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Synthesis of neurotensin(8–13)–phosphopeptide heterodimers via click chemistry

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ABSTRACT

Two neurotensin(8–13)-containing peptide heterodimers were prepared via copper(I)-mediated click chemistry. The resulting peptide dimers could be obtained in 28–31% yield after HPLC purification. Neurotensin(8–13)-containing peptide dimers were used in an in vitro binding assay to determine binding affinity towards the neurotensin receptor-1 (NTR1). The determined IC_{50} values of 8.3 μM and 0.7 μM indicate only very low binding affinity of the neurotensin(8–13)-containing peptide heterodimers towards the NTR1.

Protein phosphorylation and dephosphorylation processes play a central role in a wide variety of biological processes involved in signal transduction pathways and the control of cell growth and cell differentiation.[1–5] Aberrations in proper protein phosphorylation and dephosphorylation are associated with the development and progression of diseases such as cancer, inflammation, metabolic and neurological disorders. The study of mechanisms related to protein phosphorylation/dephosphorylation is a major effort in modern biochemistry, medicinal chemistry, and medical research.[6]

In recent years phosphopeptides have become very useful reagents for the study of protein phosphorylation and dephosphorylaton as they can mimic phosphorylated protein segments. Moreover, synthetic phosphopeptides have also become interesting drug candidates. As promising intracellular modulators phosphopeptides have been used to inhibit and stimulate various signaling cascades as exemplified for targeting SH2 domain, 14-3-3 proteins, WD-40 and the Polo-box domain (PBD) of cell cycle regulating Polo-like kinase-1 (Plk1).[7,8] Plk1 is a key enzyme in the regulation of mitotic progression, and Plk1 is overexpressed in many types of human cancer.[9,10] Recently, a series of phosphopeptides containing a Ser-[pSer/pThr]-Pro/XXX (XXX = Gly, Cys) core sequence have been identified as potential ligands for the Plk1 Polo-box domain.

However, application of phosphopeptides to study protein–protein interactions and to interfere with signaling pathways is limited by their low cell uptake capability due to the negatively charged phosphate moiety. The poor cell uptake of phosphopeptides labeled with the short-lived positron emitter fluorine-18 (^{18}F, $t_{1/2}$ = 109.8 min) was recently shown by our group.[11] Radiolabeled Plk1-PBD-binding hexaphosphopeptide H-Met-Gln-Ser-pThr-Pro-Leu-OH **1** showed only marginal internalization into both human colorectal adenocarcinoma cells (HT-29) and head and neck squamous cell carcinoma cells (FaDu).

Neurotensin (NT) is a 13 amino acid containing neuropeptide expressed within the central nervous system and in peripheral tissues like the gastrointestinal tract system. NT binds to the neurotensin receptor (NTR) whereof three different subtypes, NTR1, NTR2, and NTR3, are characterized.[12,13] NTR1 and NTR2 belong to the family of G-protein coupled receptors, and their internalization mechanism via receptor-mediated endocytosis is well described by Mazella and Vincent, 2006.[14] In the last decade, numerous studies described NTR as promising target for cancer diagnosis and therapy[15] using radiolabeled neurotensin derivatives such as ^{18}F-labeled stabilized and multimeric neurotensin(8–13)[16,17], ^{99m}Tc-labeled stabilized neurotensin analogs[18], stable tetrabranched neurotensin[19], and ^{99m}Tc/^{188}Re-labeled stable NT-XIX.[20]

In this Letter we describe the synthesis and determination of NTR binding of two peptide heterodimers consisting of NTR-binding peptide NT(8–13) **3** as molecular shuttle for NTR binding and NTR-mediated endocytosis, and Plk1-PBD-binding hexaphosphopeptide H-Met-Gln-Ser-pThr-Pro-Leu-OH **1** and the corresponding non-phosphorylated peptide H-Met-Gln-Ser-Thr-Pro-Leu-OH **2**, respectively.

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The synthesis of peptide heterodimers was accomplished through copper(I)-mediated click chemistry. The Cu(I)-mediated azide–alkyne [3+2] cycloaddition is ideally suited for the synthesis of peptide dimers due to the high reactivity and selectivity of the reaction in aqueous media.[21,22] The potential of click chemistry synthetic chemistry and medicinal chemistry was subject of various recent excellent reviews.[23,24] The application of click chemistry according to a [3+2] cycloaddition between azides and alkynes leads to the linkage of both peptides through a stable triazole moiety.

Syntheses of the phosphopeptide H-Met-Gln-Ser-pThr-Pro-Leu-OH **1** and the non-phosphorylated analog H-Met-Gln-Ser-Thr-Pro-Leu-OH **2** were accomplished via Fmoc-based orthogonal solid phase peptide synthesis (SPPS) using previously published procedures.[11] Incorporation of N-terminal azide functionality into peptides **1** and **2** was achieved through the reaction with succinimidyl-5-azidovalerate **4**, which was synthesized according to Seo et al., 2003.[25] NT(8–13) **3** was treated with succinimidyl-hex-5-ynoate **5**[26] to give the required N-terminal alkyne-functionalized NT(8–13) derivative **8** functionalization. The synthesis of azide-functionalized peptides **6** and **7** and alkyne-functionalized peptide **8** is depicted in Figure 1.

Functionalization of N-terminal ends of peptides **1**, **2**, and **3** succeeded by the reaction of 2.1 equiv of active esters **4** or **5** in a solution of acetonitrile/Kolthoff buffer (pH 8.4)[27] (3/7 (v/v)) at 40 °C for 6 h. After purification of the reaction mixture by means of HPLC and subsequent lyophilization, azide-functionalized peptides **6** and **7** were obtained 66% and 82% isolated yield, respectively. Alkyne-functionalized peptide **8** was obtained in 69% isolated yield. The purity of all peptides exceeded 95% as confirmed by HPLC analysis. The identity of all compounds as determined by means of electron spray ionization mass spectrometry (ESI-MS).

In the next step, alkyne-functionalized NT(8–13) derivative **8** was reacted with azide-modified peptides **6** and **7** under click chemistry conditions. Click chemistry involving NT(8–13) derivatives have recently been investigated and optimized by Ramenda et al., 2007.[28] The click chemistry reaction between azide-functionalized peptides **6** and **7**, and alkyne-containing NT(8–13) derivative **8** involved the use of 1.0 equiv of peptide, 0.5 equiv of $CuSO_4 \times 5H_2O$, and 5.0 equiv of sodium ascorbate as the reducing agent in 1 ml of Kolthoff's borate buffer (pH 8.4). The reaction proceeded at 40 °C for 48 h. The corresponding peptide dimers **9** and **10** were isolated in 28% and 31%, respectively, after HPLC purification and lyophilization. ESI-MS analysis of both peptide dimers gave the expected molecular masses, being $m/z = 1792$ $[M+H]^+$ for phophopeptide-containing dimer **9** and $m/z = 856.36$ $[M+H]^{2+}$ for the twofold positively charged peptide dimer **10**. The synthesis of peptide dimers via click chemistry is given in Figure 2.

Triazole-linked peptide heterodimers **9** and **10** possess a NT(8–13) motif containing a free C terminal end. Our previous work with NT(8–13) derivatives revealed that a free C-terminal end is essential for sufficient binding of NT(8–13) derivatives to the NTR1, whereas various substitutions and modifications at the N-terminal end of NT(8–13)-based compounds are accepted by the NTR1.

The binding of NT(8–13)-containing peptide heterodimers **9** and **10** towards the NTR1 was determined in a competitive in vitro binding assay using the human colon adenocarcinoma cell line HT-29.[29] HT-29 are reported to express NTR1 on their membranes, and they have been used as a suitable test system to evaluate a variety of different NT(8–13) derivatives.[30] The binding assay was performed with [^{3}H]neurotensin as reference compound and radiotracer. The peptide dimers inhibited the binding of [^{3}H]neurotensin in a dose dependent manner, showing the typical sigmoid curves (data not shown). The determined representative IC_{50} values are presented in Table 1.

In comparison to the determined IC_{50} value of 1 nM for the high affinity ligand NT(8–13), the found IC_{50} values of 8.3 μM for phosphopeptide-containing dimer **9** and 0.7 μM for non-phosphorylated peptide dimer **10** indicate a significantly diminished binding of both peptide dimers to the NTR1.

The very low affinity as expressed by the high IC_{50} value of 8.3 μM of neurotensin–phosphopeptide dimer **9** can be explained by significant structural changes of the compound through strong interactions of the positively charged guanidine groups of the arginine residues in the neurotensin(8–13) part with the negatively charged phosphate residue of the phosphopeptide part. The strong interaction between the functional groups seems to lead to significant structural changes of the otherwise linear alignment of peptide dimer **9** as depicted in Figure 2. Governed by the strong guanidine–phosphate interactions, the long linker between the NT(8–13) part and phosphopeptide part allows a close arrangement of both peptide parts resulting in a conformation of peptide dimer **9**, which is not readily recognized by the NTR1.

In contrast to phosphopeptide-containing dimer **9**, the determined IC_{50} value of 0.7 μM for non-phosphorylated peptide dimer **10** suggests a more linear alignment of the peptide dimer and, therefore, favorable conformation resulting in a much higher binding affinity (lower IC_{50} value) towards the NTR1.

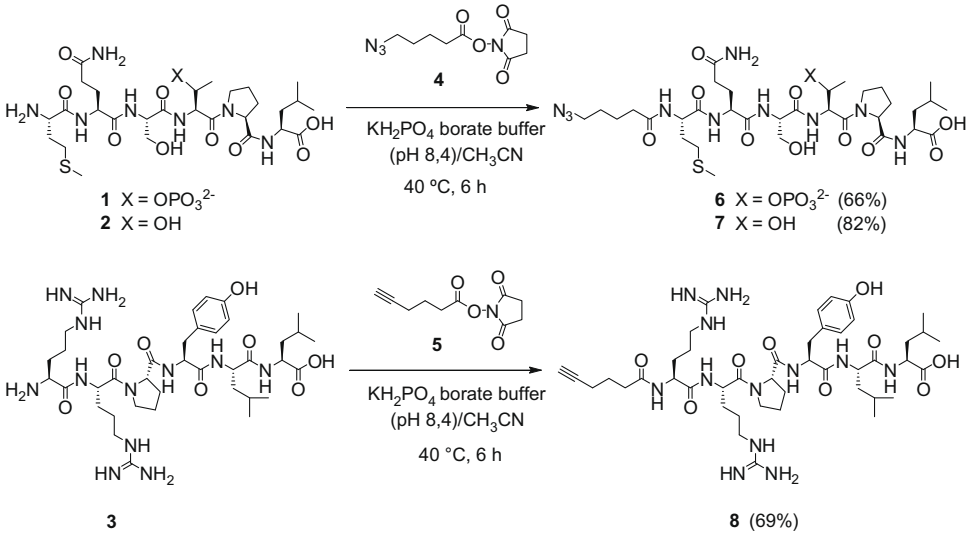

Figure 1. Synthesis of *N*-terminal functionalized peptides **6**, **7**, and **8**.

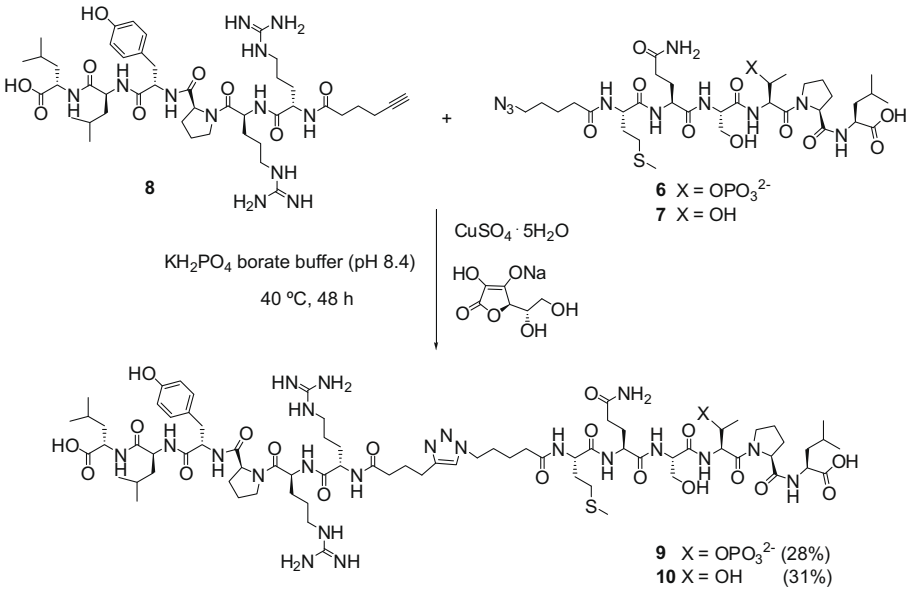

Figure 2. Click chemistry to form peptide dimers **9** and **10**.

Table 1
Binding properties of NT(8–13)–phosphopeptide dimer **9** and NT(8–13)-peptide dimer **10** in comparison in comparison with NT(8–13) in HT-29 cells

Peptide	IC_{50} (μM)
NT(8–13)	0.001
9	8.3
10	0.7

In summary, we have prepared two peptide heterodimers containing a NT(8–13) motif, and the Plk1-PBD binding hexaphosphopeptide H-Met-Gln-Ser-pThr-Pro-Leu-OH **1** or the corresponding non-phosphorylated analog **2**. Both peptide parts have been connected via formation of a triazole moiety in acceptable yields through copper(I)-mediated click chemistry under mild reaction conditions. We have demonstrated that click chemistry is a suitable tool for the formation of peptide heterodimers under mild reaction conditions. To the best of our knowledge, this is the first example of using click chemistry for the preparation of peptide heterodimers. This approach can be extended conveniently to the synthesis of a broad variety of different peptide dimers.

The NT(8–13) part was introduced for binding and internalization of the peptide dimers through the NTR1. However, based on the determined low binding affinities of neurotensin–peptide dimers **9** and **10** it can be concluded that the proposed approach for internalization of neurotensin–phosphopeptide dimers via receptor-mediated endocytosis is not feasible. Strong interaction between the positively charged guanidine groups and the negatively charged phosphate groups result in an unfavorable conformation of dimer **9** for NTR1 binding. A more rigid linker between the NT(8–13) part and the phosphopeptide part may help to circumvent this problem. Another approach for internalization of phosphopeptides is their coupling to cell-penetrating peptides as molecular shuttles to enable cell uptake. This approach is currently under investigation.

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containing 10% DMSO. The cells in the flasks were washed three times with Tris buffer. They were subsequently removed from the flask and resuspended in 8 ml of Tris buffer. The cell suspension was then homogenized in a glass homogenizer. Two hundred microliters of the cell homogenate were mixed with 100 μl of the peptide solution and 500 μl of Tris buffer. Two hundred microliters of [^{3}H]NT were added and the mixture was allowed to incubated for 30 min at room temperature. Non-specific binding was assessed in the presence of 100 mM NT(8–13).

Separation of bound and free ligand was performed by rapid filtration through Whatman GF/B glass fiber filters using a Brandel cell harvester (Gaithersburg, MD, USA). Filters were washed with three volumes of Tris buffer and were placed into vials with 4 ml scintillation cocktail (ULTIMA Gold®, Packard Instruments, USA). The [^{3}H]NT remaining on the filters was quantified by liquid scintillation spectrometry (TriCarb®, Packard Instruments, USA).

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