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Novel glycosylated endomorphin-2 analog produces potent centrally-mediated antinociception in mice after peripheral administration

Jakub Fichna [a,b,*], Marzena Mazur [c], Daria Grzywacz [d], Wojciech Kamysz [e], Renata Perlikowska [a], Justyna Piekielna [a], Marta Sobczak [a], Maciej Sałaga [a], Geza Toth [f], Anna Janecka [a], Chunqiu Chen [b], Jacek Olczak [c]

[a] Department of Biomolecular Chemistry, Faculty of Medicine, Medical University of Lodz, Mazowiecka 6/8, 92-215 Lodz, Poland
[b] Department of Gastroenterological Surgery, Tenth People's Hospital of Shanghai, School of Medicine, Tongji University, Shanghai, China
[c] TriMen Chemicals Ltd, Lodz, Poland
[d] Research and Development Laboratory, Lipopharm.pl, Zblewo, Poland
[e] Department of Inorganic Chemistry, Faculty of Pharmacy, Medical University of Gdansk, Poland
[f] Institute of Biochemistry, Biological Research Centre of Hungarian Academy of Sciences, Szeged, Hungary

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ABSTRACT

We report the synthesis and pharmacological characterization of a novel glycosylated analog of a potent and selective endogenous μ-opioid receptor (MOP) agonist, endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2, EM-2), obtained by the introduction in position 3 of the tyrosine residue possessing the glucose moiety attached to the phenolic function via a β-glycosidic bond. The improved blood–brain barrier permeability and enhanced antinociceptive effect of the novel glycosylated analog suggest that it may be a promising template for design of potent analgesics. Furthermore, the described methodology may be useful for increasing the bioavailability and delivery of opioid peptides to the CNS.

Opiates, such as morphine and other opium-derived alkaloids, have been used for centuries to alleviate moderate to severe pain. However, the side effects associated with their administration, such as respiratory depression, inhibition of gastrointestinal motility and development of physical dependence, in particular when extended in time to treat chronic conditions, severely limit the opiate application as analgesics. Recently, much effort has been put into the design of the new molecules targeting the opioid system, with potent antinociceptive action in the central nervous system (CNS) and limited effect in the periphery after systemic administration.

Most of the rational drug design studies have focused on the endogenous opioid peptides, which are important neurotransmitters and play a major role in the maintenance of homeostasis in the CNS and in the periphery. The aim is to maintain the pharmacological profile of the opioid peptides, while improve biodistribution by increasing their permeability through the blood–brain barrier (BBB) and ameliorate their stability against enzymatic degradation. The BBB, situated at the level of the endothelial cells of the brain microvascular capillaries coupled with tight junctions,[1,2] is characterized by a reduced vesicular transport, high electrical resistance and proteolytic activity, and low paracellular diffusion,[3] excluding most of the peptides from reaching the brain.

Of several approaches, which have been proposed to improve peptide delivery through the BBB, for example, increasing lipophilicity[4] or serum stability,[1] glycosylation seems to be the most promising. Glycosylation increases metabolic stability,[5] attenuates in vivo clearance,[6] and enhances pharmacological effect compared to non-glycosylated compounds, as shown for deltorphin,[7,8] cyclized Met-enkephalin analogs,[9,10] and linear Leu-enkephalin analogs.[1] However, the attachment of the carbohydrate was also shown as detrimental for opioid receptor binding affinity (Ref. 1 and citations therein).

Abbreviations: AgOTf, silver triflate; BBB, blood–brain barrier; CDMT, 2-chloro-4,6-bis[3-(perfluorohexyl)propyloxy]-1,3,5-triazine; CNS, central nervous system; DMF, dimethylformamide; EM-2, endomorphin-2; Fmoc, *N*-(9-fluorenylmethyloxycarbonyl); GPI assay, guinea pig ileum assay; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate; HR-ESI-MS, high-resolution electrospray ionization mass spectrometry; iv, intravenous; MOP, μ-opioid receptor; RP HPLC, reversed-phase high-performance liquid chromatography; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; NALME, naloxone methiodide.

* Corresponding author. Tel.: +48 42 272 57 07; fax: +48 42 272 56 94.
E-mail address: jakub.fichna@umed.lodz.pl (J. Fichna).

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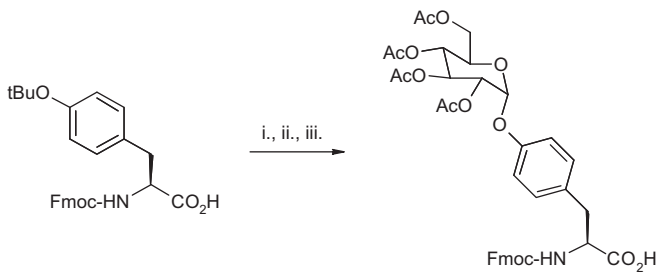
Here we report the synthesis of a novel glycosylated analog of a potent and selective endogenous μ-opioid receptor (MOP) agonist, endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2, EM-2, **1**), obtained by the introduction in position 3 of the tyrosine residue possessing the glucose moiety attached to the phenolic function via a β-glycosidic bond (Tyr(β-D-glucopyranose)). In this study we have also investigated the pharmacological properties of the new analog by using in vitro and in vivo techniques.

Fmoc-Tyr(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-OH was obtained in three consecutive steps: allyl ester protection,[11] glycosylation[12] and allyl ester deprotection[13] (Scheme 1). Glycosylated EM-2 analogs were assembled on the Rink resin using *N*-(9-fluorenylmethyloxycarbonyl) (Fmoc)-protected amino acids and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU)/*N*-methylmorpholine as coupling reagents.[14] The final peptide resin was Nα-deprotected,[15] thoroughly washed with dichloromethane, dried and divided into two portions. One portion was directly cleaved from the resin to give glycosylated EM-2 with acetylated hydroxyl groups on the glucopyranosyl moiety (**2a**) (Fig. 1), as described earlier.[14] The second portion of the peptide-resin was subjected to the action $NaOCH_3$ in DMF/MeOH, resulting in de-acetylation of the hydroxylic groups of the glucose moiety.[16] The fully de-protected peptide was then cleaved from the resin to give **2b** (Fig. 1). Glycopeptides were further purified by semi-preparative reversed-phase high-performance liquid chromatography (RP HPLC).[17] Calculated values for protonated molecular ions were in agreement with those determined by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) (see Supplementary data for analysis results).

The pharmacological profiles of EM-2 (**1**) and newly synthesized glycosylated peptides **2a** and **2b** were characterized in vitro and in vivo. Receptor binding study was performed as described earlier,[18] using [^{3}H]DAMGO as a selective MOP ligand. The functional potency at MOP was characterized in the guinea pig ileum (GPI) assay, as previously reported.[19] Antinociception was measured by the hot plate test in mice after intravenous (iv) administration of the peptides as a bolus injection at the dose of 3 mg/kg. Additionally, the peripherally restricted opioid antagonist naloxone methiodide (NALME, 1 mg/kg, ip) was used to elucidate the action of **2b** in the CNS. Serum content of **2b** in mice was analyzed using mass spectrometry.[20]

The data are expressed as mean ± SEM. Statistical analysis was performed using Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Student's *t*-test or ANOVA followed by Bonferroni post-hoc testing was used. *P* Values <0.05 were considered statistically significant.

Fmoc-tyrosine pentafluorophenyl esters carrying the sugar moieties (glucose and maltose, either of an α and β-glycosydic bond configuration) were earlier prepared by Jansson and collaborators.[21] However, we envisaged that the Pfp-ester may prove too



Scheme 1. Synthesis of Fmoc-Tyr(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-OH. Reagents and conditions: (i) allyl bromide, DIPEA; DCM, 35 °C, 4 h; 92%; (ii) 2,3,4,6-tetra-*O*-Ac-α-D-glucopyranosyl bromide, AgOTf, 3A MS; DCM, −10 °C to rt, 1 h; 76%; (iii) $Pd(PPh_3)_4$, morpholine; DCM, rt, 1 h; 38%.

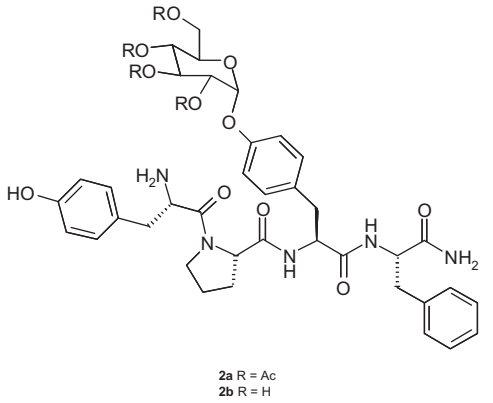


Figure 1. Structures of glycosylated endomorphin-2 analogs, **2a** and **2b**.

weakly reactive towards acylation of the amino acid residue, Phe(4), bound directly to the solid support. Having this in mind we decided to prepare the Fmoc-protected tyrosine derivative with free carboxylic group that would allow for the use of the more reactive coupling reagents like uronium salts (TBTU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate, HATU) or 2-chloro-4,6-bis[3-(perfluorohexyl)propyloxy]-1,3,5-triazine (CDMT).

Therefore, the tyrosine derivative suitable for the solid-phase peptide synthesis was prepared in three steps from the commercially available Fmoc-Tyr(*t*Bu)-OH (Scheme 1). The carboxylic group of the Fmoc-Tyr(*t*Bu)-OH was protected with the allyl ester via the reaction of the carboxylate with the allyl bromide in high yield (92%). The glycosylation of the Fmoc-Tyr(tBu)-OAll was then accomplished by reacting it with the commercially available 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide in presence of silver triflate (AgOTf) and 3Å molecular sieves in dichloromethane. Although the actual configuration of the glycosidic center was of no importance to us at this moment, we wanted the glycosylation reaction to deliver as much homogenous product as possible. It is known that the solvent strongly affects the stereochemical outcome of the glycosylation of the tyrosine: the glycosidic bond of β-configuration prevails in dichloromethane whereas acetonitrile promotes formation of the α-glycosidic bond.[22,23] Furthermore, it is also known that the *tert*-butyl protection of the phenol hydroxylic group actually enhances its nucleophilicity towards the glycosyl donor, what results in the increased yield of the glycosylation reaction. It is believed that the bulkiness of the *tert*-butyl group forces out the phenolic oxygen lone-pairs electrons out of their conjugation with the aromatic ring. Also, it was observed that the presence of the *tert*-butyl protection further increases the β/α-anomer ratio in comparison to the glycosylations run on the phenol-unprotected tyrosine residue.[24] The actual yield of the glycosylation of Fmoc-Tyr(*t*Bu)-OAll with the glucosyl donor in our hands was 76% and we were not able to detect the α-glycosylation product.

The final deprotection of the carboxylic functionality was accomplished by treating the Fmoc-Tyr(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-OAll with Pd(0) catalyst and morpholine as an allyl group scavenger. Workable, though somewhat disappointing 38% yield was encountered.

Receptor studies of the fully assembled peptides revealed a dramatic difference in the binding affinity of the new analogs at the MOP. The acetylated analog **2a** did not bind to the receptor (IC_{50} >1000 nM), while **2b** displayed a potent MOP affinity in the nanomolar range, although approximately 70-fold lower than that of the parent compound (IC_{50} 73.23 ± 3.85 vs 0.99 ± 0.08 for **2b** and **1**, respectively; data for **1** from[18]). However, **2b** showed

significantly higher affinity at MOP compared to Tyr(3)-substituted EM-2 (1100 ± 113 nM, data from Ref. 25). In the GPI assay, **2b** was only about threefold less potent than **1** (IC_{50} 16.1 ± 1.3 vs 4.7 ± 0.5 nM, respectively), while **2a** did not activate MOP (IC_{50} >1000 nM).

As shown in Figure 2A, only **2b** (3 mg/kg) produced a potent antinociception after iv administration in the hot plate test in mice. Peripherally restricted opioid receptor antagonist naloxone methiodide (1 mg/kg, ip) did not block the effect of the glycosylated EM-2, suggesting central site of action of **2b** (Fig. 2B). However, an 'off-target' activity, although unlikely, might also be suggested.

The analysis of mouse blood samples for **2b** contents produced interesting results. We have detected the peptide (M + 39 = 787.2), but we also obtained two signals that may signify the presence of its metabolites (M = 588.4 for deglycosylated and M + 23 = 759.4 for glucuronic derivative of **2b**, respectively) (see Supplementary data for analysis results). We have not observed any signals from **2b**-derived di- or tripeptides, which were expected based on previously reported degradation pathways for EM-2.

Our data show that the glycosylation of the Phe(3) aromatic moiety in EM-2 (**1**) may be an interesting approach to enhance its antinociceptive activity in the CNS, despite lower binding affinity at MOP observed in the in vitro studies. We may suggest that the augmentation of the central effect of **2b** in comparison with the parent peptide results from an increased stability against proteolytic degradation, as well as improved BBB permeability.

EMs are endogenous opioid ligands with high affinity and good selectivity at MOP compared with other opioid receptors.[26] Therefore they are regarded as good candidates for the design of novel opioid-based analgesics with potent biological activity, yet without undesired side effects in the periphery, in particular in the gastrointestinal tract (for review, see Ref. 27). Although several glycosylated analogs of endogenous opioids have been reported,[1,7–10] only some attempts have been made to increase the passage of EMs through the BBB by attachment of the carbohydrate moiety.

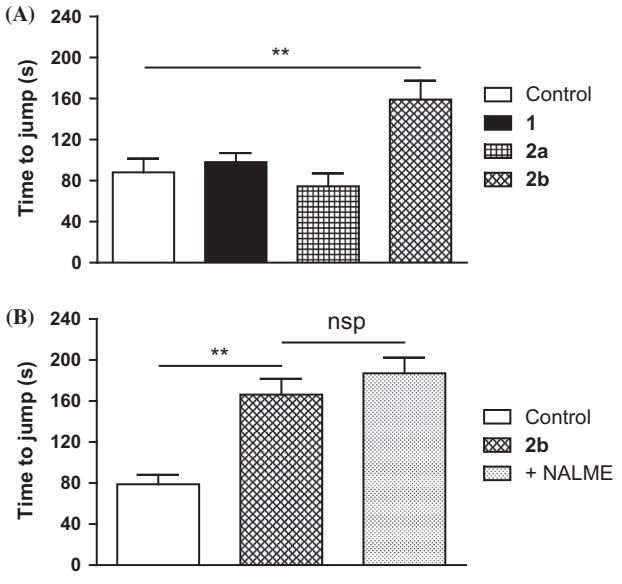


Figure 2. Pharmacological characterization of **2b** in vivo. (A) Glycosylated EM-2 analog **2b** (3 mg/kg, iv) produced a potent antinociceptive action in the hot plate test in mice as shown by the prolonged time to jump compared with vehicle-(controls) and EM-2 (3 mg/kg, iv)-treated animals. (B) The antinociceptive effect of **2b** was not blocked by the peripherally-restricted opioid antagonist naloxone methiodide (NALME, 1 mg/kg, ip), suggesting a central site of action. Results are shown as mean ± SEM of $n = 5–6$ mice for each experimental group. $^{**}p$ <0.01, as compared with control (vehicle treated mice).

Recently, Varamini and collaborators[28] showed that the modification of EM-1 with a lactose residue in position 1 resulted in a significant pain relief after iv and oral administration in rats. Interestingly, similarly to our compound **2b**, modification of EM-1 with lactose led to a decrease in MOP binding affinity and agonist activity, but was still in the nanomolar range.[28] Conjugation of EM-1 with glucose produced similar results.[29] Moreover, in our study the acetylation of hydroxyl groups of the sugar moiety, which increases lipophilicity, decreased the CNS entry of **2a**, what is in line with previous observations for glycosylated dermorphin derivatives.[30] This observation strongly argues for the presence of unsubstituted carbohydrate moiety necessary for good pharmacological activity of glycosylated analogs.

It was postulated previously that the enhancement of the BBB permeability for glycosylated compounds results from an increased uptake by the transmembrane glucose transporter GLUT1 (Ref. 9 and citations therein). However, this hypothesis has been proven incorrect[30] and the exact mechanism by which glycosylation improves BBB transport has yet to be elucidated. Among others, interaction with specific transporters, promotion of the negative membrane curvature on the surface of endothelial cells, which may increase endocytosis and result in enhanced transport through membranes via transcytosis, as well as shifting from hepatic to renal clearance have been suggested.[9,28,30]

In conclusion, the improved BBB permeability and enhanced antinociceptive effect of **2b** suggest that this glycosylated EM-2 analog is a promising template for design of potent analgesics. Furthermore, the described methodology may be useful for increasing the bioavailability and delivery of opioid peptides to the CNS.

Disclosures

The authors have nothing to disclose.

Author contributions

Study concept and design: J.F., J.O.
Peptide design and synthesis: J.F., M.M., J.P., M. S., J.O.
Acquisition and analysis of pharmacological data, statistical analysis: J.F., D.G., R.P., M.S.
Drafting of the manuscript: J.F., J.O.
Obtained funding: J.F., W.K., G.T., A.J., C.C., J.O.
Study supervision: J.F., J.O.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.10.041.

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11. To a solution of Fmoc-Tyr (*t*Bu)-OH (10 g; 21,76 mmol) in DCM, DIPEA (37 ml; 217,6 mmol) and allyl bromide (9,4 ml; 108,8 mmol) were added. The reaction was slightly heated to 35 °C for 4 h, TLC (AcOEt/hexane 1/2) indicated disappearance of the starting material. The reaction mixture was diluted with DCM, washed with 2 M HCl and brine, dried over $MgSO_4$ and concentrated. Residue was purified by column chromatography AcOEt/hexane gradient 1/10 to 1/5. Product was obtained as an yellow oil. Yield 10.0 g (92%).
^{1}H NMR ($CDCl_3$, 200 MHz) δ: 7.79–7.76 (m, 2H), 7.60–7.57 (m, 2H), 7.45–7.27 (m, 4H), 7.04–6.89 (m, 4H), 5.94–5.77 (m,1H), 5.35–5.22 (m, 2H), 4.73–4.60 (m, 3H), 4.49–4.31 (m, 2H), 4.25–4.18 (m, 1H), 3.10 (d, 2H, J = 5.5 Hz), 1.33 (s, 9H).
^{13}C NMR ($CDCl_3$, 600MHz) δ: 171.20, 155.47, 154.39, 143.77, 144.65, 141.22, 131.33, 130.36, 129.74, 127.64, 126.98, 125.02, 124.11, 119.90, 118.98, 78.34, 66.86, 65.97,47.07, 37.57, 28.74. LC/MS M+1 = 500.7.
12. To a mixture of Fmoc-Tyr(*t*Bu)-OAll (3.0 g; 6 mmol), AgOTf (2 g; 9 mmol) and 3Å molecular sieves in dry DCM under argon at −10 °C was added a solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (4.9 g, 12 mmol) in DCM. White suspension was formed. After 1 h at rt, TLC (AcOEt/hexane 1/2) showed no staring material present. The reaction mixture was neutralized with 2 equiv of DIPEA and filtered through a pad of Celite. The filtrate was concentrated and the residue was crystallized from Et_2O. Product was obtained as a white solid. Yield 3.5 g (76%).
^{1}H NMR ($CDCl_3$, 600 MHz) δ: 7.77 (d, 2H, J = 7.5 Hz), 7.56 (d, 1H, J = 7.5 Hz),7.53 (d, 1H, J = 7.5 Hz), 7.42–7.39 (m, 2H), 7.32–7.30 (m, 2H), 7.04 (d, 2H, J = 8.2 Hz), 6.89 (d, 2H, J = 8.2 Hz), 5.92–5.84 (m, 1H), 5.34–5.22 (m, 5H), 5.16–5.12 (m, 1H), 4.96 (d, 1H, J = 6.8 Hz), 4.69–4.62 (m, 2H), 4.46–4.42 (m, 1H), 4.33–4.30 (m,1H), 4.27–4.23 (m,1H), 4.20–4.17(m,1H), 4.12–4.10 (m,1H), 3.78–3.72 (m,1H), 3.16–3.04 (m, 2H), 2.05 (s, 9H), 2.03 (s, 3H). LC/MS M + 1 = 774.5.
13. Solution of Fmoc-Tyr(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-OAll (3,1 g; 4 mmol) in DCM was treated with $Pd(PPh_3)_4$ (120 mg) followed by addition of morpholine (1,3 ml; 15 mmol). After 1 h deprotection was completed. The reaction was diluted with AcOEt, washed with 10% citric acid, brine, dried over $MgSO_4$ and concentrated yielding the yellow oil. Column chromatography (DCM/MeOH, gradient elution 16/1 to 4/1) and crystallization from Et_2O gave pure product as a white solid. Yield 1.1 g (38%).
^{1}H NMR (DMSO, 600 MHz) δ: 7.86 (d, 2H, J = 7.5 Hz), 7.63 (d, 2H, J = 2.5 Hz), 7.61 (d, 2H, J = 2.5 Hz), 7.40–7.37 (m, 2H), 7.31–7.25 (m, 2H), 7.18 (d, 2H, J = 8.3 Hz), 6.85 (d, 2H, J = 8.5 Hz), 5.43–5.34 (m, 2H), 5.02–4.94 (m, 2H), 4.22–4.12 (m, 5H), 4.05–3.97 (m, 2H), 3.03 (dd, 1H, J = 13.7, 4.0 Hz), 2.8 (dd, 1H, J = 13.7, 9.6 Hz), 1.98 (s, 3H), 1.97 (s, 3H), 1.96 (s, 3H), 1.94 (s,3H).
^{13}C NMR (DMSO, 600 MHz) δ: 170.05, 169.68, 169.40, 169.20, 155.88, 155.03, 143.83, 140.72, 130.44, 127.69, 127.13, 125.38, 125.30, 120.16, 116.19, 97.44, 72.01, 70.79, 68.06, 65.57, 61.60, 56.31, 46.64, 36.07, 20.35. LC/MS M + 1 = 734.8.

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15. 20% Piperidine in dimethylformamide (DMF).
16. 8 mM $NaOCH_3$ in DMF/MeOH (17:3), 2 × 3 h.
17. Glycopeptides were further purified by semi-preparative reversed-phase high-performance liquid chromatography (RP HPLC) on a Vydac C_{18} column (10 μm, 22 × 250 mm), equipped with a Vydac guard cartridge. A solvent system of 0.1% trifluoroacetic acid (TFA) in water (A)/80% acetonitrile in water containing 0.1% TFA (B) and a linear gradient of 0–100% B over 15 min was used. Peptide purity was verified by analytical HPLC employing a Vydac C18 column (5 μm, 4.6 mm × 250 mm) and a solvent system of 0.1% TFA in water (A)/80% acetonitrile in water containing 0.1% TFA (B). A linear gradient of 0–100% solvent B over 25 min at a flow rate of 1 mL/min was used for the analysis. Final purity of both peptides was >98%.

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20. Blood samples were withdrawn from mice which received **2B** (3 mg/kg, iv) 10 min after administration of the peptide. After 4 h of precipitation at 4 °C supernatants were collected and centrifuged (3000 rpm, 1 min).
Proteins from obtained supernatants were precipitated by addition of two volumes of acetonitrile. Briefly, to 100 μl of plasma, 200 μl of acetonitrile was added with gentle vortexing. The samples were then left at room temperature for 30 min. The supernatants were retained after ultracentrifugation at 13,000*g* for 15 min and lyophilized.
The samples were then diluted with MeOH 1:1 (V/V) and acidified with 20 μl HCOOH. After preparation the samples were analyzed by electrospray ionization (ESI) and the time of flight (TOF) in micrOTOF-Q II (Bruker) mass spectrometer equipped with a quadrupole mass analyzer. Analyses were performed with the following spectrometer parameters: mass range: 50 ÷ 1200 m/z; polarity: positive; end plate offset: −500 V; capillary: 4500 V; nebulizer: 1 bar; dry gas: 6 l/min; dry temperature: 180 °C. The strong signal and low background allowed the easy identification of peptide metabolite. The experiments were repeated at least twice.

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