Letter

Modifications at Arg and Ile give neurotensin(8-13) derivatives with high stability and retained NTS1 receptor affinity

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Modifications at Arg and Ile give neurotensin(8-13) derivatives with high stability and retained NTS_1 receptor affinity

Lisa Schindler,[†] Günther Bernhardt,[†] and Max Keller*,[†]

[†]Institute of Pharmacy, Faculty of Chemistry and Pharmacy, University of Regensburg, Universitätsstr. 31, D-93053 Regensburg, Germany

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ABSTRACT: Due to its expression in various malignant tumors, the neurotensin receptor 1 (NTS_1R) has been suggested and explored as a target for tumor diagnosis and therapy. Animal model-based investigations of various radiolabeled NTS_1R ligands derived from the hexapeptide neurotensin(8-13) (NT(8-13)), e.g. ^{68}Ga- and ^{18}F-labeled compounds for PET diagnostics, give rise to optimize such radiotracers for clinical use. As NT(8-13) is rapidly degraded in vivo, structural modifications are required in terms of increased metabolic stability. In this study, the stabilization of the peptide backbone of NT(8-13) against enzymatic degradation was systematically explored by performing an N-methyl scan, replacing Ile^{12} by *tert*-butylglycine^{12} (Tle^{12}) and N-terminal acylation. N-methylation of either arginine, Arg^8 or Arg^9, combined with the Ile^{12}/Tle^{12} exchange proved to be most favorable with respect to NTS_1R affinity (K_i < 2 nM) and stability in human plasma ($t_{1/2}$ > 48 h), a valuable result regarding the development of radiopharmaceuticals derived from NT(8-13).

Introduction. The neuromodulator neurotensin (NT), a 13 amino acid peptide (Figure 1), is found in the central nervous system (CNS), mediating e.g. analgesic effects, as well as in the periphery (primarily in the gastrointestinal tract).[1-2] The carboxy-terminal hexapeptide of NT (NT(8-13) (**1**), Figure 1), is biologically equi-active to NT.[3] The physiological effects of NT are mediated by three cell-surface receptors: the NT receptors 1 and 2 (NTS_1R, NTS_2R), both G-protein coupled receptors,[4] and the NTS_3R, which belongs to the Vps10p-domain receptor family.[2, 5] The NTS_1R has increasingly gained interest as a target for tumor diagnosis and therapy, as it was reported to be (over)expressed in a variety of malignancies, among them the prognostically poor pancreatic adenocarcinoma, Ewing's sarcoma, breast cancer, and colorectal carcinoma.[6-9] Thus, radiolabeled NTS_1R ligands harbor the potential of being used as radiopharmaceuticals. The majority of such compounds (e.g. ^{68}Ga- and ^{18}F-labeled for PET diagnostics, ^{177}Lu-labeled for radioendotherapy) has been derived from the agonist **1**.[10-19] Noteworthily, also NTS_1R ligands derived from non-peptidic antagonists have been explored as radiodiagnostics and radiotherapeutics.[20-21] Recently reported data of a clinical trial on the treatment of pancreatic adenocarcinoma in men by ^{177}Lu-labeled NTS_1R antagonists give reason to develop clinical trial candidates with improved properties.[22] Therefore, peptidic NTS_1R ligands, such as radiolabeled derivatives of **1**, should be considered for clinical trials.

A major drawback of peptide **1** is its rapid degradation in vivo by peptidases (see Figure 1).[23-24] Enzymatic degradation of **1** occurs at three major sites: the Arg^8-Arg^9 bond, the Pro^{10}-Tyr^{11} bond and the bond between Tyr^{11} and Ile^{12} (*cf.* Figure 1).[24-25] The predominant approaches to stabilize the backbone of **1** are N-methylation of Arg^8 or Arg^9, N-terminal acylation and the exchange of Ile^{12} by *tert*-butylglycine (Tle).[10-15, 17, 26-38] However, for some interesting analogs of **1**, such as N-methylated derivatives, investigations on the stability are lacking.[33, 39]. It is worth mentioning that described derivatives of **1**, containing Tle^{12} instead of Ile^{12}, include additional structural modifications throughout,[10-12, 29, 31-32, 38, 40] i.e. [Tle^{12}]NT(8-13) (**2**, *cf.* Figure 1) has not been reported to date to the best of the authors' knowledge. Therefore, it is difficult to estimate the impact of the Ile^{12}/Tle^{12} exchange on the stability of Tle^{12}-containing derivatives of **1**.

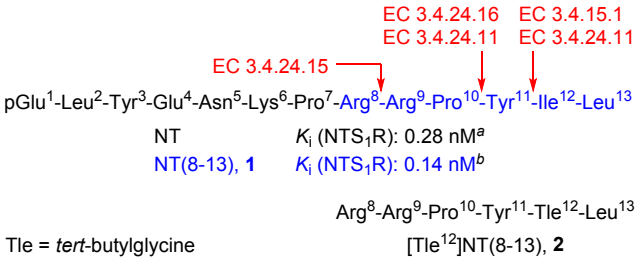


Figure 1. Amino acid sequences of neurotensin, **1** (NT(8-13), in blue) and **2**, as well as major enzymatic cleavage sites (in red) of **1**.[3, 24-25] EC 3.4.24.15: metalloendopeptidase 24.15, EC 3.4.24.16: metalloendopeptidase 24.16, EC 3.4.24.11: neutral endopeptidase 24.11, EC 3.4.15.1: angiotensin converting enzyme (ACE).[24-25] [a]Barroso *et al.*[41] [b]Keller *et al.*[42]

Aiming at a systematic study on the stabilization of the NT(8-13) core structure, we synthesized compound **2**, performed an N-methyl scan of **1**, combined N-methylation with the Ile[12]/Tle[12] exchange and, additionally, prepared N-terminally acylated derivatives of **1**. All compounds were studied with respect to NTS_1R binding and plasma stability.

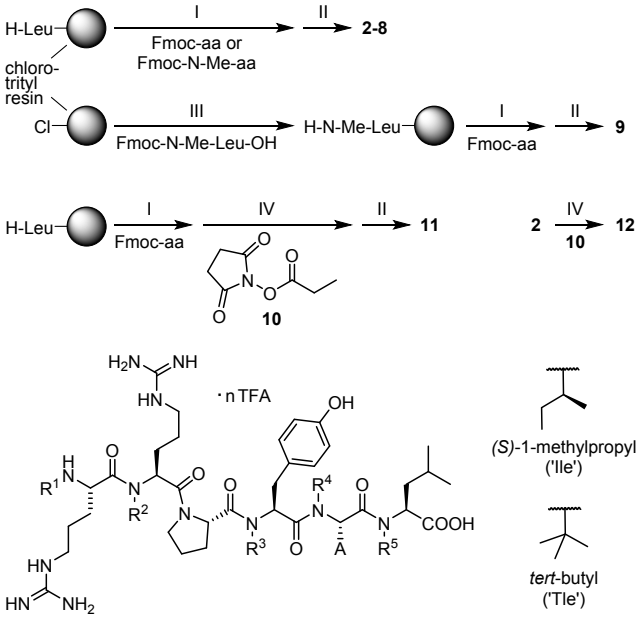
Results and Discussion. Peptides **2**, **3**,[34] **4**,[33] **5**,[33, 39] **6**,[33] **7**, **8** and **9**[33] were prepared by solid-phase peptide synthesis (SPPS) according to the 9-fluorenylmethoxycarbonyl (Fmoc) protecting group strategy using 1-hydroxybenzotriazole (HOBt)/*O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and diisopropylethylamine (DIPEA) for amide bond formation (Scheme 1). Coupling of Fmoc-protected amino acids to the secondary amino group of N-methylated amino acids turned out to be the yield limiting factor in case of **5**, **6** and **9** (overall yields: 18%, 15% and 20%, respectively). The N-terminally propionylated derivative **11** was obtained by treatment of the respective resin-bound, side chain-protected, but N-terminally deprotected precursor peptide with succinimidyl propionate (**10**) followed by cleavage off the resin and side chain deprotection. By contrast, the N-terminally propionylated peptide **12** was prepared by solution phase treatment of **2** with compound **10** (Scheme 1).

NTS_1R binding data (K_i values) were determined for **1-9**, **11** and **12** by competition binding with [^{3}H]UR-MK300[42] ([^{3}H]**13**, for structure see Figure S1, Supporting Information) at intact $hNTS_1R$ expressing HT-29 colon carcinoma cells (Table 1). The replacement of Ile[12] by Tle[12] in **1** (compound **2**) resulted in a minor decrease in NTS_1R affinity (K_i values of **1** and **2**: 0.33 *vs*. 1.17 nM, *cf*. Table 1). Regarding the N-methyl scan of **1** (peptides **3-6** and **9**), methylation at Arg[8] or Arg[9] (**3**, **4**) did not affect NTS_1R affinity (K_i < 0.5 nM, Table 1). By contrast, N-methylation of Tyr[11], Ile[12] or Leu[13] (**5**, **6**, **9**) led to a considerable decrease in NTS_1R affinity (K_i values: > 1,000 nM, 60 nM and 880 nM, respectively, *cf*. Table 1). As expected, the combination of the N-methylation at Arg[8] or Arg[9] with the replacement of Ile[12] by Tle[12] (peptides **7** and **8**) resulted in NTS_1R affinities comparable to that of **2** (Table 1). The N-terminally propionylated analogs of **1** and **2** (compounds **11** and **12**) exhibited K_i values (NTS_1R) of 1.0 and 18 nM, respectively.

Figure 2 illustrates a general decrease in NTS_1R affinity caused by the replacement of Ile[12] by Tle[12] in **1**, **3**, **4** and **11**, giving **2**, **7**, **8** and **12**, respectively, and a dependency of the extent of the decrease in affinity on the primary structure of the peptides. This is in agreement with reported NTS_1R binding data of derivatives of **1** containing Tle[12].[10-11, 27, 31, 38, 40]

In order to investigate the effect of N-methylation (**3-9**), the Ile[12]/Tle[12] exchange (**2**, **7**, **8**, **12**) and N-terminal acylation (**11**, **12**) on the stability of the peptides against enzymatic cleavage, the stability of all compounds was investigated in human plasma at 37 °C for up to 48 h (Figure 3, Table 1). Whereas N-methylation of Arg[8] or Arg[9] in **1** (compounds **3** and **4**) significantly enhanced the peptide stability in plasma compared to **1**, methylation of Tyr[11], Ile[12] and Leu[13] (**5**, **6**, **9**) did not lead to higher plasma stabilities. Strikingly, peptide **2**, which differed from **1** only with respect to the replacement of Ile[12] by Tle[12], proved to be as unstable as **1** (Figure 3, Table 1). However, the combination of the Ile[12]/Tle[12] exchange with N-methylation of Arg[8] or Arg[9] (**7**, **8**) resulted in significantly higher plasma stabilities ($t_{1/2}$ > 48 h) compared to **3** and **4**. These results confirmed that both, N-terminal (cleavage between Arg[8] and Arg[9]) and C-terminal (cleavage between Tyr[11] and Ile[12]) degradation are highly relevant, and revealed that the former occurs faster than the latter. As in case of N-terminal methylation of **1** (peptide **3**), N-terminal propionylation of **1** (peptide **11**) resulted in a moderate increase in enzymatic stability compared to **1** ($t_{1/2}$ of **11** between 1 h and 2 h, *cf*. Table 1). The combination of N-terminal propionylation with an Ile[12]/Tle[12] exchange (compound **12**) led to an excellent plasma stability as also observed in case of combining N-terminal methylation with an Ile[12]/Tle[12] exchange (peptide **7**) (Figure 3, Table 1).

Scheme 1. Syntheses of the NT(8-13) derivatives 2-9, 11 and 12.[a]



compd.	n	R^1	R^2	R^3	R^4	R^5	A
2	3	H	H	H	H	H	*tert*-butyl
3	3	CH_3	H	H	H	H	*(S)*-1-methylpropyl
4	3	H	CH_3	H	H	H	*(S)*-1-methylpropyl
5	3	H	H	CH_3	H	H	*(S)*-1-methylpropyl
6	3	H	H	H	CH_3	H	*(S)*-1-methylpropyl
7	3	CH_3	H	H	H	H	*tert*-butyl
8	3	H	CH_3	H	H	H	*tert*-butyl
9	3	H	H	H	H	CH_3	*(S)*-1-methylpropyl
11	2	CO-C_2H_5	H	H	H	H	*(S)*-1-methylpropyl
12	2	CO-C_2H_5	H	H	H	H	*tert*-butyl

[a]Reagents and conditions: (I) Fmoc strategy SPPS using HBTU/HOBt and DIPEA, solvent: DMF/NMP (80:20 v/v), 35 °C, 2 × 1 h or 2 × 2 h, Fmoc-deprotection: 20% piperidine in DMF/NMP (80:20 v/v), rt, 2 × 8-10 min; (II) (1) hexafluoro-2-propanol (HFIP)/CH_2Cl_2 (1:3 v/v), rt, 2 × 20 min, (2) TFA/H_2O (95:5 v/v), rt, 3 h; (III) DIPEA, solvent: CH_2Cl_2, 35 °C, 14 h; (IV) DIPEA, solvent: DMF/NMP (80:20 v/v), rt, 1 h; overall yields: 77% (**2**), 67% (**3**), 56% (**4**), 18% (**5**), 15% (**6**), 42% (**7**), 38% (**8**), 20% (**9**), 56% (**11**), 85% (**12**).

Figure 4 provides an overview of the major degradation fragments identified by LC-HRMS. The Arg[8]-Arg[9], Pro[10]-Tyr[11] and Tyr[11]-Ile[12] bonds were identified as the major cleavage sites (Figure 4), being in agreement with reported data on the metabolic stability of **1**.[24-25] As outlined above, the present study suggests that cleavage of Arg[8] in **1** occurs faster than its

Table 1. Peptide sequences and NTS_1R affinities of 1-9, 11 and 12, as well as stabilities of 1-9, 11 and 12 in human plasma/PBS (1:2 v/v) (37 °C).

compd.	sequence	K_i [nM] NTS_1R[a]	% intact peptide in plasma[b] after the specified incubation times:						
			10 min	30 min	1 h	2 h	6 h	24 h	48 h
1	Arg-Arg-Pro-Tyr-Ile-Leu	0.33 [0.35, 0.31] (lit. 0.14[c])	23.1 ± 0.2	n.d.	< 1	n.d.	n.d.	< 1	< 1
2	Arg-Arg-Pro-Tyr-Tle-Leu	1.17 [1.17, 1.17]	10.8 ± 0.5	n.d.	< 1	n.d.	n.d.	< 1	< 1
3	N(Me)-Arg-Arg-Pro-Tyr-Ile-Leu	0.223 ± 0.005 (lit. 0.29[d])	92.1 ± 0.1	88.2 ± 0.2	79.7 ± 0.1	70.8 ± 0.1	n.d.	n.d.	n.d.
4	Arg-N(Me)-Arg-Pro-Tyr-Ile-Leu	0.29 ± 0.03 (lit. 0.51[e])	> 99	93.6 ± 0.1	83.7 ± 0.3	66.4 ± 0.1	n.d.	n.d.	n.d.
5	Arg-Arg-Pro-N(Me)-Tyr-Ile-Leu	> 1,000 (lit. 5100[e])	22.9 ± 0.2	< 1	< 1	< 1	n.d.	n.d.	n.d.
6	Arg-Arg-Pro-Tyr-N(Me)-Ile-Leu	60 ± 5 (lit. 160[e])	2.6 ± 0.5	< 1	< 1	< 1	n.d.	n.d.	n.d.
7	N(Me)-Arg-Arg-Pro-Tyr-Tle-Leu	0.88 ± 0.13	n.d.	n.d.	> 99	n.d.	> 99	98.3 ± 0.8	86.8 ± 0.3
8	Arg-N(Me)-Arg-Pro-Tyr-Tle-Leu	1.6 ± 0.1	n.d.	n.d.	> 99	n.d.	> 99	> 99	> 99
9	Arg-Arg-Pro-Tyr-Ile-N(Me)-Leu	880 ± 260 (lit. 190[e])	39.9 ± 0.9	< 1	< 1	< 1	n.d.	n.d.	n.d.
11	Propionyl-Arg-Arg-Pro-Tyr-Ile-Leu	1.0 ± 0.2	> 99	84.0 ± 0.1	71.8 ± 0.2	32.4 ± 0.1	n.d.	n.d.	n.d.
12	Propionyl-Arg-Arg-Pro-Tyr-Tle-Leu	18 ± 2	n.d.	n.d.	> 99	n.d.	> 99	> 99	92.5 ± 0.9

[a]Determined by radioligand competition binding with [^{3}H]**13** at HT-29 cells; mean values from two (**1**, **2**), three (**3**, **4**) or four (**6-9**, **11**, **12**) independent experiments, each performed in triplicate (for n > 2 K_i values are given ± SEM, in case of n = 2 individual K_i values are given in square brackets). [b]The initial concentration of the peptides in plasma/PBS (1:2 v/v) was 100 µM; presented are mean values ± SEM from three independent experiments (SEM not given if no decomposition was observed). [c]Keller *et al.*[42] [d]Orwig *et al.*[34] [e]Härterich *et al.*[33] n.d. = not determined.

C-terminal degradation. This is, on one hand, in agreement with reports in the literature,[24] on the other hand it is in disagreement with other reports, which suggest an Ile[12]/Tle[12] exchange as the most crucial structural modification with respect to metabolic stabilization.[27-28]

In conclusion, the synthesis and investigation of N-methylated derivatives of NT(8-13) (**1**), N-terminally acylated derivatives of **1** and analogs containing Tle[12] instead of Ile[12], revealed that only the combination of appropriate N-terminal (e.g. N-methylation of Arg[8]) and C-terminal (replacement of Ile[12] by Tle[12]) structural modifications in **1** affords highly stable (plasma half-live > 48 h) congeners of **1** (compounds **7**, **8** and **12**). Fortunately, two of the most stable compounds (**7**, **8**) exhibited the highest NTS_1R affinities of the investigated analogs of **1**. This work answers open questions concerning the controversially discussed impact of various structural modifications of **1** on the enzymatic stability, thus supporting the development of stable radiolabeled derivatives of **1**, which harbor the potential of being used as radiopharmaceuticals.

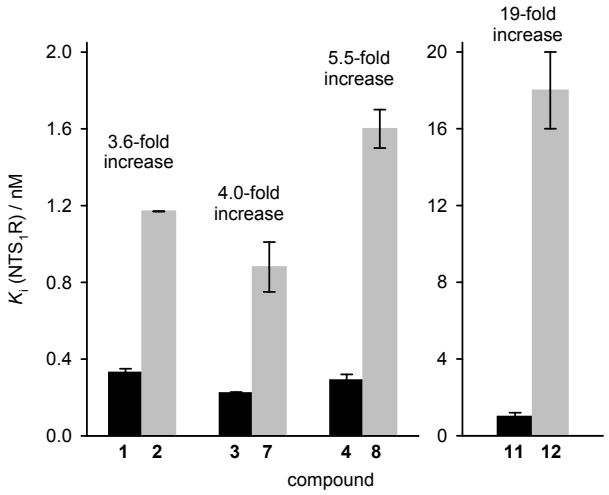


Figure 2. Decrease in NTS_1R affinity (increase in K_i) resulting from the exchange of Ile[12] by Tle[12] in **1**, **3**, **4** and **11** (black bars)

giving **2**, **7**, **8** and **12** (grey bars), respectively. Note: the scales of the Y-axes are different.

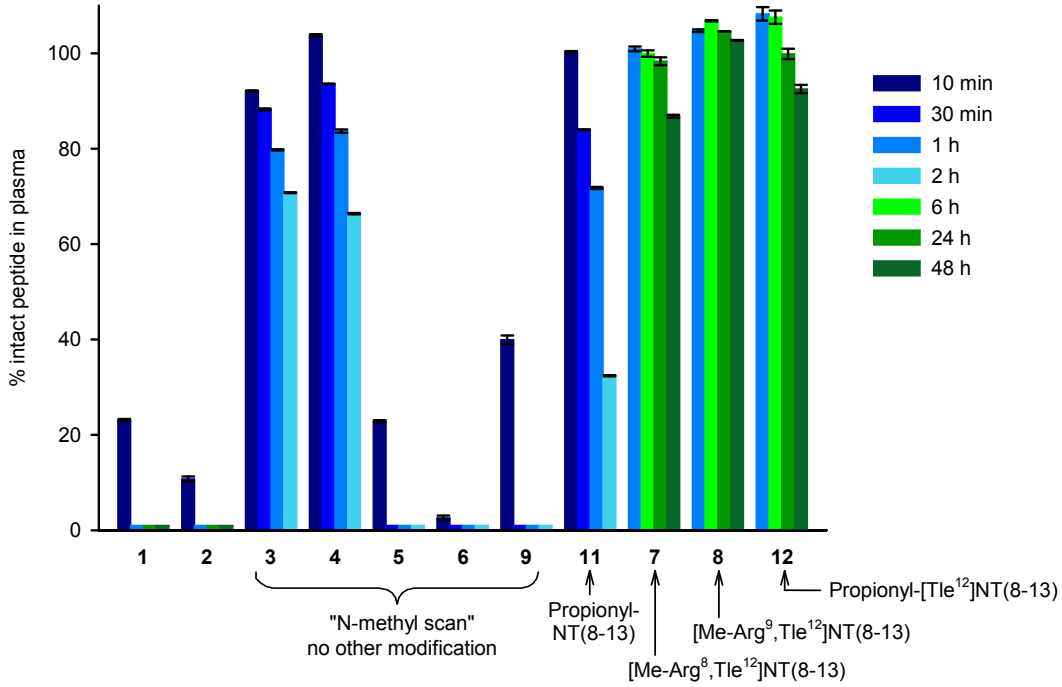


Figure 3. Stabilities of **1-9**, **11** and **12** in human plasma/PBS (1:2 v/v) at 37 °C investigated for up to 48 h. Data represent mean values ± SEM from three independent experiments.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

General experimental conditions; experimental synthetic protocols and analytical data of compounds **2-9**, **11** and **12**; radioligand competition binding assay; experimental protocol for the investigation of the stability of **1-9**, **11** and **12** in human plasma; Figures S1 and S2; RP-HPLC chromatograms of compounds **2-9**, **11** and **12**; ^{1}H-NMR spectra of compounds **2-9**, **11** and **12** in DMSO-d_6 and DMSO-d_6/D_2O (PDF)

Molecular formula strings (CSV)

AUTHOR INFORMATION

Corresponding Author

* E-mail: max.keller@ur.de. Phone: (+49)941-9433329. Fax: (+49)941-9434820

Author Contributions

L.S. performed any syntheses, radioligand competition binding experiments and plasma stability studies. M.K. initiated and planned the project. M.K. and G.B. supervised the research. L.S., M.K. and G.B. wrote the manuscript. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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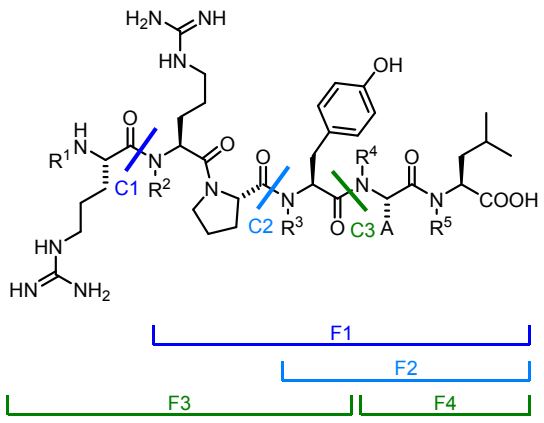
ABBREVIATIONS

2-ClTrt, 2-Chlorotrityl; 2-ClTrt-Cl, 2-Chlorotrityl-chloride; DIPEA, diisopropylethylamine; FCS, fetal calf serum; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; HOBt, 1-hydroxybenzotriazole; HT-29, human colorectal adenocarcinoma cell line; IC_{50}, inhibitor/antagonist concentration which suppresses 50% of an agonist induced effect, or displaces 50% of a labeled ligand from the binding site; *k*, retention (or capacity) factor (HPLC); K_d, dissociation constant obtained from a saturation binding experiment; K_i, dissociation constant obtained from a competition binding experiment; NT, neurotensin; NT(8-13), neurotensin(8-13); NTS_1R, neurotensin receptor 1; NTS_2R,

neurotensin receptor 2; RP, reversed phase; SEM, standard error of the mean; SPPS, solid-phase peptide synthesis; Tle, *tert*-butylglycine.



Identified fragments after incubation in human plasma/PBS (1:2 v/v):

compd.	R^1	R^2	R^3	R^4	R^5	A	F1	F2	F3	F4
1	H	H	H	H	H	*(S)*-1-methylpropyl	x	x		
2	H	H	H	H	H	*tert*-butyl	x	x		
3	CH_3	H	H	H	H	*(S)*-1-methylpropyl			x	x
4	H	CH_3	H	H	H	*(S)*-1-methylpropyl			x	x
5	H	H	CH_3	H	H	*(S)*-1-methylpropyl	x			
6	H	H	H	CH_3	H	*(S)*-1-methylpropyl	x	x		
7	CH_3	H	H	H	H	*tert*-butyl				
8	H	CH_3	H	H	H	*tert*-butyl				
9	H	H	H	H	CH_3	*(S)*-1-methylpropyl	x	x		x
11	$CO\text{-}C_2H_5$	H	H	H	H	*(S)*-1-methylpropyl			x	x
12	$CO\text{-}C_2H_5$	H	H	H	H	*tert*-butyl		x		x

Figure 4. Major enzymatic cleavage sites (C1-C3) of compounds **1-9**, **11** and **12** as well as corresponding fragments F1-F4, identified by LC-HRMS analysis after incubation in human plasma at 37 °C for up to 48 h.

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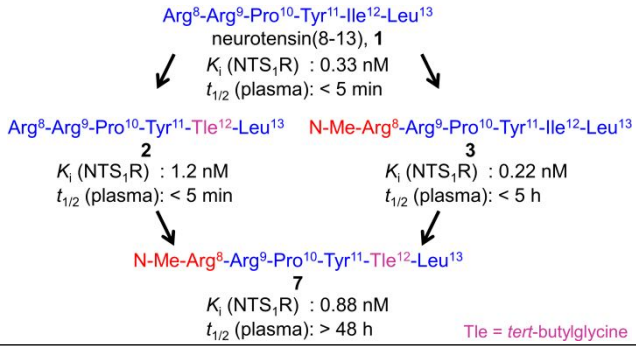
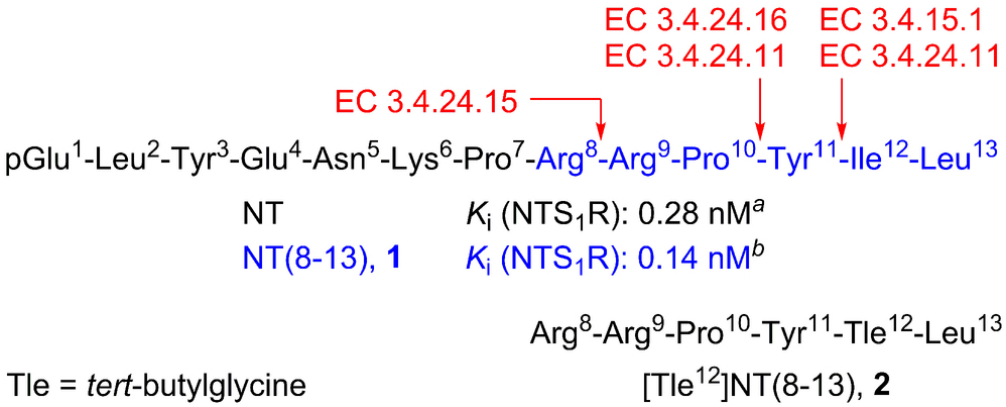
Table of Contents Graphic





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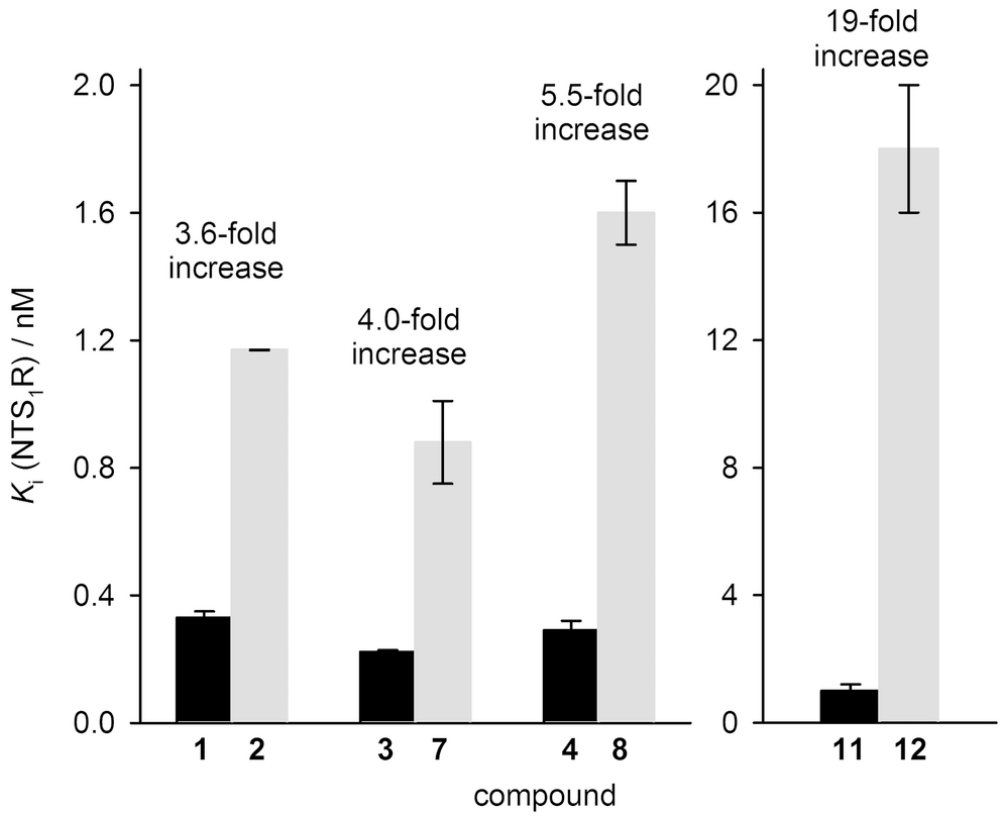

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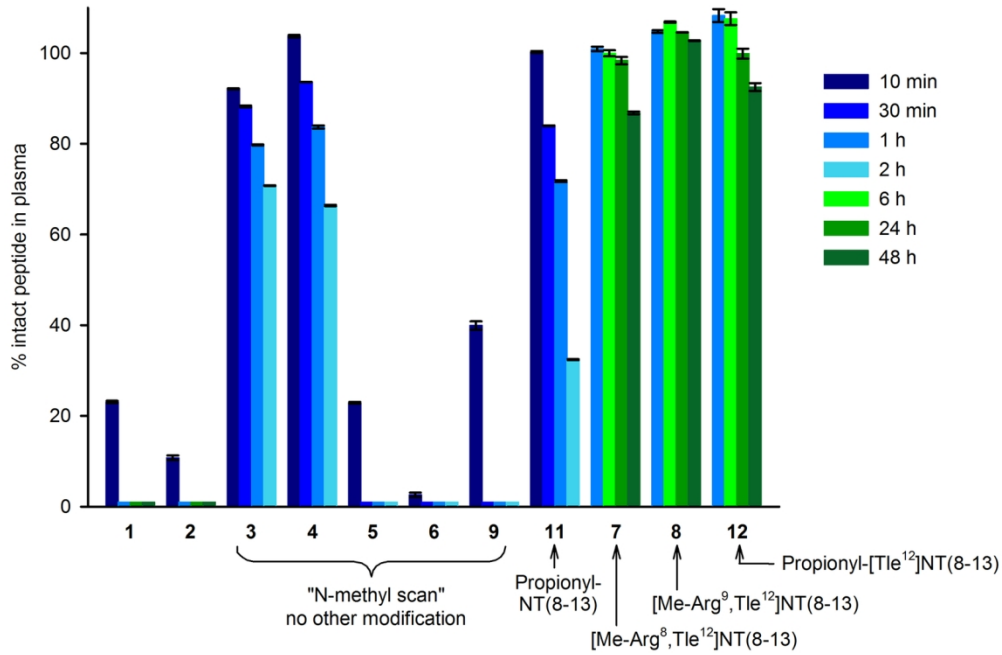

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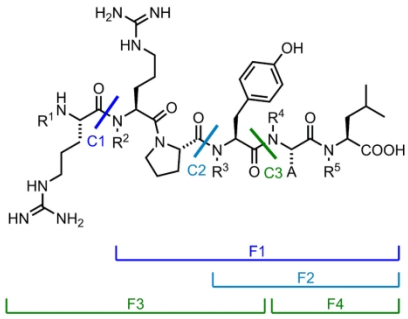

Identified fragments after incubation in human plasma/PBS (1:2 v/v):

compd.	R^1	R^2	R^3	R^4	R^5	A	F1	F2	F3	F4
1	H	H	H	H	H	*(S)*-1-methylpropyl	x	x		
2	H	H	H	H	H	*tert*-butyl	x	x		
3	CH_3	H	H	H	H	*(S)*-1-methylpropyl			x	x
4	H	CH_3	H	H	H	*(S)*-1-methylpropyl			x	x
5	H	H	CH_3	H	H	*(S)*-1-methylpropyl	x			
6	H	H	H	CH_3	H	*(S)*-1-methylpropyl	x	x		
7	CH_3	H	H	H	H	*tert*-butyl				
8	H	CH_3	H	H	H	*tert*-butyl				
9	H	H	H	H	CH_3	*(S)*-1-methylpropyl	x	x		x
11	$CO\text{-}C_2H_5$	H	H	H	H	*(S)*-1-methylpropyl			x	x
12	$CO\text{-}C_2H_5$	H	H	H	H	*tert*-butyl		x		x

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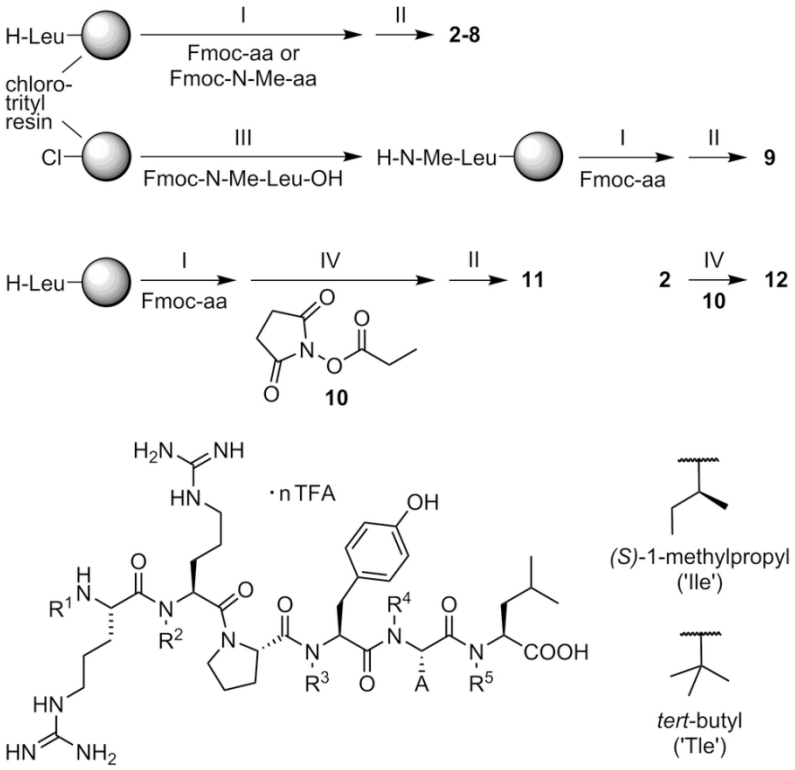

compd.	n	R^1	R^2	R^3	R^4	R^5	A
2	3	H	H	H	H	H	*tert*-butyl
3	3	CH_3	H	H	H	H	*(S)*-1-methylpropyl
4	3	H	CH_3	H	H	H	*(S)*-1-methylpropyl
5	3	H	H	CH_3	H	H	*(S)*-1-methylpropyl
6	3	H	H	H	CH_3	H	*(S)*-1-methylpropyl
7	3	CH_3	H	H	H	H	*tert*-butyl
8	3	H	CH_3	H	H	H	*tert*-butyl
9	3	H	H	H	H	CH_3	*(S)*-1-methylpropyl
11	2	$CO\text{-}C_2H_5$	H	H	H	H	*(S)*-1-methylpropyl
12	2	$CO\text{-}C_2H_5$	H	H	H	H	*tert*-butyl

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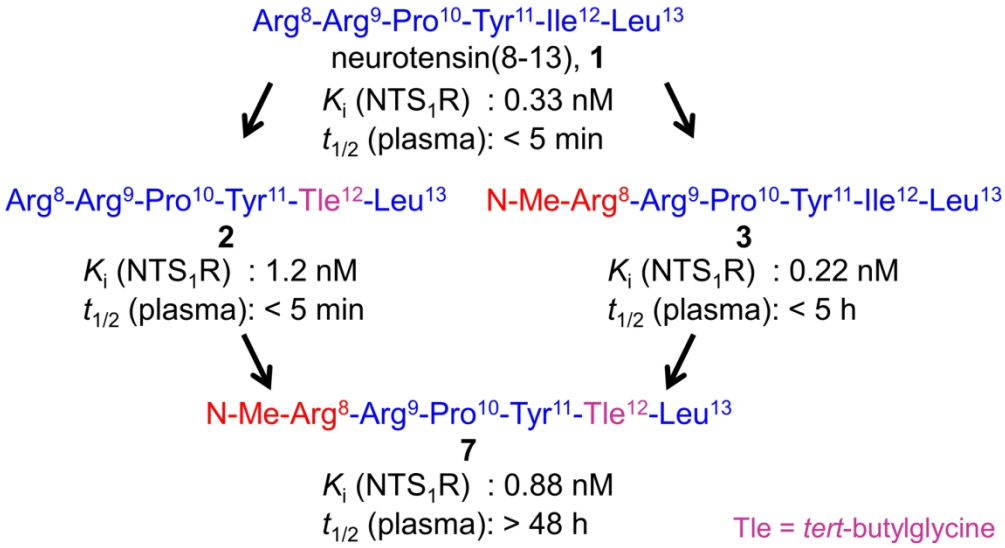

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