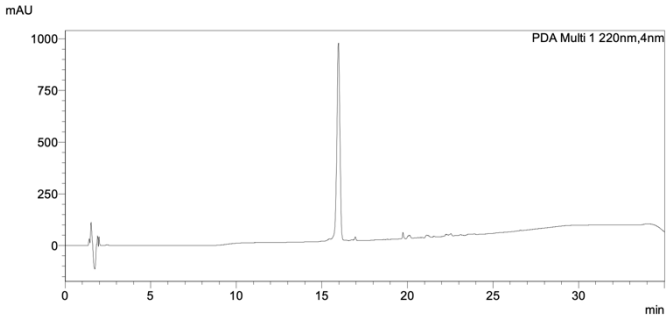
Peptide **3**: purity: 99%, t_R: 16.8 min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 20 min, flow rate of 1 mL/min), molecular formula: $C_{50}H_{66}N_{10}O_{10}S_2$, calculated mass; 1031.2500, found: 1031.4493



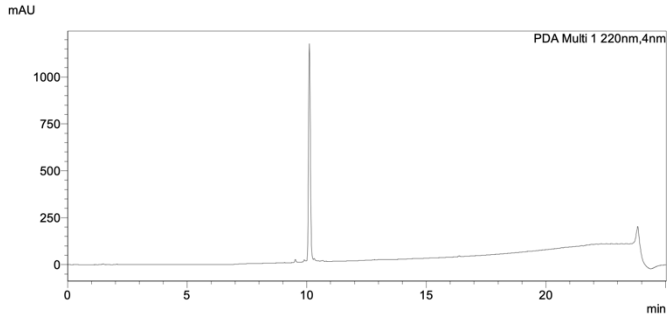
Peptide **4**: purity: 99%, t_R: 14.8 min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 20 min, flow rate of 1 mL/min), molecular formula: $C_{50}H_{66}N_{10}O_{10}S_2$, calculated mass; 1031.2500, found: 1031.4493



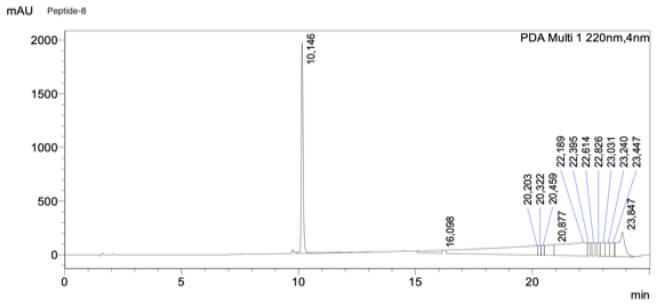
Peptide **5**: purity: 99%, t_R: 14.9 min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 20 min, flow rate of 1 mL/min), molecular formula: $C_{50}H_{66}N_{10}O_{10}S_2$, calculated mass; 1031.2500, found: 1031.4493



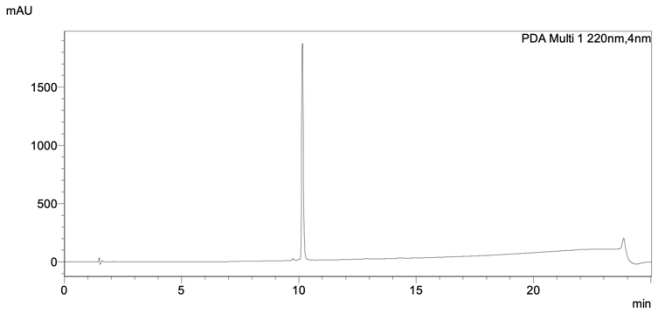
Peptide **6**: purity: 99%, t_R: 16 min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 20 min, flow rate of 1 mL/min), molecular formula: $C_{50}H_{66}N_{10}O_{10}S_2$, calculated mass; 1031.2500, found: 1031.4493



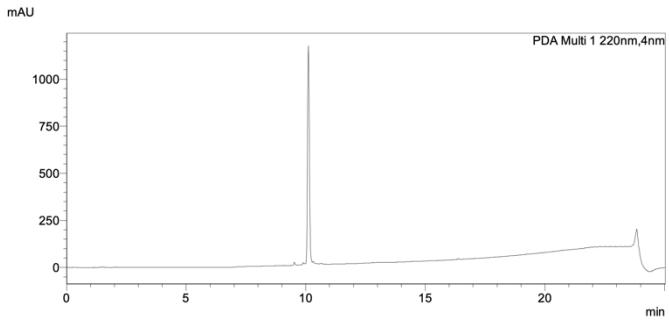
Peptide **7**: purity: 99%, t_R: 10.10 min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 25 min, flow rate of 1 mL/min), molecular formula: $C_{50}H_{66}N_{10}O_{10}S_2$, calculated mass; 1145.47, found: 1166.80



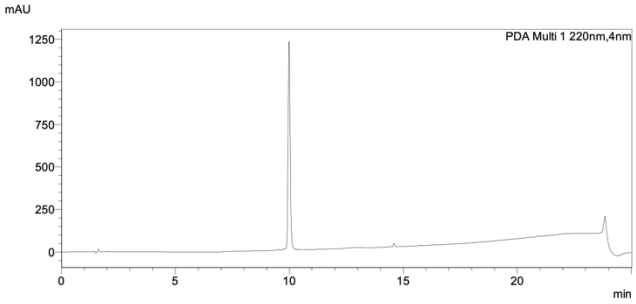
Peptide **8**: purity: 99%, t_R: 10.146 min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 25 min, flow rate of 1 mL/min), molecular formula: $C_{50}H_{66}N_{10}O_{10}S_2$, calculated mass; 1145.47, found: 1166.80



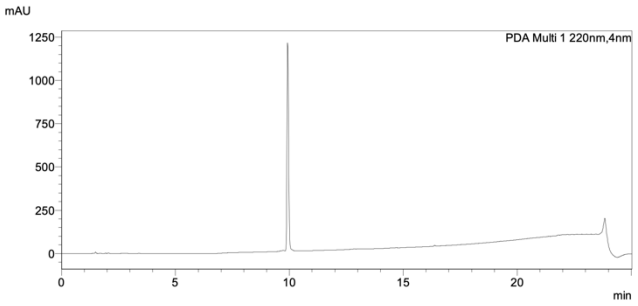
Peptide **9**: purity: 99%, t_R: 10.14 min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 25 min, flow rate of 1 mL/min), molecular formula: $C_{50}H_{66}N_{10}O_{10}S_2$, calculated mass; 1145.47, found: 1166.80



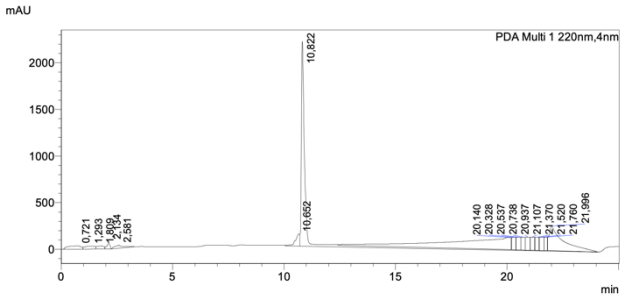
Peptide **10**: purity: 99%, t_R: 10.09 min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 25 min, flow rate of 1 mL/min), molecular formula: $C_{50}H_{66}N_{10}O_{10}S_2$, calculated mass; 1145.47, found: 1166.80



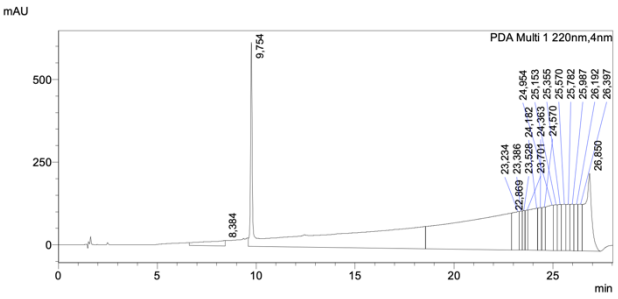
Peptide **11**: purity: 99%, t_R: 9,81 min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 25 min, flow rate of 1 mL/min), molecular formula: $C_{50}H_{66}N_{10}O_{10}S_2$, calculated mass; 1145.47, found: 1166.80



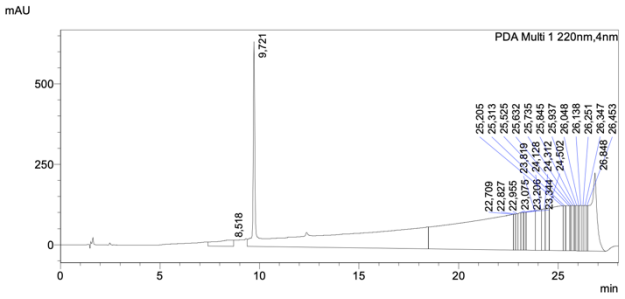
Peptide **12**: purity: 99%, t_R: 9,92 min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 25 min, flow rate of 1 mL/min), molecular formula: $C_{50}H_{66}N_{10}O_{10}S_2$, calculated mass; 1145.47, found: 1166.80



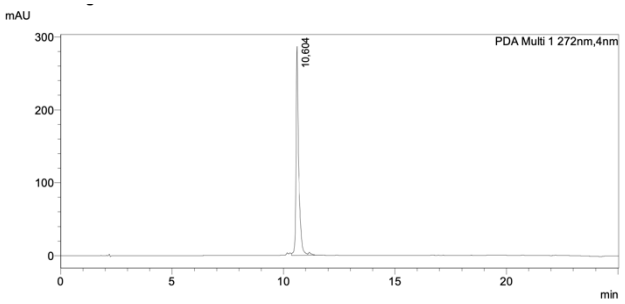
Peptide **13**: purity: 99%, t_R: 10.82 min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 25 min, flow rate of 1 mL/min), molecular formula: $C_{58}H_{81}N_{13}O_{16}S_2$, calculated mass; 1280.4, found: 1281.6



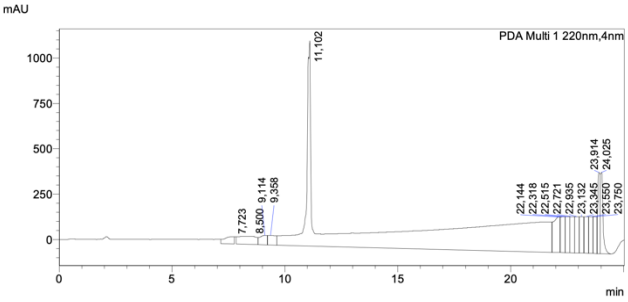
Peptide **14**: purity: 99%, t_R: 9,75min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 25 min, flow rate of 1 mL/min), molecular formula: $C_{58}H_{84}N_{12}O_{19}S_2$, calculated mass; 1317.5, found: 1318.4



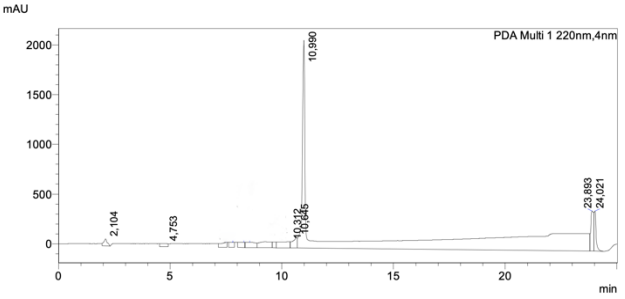
Peptide **15**: purity: 99%, t_R: 9.72min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 25 min, flow rate of 1 mL/min), molecular formula: $C_{56}H_{82}N_{12}O_{17}S_2$, calculated mass; 12585, found: 1259.6



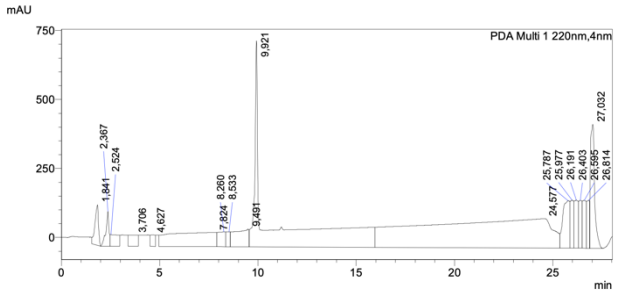
Peptide **16**: purity: 99%, t_R: 10,6min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 25 min, flow rate of 1 mL/min), molecular formula: $C_{63}H_{97}N_{15}O_{22}S_2$, calculated mass; 1288.5, found: 1289.8



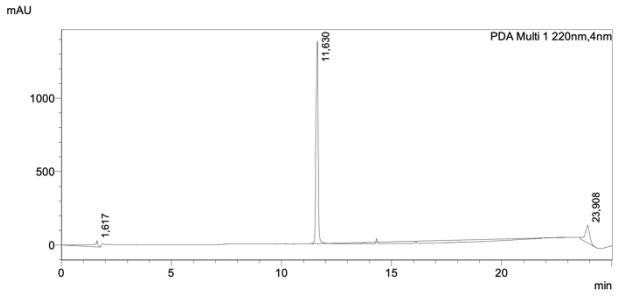
Peptide **17**: purity: 99%, t_R: 11.1min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 25 min, flow rate of 1 mL/min), molecular formula: $C_{57}H_{85}N_{13}O_{17}S_2$, calculated mass; 1288.5, found: 1289.8



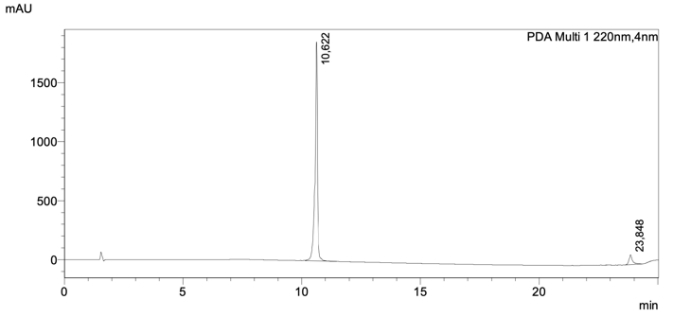
Peptide **18**: purity: 99%, t_R: 10.99min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 25 min, flow rate of 1 mL/min), molecular formula: $C_{56}H_{83}N_{13}O_{17}S_2$, calculated mass; 1254.7, found: 1255.8



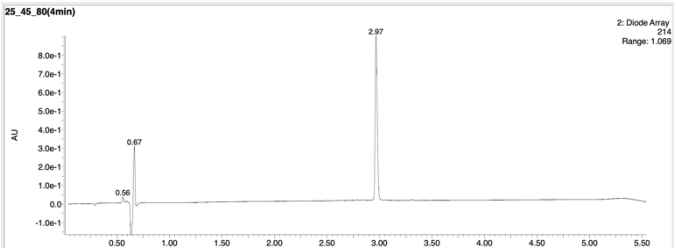
Peptide **19**: purity: 99%, t_R: 9,92min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 25 min, flow rate of 1 mL/min), molecular formula: $C_{62}H_{96}N_{14}O_{22}S_2$, calculated mass; 1289.5, found: 1290.7



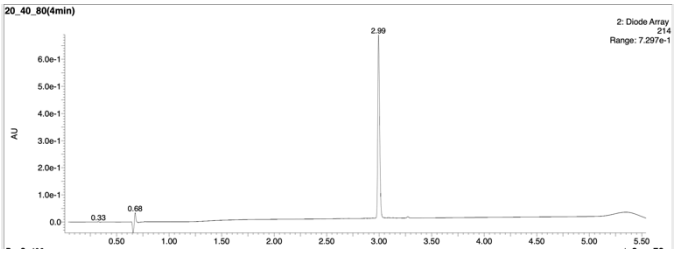
Peptide **20**: purity: 99%, t_R: 11.63min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 25 min, flow rate of 1 mL/min), molecular formula: $C_{62}H_{96}N_{14}O_{22}S_2$, calculated mass; 1215.5, found: 1216.



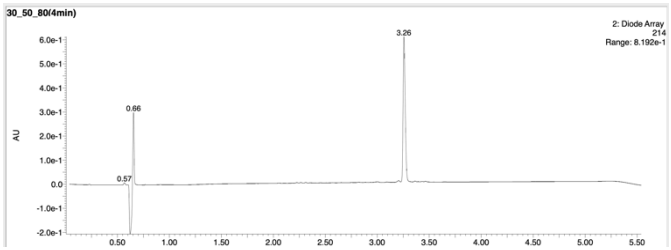
Peptide **21**: purity: 99%, t_R: 10.62min (analytical UHPLC, 10 to 90% acetonitrile in water (0.1% TFA) over 25 min, flow rate of 1 mL/min), molecular formula: $C_{62}H_{96}N_{14}O_{22}S_2$, calculated mass; 1275.4, found: 1276.9



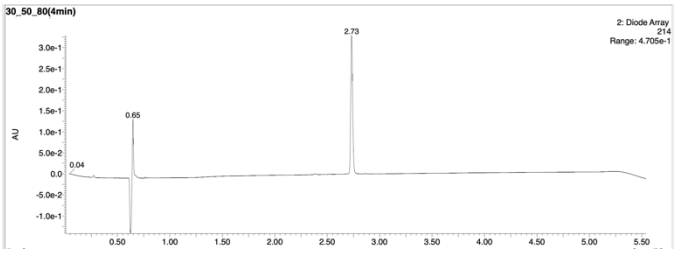
Peptide **22**: purity: 99%, t_R: 2.97 min (analytical UHPLC, 25 to 45% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{53}H_{68}N_{10}O_{14}S_2$, calculated mass; 1132.4, found: 1133.2



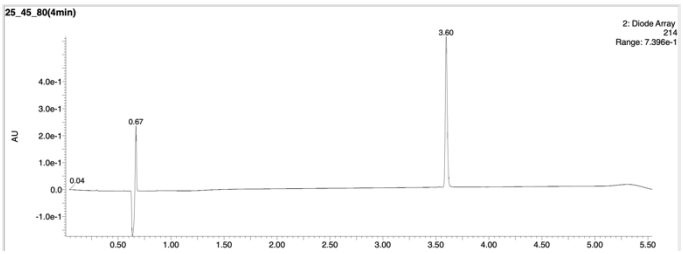
Peptide **23**: purity: 99%, t_R: 2.99 min (analytical UHPLC, 20 to 40% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{53}H_{71}N_{11}O_{12}S_2$, calculated mass; 1117,4, found: 1118.2



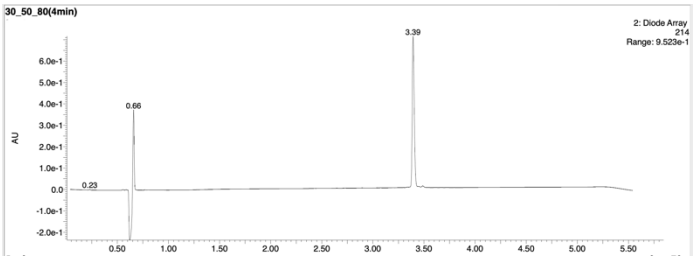
Peptide **24**: purity: 99%, t_R: 3.26 min (analytical UHPLC, 30 to 50% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{52}H_{68}N_{10}O_{11}S_2$, calculated mass; 1072,4 found: 1073.4



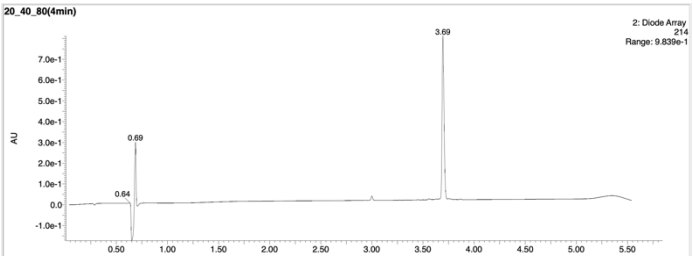
Peptide **25**: purity: 99%, t_R: 2.73 min (analytical UHPLC, 30 to 50% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{51}H_{65}IN_{10}O_{12}S_2$, calculated mass; 1200,3 found: 1201.3



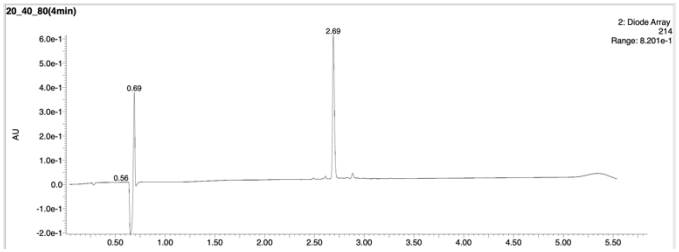
Peptide **26**: purity: 99%, t_R: 3,60 min (analytical UHPLC, 25 to 45% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{51}H_{65}IN_{10}O_{12}S_2$, calculated mass; 1200,3 found: 1201.1



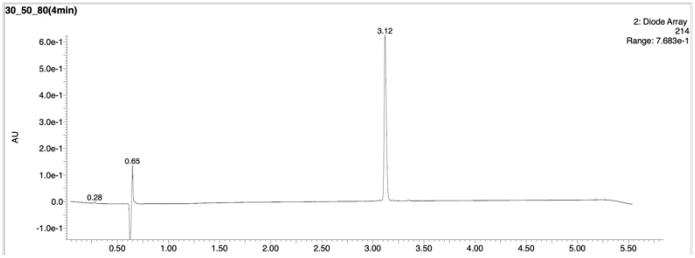
Peptide **27**: purity: 99%, t_R: 3.39 min (analytical UHPLC, 30 to 50% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{51}H_{64}I_2N_{10}O_{12}S_2$, calculated mass; 1322.2 found: 1327.0



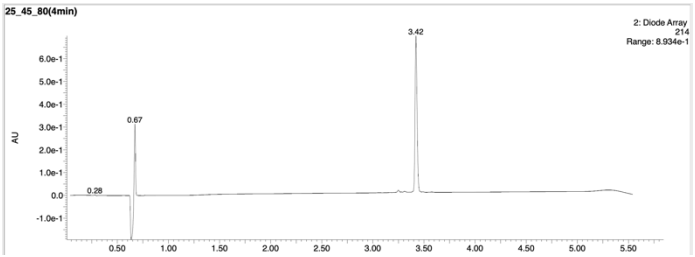
Peptide **28**: purity: 99%, t_R: 3.69 min (analytical UHPLC, 20 to 40% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{52}H_{66}N_{10}O_{14}S_2$, calculated mass; 1118.4 found: 1119.1



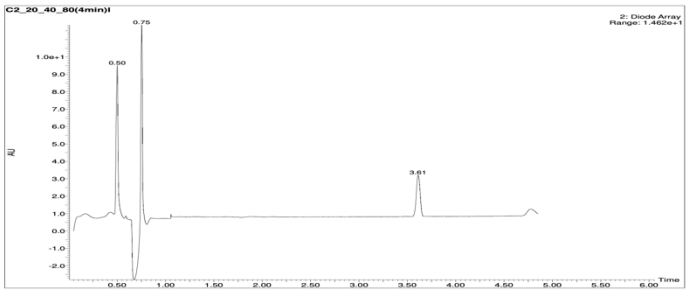
Peptide **29**: purity: 99%, t_R: 2.69 min (analytical UHPLC, 20 to 40% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{52}H_{69}N_{11}O_{12}S_2$, calculated mass; 1103,4 found: 1104.4



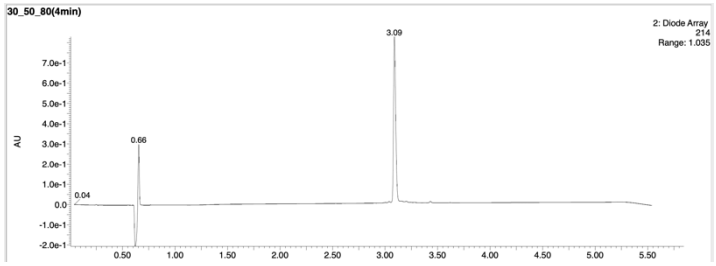
Peptide **30**: purity: 99%, t_R: 3.12 min (analytical UHPLC, 30 to 50% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{51}H_{66}N_{10}O_{11}S_2$, calculated mass; 1058.4 found: 1059.3



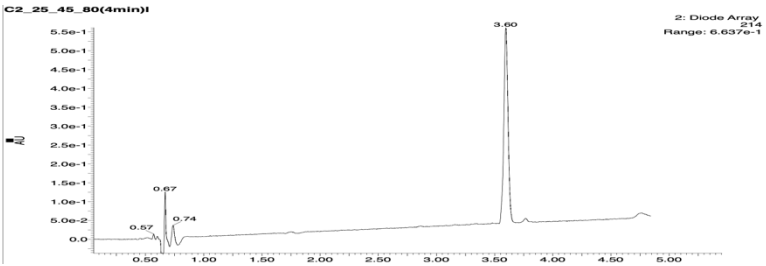
Peptide **31**: purity: 99%, t_R: 3.42 min (analytical UHPLC, 25 to 45% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{50}H_{63}IN_{10}O_{12}S_2$, calculated mass; 1186.3 found: 1187.2



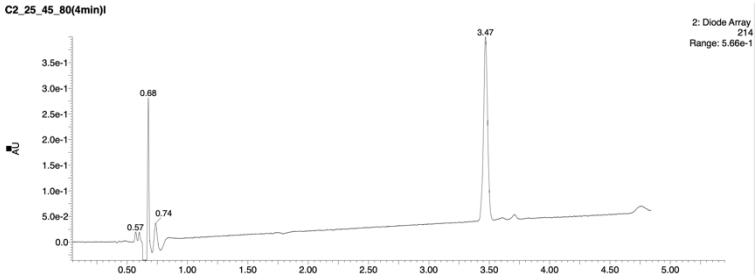
Peptide **32**: purity: 99%, t_R: 3.61 min (analytical UHPLC, 20 to 40% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{50}H_{63}IN_{10}O_{12}S_2$, calculated mass; 1186.3 found: 1187.2



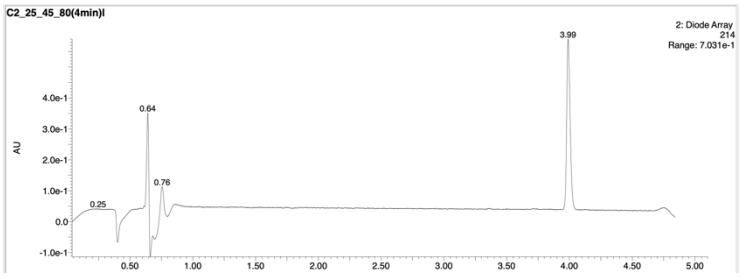
Peptide **33**: purity: 99%, t_R: 3.09 min (analytical UHPLC, 30 to 50% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{50}H_{62}I_2N_{10}O_{12}S_2$, calculated mass; 1312.2 found: 1313.1



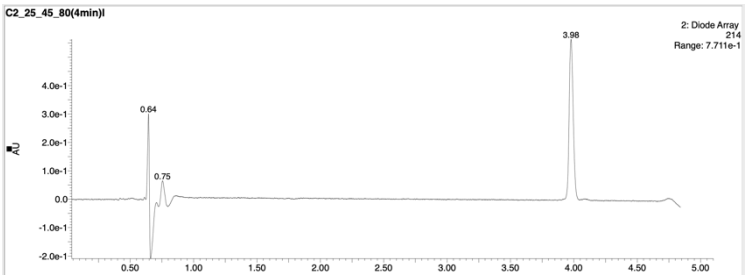
Peptide **34**: purity: 99%, t_R: 3.60 min (analytical UHPLC, 25 to 45% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{52}H_{65}F_3N_{10}O_{11}S_2$, calculated mass; 1124.4 found: 1125.8



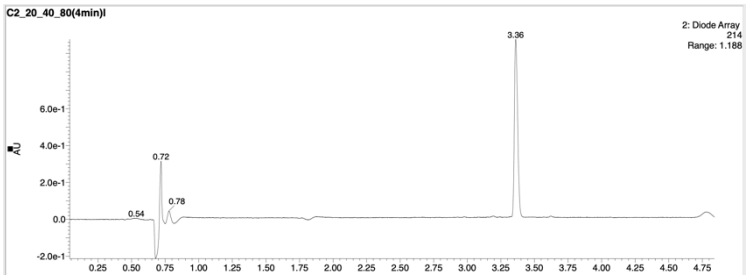
Peptide **35**: purity: 99%, t_R: 3.47 min (analytical UHPLC, 25 to 45% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{52}H_{65}F_3N_{10}O_{11}S_2$, calculated mass; 1110.4 found: 1111.8



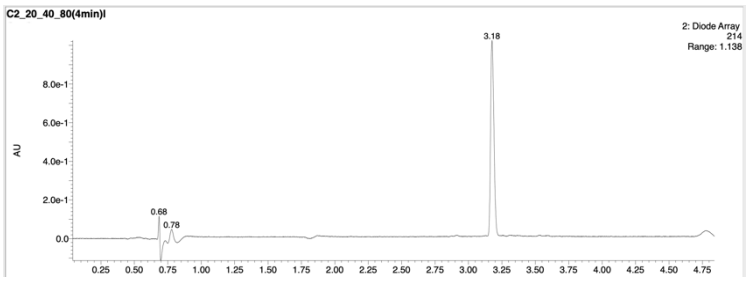
Peptide **36**: purity: 99%, t_R: 3.99 min (analytical UHPLC, 25 to 45% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{52}H_{65}F_3N_{10}O_{11}S_2$, calculated mass; 1126.4 found: 1127.8



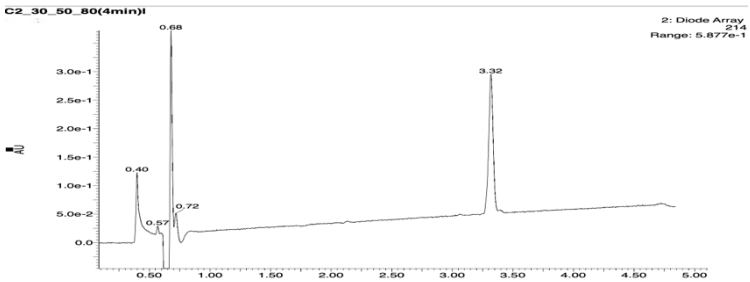
Peptide **37**: purity: 99%, t_R: 3.98 min (analytical UHPLC, 25 to 45% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{51}H_{63}F_3N_{10}O_{11}S_2$, calculated mass; 1112.4 found: 1114.0



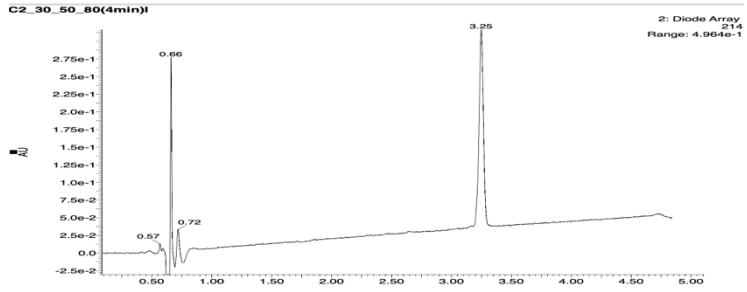
Peptide **38**: purity: 99%, t_R: 3.36 min (analytical UHPLC, 20 to 40% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{51}H_{66}N_{10}O_{12}S_2$, calculated mass; 1074.4 found: 1075.8



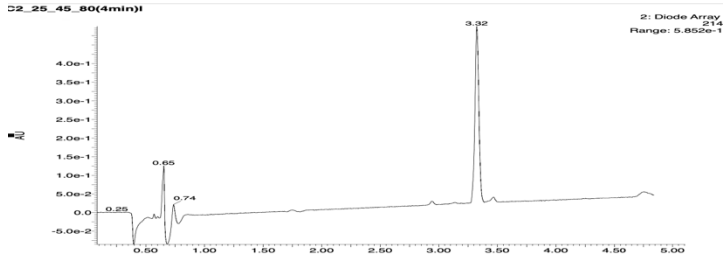
Peptide **39**: purity: 99%, t_R: 3.18 min (analytical UHPLC, 20 to 40% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{50}H_{64}N_{10}O_{12}S_2$, calculated mass; 1060.4 found: 1061.8



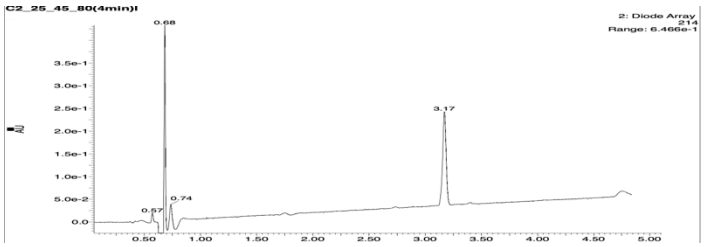
Peptide **40**: purity: 99%, t_R: 3.32 min (analytical UHPLC, 30 to 50% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{52}H_{68}N_{10}O_{12}S_2$, calculated mass; 1134.4 found: 1135.8



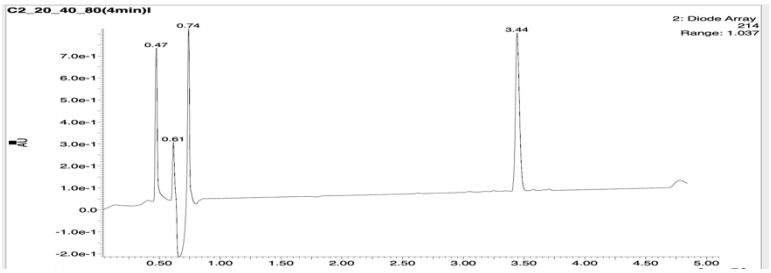
Peptide **41**: purity: 99%, t_R: 3.25 min (analytical UHPLC, 30 to 50% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{52}H_{68}N_{10}O_{12}S_2$, calculated mass; 1120.4 found: 1121.8



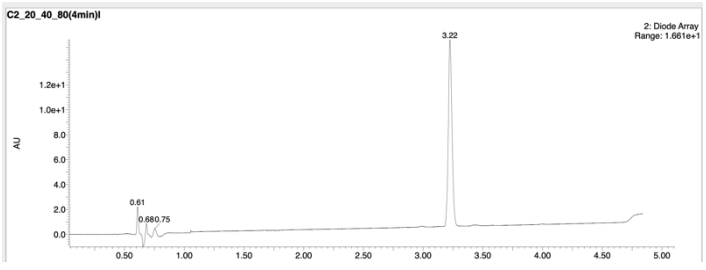
Peptide **42**: purity: 99%, t_R: 3.32 min (analytical UHPLC, 25 to 45% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{52}H_{68}N_{10}O_{12}S_2$, calculated mass; 1150.4 found: 1151.8



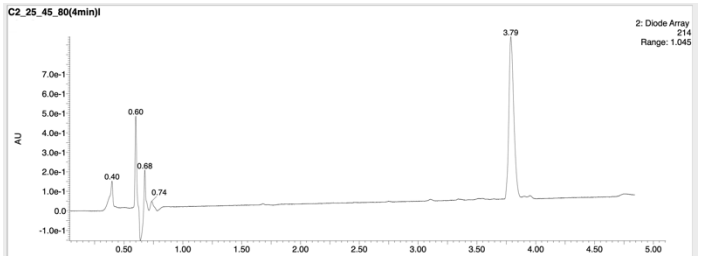
Peptide **43**: purity: 99%, t_R: 3.17 min (analytical UHPLC, 25 to 45% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{52}H_{68}N_{10}O_{12}S_2$, calculated mass; 1088.4 found: 1089.8



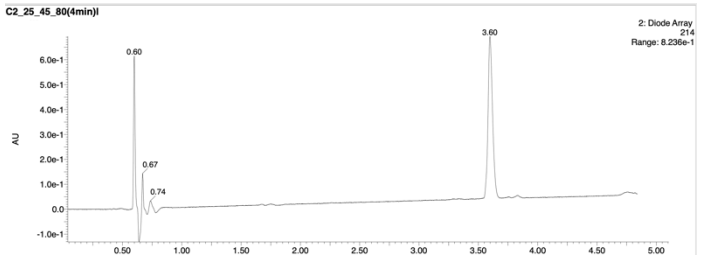
Peptide **44**: purity: 99%, t_R: 3.44 min (analytical UHPLC, 20 to 40% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{52}H_{68}N_{10}O_{12}S_2$, calculated mass; 1088.4 found: 1089.8



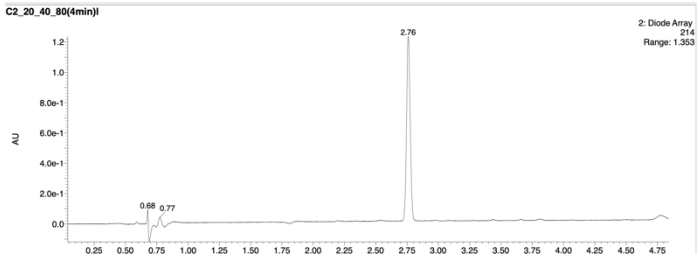
Peptide **45**: purity: 99%, t_R: 3.22 min (analytical UHPLC, 20 to 40% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{52}H_{68}N_{10}O_{12}S_2$, calculated mass; 1074.3 found: 1075.9



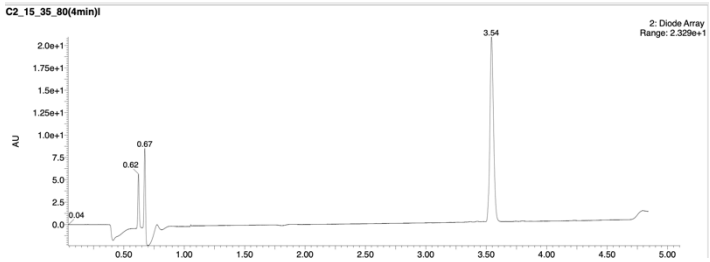
Peptide **46**: purity: 99%, t_R: 3.79 min (analytical UHPLC, 25 to 45 % acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{52}H_{68}N_{10}O_{12}S_2$, calculated mass; 1131.4 found: 1132.8



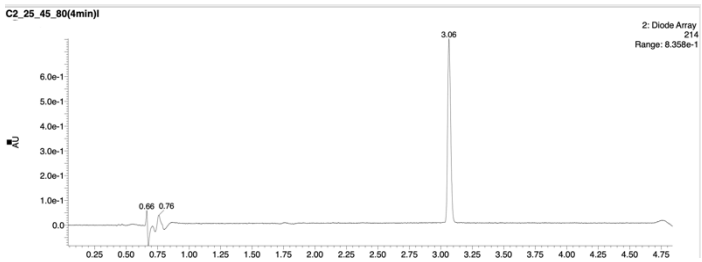
Peptide **47**: purity: 99%, t_R: 3.60 min (analytical UHPLC, 25 to 45% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{54}H_{72}N_{10}O_{12}S_2$, calculated mass; 1116.5 found: 1118.0



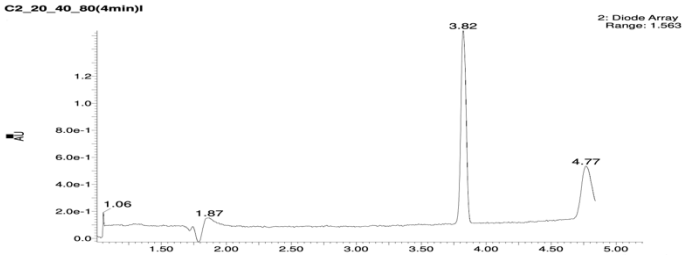
Peptide **48**: purity: 99%, t_R: 2.76 min (analytical UHPLC, 20 to 40% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{51}H_{66}N_{10}O_{13}S_2$, calculated mass; 1090.4 found: 1091.9



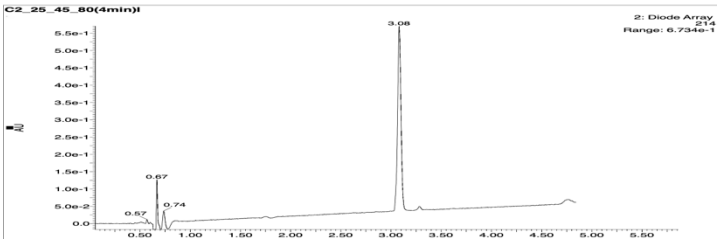
Peptide **49**: 99%, t_R: 3.54 min (analytical UHPLC, 15 to 35% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{50}H_{64}N_{10}O_{13}S_2$ calculated mass; 1074.4 found: 1077.9



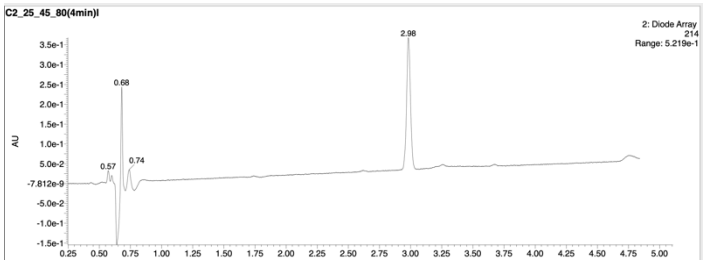
Peptide **50**: purity: 99%, t_R: 3.06 min (analytical UHPLC, 25 to 45% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{51}H_{65}N_{11}O_{14}S_2$, calculated mass; 1119.4 found: 1120.8



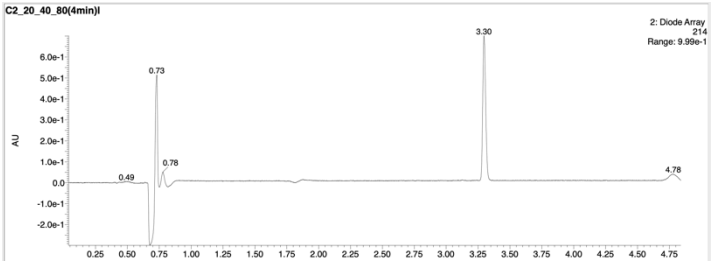
Peptide **51**: purity: 99%, t_R: 3.82 min (analytical UHPLC, 20 to 40% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{51}H_{65}N_{11}O_{14}S_2$, calculated mass; 1105.4 found: 1106.8



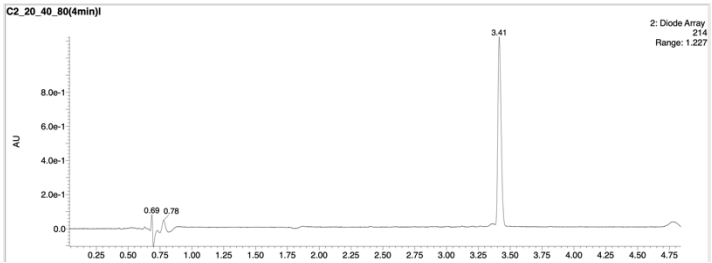
Peptide **52**: purity: 99%, t_R: 3.08 min (analytical UHPLC, 25 to 45% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{51}H_{65}N_{11}O_{14}S_2$, calculated mass; 1188.4 found: 1189.9



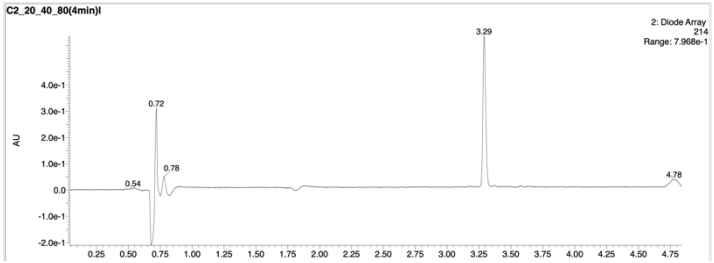
Peptide **53**: purity: 99%, t_R: 2.98 min (analytical UHPLC, 25 to 45% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{51}H_{65}N_{11}O_{14}S_2$, calculated mass; 1074.4 found: 1075.9



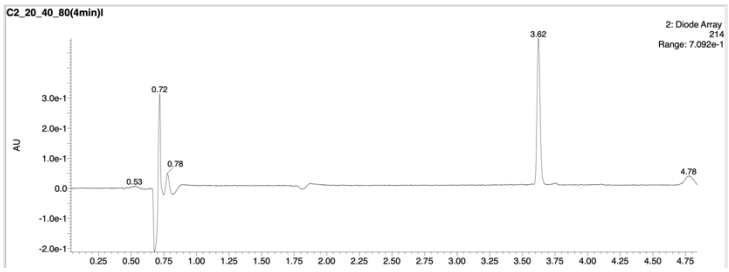
Peptide **54**: purity: 99%, t_R: 3.30 min (analytical UHPLC, 20 to 40% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{56}H_{68}N_{10}O_{12}S_2$, calculated mass; 1136,4 found: 1138.8



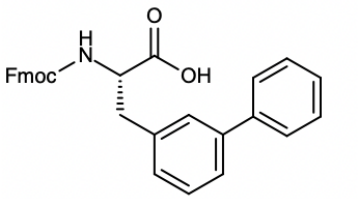
Peptide **55**: purity: 99%, t_R: 3.41 min (analytical UHPLC, 20 to 40% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{56}H_{68}N_{12}O_{12}S_2$, calculated mass; 1164.4 found: 1165.8



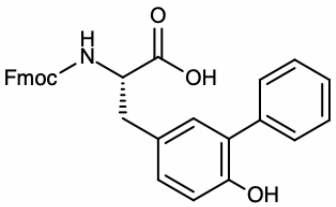
Peptide **56**: purity: 99%, t_R: 3.29 min (analytical UHPLC, 20 to 40% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{55}H_{66}N_{10}O_{12}S_2$, calculated mass; 1123.4 found: 1124



Peptide **57**: purity: 99%, t_R: 3.62 min (analytical UHPLC, 20 to 40% acetonitrile in water (0.1% TFA) over 4 min, flow rate of 0.5 mL/min), molecular formula: $C_{56}H_{68}N_{10}O_{12}S_2$, calculated mass; 1137.4 found: 1138,3



$_1$H-NMR (600 MHz, CDCl3): δ (ppm) = 3.06 (dd, *J* = 5.4 *J* = 13.7, 1 H), 3.15 (dd,*J* = 5.1, *J* = 13.8, 1 H), 4.17 (t, *J* = 7.0, 1 H), 4.30-4.34 (m, 1 H), 4.38-4.43 (m,1 H), 4.64-4.70 (m, 1 H), 5.41 (t, *J* = 7.9, 1 H), 6.86 (d, *J* = 8.0, 1 H), 6.99 (d,*J* =8.3, 1 H), 7.05 (s, 1 H), 7.22 – 7.25 (m, 2 H), 7.29-7.34 (m, 4 H), 7.41-7.45 (m, 3 H),7.56 (d, *J* = 7.3, 2 H), 7.72-7.76 (m, 2 H).



$_1$H-NMR (600 MHz; $CDCl_3$): δ (ppm)=3.05-3.17 (2H,m), 4.20 (1H, m), 4.33-4.43 (2H, m), 4.67-4.70 (1H, m,), 5.32 (1H, d, *J*=9.8), 6.98 (1H, t, J=8.4), 7.05 (1H, d, *J*=9.1), 7.13 (1H, s), 7.25-7.30 (3H, m), 7.35-7.41 (4H, m), 7.52 (2H, d, *J*=7), 7.55-7.58 (2H, m), 7.76 (2H, d, *J*=9.1)

Acknowledgements

The opportunity to carry out important goals it's almost impossible without the support of other people, and I at the end of my course I would to thanks many people.

Professor Paolo Grieco I would to thanks for the opportunity to work in yours groups, you haven't been only a scientific guide but a solid reference point.

Professor Mauro Adamo my sincere gratitude at my second supervisor for welcoming me in yours research groups and introduce me a different point of view of scientific word

Elisabetta Bianchi, Federica Orvieto e Stefania Colarusso profound gratitude at my supervisor in the company experience for spurring me to do my best and for believing in me.

A **Francesco Merlino** who thanks to his guidance he has contributed significantly to my training.

Rosita Bellavita more than a colleague a great friend, thank you for covering my shoulders on this path.

Simone, **Laura**, **Deborah**, **Grazia**, **Francesco**, **Daniele**, **Caterina** my colleges in Dublin, it is thanks to you if I have this experience in my heart and I felt at home even in a foreign country.

Daniele, **Federica T** and **Federica R** colleagues in my experience in the company, you were like a second family for me.

Finally, I would like to express my gratitude to my family, for always encouraging me to give my best in every situation.

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