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ORIGINAL ARTICLE

Physicochemical characteristics and in vitro release from oil-based vehicles of peptidomimetics: parenteral depots for intra-articular administration

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Abstract

Results: Basic physicochemical properties including their apparent solubility in aqueous buffer and vegetable oils of a series of 11 peptidomimetics varying with respect to chain length and degree of N-methylation were estimated. It was observed that the compounds in contact with water transformed into sticky, slowly dissolving semisolid materials. Based on these observations, the in vitro release behavior of selected peptide derivatives from oil solutions and in situ formed precipitates was investigated using a validated in vitro release model. *Conclusion*: The results of this investigation suggest that both types of oil-based drug delivery systems might constitute alternative sustained release formulation principles of such amorphous peptide derivatives for the intra-articular route of administration.

Key words: Depot formulation, in situ forming DDS, intra-articular drug delivery, oil solution, semisolid precipitates

Introduction

The design of suitable dosage forms for small polar peptides may constitute a significant challenge. In addition, transport to the site of action of such active agents is often hampered by extensive enzymatic degradation and poor biomembrane transport properties[1,2]. Improved biopharmaceutical properties may result from using the prodrug approach[3–5] or the design of peptidomimetics, for example by incorporation of D-amino acids or backbone modifications in the form of N-methylation and amide bond isosteres[6–10]. To this end, focus has mainly been directed toward enhancing oral bioavailability. However, it has recently been reported that epidermal growth factor and other endogenous peptidic growth factors may play a major role in restoration of the articular cartilage. Intra-articular (IA) administration of such peptides or peptidomimetics hereof might therefore be of potential interest in the management of osteoarthritis[11]. Small-molecule drugs are rapidly cleared from the synovial space after injection. Maintenance of therapeutic drug concentrations over extended time periods can be achieved by repeated IA administrations or, more ideally, by immobilization of the active agent in the form of an injectable depot formulation from which the drug is released in a controlled manner[12]. The majority of the experimental depot formulation principles investigated after injection into the joint cavity belongs to the group of microparticulate drug delivery systems (DDSs)[12], the development of which may encounter problems with poor physical stability and sterile manufacture. Hence, oil solutions and liquid in situ forming DDS approaches[13–15] might therefore constitute alternative IA depot formulation types.

The influence of structural features on biomembrane permeability of small peptides has been the subject of several studies[1,6–10,16]. As concerns basic characteristics needed for formulation design, including solubility in

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aqueous as well as nonaqueous media, only sparse information is available. In this study, basic physicochemical properties of 11 synthesized model tri-, tetra-, and pentapeptidomimetics varying with respect to degree of N-methylation have been estimated. Furthermore, the in vitro release behavior of selected peptide derivatives from oil solutions and in situ formed semisolid precipitates using a validated in vitro release model is reported. This dialysis membrane-based model allows for the study of solute transport processes in an environment mimicking that therapeutic agents may be exposed to after IA administration[17].

Materials and methods

Materials

Chemicals used in the synthesis work were purchased from Sigma (St. Louis, MO, USA), BaChem AG (Bubendorf, Switzerland), Advanced Chemtech (Louisville, KY, USA), or Merck (Darmstadt, Germany) and used without further purification with the exception of tetrahydrofuran (THF) and *N*,*N*-dimethyl-formamide (DMF), which were dried and stored over 3 Å molecular sieves.

2-Propanol (>99.7%) was purchased from Merck (Darmstadt, Germany) and *N*-methyl-2-pyrrolidone (NMP), sesame oil, castor oil, 20% technical grade solution poly(diallyldimethylammonium chloride) (PDMAC) M_w 400,000–500,000, and 25% technical grade solution poly(vinylsulfonate) sodium salt (PVS) M_w 4000–6000 were purchased from Sigma-Aldrich (Steinheim, Germany). Chemicals for preparation of buffers and high-performance liquid chromatography (HPLC)-mobile phases were of at least analytical grade. For the solubility, distribution, and release experiments a 67 mM phosphate buffer pH 7.4 was used unless otherwise stated. Purified water from a Milli-Q deionization unit (Millipore, Bedford, MA, USA) was used for the capillary electrophoresis (CE) measurements. For all other experiments deionized water was used. Visking dialysis, tubing size 27/32, 21.5 mm, MW cut-off 12–14 kDa (VWR International, West Chester, PA, USA), was employed in the dialysis experiments.

Synthesis of model peptides

Conventional solution-phase methodologies were used for the preparation of 12 peptide analogs as illustrated in Figure 1. The yield of peptide analog 5,4 was very low

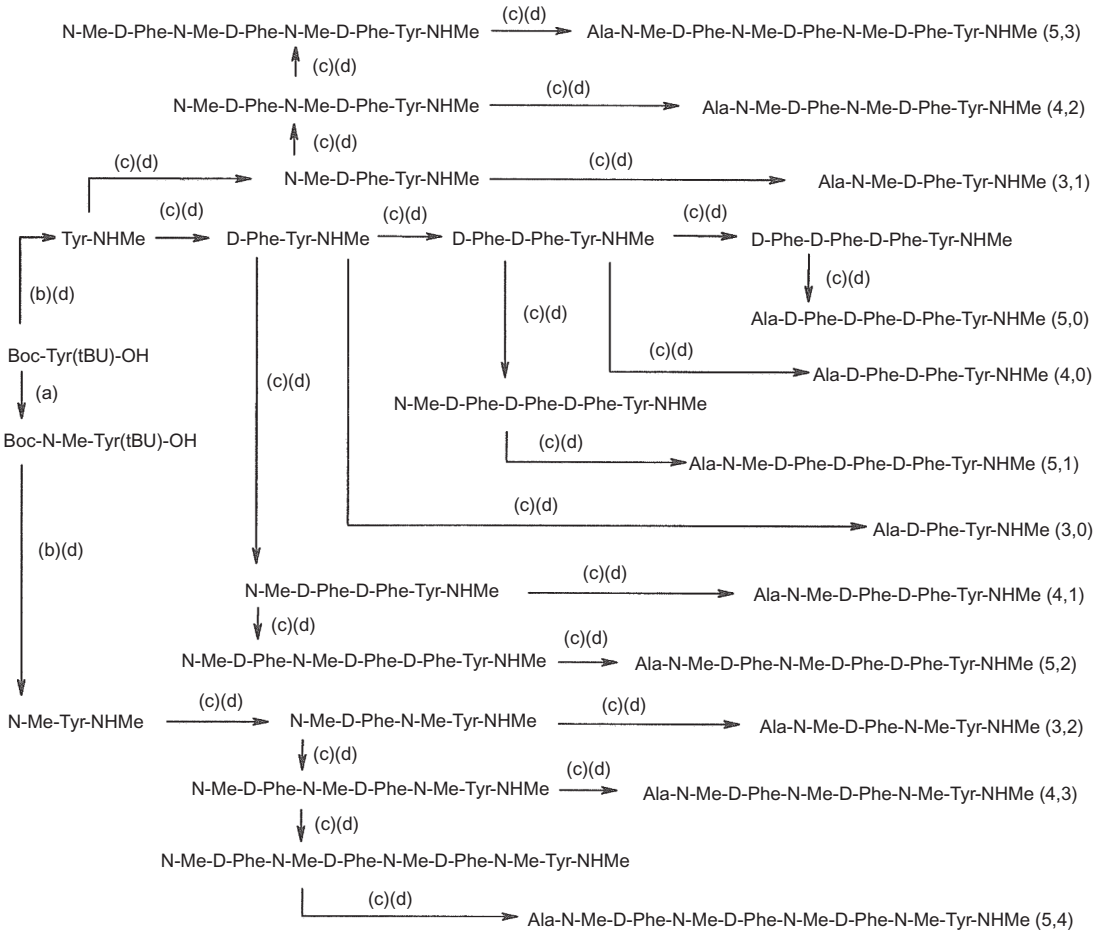
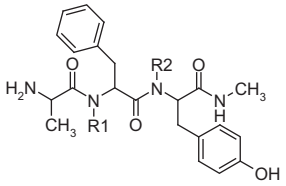
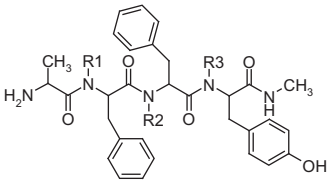
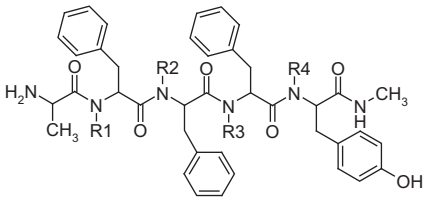


Figure 1. Synthesis of peptidomimetics. (a) N-Methylation of tyrosine; (b) coupling of amide bonds (I); (c) coupling of amide bonds (II); and (d) removal of protection of Boc group. Please refer to text for details and general procedures.

Table 1. Sequence and structure of the investigated *N*-methyl amide peptidomimetics.

Peptide	Sequence	R1	R2	R3	R4	Structure
3,0	Ala-D-Phe-Tyr-NHMe	H	H			
3,1	Ala-ΨD-Phe-Tyr-NHMe	CH_3	H			
3,2	Ala-ΨD-Phe-ΨTyr-NHMe	CH_3	CH_3			
4,0	Ala-D-Phe-D-Phe-Tyr-NHMe	H	H	H		
4,1	Ala-ΨD-Phe-D-Phe-Tyr-NHMe	CH_3	H	H		
4,2	Ala-ΨD-Phe-ΨD-Phe-Tyr-NHMe	CH_3	CH_3	H		
4,3	Ala-ΨD-Phe-ΨD-Phe-ΨTyr-NHMe	CH_3	CH_3	CH_3		
5,0	Ala-D-Phe-D-Phe-D-Phe-Tyr-NHMe	H	H	H	H	
5,1	Ala-ΨD-Phe-D-Phe-D-Phe-Tyr-NHMe	CH_3	H	H	H	
5,2	Ala-ΨD-Phe-ΨD-Phe-D-Phe-Tyr-NHMe	CH_3	CH_3	H	H	
5,3	Ala-ΨD-Phe-ΨD-Phe-ΨD-Phe-Tyr-NHMe	CH_3	CH_3	CH_3	H	

Abbreviation: Ψ, *N*-methyl amide isostere (Ψ[$CONCH_3$]).

thus preventing the use of this peptide analog for further study and hence this peptide analog is not included in Table 1. All peptide analogs were purified by preparative HPLC. The purity of the final products (95.0–98.8%) was evaluated by ^{1}H nuclear magnetic resonance (NMR) spectroscopy, analytical reversed-phase HPLC (two different gradient systems with UV detection at 214, 254, 276, and 301 nm), liquid chromatography-mass spectrometry (LC-MS) (single quadrupole mass spectrometer detector and electrospray ionization in positive ion made mode), and thermogravimetric analysis.

The following are the general synthetic methods for preparation of peptide analogs:

1. N-Methylation of tyrosine. General procedure: 37 mL of methyl iodide (592 mmol, 8 equiv.) was added to a stirred solution of Boc-Tyr-OtBu (25.0 g, 74.0 mmol) in dried THF (250 mL), the mixture was ice cooled and 88.8 g sodium hydride (60% dispersion in oil, 222 mmol, 3.0 equiv.) was added and the mixture was stirred overnight. Two hundred and fifty milliliters of water was added and the aqueous phase was washed with diethylether. $NaHSO_4$ (10%) was added until pH 2, and the product was extracted with ethyl acetate (EtOAc). The combined organic phases were washed with water, dried ($MgSO_4$) and concentrated in vacuo to afford Boc-N-Me-Tyr(tBu)-OH.
2. Coupling of amide bond (i). General procedure: Crude Boc-N-Me-Tyr(tBu)-OH (23.6 g, 67 mmol) and 1-hydroxybenzotriazole hydrate (HOBT) (9.05 g, 67 mmol) were dissolved in DMF (400 mL) and cooled in an ice-bath. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) (13.4 g, 70 mmol) was added and the mixture was stirred for 30 minutes. Methylamine [9.5 g, 33% (w/w) in methanol, 100 mmol] was added and the mixture was stirred for 2 days. EtOAc (500 mL) and water (500 mL) were added and the phases were separated. The aqueous phase was washed with EtOAc (500 mL). The combined organic phases were washed with saturated $NaHCO_3$ (250 mL), $NaHSO_4$ (400 mL, 10%), and water (400 mL). The organic phase was dried ($MgSO_4$) and concentrated in vacuo to afford Boc-N-Me-Tyr(tBu)-NHMe.
3. Coupling of amide bond (ii). General procedure: A solution of Boc-N-Me-D-Phe-OH (17.5 g, 62.8 mmol) in 150 mL of DMF was placed in a one-necked round-bottomed flask equipped with a magnetic stirrer and a nitrogen bubbler. 3,4-Dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (DHOBT) (10.2 g, 62.8 mmol)) and *N*-methylmorpholine (NMM) (7.1 mL, 62.8 mmol) was added to the solution at room temperature. Immediately after 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) (12.6 g, 65.9 mmol), NMM (14.3 mL, 126 mmol), and N-Me-Tyr-NHMe (crude 62.8 mmol) were added. The reaction was stirred overnight at room temperature. The mixture was added to 750 mL of EtOAc and

washed with 300 mL of aqueous $NaHSO_4$ (10%). The layers were separated, and the aqueous layer was re-extracted with an additional 500 mL of EtOAc. The combined organic layers were washed with 100 mL of water, 300 mL of aqueous $NaHCO_3$ (saturated), 100 mL of water, dried over $MgSO_4$, filtered and concentrated in vacuo to afford Boc-N-Me-D-Phe-N-Me-Tyr-NHMe.

4. The Boc-protecting groups were removed by treatment with either hydrogen chloride in ethyl acetate or hydrogen chloride in dioxane. General procedure: To crude Boc-N-Me-D-Phe-N-Me-Tyr-NHMe dissolved in 30 mL of dioxane 47 mL of 4N HCl in dioxane was added. The mixture was stirred under nitrogen until gas production was no longer observed. The volatiles were removed in vacuo and the residue was dissolved in water (200 mL) and the aqueous layer was washed with EtOAc (2 × 200 mL). The combined organic layers were washed with water (100 mL, pH 3). The combined aqueous layers were adjusted to pH 8 with $NaHCO_3$ and extracted with EtOAc (3 × 200 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated in vacuo to afford N-Me-D-Phe-N-Me-Tyr-NHMe.

The peptides synthesized using the outlined procedures are depicted in Table 1. No efforts were given to optimize the syntheses.

Peptide analog 3,0. ^{1}H NMR (400 MHz, CD_3OD) δ: 1.07–1.17 [d, 3H, CH_3 (Ala side chain)], 2.60 [s, 3H, CH_3 (Tyr-NH-CH_3)], 2.58–3.10 (m, 4H, 2 × CH_2), 3.43–3.36 (q, 1H, α1), 4.33–4.69 (m, 2H, α2 and α3), 6.56–7.37 (m, 9H, aromatic protons); LC-MS: m/z = 413.2 (m + 1).

Peptide analog 3,1. ^{1}H NMR (400 MHz, CD_3OD) δ: 0.66–0.77 [d, 3H, CH_3 (Ala side chain)], 2.62–3.21 [m, 10H, CH_3 (N-CH_3), CH_3 (Tyr-NH-CH_3), 2 × CH_2], 3.67–3.80 (q, 1H, α1), 4.45–4.58 (dd, 1H, α3), 5.30–5.45 (dd, 1H, α2), 6.63–7.36 (m, 9H, aromatic protons); LC-MS: m/z = 427.1 (m + 1).

Peptide analog 3,2. ^{1}H NMR (400 MHz, CD_3OD) δ: 0.54–0.86 [m, 3H, CH_3 (Ala side chain)], 2.44–3.24 [m, 13H, 2 × CH_3 (2 × N-CH_3), CH_3 (Tyr-NH-CH_3), 2 × CH_2], 3.35–3.84 (m, 1H, α1), 4.60–5.76 (m, 2H, α2 and α3), 6.63–7.28 (m, 9H, aromatic protons); ^{1}H NMR (400 MHz, DMSO-d6, 110°C) δ: 0.9 [s, 3H, CH_3 (Ala side chain)], 2.5–3.2 (m, 13H, 3 × N-CH_3), 2 × CH_2), 3.65 (s, 1H, α1), 5.1 and 5.5 (2s, 2H, α2 and α3), 6.6–7.3 (m, 9H, aromatic protons); LC-MS: m/z = 441.1 (m + 1).

Peptide analog 4,0. ^{1}H NMR (400 MHz, CD_3OD) δ: 1.04–1.12 [d, 3H, CH_3 (Ala side chain)], 2.64–3.10 [m, 9H, 3 × CH_2 and CH_3 (Tyr-NH-CH_3)], 3.44–3.58 (q, 1H, α1), 4.38–4.59 (m, 3H, α2, α3 and α4), 6.62–7.31 (m, 14H, aromatic protons); LC-MS: m/z = 560.2 (m + 1).

Peptide analog 4,1. ^{1}H NMR (400 MHz, CD_3OD) δ: 0.63–0.95 [m, 3H, CH_3 (Ala side chain)], 2.56–3.28 [m, 12H, 3 × CH_2 and 2 × CH_3 (N-CH_3 and Tyr-NH-CH_3)], 3.57–3.69 (q, 1H, α1), 4.42–4.63 (m, 2H, α3 and α4), 4.64–4.71 (m, 1H, α2), 6.64–7.34 (m, 14H, aromatic protons); LC-MS: m/z = 574.2 (m + 1).

Peptide analog 4,2. ^{1}H NMR (400 MHz, CD_3OD) δ: 0.52–0.71 [m, 3H, CH_3 (Ala side chain)], 2.16–3.21 [m, 15H, 3 × CH_2 and 3 × CH_3 (2 × N-CH_3 and Tyr-NH-CH_3)], 3.35–3.68 (m, 1H, α1), 4.45–4.59 (m, 1H, α4), 5.12–5.76 (m, 2H, α2 and α3), 6.66–7.39 (m, 14H, aromatic protons); LC-MS: m/z = 588.3 (m + 1).

Peptide analog 4,3. ^{1}H NMR (400 MHz, CD_3OD) δ: 0.53–0.83 [m, 3H, CH_3 (Ala side chain)], 2.08–3.23 [m, 18H, 3 × CH_2 and 4 × CH_3 (3 × N-CH_3 and Tyr-NH-CH_3)], 3.38–3.69 (m, 1H, α1), 5.02–5.85 (m, 3H, α2, α3, and α4), 6.62–7.38 (m, 14H, aromatic protons); LC-MS: m/z = 602.2 (m + 1).

Peptide analog 5,0. ^{1}H NMR (400 MHz, CD_3OD) δ: 1.01–1.10 [d, 3H, CH_3 (Ala side chain)], 2.62–3.10 [m, 11H, 4 × CH_2 and CH_3 (Tyr-NH-CH_3)], 3.49–3.59 (q, 1H, α1), 4.37–4.68 (m, 4H, α2, α3, α4, and α5), 6.59–7.33 (m, 19H, aromatic protons); LC-MS: m/z = 707.2 (m + 1).

Peptide analog 5,1. ^{1}H NMR (400 MHz, CD_3OD) δ: 0.59–0.93 [m, 3H, CH_3 (Ala side chain)], 2.44–3.24 [m, 14H, 4 × CH_2 and 2 × CH_3 (N-CH_3 and Tyr-NH-CH_3)], 3.53–3.65 (q, 1H, α1), 4.40–4.72 (m, 3H, α3, α4, and α5), 5.34–5.46 (dd, 1H, α2), 6.62–7.36 (m, 19H, aromatic protons); LC-MS: m/z = 721.3 (m + 1).

Peptide analog 5,2. ^{1}H NMR (400 MHz, CD_3OD) δ: 0.40–0.70 [m, 3H, CH_3 (Ala side chain)], 2.06–3.20 [m, 17H, 4 × CH_2 and 3 × CH_3 (2 × N-CH_3 and Tyr-NH-CH_3)], 3.33–3.43 (m, 1H, α1), 4.38–4.78 (m, 2H, α4 and α5), 5.13–5.61 (m, 2H, α2 and α3), 6.58–7.35 (m, 19H, aromatic protons); LC-MS: m/z = 735.3 (m + 1).

Peptide analog 5,3. ^{1}H NMR (400 MHz, CD_3OD) δ: 0.45–0.99 [m, 3H, CH_3 (Ala side chain)], 1.79–3.22 [m, 20H, 4 × CH_2 and 4 × CH_3 (3 × N-CH_3 and Tyr-NH-CH_3)], 3.33–3.63 (m, 1H, α1), 4.43–4.63 (m, 1H, α5), 5.07–5.77 (m, 3H, α2, α3, and α4), 6.64–7.43 (m, 19H, aromatic protons); LC-MS: m/z = 749.2 (m + 1).

Peptide analog 5,4. ^{1}H NMR (400 MHz, CD_3OD) δ: 0.82–1.20 [m, 3H, CH_3 (Ala side chain)], 1.80–3.22 [m, 23H, 4 × CH_2 and 5 × CH_3 (4 × N-CH_3 and Tyr-NH-CH_3)], 3.59–3.82 (m, 1H, α1), 4.99–5.90 (m, 4H, α2, α3, α4, and α5), 6.64–7.41 (m, 19H, aromatic protons); LC-MS: m/z = 763.3 (m + 1).

Stability in aqueous buffer solution

The stability of the model peptides was investigated in 67 mM phosphate buffer pH 7.4 (PBS) at 37 ± 0.5°C. The reactions were initiated by dissolving an amount of peptide in 10 mL of preheated buffer or by adding 100 µL of peptide stock solution in methanol or acetonitrile to 10 mL of preheated PBS to give an initial concentration of approximately 1×10^{-4} M (or less in case of poorly soluble derivatives). At appropriate time intervals, samples were withdrawn and analyzed immediately by HPLC. For peptidomimetics undergoing degradation, pseudo-first-order degradation rate constants (k_{obs}) were determined from the slopes of linear plots of the logarithm of

intact compound against time. All experiments were carried out in triplicate.

Determination of apparent solubility and distribution coefficients

The apparent solubility of the peptides was investigated in PBS, sesame oil, and castor oil at 37 ± 0.5°C. Excess model peptide was added to PBS and the mixture rotated at 37°C in an TH30 incubator hood (Johanna Otto, Hechingen, Germany) until attainment of a constant concentration (1–2 days). When needed pH was adjusted to 7.40 by addition of 4M HCl. A sample was withdrawn and centrifuged at 6700 × g for 10 minutes. An aliquot of the supernatant was diluted with PBS prior to HPLC analysis. The apparent solubility of the peptides in sesame oil was examined in a similar fashion except that rotation for 4 days was necessary for attainment of equilibrium and samples were diluted with 2-propanol prior to HPLC analysis. The solubility experiments were done in triplicate. The apparent solubility of the peptides in castor oil was estimated by adding accurately weighed portions of peptide (approximately 2 mg) successively to 1.0 mL of castor oil in screwcapped vials until undissolved peptide was observed by visual inspection.

The distribution of the model peptides between (i) 1-octanol and PBS, (ii) sesame oil and PBS, and (iii) castor oil and PBS was investigated at 37 ± 0.5°C. The distribution coefficients were calculated from the concentration of the peptides in the aqueous phase as measured by HPLC before and after the distribution process. The sample preparations were carried out in the incubator hood and all equipment used were preheated to 37 ± 0.5°C. The distribution experiments were done in triplicate.

Determination of pK_a values using capillary electrophoresis

Sample and buffer preparation

Stock solutions of the peptides were prepared in dimethylsulfoxide (DMSO). Sample solutions were obtained by dilution of the stock solutions with water to give a DMSO content of 1% (v/v) and peptide concentrations of about 20 μg/mL. Buffer solutions covering the pH range 1.3–12 were prepared by mixing buffer stock solutions to give the desired pH and an ionic strength of 0.05 M essentially as described by Ishihama et al.[18] CE buffer solutions were filtered through 0.45 μm nylon filters (Chromacol LTD, Herts, UK) prior to use.

Instrumentation and capillary electrophoresis procedures

Capillary electrophoresis (CE) experiments were performed on a Hewlett-Packard ^{3D}CE (Avondale, PA, USA) equipped with a diode-array detector. Fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA) and dynamically coated with PDMAC and PVS as described previously[19]. A PDMAC/PVS-coated capillary of 32 cm × 50 μm ID, with a length of 24 cm to the detector was used. UV detection was performed at 200, 214, 230, and 250 nm. Injection was performed from the short end of the capillary by applying pressure (50 mbar) for 2 seconds. The capillary cassette temperature was set to 20°C. The applied voltage in the normal polarity mode was adjusted (6.5–20 kV) to give a mean capillary temperature of 25°C as calculated by the method of Kok[20]. Between measurements the capillary was flushed with 0.1% PVS solution and electrophoresis buffer solution for 1 minute each.

Data analysis

The effective electrophoretic mobility μ_{eff} was calculated from

$$\mu_{\text{eff}} = \frac{l_c l_d}{V}\left(\frac{1}{t} - \frac{1}{t_0}\right), \tag{1}$$

where l_c is the total capillary length; l_d is the length of the capillary from the inlet end to the detector; V is the applied voltage; t and t_0 are the measured migration times of the analyte and the electroosmotic flow, respectively.

The pK_a values were determined from Equation (2) by nonlinear regression analysis using the SigmaPlot 2000 software package (SPSS Inc., Chicago, IL, USA):

$$\mu_{\text{eff}} = \frac{10^{pK_1 - pH} \cdot \mu_{BAH_2^+} + 10^{pH - pK_2} \cdot \mu_{BA^-}}{1 + 10^{pK_1 - pH} + 10^{pH - pK_2}}, \tag{2}$$

where pK_1 and pK_2 are the ionization constants and $\mu_{BAH_2^+}$ and μ_{BA^-} are the electrophoretic mobilities of the fully protonated and deprotonated species, respectively.

Release experiments

The release studies were performed at 37 ± 0.5°C using the rotating dialysis cell described previously[17,21,22]. At time zero, the dialysis cell containing PBS (5 mL) and an aliquot of the test formulation was placed inside a round-bottomed vessel containing 1000 mL of the release medium (PBS) preheated to 37 ± 0.5°C. The revolution speed of the dialysis cell was set at 50 rpm. At appropriate times, 1500 μL samples for HPLC analysis were withdrawn from the acceptor phase. The cumulated amount of diffusant appearing in the acceptor phase ($M_{A,t}$) was calculated according to

$$M_{A,t} = V_S \sum_{i=1}^{i} C_{i-1} + V_A C_i, \tag{3}$$

where V_A and V_S are the volumes of acceptor phase and samples withdrawn from the acceptor phase, respectively. C_i is the drug concentration in sample i. All release experiments were followed until equilibrium was

attained in the system and carried out in triplicate unless otherwise indicated.

Analysis

For characterization of the peptide synthesis products, NMR spectroscopy was carried out on a Bruker AMX2 spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 400 MHz for ^{1}H, and shifts (δ) are given in parts per million (ppm). The peptide analogs (4.5–8.1 mg) were dissolved in 600 µL CD_3OD (99.8% D) spiked with 0.05% Tetramethylsilane (TMS) (Lab-Science ApS, Copenhagen, Denmark). The experiments were performed at room temperature. At room temperature the peptides containing methylated amide bonds gave rise to multiple peaks resulting from different conformers of the peptides. For peptide 3,2 NMR spectra was recorded in DMSO-d6 at increased temperatures (27°C, 50°C, 70°C, 90°C, 110°C) and it was observed that the multiple peaks were joined to single peaks as a result of the sufficient energy available to overcome the rotational barrier. Examination of a final spectrum of peptide 3,2 conducted at room temperature revealed that the peptide was stable during the experiments.

For characterization of the peptide degradation products, NMR spectroscopy of the reaction mixtures was performed on a Bruker AV 400 WB (Bruker Biospin, Rheinstetten, Germany) using suppression of the water signal (presaturation). d_4-TSP (Trimethylsilyl-2,2,3,3-tetradeuteropropionic acid) was used as chemical shift reference. From the chemical shift of 2.38 ppm for the alanine CαH it can be deduced that the CαH is close to the face of the aromatic ring of phenylalanine (i.e., in the shielding cone of the phenyl ring[23]), which is only possible if a ring system is formed and one amino acid is D and the other L. All other signals are also consistent with the compound being a diketopiperazine. All unstable peptides were characterized by forming a diketopiperazine structure.

Samples from stability and release experiments as well as for physicochemical characterization were analyzed using HPLC. The HPLC system consists of a Merck-Hitachi L-7100 pump connected to a Merck-Hitachi L-7400 UV detector (VWR International, Tokyo, Japan). A Merck-Hitachi 7200 autosampler was connected to the system. Reversed-phase chromatography was performed using a Varian RP-C18 Chromsep SS column (5 µm, 4.6 id × 150 mm) (Chrompack Varian, Middelburg, The Netherlands) equipped with a Varian precolumn filter. The flow rate was 1.0 mL/min and the mobile phases used consisted of acetonitrile and 0.1% trifluoroacetic acid in demineralized water. The content of acetonitrile was adjusted to 15–45% (v/v) to give appropriate retention times for the various peptides. A wavelength of 204 nm was used for monitoring the column effluent. Quantitation of the compounds was done from peak area measurements in relation to those of standard solutions prepared in the respective solvents and measured under the similar conditions.

Results and discussion

Preparation of peptidomimetics

A series of 11 peptidomimetics varying with respect to chain length and degree of N-methylation was designed and synthesized (Table 1; Figure 1). As access to considerable amounts of the derivatives was desirable, conventional solution-phase peptide syntheses were carried out.

The structures were built using the amino acids alanine, phenylalanine, and tyrosine allowing for easy UV detection and gamma scintigraphy detection after ^{125}I labeling of the tyrosine residue (to be published elsewhere). Enhanced metabolic stability was attempted by incorporation of D-phenylalanine in the peptides and by converting the C-terminal into the *N*-methyl amide. In addition, masking of the C-terminal carboxyl group enabled the isolation of the peptidomimetics as unionized free bases. Furthermore, different degrees of N-methylation of the peptide bonds were introduced thus varying the number of hydrogen bond donor sites in the peptide backbone (Table 1). The first step in the purification of the peptides involved preparative HPLC. The aqueous effluents were made slightly alkaline and the uncharged peptidomimetics were extracted into ethyl acetate. Following concentration of the organic phases in vacuo, the peptidomimetics were obtained as amorphous powders (as assessed by DSC measurements). Attempts to obtain crystalline forms of the peptidomimetics were unsuccessful.

Physicochemical characterization

Apparent solubility

The apparent solubilities determined for the amorphous peptidomimetics are presented in Table 2. In this context, the term apparent solubility has been used since the isolation of peptide derivatives as amorphous material excluded determination of thermodynamic solubilities. The apparent solubility in aqueous buffer tends to decrease with the incorporated number of phenylalanine residues where the amino acid side chains add to the hydrophobicity of the derivatives[7]. Compared to the parent peptides it seems that N-methylation of the peptide backbone leads to derivatives exhibiting enhanced aqueous as well as castor oil solubility (Table 2), possibly due to a reduction of intra- as well as intermolecular hydrogen bond formation interactions. The relatively high peptide solubilities observed in castor oil compared to those in sesame oil results most likely from the fact that castor oil, unlike sesame oil, possesses hydrogen bond donor properties[24].

Distribution coefficients

The partitioning of the model peptidomimetics between PBS and (i) octanol, (ii) sesame oil, and (iii) castor oil were investigated at 37°C (Table 2). For all systems investigated, it is observed that for each additional phenylalanine residue incorporated in the peptide

Table 2. Physicochemical properties of the model peptidomimetics. Apparent peptide solubility (mM) in 67 mM phosphate buffer pH 7.4 (S_{PBS}), sesame oil ($S_{s.o.}$), and castor oil ($S_{c.o.}$) and partitioning between 67 mM phosphate buffer pH 7.4 and (i) octanol ($D_{oct:PBS}$), (ii) sesame oil ($D_{s.o.:PBS}$), and (iii) castor oil ($D_{c.o.:PBS}$) at 37°C.

Peptide	S_{PBS}	$S_{s.o.}$	$S_{c.o.}$	$D_{oct:PBS}$	$D_{s.o.:PBS}$	$D_{c.o.:PBS}$	pK_a[a]
3,0	24[b]	<LOD	<4.8	1.8 (5.4)	nd	nd	7.83;9.36
3,1	48 (5.8)	<LOD	47 < S < 61	2.0 (0.7)	nd	nd	nd
3,2	>100	0.51 (11)	>80	3.3 (2.6)	0.013 (16)	1.1 (0.8)	7.82;9.30
4,0	0.042 (4.8)	nd	nd	39 (2.8)	nd	nd	nd
4,1	7.9 (3.5)	<LOD	14 < S < 22	39 (1.6)	nd	nd	nd
4,2	0.061 (3.8)	<LOD	>64	89 (3.5)	nd	16 (2.0)	nd
4,3	7.7 (3.4)	<LOD	>61	61 (3.4)	0.34 (21)	12 (13)	7.81;9.25
5,0	0.0044 (20)	nd	nd	703 (5.4)	nd	nd	nd
5,1	0.62 (18)	<LOD	<3.1	474 (1.8)	nd	nd	nd
5,2	0.53 (4.5)	nd	>49	910[b]	nd	172 (9.3)	nd
5,3	0.60[b]	<LOD	>53	701 (2.1)	0.88 (8.5)	86 (2.6)	nd

Numbers in brackets are RSDs given in % (n = 3). <LOD: below the limit of detection (0.02–0.1 mM). nd: not determined. [a]pK_a values (R.S.D. < 2%) are given for the amine and phenol group (amine;ph). [b]n = 2.

backbone the distribution coefficient increases about one log unit. Similar findings have previously been reported for a homologous series of peptidomimetics[1]. Compared to the sesame oil partitioning system higher distribution coefficients were determined in the castor oil–PBS system. This is in accordance with the fact that the peptides exhibit by far the highest solubility in castor oil. In octanol–water systems[25] and oil–water systems[26], reasonable estimates of drug distribution coefficients have been calculated from the ratio of drug solubilities in the respective immiscible phases.

pK_a Values

pK_a Values were determined for three of the peptidomimetics varying with respect to degree of methylation and chain length. As apparent, similar pK_a values were obtained for the three investigated peptidomimetics amounting to approximately 7.8 and 9.3 for the amine and phenol functional groups, respectively (Table 2). The N-terminal amino group of the peptides is therefore only partly ionized (protonated) at physiological pH 7.4.

Peptide stability

The stability of the peptides in 67 mM phosphate buffer pH 7.4 (37°C) was followed for a minimum of 3 days. No significant degradation was observed for the peptides 3,0; 3,2; 4,0; 4,2; 4,3; 5,0; 5,2; and 5,3. In contrast, the peptides 3,1; 4,1; and 5,1 were observed to be unstable under these conditions. Degradation was followed for 11–17 days and it was observed that disappearance rates conformed to pseudo-first-order kinetics. Half-lives were calculated to 13, 3.9, and 5.5 days for peptide 3,1; 4,1; and 5,1, respectively (RSDs < 2.4%, n = 3). Product analysis (by NMR) revealed that the peptide degradation was accompanied by a 2,5-diketopiperazine (DKP) formation. It is well-documented that dipeptide esters in an aqueous environment readily cyclize to their corresponding DKPs[27] (and references cited therein). Also dipeptide amides have been found to undergo cyclization reactions to yield DKPs[28]. The mechanism of the observed DKP formation involves most likely a nucleophilic attack of the N-terminal nitrogen on the amide carbonyl between the second and third amino acids. In contrast to their mono N-methylated counterparts, the parent tri-, tetra-, and pentapeptides undergo no or only very slow cyclization under the present conditions. This observation, together with the fact that the intramolecular aminolysis reactions of the peptides 3,1; 4,1; and 5,1 proceed at different rates, may strongly suggest that structural factors influence the formation of the six-membered cyclic transition state.

Release experiments

In the rotating dialysis cell model, the selected peptide formulations were applied to the aqueous donor compartment (5.0 mL PBS) that was separated from the acceptor phase (1000 mL PBS) by a dialysis membrane. The model has been proposed to be useful for simulation of events potentially influencing drug residence time in the knee joint cavity after IA instillation of depot injectables[17,29], that is the situation where (i) a depot formulation is administered into the aqueous synovial fluid and (ii) the drug residence time in this small compartment is dependent on the interrelated processes involving the rate of drug release from the immobilized depot and passive transport of the drug out of this compartment into the blood[12].

Release from aqueous and oil solutions

Only four peptidomimetics were selected for the release experiments. The remaining seven derivatives were not investigated due to either low yield from synthesis or instability in aqueous solution (peptides 3,1, 4,1, and 5,1). Basic release characteristics of the four peptides were determined at 37°C after applying a PBS solution of

Table 3. The obtained apparent first-order rate constant, k_{app}, half-life, $t½$, and permeability coefficient, P, after instillation of aqueous and oil solutions of model peptides into the donor cell of the rotating dialysis cell model at 37°C. A 67 mM phosphate buffer pH 7.4 was used as donor and acceptor media. The volumes of the donor and acceptor phases were 5 and 1000 mL, respectively, and the area of diffusion 22 cm^2.

Peptide	Formulation type	k_{app} (min^{-1})	$t½$ (hours)	P (cm/min)
3,0	Aqueous solution	1.1×10^{-2} (5.0)	1.1	2.4×10^{-3}
3,2	Aqueous solution	1.3×10^{-2} (7.3)	0.9	3.1×10^{-3}
4,3	Aqueous solution	1.1×10^{-2} (2.9)	1.1	2.4×10^{-3}
5,3	Aqueous solution	7.4×10^{-3} (26)[a]	1.6	1.7×10^{-3}
5,3	Castor oil solution[b]	1.3×10^{-3} (7.5)	8.7	2.7×10^{-3}

Numbers in brackets are RSDs given in % (n = 3). In the experimental runs comprising aqueous and oil solutions, the model peptides were dissolved in the aqueous donor phase and in 0.5 mL oil, respectively. [a]The high RSD is due to the low solubility of this compound. [b]Dose = 8 mg.

the respective derivative to the donor cell (Table 3). Under these conditions, it can be shown that the rate of diffusant appearance into the acceptor phase obeys mathematically the first-order kinetics under sink conditions[17]. The apparent first-order rate constant, k_{app}, was obtained from the relationship

$$\ln(M_{A,\infty}-M_{A,t})=\ln(M_{A,\infty})-k_{app}t, \quad (4)$$

where $M_{A,\infty}$ and $M_{A,t}$ refer to the cumulated amount of diffusant in the acceptor phase at infinity and time t, respectively. The permeability coefficient P can be calculated from the expression

$$k_{app}=\frac{PA}{V_{Dw}}, \quad (5)$$

where A is the effective membrane area for diffusion (22 cm^2) and V_{Dw} is the volume of the aqueous donor phase[17]. The data presented in Table 3 indicate that diffusion across the membrane only to a minor extent is influenced by differences in the hydrodynamic volume of the diffusants. In case an oil solution of a drug initially is added to the aqueous donor phase, the drug is released from the formulation by partitioning from the oil into the aqueous phase. Under the experimental conditions used, the transport of the diffusant from the donor compartment into the acceptor phase applies to apparent first-order kinetics according to the rate expression

$$\frac{dM_{A,t}}{dt}=PA\left(\frac{1}{V_{Dw}+V_oD_{7.4}}+\frac{1}{V_A}\right)(M_{A,\infty}-M_{A,t}) \quad (6)$$

or in integrated form

$$\ln(M_{A,\infty}-M_{A,t})=-PA\left(\frac{1}{V_{Dw}+V_oD_{7.4}}+\frac{1}{V_A}\right)t+\ln(M_{A,\infty}), \quad (7)$$

where V_o is the oil volume injected, V_A is the volume of the acceptor phase, and $D_{7.4}$ refers to the pH-dependent distribution coefficient of the drug[30]. Accordingly, the apparent first-order rate constant k_{app} can be expressed as

$$k_{app}=PA\left(\frac{1}{V_{Dw}+V_oD_{7.4}}+\frac{1}{V_A}\right). \quad (8)$$

Based on the surprisingly high solubility of the peptidomimetics in castor oil (Table 2), release from a castor oil solution, after instillation into the donor cell, was investigated using peptidomimetic 5,3. The release profiles obtained from applying peptidomimetic 5,3 into the donor phase in the form of an aqueous solution (approximately 1.5 mg in 5 mL) and a castor oil solution (approximately 8 mg in 0.5 mL) are depicted in Figure 2. The feasible agreement between the experimental release data and the release curves constructed from employing the apparent first-order rate constants (from Equations (4) and (7), respectively) strongly indicates that in both experiments release of 5,3 was adequately

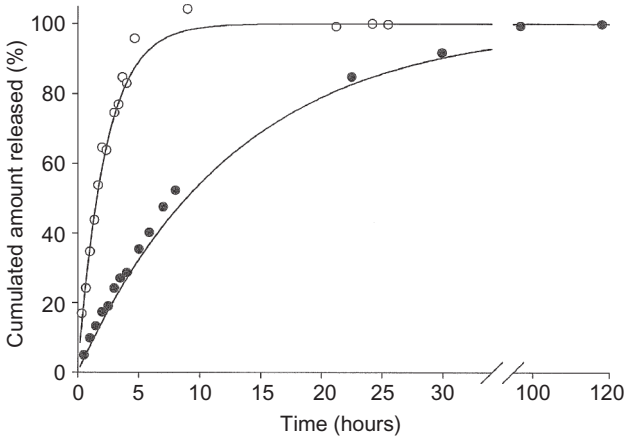


Figure 2. Representative release profiles from single experiments in 67 mM phosphate buffer at pH 7.4 and 37°C obtained after applying an aqueous solution of peptide 5,3 (1.5 mg in 5 mL PBS) (○) and a solution of peptide 5,3 in castor oil (8 mg 5,3 in 0.5 mL) (●) to the donor cell.

described by apparent first-order kinetics. Consequently, Equation (8) can be used to estimate the release rates of the other peptide derivatives dissolved in castor oil with the knowledge of their castor oil–PBS distribution coefficients. Transfer into the acceptor phase proceeds faster from the aqueous peptide solution. In fact, the castor oil solution affords a half-life of release (8.7 hours) more than five times that obtained from an aqueous solution (1.6 hours) (Table 3). It should be noticed that the in vitro model is likely to underestimate the in vivo depot effect as the oil phase, due to rotation of the dialysis cell, is effectively dispersed into small droplets in the aqueous buffer in the donor compartment thus creating a large interface between the oil and the aqueous phase[30,31]. The net rate of drug transfer from the oil phase into the aqueous donor phase is governed by the solute distribution coefficient between the two respective phases and the interfacial area[24,32]. Under the conditions employed, it is therefore assumed that partitioning equilibrium between the immiscible phases is established instantaneously[30,31].

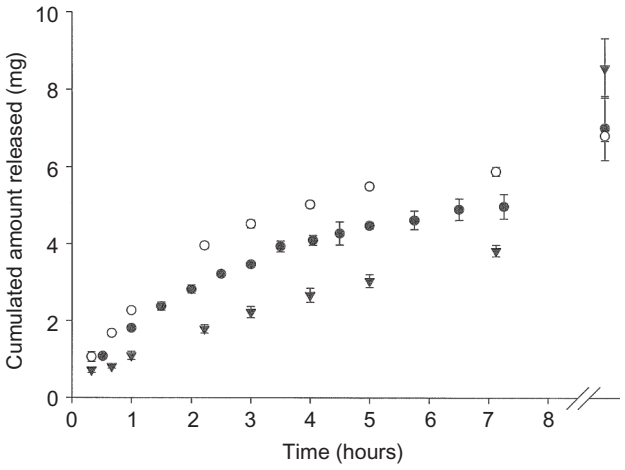


Figure 3. Release profiles (37°C) of in situ formed precipitates of peptides 5,3 resulting from addition of (i) 1 mL solution (NMP–sesame oil 1:1) of 5,3 (8 mg) (○); (ii) 0.1 mL NMP solution of 5,3 (8 mg) (●); and (iii) a preformed precipitate in sesame oil (10 mg 5,3 in 0.5 mL) (▼) to 5 mL of the donor phase (PBS). The last data points were obtained after washing of the donor cell with ethanol.

Release from in situ formed precipitates

As regards marketed products, glucocorticoids represent the only drug class that has been injected intra-articularly in the form of depot suspensions[33]. A priori, aqueous as well as oil suspensions of new drug candidates are of potential interest for the IA route of administration due to the relative simplicity of such formulations and the ability to incorporate high drug loads. Recent observations (in vitro) imply that the drug release mechanism of both suspension types is the same with drug release rates governed by the dissolution of the solid particles[26]. Depot oil suspensions are particularly attractive for peptides susceptible to degradation in aqueous media[34] (and references cited therein).

During the apparent solubility experiments in aqueous buffer, the solid amorphous peptide derivatives were transformed into sticky semisolid materials. The slow dissolution observed may, at least in part, be ascribed to the combination of poor solubility and relatively small total water-accessible surface areas of the semisolids. Hence, in preliminary experiments potential sustained release properties of in situ formed precipitates (ISFP) of peptide 5,3 (the least water-soluble derivative of the four selected) were investigated. NMP, in which the peptides were highly soluble, was used as cosolvent in the in situ precipitation forming systems. This cosolvent has been found to be miscible with both vegetable oils and water[35]. In oil–water two-phase systems, NMP exhibits much higher affinity for the aqueous phase and this cosolvent may therefore be of potential interest in the design of in situ suspension-forming DDSs[36]. In the donor compartment (5 mL PBS), ISFPs were formed after addition of peptide 5,3 (approximately 8 mg) in the form of (i) a NMP solution (0.1 mL) and (ii) a solution of NMP and sesame oil 1:1 (in total 1 mL). The latter vegetable oil was chosen as peptide 5,3 was poorly soluble in this vehicle (Table 2). Qualitatively, the shapes of the peptide release profiles from the two formulation principles are comparable. However, direct comparison of the initial release profiles is not possible as the exact total amount of peptide used in each experiment could not be determined (Figure 3). After an initial period with relatively fast release (up to about 3 hours), the peptide appearance rate in the acceptor phase declined significantly. After 4 days, semisolid material was still present in the donor cell (adhesion mainly to the metal parts of dialysis cell). After washing the donor cell with ethanol, about 90% of the instilled dose of peptide 5,3 could be accounted for. As apparent from Figure 3, similar initial release characteristics of peptide 5,3 for the two formulation types were observed after adding a preformed oil precipitate to the donor cell.

Conclusions

A series of 11 tri-, tetra-, and pentapeptides were synthesized and isolated as amorphous solids and their basic physicochemical properties were estimated. It was observed that N-methylation of the peptide amide bond closest to the N-terminal led to unstable derivatives that in PBS underwent breakdown and cyclization reactions to their corresponding 2,5-diketopiperazine (DKP) derivatives. It was observed that the compounds in contact with water transformed into sticky semisolid materials suggesting that oil-based (depot) formulations of the derivatives might be of potential interest for the IA route of administration. Results of this in vitro study indicate that IA sustained release of such amorphous small peptides might be accomplished from castor oil solutions or mixed sesame oil–NMP solutions with the latter

enabling in situ formation of semisolid peptide precipitates upon contact with aqueous media. In vivo properties of the formulation principles are presently under investigation in our laboratory.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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