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In Vitro Enzymatic Stabilities of Methionine-enkephalin Analogues Containing an Adamantane-type Amino Acid

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The enzymatic stability of synthetic methionine-enkephalin peptide analogues containing an unnatural amino acid of the adamantane-type **3**-**5** was examined in human serum, at 37 °C, and compared with the results of the degradation of the parent endogenous pentapeptide **1** and the tripeptide, Tyr-Gly-Gly (**2**). Methionine-enkephalin (Tyr-Gly-Gly-Phe-Met, **1**) and tripeptide **2** are rapidly degraded in 80 % human serum with half-lives of 12.2 and 23.0 minutes, respectively, preferably by aminopeptidase cleavage of the *N*-terminal Tyr-Gly peptide bond. Incorporation of the rigid and sterically hindered 1-adamantylglycine moiety into the peptide sequence resulted in increased stability of compound **3**, while compounds **4a** and **5a** were not at all susceptible to the enzymes present in human serum. Strong binding of peptides **3**-**5** to human serum proteins was demonstrated.

INTRODUCTION

Opioid peptides act as cell growth factors, in addition to regulating neurotransmission/neuromodulation in the nervous system. The native opioid growth factor (OGF), methionine-enkephalin (Tyr-Gly-Gly-Phe-Met, **1**), plays a role in cell proliferation and tissue organization during development, cellular renewal, wound healing, and angiogenesis, but also in cancer.[1,2]

To obtain more selective opioid peptides with improved or novel activity profiles toward malignant diseases, modifications using lipophilic moieties may be of particular benefit to passive or active cellular absorption by membrane penetration or attachment. The susceptibility of the modified peptides to enzymatic degradation may also decrease. Recently, we designed and synthesized new analogues of methionine-enkephalin (**1**) by substituting either Gly^2 or Gly^2-Gly^3 in **1** and/or in its shorter *N*-terminal fragments by unnatural adamantane-derived amino acids.[3] Exclusively, the peptide analogues containing $C^{\alpha\alpha}$-dialkylated glycine or C^{α}-alkylated glycine residues showed *in vitro* antitumor activity in the cell lines HEp-2, HBL, SW-620 and Caco-2. In particular, the pentapeptide Tyr-(*R,S*)-Aaa-Gly-Phe-Met (**3**) [Aaa = (*R,S*)-(1-adamantyl)glycine] (Figure 1) showed the most potent tumoricidal activity by inducing apoptosis in the cell lines HEp-2 and SW-620. The obtained data suggested synergism of [(*S*)- and [(*R*)-Aaa]-**3** when investigated together as a racemate. Hence, racemic **3** appears to represents formulation of choice for future screening of this molecule for cytotoxic properties. In addition, the diastereomeric tripeptides Tyr-(*S*)-Aaa-Gly (**4a**) and Tyr-(*R*)-Aaa-Gly (**4b**) showed similar, cell-selective, cytotoxic effects on tumor cell lines irrespective of Aaa chirality.[3]

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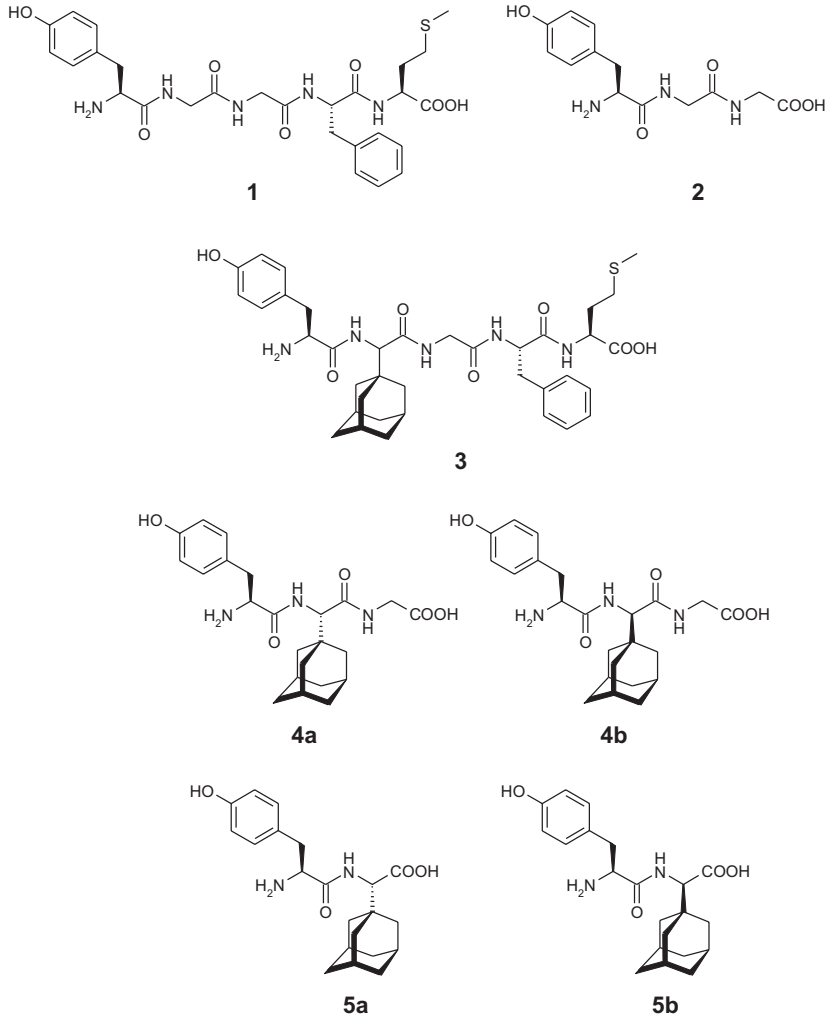

Figure 1. Structure of peptides **1**-**5**.

In this paper we compare the metabolic stability of pentapeptide **3**, of the tripeptides **4** and of the dipeptides Tyr-Aaa (**5**) in diluted human serum with serum stability of methionine-enkephalin (**1**) and its shorter *N*-terminal tripeptide fragment, Tyr-Gly-Gly (**2**).

EXPERIMENTAL

Methionine-enkephalin (**1**) and Tyr-Gly-Gly (**2**) were purchased from Bachem. Human serum and *p*-nitro-L-phenylalanine were obtained from Sigma-Aldrich. *o*-Hydroxyphenylacetic acid was obtained from Fluka. Literature procedures were used for the synthesis of Tyr-(*R,S*)-Aaa-Gly-Phe-Met (**3**) [Aaa = (*R,S*)-(1-adamantyl)glycine], Tyr-(*S*)-Aaa-Gly (**4a**), Tyr-(*R*)-Aaa-Gly (**4b**), Tyr-(*S*)-Aaa (**5a**) and Tyr-(*R*)-Aaa (**5b**).[3]

*Stability of Peptides **1**-**3**, **4a** and **5a** in Human Serum*

For serum stability studies, an aliquot (8×10^{-4} mol dm^{-3}) of the respective peptide (**1**-**3**, **4a**, **5a**), supplemented with the internal standards, *o*-hydroxyphenylacetic acid (40 μg/mL) and *p*-nitro-L-phenylalanine (20 μg/mL), was dissolved in 80 % human serum (5 mL, diluted with water), and kept at 37 °C in a teflon lined screw-cap test tube. Three samples (0.1 mL) were removed at appropriate time intervals and deproteinized by addition of 48 % aqueous TFA (0.02 mL). The samples were briefly vortexed and frozen. The thawed samples were centrifuged for 10 min (15000 g) and the concentration of the starting compounds and metabolites in the supernatants was monitored by RP HPLC on an analytical column (250 × 4 mm I.D., 5 μm) at a flow rate of 0.5 mL/min, with 40 % MeOH/0.1 % TFA for compound **1**, 20.25 % MeOH/0.1 % TFA for compound **2** and tyrosine, and 50.5 % MeOH/0.1 % TFA for compounds **3**, **4a** and **5a**, using a HP 1090 system equipped with a diode-array detector. UV absorbance was monitored at 280 and 215 nm.

*Binding of Peptides **1**-**3**, **4a** and **5a** to Human Serum Proteins*

The affinity of serum proteins for peptides **1**-**3**, **4a** and **5a** was analyzed by dissolving peptide compound (8×10^{-4} mol dm^{-3}), and the internal standards, *o*-hydroxyphenylacetic acid (40 μg/mL) and *p*-nitro-L-phenylalanine (20 μg/mL),

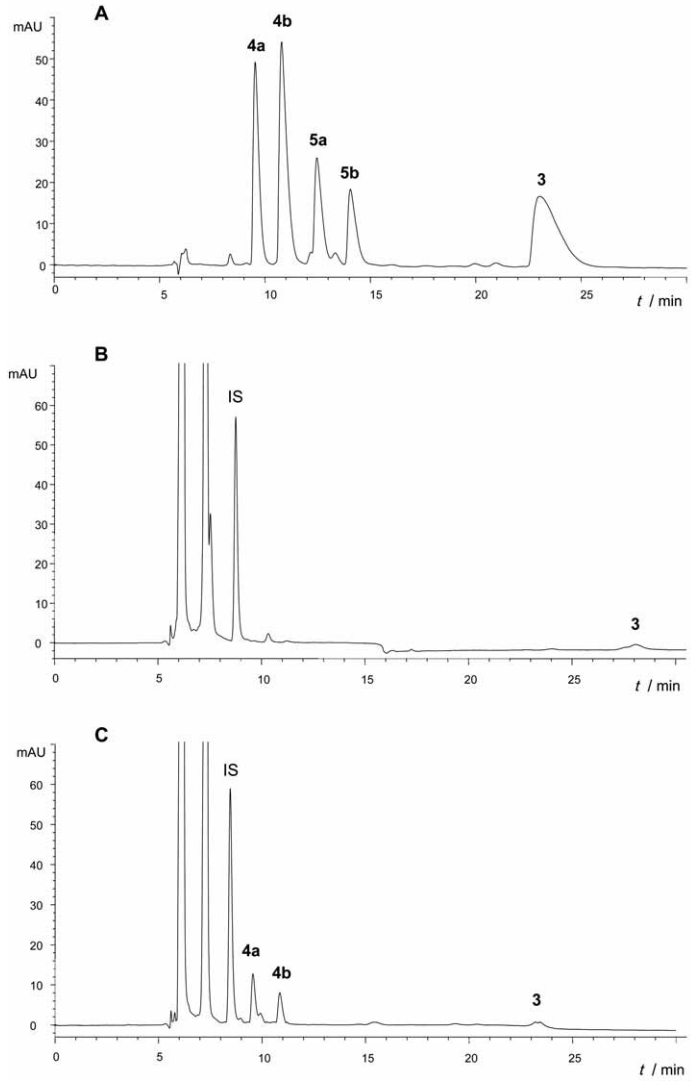

Figure 2. RP HPLC elution profile of (A) peptides **3**-**5**; (B) deproteinized solution of compound **3** in 80 % human serum at $t = 0$; (C) deproteinized solution of compound **3** in 80 % human serum after 4 days of incubation at 37 °C. The mobile phase was 50.5 % MeOH/0.1 % TFA at a flow of 0.5 mL/min. Each compound was dissolved individually in phosphate buffer (pH = 7.4) (panel A) or in human serum (panels B and C) at concentration of 8×10^{-4} mol dm^{-3}. The injection volume was 20 μL. The absorbance was recorded at 280 nm. IS = internal standard (o-hydroxyphenylacetic acid). Observe the decreased absorbance of peptide **3** in panels B and C due to binding to serum proteins (see Experimental and Figure 3 for details).

in water, in 80 % human serum or in 0.0008 mol dm^{-3} phosphate buffer/0.1 mol dm^{-3} NaCl (pH = 7.4) containing human serum albumin (HSA) (50 mg/mL). The samples dissolved in human serum or in HSA were deproteinized as described above and analyzed by RP HPLC in solvent systems indicated in the previous section. The concentration of the unbound peptide was determined by electronic integration of the peak areas and calculation of the analyte/internal standard peak-ratios.

RESULTS AND DISCUSSION

It is known that enkephalins and their shorter fragments are rapidly degraded in human blood plasma, the predominant (≈ 80 %) route for the degradation being hydrolysis of the *N*-terminal Tyr^1-Gly^2 bond by aminopeptidases.[4,5]

Proteolytic degradations of peptides **1**-**5** was examined at 37 °C in 80 % human serum. In agreement with published results, we found rapid disappearance of peptides **1** and **2** in human serum. Specifically, the half-lives for methionine-enkephalin (**1**) and Tyr-Gly-Gly (**2**) were 12.2 min and 23.0 min, respectively. While methionine-enkephalin (**1**) itself was rapidly degraded, the pentapeptide analogue Tyr-(*R,S*)-Aaa-Gly-Phe-Met (**3**) was very slowly converted to tripeptides **4a** and **4b** (Figures 2B, 2C) indicating that only dipeptidyl carboxypeptidase(s) are involved in the hydrolysis of peptide **3**. After 4 days of incubation in human serum, the relative amounts of tripeptides **4a** and **4b** reached only 19 % and 16 %, re-

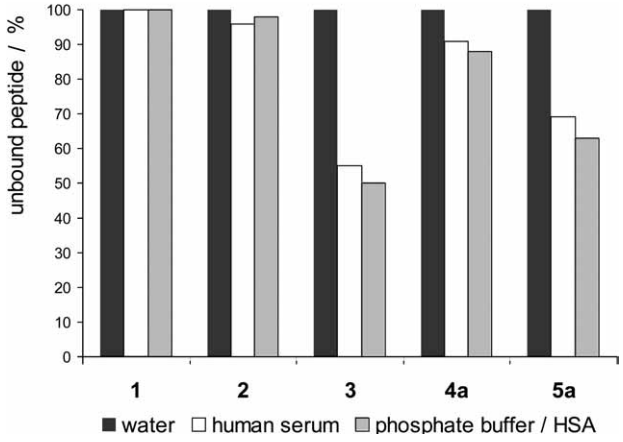

Figure 3. Comparison of the binding affinities of peptides **1**-**3**, **4a** and **5a** for serum proteins. The relative concentrations of unbound peptides were determined by RP HPLC.

spectively. The structurally related smaller peptides Tyr-(*S*)-Aaa-Gly (**4a**) and Tyr-(*S*)-Aaa (**5a**), under identical experimental conditions, showed no sign of degradation over the time span investigated (14 days). The stability of the corresponding diastereomers **4b** and **5b** could not be determined because of their water-insolubility.

Binding to serum proteins profoundly affects the suitability of bioactive peptides for biomedical application. Based on the observation that the absorbance of Tyr-(*R,S*)-Aaa-Gly-Phe-Met (**3**) at 280 nm decreased after dissolution in human serum (Figures 2B, 2C), the absorbances of peptides **1**-**3**, **4a** and **5a** were compared following dissolution in water, 80 % human serum or in a solution of human serum albumin (HSA). As illustrated in Figure 3, after dissolution in human serum, and deproteinization, the absorbance of compounds **3**, **4a** and **5a** decreased by 45 %, 9 % and 31 %, respectively, while the absorbance of methionine-enkephalin (**1**) and Tyr-Gly-Gly (**2**) remained unchanged. Almost identical results were obtained when the respective peptides were dissolved in a solution of HSA; this suggests that the observed decrease in absorbance is likely due to binding to the latter (major) serum protein.

In conclusion, the enzymatic stability and serum protein affinity of methionine-enkephalin and its analogues **3**-**5** were compared. The incorporation of the rigid and sterically hindered 1-adamantylglycine moiety into the peptide sequence resulted in increased stability of compound **3**, while compounds **4a** and **5a** were totally insusceptible to the enzymes present in human serum. Strong binding of peptides **3**-**5** to human serum proteins was demonstrated.

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SAŽETAK

In Vitro enzimska stabilnost analoga metionin-enkefalina s ugrađenom neprirodnom aminokiselinom adamantanske strukture

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Ispitana je enzimska stabilnost sintetskih peptidnih analoga metionin-enkefalina, koji sadrže neprirodnu aminokiselinu adamantanske strukture **3**-**5**, u humanom serumu pri 37 °C, te uspoređena s rezultatima enzimske razgradnje ishodnih peptida, endogenog pentapeptida **1** i tripeptida, Tyr-Gly-Gly (**2**). Ustanovljena je vrlo brza hidroliza metionin-enkefalina (**1**) ($t_{1/2}$ = 12,2 min) i tripeptida **2** ($t_{1/2}$ = 23,0 min) u humanom serumu, mehanizmom koji se zbiva ponajprije djelovanjem aminopeptidaze na Tyr-Gly peptidnu vezu. Ugradnja rigidnog i voluminoznog 1-adamantilglicinskog ostatka u peptidnu strukturu uzrokuje povećanu stabilnost spoja **3** i potpunu stabilnost spojeva **4a** i **5a** prema enzimima prisutnim u humanom serumu. Uočena je sklonost vezanja peptida **3**-**5** na proteine humanog seruma.