SYNTHESIS AND METABOLIC STABILITY OF A TRITIUM-LABELLED SUBSTANCE P ANALOGUE

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1. Introduction

We have described the synthesis of a substance P analogue, <Glu–Gln–Phe–MePhe–MeGly–Leu–Met-NH_2 [1]. The peptide, referred to herein as DiMe-C7, was designed to be resistant to a substance P degrading enzyme purified from brain [2]. Evidence for the increased stability of DiMe-C7 in rat brain preparations has been presented [1]. Moreover, DiMe-C7 exhibited a high potency in competing for tritiated substance P binding to rat brain membranes as well as retaining biological activities in peripheral substance P bioassays [1]. This report describes the synthesis of the precursor, production of the radiolabelled peptide by catalytic dehalogenation and further evidence for enhanced resistance of DiMe-C7 to in vitro and in vivo digestion by rat brain compared with substance P.

2. Materials and methods

2.1. *Materials*

Polyacrylamide resin [4] (0.3 mmol sarcosine/g resin) was from Imperial Chemical Industries Ltd. Fmoc-Gln-*p*-nitrophenylester and Fmoc-Leu were purchased from Chemical Dynamics Co. For the preparation of Fmoc-MeGly and Fmoc-MePhe see [1] and for Fmoc-Phe (I) see [5].

Abbreviations: DCC, dicyclohexylcarbodiimide; DMF, *N,N*-dimethylformamide; Fmoc, fluorenylmethoxycarbonyl; <Glu, pyroglutamic acid; HPLC, high-pressure liquid chromatography; MeGly, *N*-methyl-glycine (sarcosine); MePhe, *N*-methyl-L-phenylalanine; ND, not determined; Phe(I), 4-iodo-L-phenylalanine; TLC, thin-layer chromatography; VTA, ventral tegmental area

Dimethylacetamide was obtained from East Anglia Chemicals and redistilled under vacuum. Piperidine was redistilled over KOH and kept in the dark. Tritium gas (98%) was purchased from the Radiochemical Centre, Amersham, Bucks. Solid phase synthesis was carried out using a VEGA model 50 peptide synthesizer.

2.2. *Analytical methods*

High-pressure liquid chromatography (HPLC) of the unlabelled peptide was carried out using a Du Pont model 850 HPLC system with a spectrophotometer model 852 and a column compartment model 850 in conjunction with one of the following isocratic reverse-phase systems: (A) μ-Bondapak C_{18}, CH_3CN/50 mM H_3PO_4, 31:69 (v/v); (B) μ-Bondapak C_{18}, CH_3CN/10 mM NH_4OAc (pH 4.0), 35:65 (v/v); (C) Zorbax TMS, CH_3CN/50 mM H_3PO_4, 18:82 (v/v). All runs were performed at 35°C at a flow rate of 2 ml/min.

Analysis by HPLC of the radioactive product was effected on columns (0.46 × 25 cm) of the stated phases using constant volume (20 ml) gradients with the following solvent systems: (D) Nucleosil 10 C_{18}, CH_3CN/18 mM H_3PO_4 (from 25:75 to 50:50, v/v); (E) Nucleosil 10 CN, CH_3CN/18 mM H_3PO_4 (from 25:75 to 50:50, v/v); (F) Ph-bondapak, CH_3CN/0.2 N triethylammonium phosphate (pH 3.0) (from 25:75 to 40:60, v/v). Flow rate was in the range of 1.5–2.0 ml/min. TLC analysis was on silica gel (Merck F254) using the following solvent systems: (G) *n*-BuOH:HOAc:H_2O, 12:5:3; (H) *n*-PrOH:H_2O, 7:3; (I) $CHCl_3$:CH_3OH:NH_4OH, 60:45:20 and (J) EtOAc:C_5H_5N:HOAc:H_2O, 5:5:1:3.

Amino acid analysis of the unlabelled peptide was performed as described in [1] and of the tritiated peptide as in [6].

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2.3. *Solid phase synthesis: Gln–Gln–Phe(I)–MePhe–MeGly–Leu–Met–resin (I)*

Boc-methionyloxycarbonylbenzoyl–alanyl–resin (0.30 mmol Met/g; 800 mg) was deprotected, neutralized and washed [1,7]. Fmoc–Leu, Fmoc–MeGly, Fmoc–MePhe, Fmoc–Phe(I) (12 equiv.) were pretreated with DCC (6 equiv.) in dichloromethane to give symmetrical anhydrides. The solvent was removed, and the anhydrides were dissolved in dimethylacetamide and added to the peptide–resin. Coupling of Fmoc–MePhe was followed by acetylation and in the case of Fmoc–Phe(I) a double coupling was performed prior to acetylation. Glutamine was incorporated via an active ester coupling. The peptide–resin was treated with hydroxybenzotriazole (6 equiv.) before addition of Fmoc–Gln–*p*-nitrophenyl ester (6 equiv.). Completion of coupling reactions was demonstrated by the ninhydrin test in [8]. After complete synthesis, 900 mg peptide–resin was recovered.

2.4. *<Glu–Gln–Phe(I)–MePhe–MeGly–Leu–Met–NH_2 (II)*

A portion (300 mg) of the heptapeptide–resin was subjected to amino-lysis for 25 h [5]. The peptide-material was dissolved in CH_3CN/10 mM NH_4OAc (pH 4.0), 5:95 (v/v) (10 ml) + HOAc (3 ml) and left at 64°C. After 48 h, virtually all heptapeptide was converted to <Glu–heptapeptide with no detectable side reaction. After evaporation the peptide material was dissolved in CH_3OH (2 ml) and purified on a semi-preparative C_{18} (0.79 × 30 cm) column in portions (300–400 μl), using a 15 min linear gradient of CH_3CN/10 mM NH_4OAc (pH 4.0) (from 35:65 to 58:42, v/v) and flow rate of 4 ml/min (fig.1). The material corresponding to the main peak was collected, the CH_3CN was removed by evaporation and the sample was lyophilized twice giving 20.0 mg (40%) analytically pure peptide. Amino acid analysis: Glu + Gln, 2.10; Phe(I), 0.91; MePhe, ND; Sar, ND; Leu, 0.96; Met, 1.02.

2.5. *<Glu–Gln–[^{3}H]Phe–MePhe–MeGly–Leu–Met-NH (III)*

A solution of compound (II) (25.4 mg; 2.5 μmol) in DMF (0.5 ml) was stirred with 98% $^3H_2$ gas (3.2 ml, 8 Ci) in the presence of 10% Pd/C (6.3 mg) and 5% $Rh/CaCO_3$ (6.8 mg). After 40 min, the catalysts were removed by filtration through TLC-grade cellulose and the filtrate was evaporated. The residue was dissolved in H_2O (1 ml), HOAc (50 μl), DMF (50 μl),

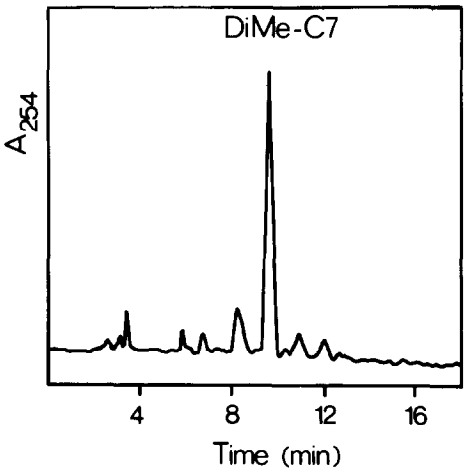

Fig.1. High-pressure liquid chromatography of crude <Glu–Gln–Phe(I)–MePhe–MeGly–Leu–Met-NH_2 on a semi-preparative C_{18} μ-Bondapak reverse-phase (0.79 × 30 cm) column. Elution was done at room temperature, with a 15 min linear gradient of CH_3CN/10 mM NH_4OAc pH 4.0 from 36:65 to 58:42 (v/v) at a flow rate of 4 ml/min. For preparative runs, material corresponding to the main peak (eluting at 9.6 min) was collected and pooled.

CH_3OH (100 μl) and put through a 0.5 ml column of Dowex-1 (acetate). After evaporation the residue was dissolved in H_2O/HOAc (4:1, v/v, 0.5 ml) and applied to a column (0.7 × 50 cm) of Nucleosil 10 C_{18}. The column was eluted at 6 ml/min with constant volume gradient (100 ml) of $CH_3CN/H_2O/H_3PO_4$ (from 250:750:1 to 500:500:1). The elute was monitored at 210 nM and fractions (0.5 min) were collected automatically. Fractions containing the main peak judged by the UV trace, were pooled and the volume was reduced by evaporation. The sample was passed through a column (1 ml) of Dowex 1 (acetate form) resin. After evaporation the sample was dissolved in H_2O and stored in liquid N_2 at 3.63 mCi/ml. Amino acid analysis: Glu + Gln, 2.02 (–); Phe, 1.00 (–); MePhe, ND (–) MeGly ND (–); Leu, 1.01 (1.01); Met, 0.95 (1.00); NH_3, 3.16 (1.28). (Figures in brackets are after enzyme hydrolysis.)

2.6. *Analytical characterization of II and III*

The purity of II was assessed by HPLC using the reverse-phase systems A–C (see section 2.2). The only impurity detectable was the sulphoxide. The purity of the preparation was also confirmed by thin-layer chromatography (TLC) using the systems G–H.

Chromatographic purity of III after 28 days storage

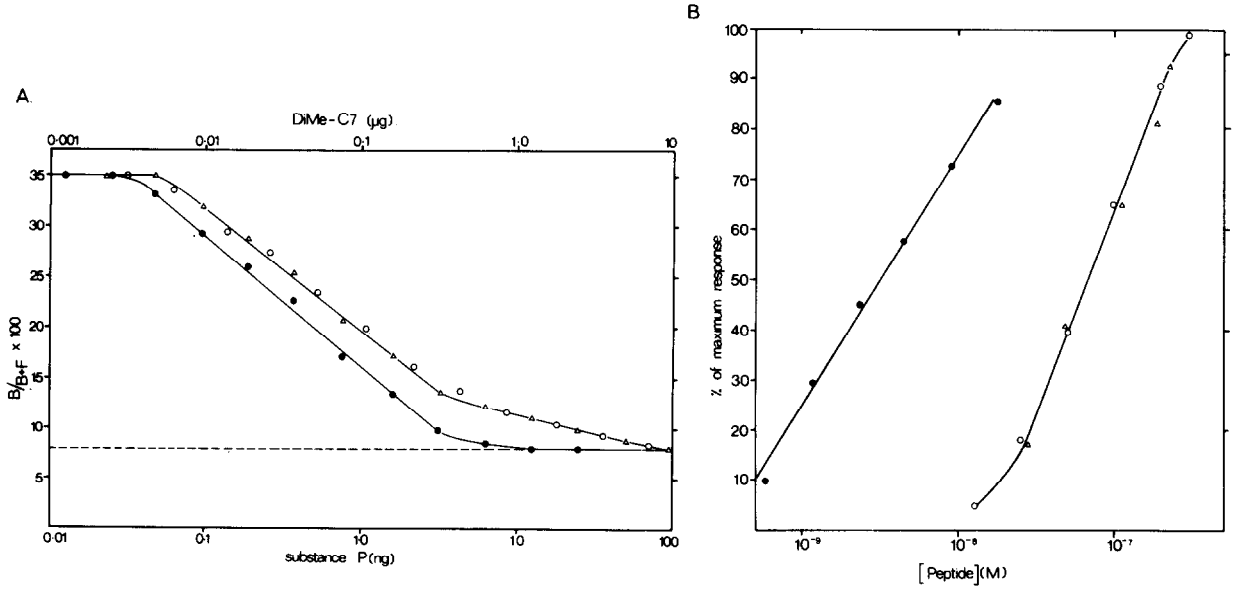

Fig.2. (A) Cross-reactivity of standard DiMe-C7 and [^{3}H]DiMe-C7 in a substance P radioimmunoassay. The dilution curves for DiMe-C7 (–○–○–) and [^{3}H]DiMe-C7 (–△–△–) are identical, but not parallel to that for standard substance P (–●–●–). The cross-reactivity relative to substance P against the C-terminal-directed guinea pig antiserum [10] is 0.005. Concentrations for standard substance P and DiMe-C7 were determined by amino acid analyses of stock solutions. The dotted line marks blank values (8%): B, antiserum bound tracer; F, free (unbound) tracer. (B) Contractile potencies of DiMe-C7 (–○–○–) and [^{3}H]DiMe-C7 (–△–△–) compared with standard substance P (–●–●–) on the guinea pig ileum. Potencies were tested in the presence of 1 μM atropine sulphate, 1μM mepyramine maleate, and 10 μM tryptamine hydrochloride.

at –196°C was determined by systems D–F. The purity was estimated to be 95.6–97.0% with the main impurity being sulphoxidized peptide (2.1–2.4%). TLC in solvent systems H–J revealed similar value, 94.5–97.3% and 2.4–3.7% sulphoxidized peptide. The distribution of radioactivity amongst the amino acid residues was 98.0% in Phe and 0.4% in Met.

2.7. *Guinea pig ileum assay*

The experimental details for assessing spasmogenic activity on guinea pig ileum have been given in [2]. In this assay, [^{3}H]DiMe-C7 was equipotent to the unlabelled material. The dose–response curves were superimposable (fig.2B), and the potency relative to substance P was 0.046*.

2.8. *Radioimmunoassay (RIA)*

RIA was performed as in [9] using a substance P C-terminus directed antiserum raised in guinea pig. In this assay the tritiated peptide behaved exactly as the non-radioactive material (fig.2A). The cross-reactivity relative to substance P against the C-terminal-directed guinea pig antiserum was 0.005.

2.9. *Metabolic stability – rat brain synaptosomes*

[^{3}H]DiMe-C7 (198 pmol) was added to 20 mM Hepes (pH 7.4) (500 μl) containing 0.5% bovine serum albumin and 1 mg membrane protein of a rat whole brain P_2B fraction [10]. After incubation for 30 min at 37°C, 6 M HCl (100 μl) was added to the sample and the sample was clarified by centrifugation through a 0.2 μm Teflon filter (Bio-analytical Systems). The clarified supernatant (25 μl) was analysed by reverse-phase HPLC on a μBondapak C_{18} (0.39 × 30 cm) column. Samples were eluted with a 20 min linear gradient of CH_3CN/50 mM H_3PO_4 (from 5.95 (v/v) to 65:35 (v/v)) at a flow rate of 2 ml/min at room temperature. The elution position of DiMe-C7 was determined by calibration runs using synthetic DiMe-C7 detected by absorbance at 220 nm. Fractions (0.2 min) were counted for radioactivity by liquid scintillation and corrected for quenching.

* Note that the value was obtained in the presence of 1 μM atropine; a higher potency is obtained in the absence of atropine, as reported in [1]

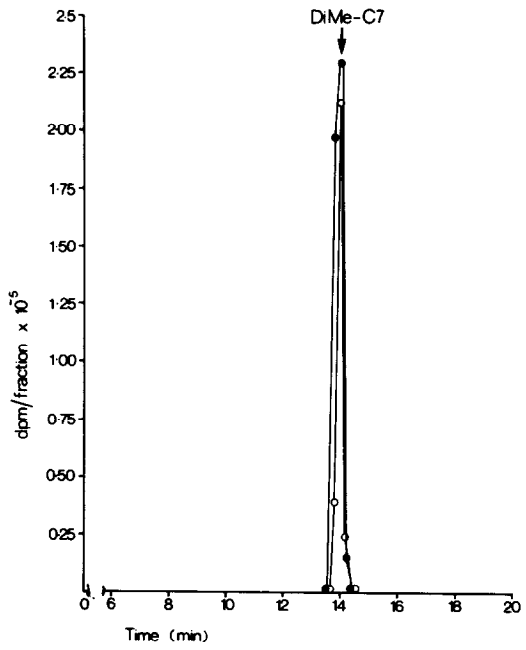

Fig.3. HPLC analysis of [^{3}H]DiMe-C7 before (–•–•–) and after (–o–o–) incubation with a rat brain P_2B fraction. The arrow marks the elution position of standard synthetic DiMe-C7. The radiochemical recoveries of the applied samples were 110% of 9.9 pmol for the 'before' sample, and 82% of 8.25 pmol for the 'after' sample.

2.10. *Metabolic stability – in vivo brain microinfusion*

A solution 1.2 μg/ml (2 μl) of [^{3}H]DiMe-C7 (diluted with standard synthetic DiMe-C7 to spec. act. 2.95 Ci/mmol) in 0.9% saline was infused into the rat brain ventral tegmental area (VTA) using an implanted guide cannula [11]. The sample was injected over 2 min and then at least 1 min was allowed for sample diffusion. Rats were killed by decapitation at intervals after injection, and the VTA was quickly dissected on ice and frozen. The frozen regions were weighed, homogenised and extracted for 5 min in boiling 1 M HOAc (10 vol.). The extract was centrifuged for 5 min at 10 000 × *g* at room temperature in a Beckman 'Microfuge'. The supernatant was freeze-dried and the residue extracted with absolute ethanol (100 μl). Duplicate samples (20 μl) of the ethanol extract were counted by liquid scintillation, and a sample (20 μl) was applied to LQD silica gel plates (Quantum Industries). Plates were developed in TLC system H, dried, and scanned for radioactivity with a Berthold thin-layer chromatogram scanner. Markers [^{3}H]DiMe-C7 and standard DiMe-C7 were included on each plate. The latter was visualised by chlorine/*o*-tolidine treatment. Both unlabelled and labelled markers were largely oxidised during application to the thin-layer plate: thus, extracted samples were expected to be largely, if not wholly, in the oxidised form. Possible degradation first appeared at 50 min, as determined by the appearance of radioactive products which did not co-migrate with the oxidised or unmodified DiMe-C7 standards.

3. Results and discussion

A radiolabelled enzyme-resistant substance P analogue [^{3}H]DiMe-C7, was synthesised and compared to the unlabelled peptide. The identity of the compound was confirmed by physico–chemical and biological criteria. In all cases the radiolabelled peptide and the non-labelled compound exhibited identical properties. The specific radioactivity (18.3 Ci/mmol) was lower than theoretical, but high enough to be used as a radioligand in receptor-binding studies [3]. DiMe-C7 has been demonstrated to be completely stable when exposed to substance P-degrading enzyme as well as to a rat hypothalamic slice preparation [1]. However, in the latter case a relatively high concentration of the peptide had to be used because peptide levels were monitored by immunoreactivity and DiMe-C7 cross-reacted poorly with the C-terminus directed substance P antiserum. With the tritiated peptide we have been able to test metabolic stability

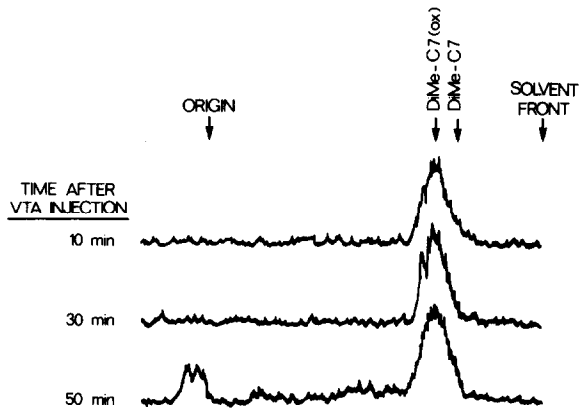

Fig.4. Radioactivity scans of thin-layer chromatograms of [^{3}H]DiMe-C7 and other products recovered after in vivo infusion into the rat brain ventral tegmental area. Reanalysis by HPLC of chromatographed material confirmed the TLC results.

at much lower peptide concentrations. When substance P is treated with a rat synaptosomal preparation, the peptide is split by endopeptidase attack [2] and the inactivation of substance P is complete within minutes. When DiMe-C7 was exposed to the same conditions no degradation of the peptide was detectable up to 30 min.

Stereotactic injection of [^{3}H]DiMe-C7 into VTA of the rat brain showed that the injected peptide remained substantially intact even 50 min after the infusion whereas the same amount of substance P was completely degraded after <10 min. The combination of high biological potency, enzymatic resistance and lack of charged groups (likely to decrease some types of adsorption), makes the radiolabelled version of DiMe-C7 potentially useful probe of substance P receptors.

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