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Short Communication

Solvent system for the counter-current purification of hydrophobic bombesin analogues

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ABSTRACT

A two-phase solvent system has been developed for the fractionation of some highly hydrophobic bombesin analogues prepared by solid-phase peptide synthesis. The solvent system, composed of toluene, ethyl acetate, dichloromethane, methanol and aqueous triethylammonium acetate, has been proven useful for the purification of these water-insoluble peptides.

INTRODUCTION

The tetradecapeptide bombesin (BN), orginally isolated from frog skin, exerts a series of physiological effects in humans [1,2], and BN-like peptides have been shown to act as autocrine growth factors in cell lines of a malignant tumour, the small cell lung carcinoma (SCLC) [3]. This finding greatly stimulated the search for BN antagonists, and several classes of peptides with antagonistic properties have since been described [4,5]. We have prepared a series of analogues having a hybrid structure between that of BN itself and [D-Pro^2]-spantide, an antagonist of substance P, which is also a weak BN antagonist [6].

Peptides I and IV (see Table I) are two analogues in this series, which appeared heterogeneous by reversed-phase high-performance liquid chromatography (RP-HPLC), as obtained by solid-phase synthesis. Both of them are N- and C-terminal blocked peptides, spanning a highly hydrophobic sequence. Similar BN-related, water-insoluble peptides have recently been purified by counter-current chromatography in horizontal flow-through coil planet centrifuge, using the aqueous phase of a chloroform–acetic acid–water (2:2:1) system as the mobile phase [7]. Preliminary partition tests in this system, and also in other systems of relevance for peptide counter-current purification [8,9], showed that they were not suitable in this instance. In this

TABLE I

AMINO ACID SEQUENCES OF BN ANALOGUES I–V

<Glu = pyroglutamic acid; [ ] = deleted residue.

No.	Amino acid sequence
I	Boc–D-Gln–Trp–Ala–D-Trp–Phe–D-Trp–Leu–Nle–NH_2
II	D-<Glu———[ ]———NH_2
III	Boc———[ ]———NH_2
IV	Boc———-D- His———NH_2
V	Boc———[ ]-D- His———NH_2

paper, the particular counter-current conditions developed for the purification of such peptides in a classical apparatus are described.

EXPERIMENTAL

All reagents were of analytical-reagent grade and solvents were of analytical-reagent or HPCL grade. N,N-Dimethylformamide (DMF) was distilled over ninhydrin and stored in the dark over 4 Å molecular sieves. Trifluoroacetic acid (TFA) was of Sequanal grade from Pierce (Rockford, IL, USA). The amino acid derivatives were obtained from Bachem Feinchemikalien (Bubendorf, Switzerland) or Peninsula Labs. (Belmont, CA, USA) and Merrifield resin (0.88 mequiv./g) from Bio-Rad Labs. (Richmond, CA, USA).

Synthesis

The bombesin analogues I and IV were synthesized in a semi-automatic laboratory-made apparatus starting with N^{α}-*tert*-.-butyloxycarbonyl (Boc)–Nle–OCH_2 resin (0.56 mmol/g), prepared by esterification of Merrifield resin by Gisin's method [10]. The protocol of the synthesis involved the following steps: washing with dichloromethane (DCM); deprotection with 40% TFA in DCM or, following the insertion of Trp, with 40% TFA in DCM containing 2.5% anisole and 2.5% 2-mercaptoethanol; washing; neutralization with 10% N-methylmorpholine in DCM; washing; double coupling in DCM or DMF for 1 h with the preformed symmetrical anhydride of the Boc-amino acid or, in the case of Boc-D-glutamine, with the 1-hydroxybenzotriazole (HOBt) ester in DMF for 4 h, then washing with DCM (or DMF and DCM), 2-propanol and again DCM. The imidazole ring of Boc-D-histidine was protected as the 4-toluenesulphonyl (Tos) derivative. The peptides were cleaved from the resin by treatment with saturated anhydrous ammonia in methanol–DMF (1:1, v/v) at 4°C for 4 days. After evaporation of the filtrate, the product was precipitated from methanol solution with diethyl ether. The yield of crude product was 1.35 g for I and 0.91 for IV.

Counter-current chromatography

A Craig-type apparatus (Labortec, Bubendorf, Switzerland) consisting of 120 distribution tubes (volume of the phase = 25 ml) was employed. Crude peptides were dissolved in methanol and the solution was evaporated to an oil, which was taken up with 25 ml of the lower phase obtained by partitioning at room temperature of the following mixture: toluene–ethyl acetate–DCM–methanol–triethylammonium acetate (0.1 *M* triethylamine adjusted to pH 4 with glacial acetic acid) (10:3:5:12:6). The solution was charged into the first tube of the apparatus filled with the lower phase, and distribution was allowed to proceed over the whole series of tubes. Usually, the fractions were analysed by thin-layer chromatography (TLC) on silica gel plates (Merck, Darmstadt, Germany) with the eluent benzene–ethyl acetate–acetic acid–water (5:5:2:0:75), upper phase, or *n*-butanol–acetic acid–water (4:1:1) and detection with Ehrlich's reagent [11]), and peak tubes were combined and evaporated under vacuum. The products were finally recovered by precipitation with water (I–III) or diethyl ether (IV and V) from methanol solution.

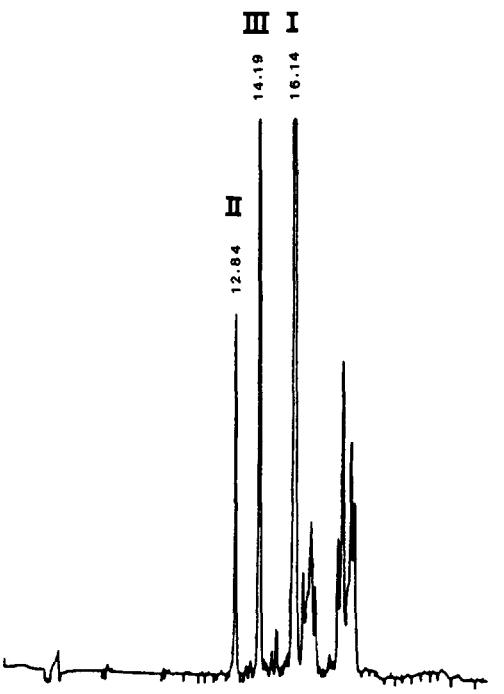

Fig. 1. RP-HPLC profile of crude I. Conditions: eluent A = 0.05% TFA in water, eluent B = 0.05% TFA in acetonitrile gradient from 20% to 70% B over 15 min; sample size, *ca.* 20 μg; detection wavelength, 230 nm at 0.4 a.u.f.s. Arabic numbers at peaks indicate retention times in min.

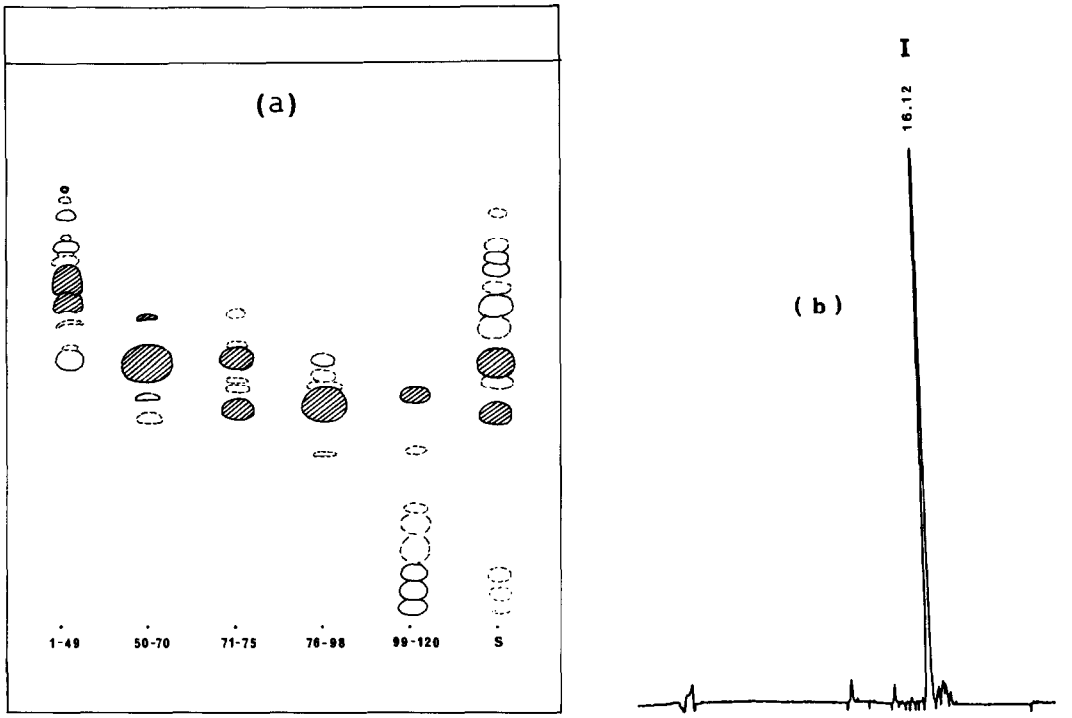

Fig. 2. (a) TLC of fraction pools from the counter-current distribution (CCD) of crude analogue I. Eluent, benzene–ethyl acetate–acetic acid–water (5:5:2:0.75), upper layer; detection, with Ehrlich's reagent [11]; sample size, *ca.* 80 μg. (b) RP-HPLC profile of fractions 50–70 from the same CCD run. Conditions as in Fig. 1, except sample size, *ca.* 8 μg.

Analysis

The purified peptides were analysed for homogeneity by HPLC using a Hewlett-Packard (Avondale, PA, USA) Model 1084 apparatus equipped with a variable-wavelength detector (HP 79875A) and a Model HP79850B LC terminal, on a LiChrosorb RP-18 column (25 × 0.4 cm, I.D. particle diameter 5 μm) (Merck) at 1 ml/min (for other conditions, see the legends of the figures). Amino acid analysis was performed on a 3 *M* mercaptoethanesulphonic acid hydrolysate. The structure of each of the purified peptides was confirmed by fast atom bombardment mass spectrometry (FAB-MS).

RESULTS AND DISCUSSION

BN-antagonist I was obtained as heterogeneous material, as shown in Fig. 1, which reproduces the HPLC trace of the crude product. The major peak

TABLE II

PARTITION COEFFICIENTS, K_n[a], OF BN ANALOGUES IN DIFFERENT SOLVENT SYSTEMS

Solvent composition	Volume ratio	K_n
Chloroform–acetic acid–water	2:2:1	25
Toluene–ethyl–acetate–DCM–methanol–0.1 *M* hydrochloric acid	10:3:5:12:6	4.1
Toluene–ethyl acetate–DCM–methanol–0.1 *M* acetic acid	10:3:5:12:6	2.7
Toluene–ethyl acetate–DCM–methanol–triethyl–ammonium acetate[b]	10:3:5:12:6	1.05; 0.12[c]

[a] K_n = solute concentration in the non-aqueous phase/solute concentration in the aqueous phase.
[b] 0.1 *M* triethylamine adjusted to pH 4 with glacial acetic acid.
[c] The frist value refers to peptide I and the second to peptide IV.

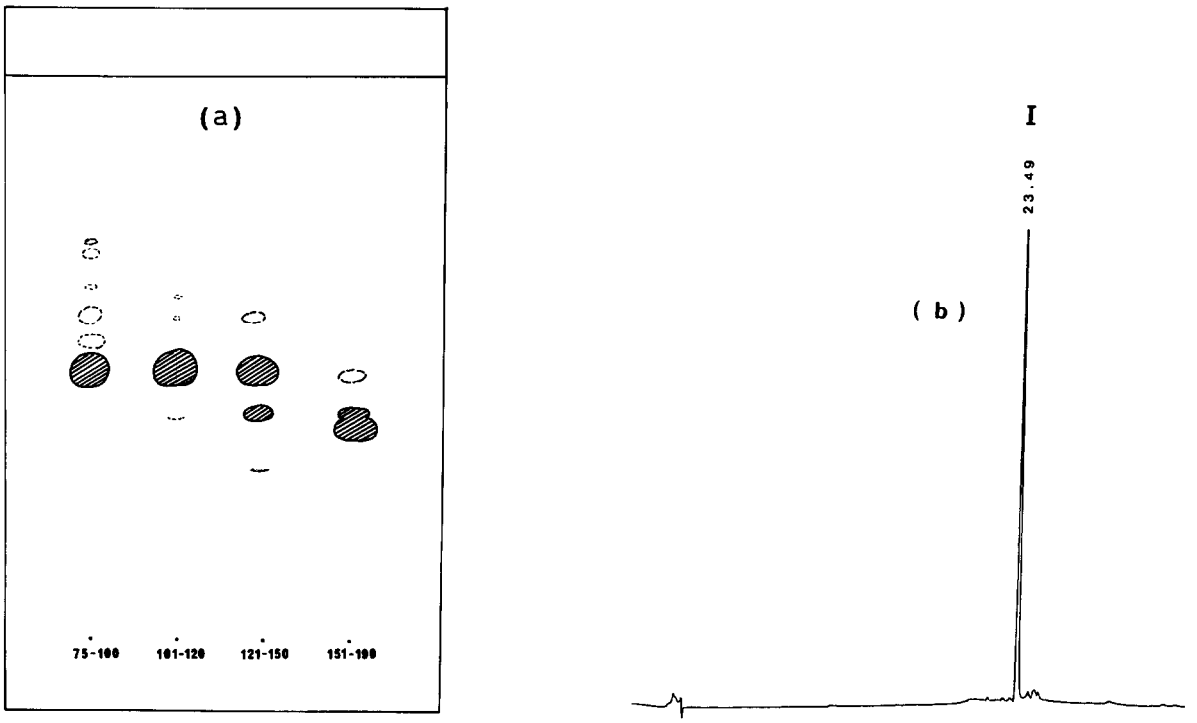

Fig. 3. (a) TLC of fraction pools from the CCD of semi-purified analogue I. Conditions as in Fig. 2a. (b) RP-HPLC profile of fractions 101–120 from the same CCD run. Conditions: eluent A = 20 m*M* KH_2PO_4 (pH 3.5)–acetonitrile (9:1), eluent B = 20 m*M* KH_2PO_4 (pH 3.5)–acetonitrile (3:7), isocratic elution with 30% B for 1 min, then gradient from 30% to 90% B over 20 min; sample size, *ca.* 5 μg; detection wavelength, 210 nm at 0.4 a.u.f.s.

in this chromatogram accounts for *ca.* 41% of the mixture. For the counter-current purification of this synthetic peptide, the usual polar *n*-butanol solvent systems proved totally inadequate. To select a suitable solvent system, the partition coefficient of the sample (defined here as K_n, the solute concentration in the non-aqueous phase divided by that in the aqueous phase) was measured by a traditional micro shake-flask test coupled with HPLC. The screening of relatively non-polar systems started with chloroform–acetic acid–water (2:2:1), which has already been found useful for the purification of similar water-insoluble peptides [7]. In contrast, in this instance the partition coefficient of the sample in that system was very high (Table II), thus rendering its use impractical.

Several other systems that have been suggested for poorly polar peptides were tried [8,9]: they were composed of toluene, and/or DCM (or chloroform), optionally ethyl acetate, methanol and an acidic aqueous component such as dilute hydrochloric or acetic acid, in a range of proportions. The K_n value of peptide I was considerably lowered in some of these systems containing ethyl acetate (see Table II), but still high, considering the large amount of heterogeneous more hydrophobic material which had to be removed in this purification (see Fig. 1). The introduction of triethylammonium acetate as an aqueous component resulted in a critical adjustment of the partition coefficient, which approached unity (see Table II). The whole crude could then be purified by counter-current chromatography with an optimized solvent system, consisting of toluene–ethyl acetate–DCM–methanol–aqueous triethylammonium acetate (10:3:5:12:6, v/v).

After 120 transfers, fractions 50–70 were found to contain the peak of the main product and were pooled, yielding 0.61 g of I with a degree of purity of 84% by HPLC (Fig. 2). The material obtained

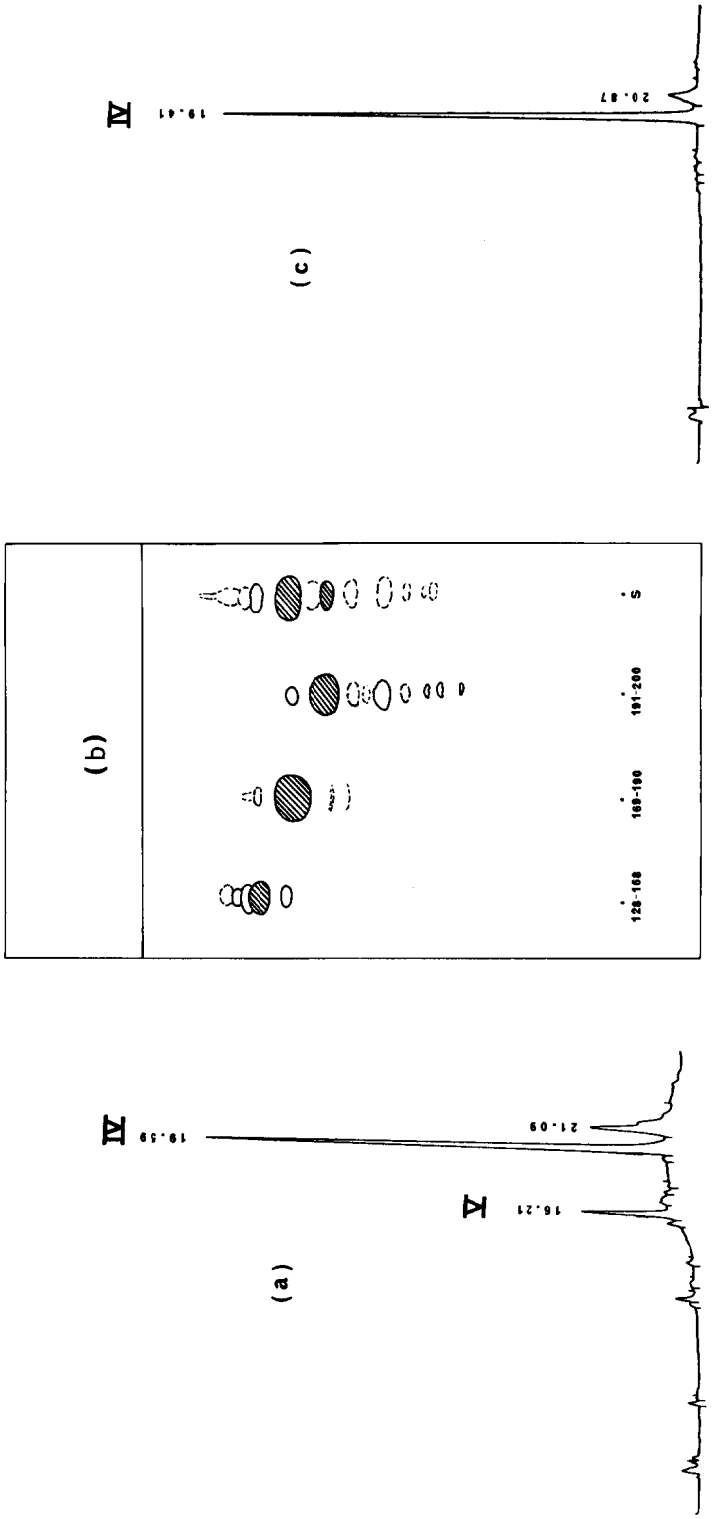

Fig. 4. RP-HPLC profiles of (a) crude IV and (c) fractions 169–190 from CCD performed on this product. Conditions: sample size, (a) *ca.* 10 μg and (c) *ca.* 5 μg; eluents as in Fig. 3b, gradient from 30% to 70% B over 15 min, followed by isocratic elution with 70% **B** for 10 min; detection wavelength, 210 nm at 0.4 a.u.f.s. (b) TLC of fraction pools from the same CCD. Eluent, *n*-butanol–acetic acid–water (4:1:1); other conditions as in Fig. 2a.

from fractions 76–98 (0.28 g) was also analysed by HPLC and it consisted of the two side-products, II and III, detected in Fig. 1 as peaks with retention times of 12.8 and 14.2 min, respectively. Interestingly, these products were both identified by amino-acid analysis as deletion peptides lacking Phe^5. FAB-MS analysis enabled us to ascertain that II, the earlier eluting side-product, bore an additional structural modification,consisting of the presence of a D-pyroglutamic residue at the amino terminus instead of the starting Boc-protected D-glutamine. This product may have been formed during the cleavage treatment, performed by prolonged ammonolysis, owing to a sequence-specific lability of the Boc group in the deletion heptapeptide. The pool from fractions 71–75, which was found to be a 1:1 mixture of peptides I and III, the later eluting deletion peptide, yielded a very small amount of material (22 mg).

Hence a single counter-current run with the selected solvent system turned out to be very effective in resolving such products, bearing slight structural modifications. In addition, peptide I could be further purified by subjecting the semi-purified material (0.53 g) to a second counter-current distribution in the same solvent system (Fig. 3a). In this instance, 95 fractions of the head effluent were collected in external tubes, so that a total of 215 transfers was performed. Fractions 101–120 were combined, yielding 0.25 g of fairly pure peptide I (Fig. 3b). In addition, 0.14 g of 75% pure product was recovered from head and tail fractions, possibly to be recycled.

The same solvent system as successfully used for analogue **I** was tried for the purification of analogue **IV**, which also appeared as a composite crude on HPLC analysis (Fig. 4a). The partition coefficient of the product was much lower in this instance (see Table II), owing to the presence of a basic residue (D-His^6) in the molecule. The crude product was submitted to a total of 200 transfers (80 fractions of the effluent were collected in external tubes), after which (Fig. 4b) fractions 169–190 were found to contain the peak of the main product and pooled, yielding 0.34 g of IV with a degree of purity near 90% (Fig. 4c). The peptide was still contaminated with more hydrophobic material, but had been completely resolved from the main impurity, V (peak which retention time 16.2 min in Fig. 4a). This side-product was isolated from fractions 191–200, and, similarly to what was found with analogue I, it was identified as the deletion peptide lacking Phe^5. This may reflect a particular difficulty in coupling this amino acid to the tripeptidyl resin, namely H–D-Trp–Leu–Nle–OCH_2 resin for peptide I and H–D-His(Tos)–Leu–Nle–OCH_2 resin for peptide IV.

The results obtained in the counter-current chromatography of the two analogues show that the solvent system containing triethylammonium acetate as an aqueous component can be very helpful for fractionating water-insoluble peptides. In particular, it enabled us to achieve effectively the purification both of peptide I, which displays a strongly hydrophobic structure and had a negligible solubility in the aqueous phase of a number of solvent systems, and peptide IV, which is also water insoluble but contains a residue bearing a dissociable group in the side-chain. In addition, for hydrophobic peptides containing other dissociable functions, one might take advantage of the possibility of adjusting the pH of the aqueous component over a wide range of values, while maintaining its ability to sulubilize the peptides efficiently, and thus possibly conferring on them a suitable partition coefficient.

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