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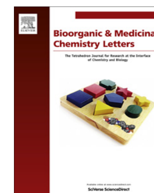

Novel glycosylated endomorphin-2 analog produces potent centrally-mediated antinociception in mice after peripheral administration

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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.

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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.

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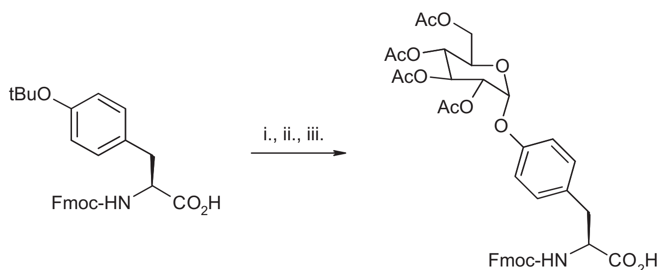
Here we report the synthesis of a novel glycosylated analog of a potent and selective endogenous μ-opioid receptor (MOP) agonist, endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2, EM-2, **1**), obtained by the introduction in position 3 of the tyrosine residue possessing the glucose moiety attached to the phenolic 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%.

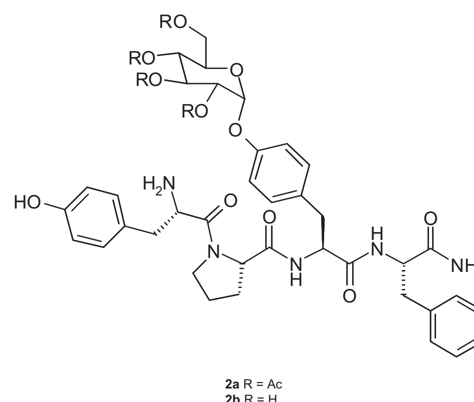


Figure 1. Structures of glycosylated endomorphin-2 analogs, **2a** and **2b**.

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

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