Journal of Peptide Science
J. Peptide Sci. **4**: 294–299 (1998)

Coloured Peptides: Synthesis, Properties and Use in Preparation of Peptide Sub-library Kits

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Received 2 September 1997

Accepted 10 October 1997

Abstract: Several methods were developed for the solid-phase synthesis (SPPS) of coloured peptides and peptide libraries. At first a bifunctional red compound, 4-(4-(*N*-ethyl-*N*-(3-(*tert*-butyloxycarbonyl)aminopropyl)amino)phenylazo)benzoic acid (Boc-*EPAB*), was coupled with chloromethyl resin to obtain a new solid support suitable for SPPS using Boc chemistry. Peptides synthesized on this coloured resin had the chromophore at their C-termini. N-terminally coloured peptides were synthesized on a traditional solid support, coupled with chromophoric carboxylic acid before cleavage. A model pentapeptide, Phe-Ala-Val-Leu-Gly, and its ten derivatives were synthesized and their properties studied. It was found that the presence of chromophores decreases the water solubility of peptides. However, insertion of solubilizing tags (penta-lysine sequences or polyoxyethyl chains) into the molecule of any coloured derivative resulted in enhancement of the solubility. The RP-HPLC hydrophobicity indexes (φ_0) of the coloured peptides were also determined because φ_0 values are closely related to their water solubility. A coloured pentapeptide library was synthesized using the portioning-mixing method. Each component of this library contained the red azo dye (*EPAB*) and the penta-lysine tag. Before the last coupling step the samples were not mixed. All of the 19 sub-libraries obtained after cleavage were readily soluble in water, giving intense red solutions.

The effect of chromophore (*EPAB*) and/or penta-lysine solubilizing tag on the biological activity was also studied. Potencies of the bovine neurotensin 8–13 fragment and its different coloured and penta-lysine derivatives were compared in isolated longitudinal muscle strips of guinea pig ileum. It was shown that the hexapeptide with penta-lysine tag had almost the same activity as the 8–13 fragment itself. The activity of the *EPAB*-derivative was found to be rather low. However, the presence of the solubilizing tag in the coloured hexapeptide compensated the negative effect of the chromophore. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Coloured neurotensin analogues; coloured peptides; coloured peptide libraries; peptide labelling with chromophores; peptide synthesis on coloured support; solubilizing tags

Abbreviations*: ANAB*, 4-((9,10-dehydro-9,10-dioxo-1-(4-amino)anthracenyl)-amino)benzoic acid; Boc-*EPAB*, 4-(4-(*N*-ethyl-*N*(-3-(*tert*-butyloxycarbonyl)aminopropyl)amino)phenylazo)benzoic acid; *DPBA*, 4-(4-(diethylamino)phenylazo)benzoic acid; Gaba, γ-amino-butyric acid; TFMSA, trifluromethanesulfonic acid.

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Contract grant sponsor: Hungarian Scientific Research Foundation; Contract grant number: T15718, T19306, T19858

CCC 1075–2617/98/040294-06$17.50

INTRODUCTION

Since 1988 when an effective combinatorial technique was introduced – the portioning–mixing (PM) method for the synthesis of multicomponent peptide mixtures [1–4] numerous oligopeptide libraries and other oligomer libraries have been prepared using this procedure. These libraries became the main source of compounds for drug discovery, opening a new era in pharmaceutical research [5–10].

Using the PM method, support-bound or free peptide libraries can be prepared, depending on the type of the starting resin. Support-bound libraries have usually been screened by binding tests using labelled soluble receptors. Free peptide libraries, however, can be utilized in a wider range, e.g. in the identification of active peptides bound to different types of insoluble target molecules. The binding may be visualized if one uses free peptide sub-library kits [11] containing coloured components. By the use of colour labels both the number of the components of the kit and the number of the screening experiments can be substantially reduced [12]. In this paper we describe the synthesis and some physical properties of free peptides and peptide libraries labelled with chromophores [13].

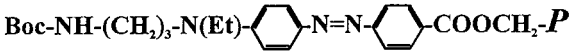
THEORETICAL

The effect of colour labelling on a model peptide, Phe-Ala-Val-Leu-Gly (FAVLG in one-letter coding), was studied. Synthesis of FAVLG was carried out with a usual Boc chemistry using chloromethyl resin as solid support. The peptide was cleaved with TFMSA, and the crude product was purified by RP-HPLC. The pure peptide was identified by FAB-MS (Table 1). FAVLG containing no polar side chain was moderately soluble in water. Its RP-HPLC hydrophobicity index (φ_0; Table 2) was determined. This value is the concentration of the organic component of the mobile phase at which the retention time is double the dead time (at log $k' = 0$). It depends on pH, temperature and the organic component of the solvent. It is independent, however, of other factors, for example, column size and flow rate [14].

Two versions of labelling were worked out: modification of the peptide at the C-terminal and at the N-terminal residue.

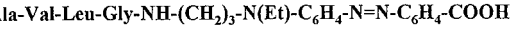
Peptide Labelling with Chromophores

For C-terminal labelling a modified solid support was used. An azo dye, Boc-*EPAB* [15], was coupled with chloromethyl resin in the presence of anhydrous potassium fluoride [16] to produce a new coloured support, Boc-*EPAB*-hydroxymethyl resin (**I**).



I (*P* denotes polymer matrix of resin)

Using this red resin (**I**) the apolar model sequence was synthesized with Boc chemistry. After cleavage from the resin with TFMSA, the peptide was precipitated with water, the red-violet precipitate was purified by RP-HPLC. The peptide (FAVLG-*EPAB*; **II**) (Table 1) holding the label at its C-terminus was almost insoluble in water: the solvent remained colourless after standing several hours with the coloured peptide.



II

Table 1 Identification of FAVLG, RRPYIL and Their Derivatives by Mass Spectrometry[a]

Peptide or peptide derivative	Monoisotopic molecular mass calculated	Protonated molecular mass measured
FAVLG	505.3	506.2
FAVLG-*EPAB*	813.4	814.3
DPBA-FAVLG	784.4	785.3
FAVLG-$[Lys]_5$	1145.8	1146.9
ANAB-FAVLG	845.4	846.4
FAVLG-$[Lys]_5$-*EPAB*	1453.9	1455.0
FAVLG-Gaba-*EPAB*-$[Lys]_5$	1538.9	1539.7
DPBA-FAVLG-$[Lys]_5$	1424.9	1425.7
DPBA-$[Lys]_5$-FAVLG	1424.9	1425.7
ANAB-FAVLG-$[Lys]_5$	1485.8	1486.7
ANAB-$[Lys]_5$-FAVLG	1485.8	1487.2
RRPYIL	816.5	817.3[b]
RRPYIL-*EPAB*	1124.6	1125.4[b]
RRPYIL$[Lys]_5$	1457.0	1457.8[b]
RRPYIL-$[Lys]_5$-*EPAB*	1765.1	1766.0[b]

[a] FAVLG-peptides and RRPYIL-peptides were measured by FAB-MS and electrospray ionization (ESI) technique, respectively.
[b] The protonated molecular ion data were calculated from the multiply-charged ions obtained by ESI method.

Table 2 HPLC Retention Times (RT) and Hydrophobicity Indexes (φ_0)[a] of FAVLG and Its Derivatives

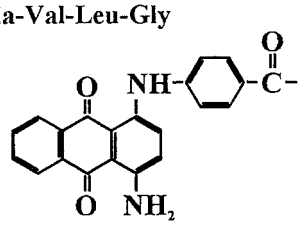
Peptide or peptide derivative	RT (min)	φ_0 (%)
FAVLG	3.0[b]	23.0
FAVLG-*EPAB*	14.9[b]; 23.4[c]	45.5
DPBA-FAVLG	21.2[b]	54.5
FAVLG-$[Lys]_5$	2.1[c]	13.5
ANAB-FAVLG	32.5[b]	57.4
FAVLG-$[Lys]_5$-*EPAB*	14.6[c]	30.5
FAVLG-Gaba-*EPAB*-$[Lys]_5$	14.5[c]	28.0
DPBA-FAVLG-$[Lys]_5$	24.5[c]	36.1
DPBA-$[Lys]_5$-FAVLG	12.6[c]	25.5
ANAB-FAVLG-$[Lys]_5$	29.7[c]	40.8
ANAB-$[Lys]_5$-FAVLG	17.6[c]	30.7

[a] Hydrophobicity index is the concentration of organic solvent at $\log k' = 0$ (i.e. at the double of the dead time) [12].
[b] B: 30–70% in 45 min.
[c] B: 20–70% in 45 min.

When labelling at the N-terminus, chromophores were attached to FAVLG molecule at the end of the synthesis, before cleavage of the peptide from the resin. One of two coloured monocarboxylic acids, a blue anthraquinone dye (*ANAB*) and a red azo compound (*DPBA*), was used as an acylating agent. Having cleaved them from the resin with TFMSA, the labelled peptides were precipitated with water. *ANAB*-FAVLG (**III**) and *DPBA*-FAVLG (**IV**) had dark blue and red colours, respectively, and they did not dissolve in water.

X-Phe-Ala-Val-Leu-Gly

III: X = *ANAB*- =



IV: X = *DPBA*- = $(Et)_2N$-C$_6$H$_4$-N=N-C$_6$H$_4$-CO-

Table 2 shows that φ_0 values of FAVLG-*EPAB*, *DPBA*-FAVLG and *ANAB*-FAVLG are higher than that of unmodified pentapeptide, i.e. these derivatives are more hydrophobic than FAVLG. In other words, the presence of the chromophores both in C-terminal and N-terminal positions considerably increases the HPLC retention times.

Enhancement of the Solubility of Coloured Peptides

It was supposed that inserting into the molecule a strongly polar (hydrophilic) sequence, for example, an oligolysine chain, results in the increase of the solubility of coloured peptides. Starting with Boc-Lys(Z)-resin ester, a decapeptide, FAVLG-$[Lys]_5$ was prepared. As we had expected, this peptide was more water-soluble than FAVLG itself (φ_0 value of FAVLG-$[Lys]_5$ was lower than that of FAVLG (see Table 2). Using $[Lys]_5$ as solubilizing tag, two versions were developed for the enhancement of water solubility. In the first case, starting with Boc-*EPAB*-hydroxymethyl resin (**I**) first $[Lys(Z)]_5$ then the FAVLG sequence were synthesized. The cleaved product (FAVLG-$[Lys]_5$-*EPAB*; **V**) was soluble in water and gave an intense red solution.

Phe-Ala-Val-Leu-Gly-$[Lys]_5$-NH-$(CH_2)_3$-N(Et)-C_6H_4-N=N-C_6H_4-COOH

V

In the second case Boc-Lys(Z)-resin ester was four times successively coupled with Boc-(Z)Lys, then with Boc-*EPAB*, finally with Boc-Gaba to produce a new red support, Boc-Gaba-*EPAB*-$[Lys]_5$ resin (**VI**). Gaba was inserted into the molecule as a spacer between the aromatic label and the peptide sequence.

Boc-Gaba-NH-$(CH_2)_3$-N(Et)-C_6H_4-N=N-C_6H_4-CO-$[Lys(Z)]_5$-OCH_2-*P*

VI

Using this resin FAVLG sequence was synthesized. Having cleaved from the support a red, water-soluble product was obtained (FAVLG-Gaba-*EPAB*-$[Lys]_5$; **VII**).

Phe-Ala-Val-Leu-Gly-Gaba-NH-$(CH_2)_3$-N(Et)-C_6H_4-N=N-C_6H_4-CO-$[Lys]_5$

VII

Both labelled peptides (**V** and **VII**) contained $[Lys]_5$ as solubilizing tag, though in different part of their molecules. Being a highly polar cationic sequence, $[Lys]_5$ can make apolar molecules hydrophilic. The presence of the solubilizing tag significantly alters the HPLC retention time and the φ_0 value of the model peptide (Table 2).

Insertion of $[Lys]_5$ into the N-terminally coloured peptides, *ANAB*-FAVLG (**III**) and *DPBA*-FAVLG (**IV**) also resulted in the expected changes in their physical properties: all the four derivatives, *DPBA*-FAVLG-$[Lys]_5$, *DPBA*-$[Lys]_5$-FAVLG, *ANAB*-FAVLG-$[Lys]_5$ and *ANAB*-$[Lys]_5$-FAVLG dissolved in water readily and had relatively low φ_0 values.

Some derivatives of *ANAB*-FAVLG and *DPBA*-FAVLG were synthesized using the polyoxyethyl group *POE* as solubilizing tag at their C-termini. Syntheses were performed on TentaGel PAP resin with Fmoc chemistry. After cleaving *ANAB*-FAVLG-*POE* (**VIII**) and *DPBA*-FAVLG-*POE* (**IX**) were obtained as coloured and water-soluble products (blue and red, respectively).

***ANAB*-Phe-Ala-Val-Leu-Gly-$[OCH_2CH_2]_n$-OH**

VIII; n ≈ 70

***DPBA*-Phe-Ala-Val-Leu-Gly-$[OCH_2CH_2]_n$-OH**

IX; n ≈ 70

Coloured Peptide Library Solubilized by Penta-lysine Tag

Using one of our red supports (**VI**) a 'normal' pentapeptide library [17] was synthesized applying the PM method [4], varying 17 common proteinogen amino acids in every coupling position (Cys, His and Trp were omitted). After the last coupling step the samples were not mixed, resulting in 17 sub-libraries, each of them formally consisting of $17^4 = 83521$ peptides. Every sub-library had a different amino acid residue in N-terminal position. All of these red samples were readily soluble in water.

Effect of *EPAB* and $(Lys)_5$ on the Biological Activity of Neurotensin 8–13 Fragment

Synthetic 8–13 fragment of bovine neurotensin (Arg-Arg-Pro-Tyr-Ile-Leu; RRPYIL) was chosen as a model compound to study the effect of the presence of chromophore and/or $[Lys]_5$ solubilizing tag on the biological activity. This hexapeptide was found to possess the full potency and 70% of the activity of neurotensin (1–13) on perfused rat heart, and in rat stomach strips, respectively [18], and had a receptor binding affinity similar to that of native neurotensin in neonatal mouse brain [19]. RRPYIL and its three derivatives RRPYIL-*EPAB*, RRPYIL-$[Lys]_5$ and RRPYIL-$[Lys]_5$-*EPAB* were synthesized with SPPS, Boc chemistry (MS data are in Table 1). All of these peptides were water soluble. In the case of RRPYIL-EPAB it was due to the effect of two strongly polar arginine residues in the molecule.

In our experiments the assay was based on the effect that neurotensin releases acetylcholine from cholinergic interneruons of the Auerbach plexus which, in fact, contracts smooth muscle cells [20]. Effect of RRPYIL, RRPYIL-*EPAB*, RRPYIL-$[Lys]_5$ and RRPYIL-$[Lys]_5$-*EPAB* were measured in isolated longitudinal muscle strips of guinea-pig ileum. ED_{50} values obtained are in Table 3. The results show that RRPYIL-$[Lys]_5$ has almost the same activity as RRPYIL. The presence of chromophore results in a significant decrease: potency of RRPYIL-*EPAB* is much less than that of 8–13 peptide. However, $[Lys]_5$ seems to compensate for the influence of *EPAB* since the activity of RRPYIL-$[Lys]_5$-*EPAB* surpasses that of the merely coloured peptide. Therefore it seems advisable to apply coloured peptides in biological studies only with polar appendix, e.g. $[Lys]_5$ in their molecules.

EXPERIMENTAL PART

Chloromethylated styrene-divinyl benzene (99:1) resin was obtained from Bio-Rad, Richmond, CA, USA (Bio-Beads SX1, 200–400 mesh, 1.26 mequiv. Cl/g). TentaGel PAP resin (No. J 1002; capacity 0.23 mmol) was a product of Rapp Polymere, Tübingen, Germany. Boc-*EPAB*-hydroxymethyl resin (**I**) (2.8%

Table 3 ED_{50} Values of Neurotensin 8–13 Fragment and Its Analogues Obtained in Isolated Guinea Pig Longitudinal Muscle Strips

Peptide or peptide derivative	ED_{50} values[a]
RRPYIL	3.8×10^{-6} M
RRPYIL-*EPAB*	1.1×10^{-4} M
RRPYIL$[Lys]_5$	7.7×10^{-6} M
RRPYIL-$[Lys]_5$-*EPAB*	8.6×10^{-5} M

[a] ED_{50} values are the concentrations inducing 50% of the maximal contractions.

N; 0.5 mmol Boc-NH/g) was prepared from chloromethylated resin and Boc-*EPAB* [15] according to Horiki *et al.* [16]. Boc-Gaba-*EPAB*-[Lys(Z)]$_5$-resin (**VI**) (4.9% N; 0.24 mmol Boc-NH/g) was synthesized starting from Boc-Lys(Z)-resin ester (Sigma-Aldrich Kft, Budapest, Hungary; Sigma No. B-0147, Lot 47F-5805; 0.46 mmol Boc-Lys(Z)/g).

Synthesis of *ANAB* has been described elsewhere [15]. Preparation of *DPBA* was carried out by a usual coupling reaction of diazotized 4-amino-benzoic acid and *N*,*N*-diethyl aniline. Boc- and Fmoc-amino acids were purchased from Bachem, Bubendorf, Switzerland. Reagents and solvents for peptide synthesis were obtained from Fluka AG, Buchs, Switzerland.

For Boc chemistry a coupling protocol of Gutte and Merrifield [21] was adapted with slight modifications [22]. This protocol was also used when *ANAB* and *DPBA* were attached to the N-termini of peptides. The peptides were cleaved treating the resin with a mixture of TFMSA and TFA (1:9, v/v) for 1 h at room temperature. Three peptides (FAVLG-*EPAB*, *DPBA*-FAVLG and *ANAB*-FAVLG) were precipitated with water and the others with ether. The precipitates were filtered and washed with the precipitating solvent, and then dried over potassium hydroxide. The water-soluble samples were dissolved in water and lyophilized.

When the FAVLG sequence was synthesized on TentaGel PAP resin (0.2 g; 0.046 mmol), the Fmoc-protocol of Chang *et al.* [23] was applied. At the end of the synthesis, the resin was divided into two equal parts. One part was coupled with *ANAB*, the other one with *DPBA*, then the peptides were cleaved from the resin with TFA. After reducing the volume of the acidic, coloured solutions *in vacuo*, the peptides were precipitated with ether, filtered and dried over KOH, and lyophilized.

The peptides were purified by RP-HPLC using a C18 (10 μm) Nucleosil (BST, Budapest, Hungary) semi-preparative column (9.0 × 250 mm; flow rate, 3.0 ml/min) and a Knauer 64 apparatus. A linear gradient of 95% acetonitrile in 0.1% aqueous TFA was applied. Analytical chromatographic experiments were made on a 4.0 × 150 mm C18 column (5 μm) with a flow rate of 1.0 ml/min. The absorbance at 254 mm was recorded. The RP-HPLC hydrophobicity indexes (φ_0) were determined according to the method of Valkó and Slégel [14] using an analytical column and isocratic conditions, and calculated from retention times (RT) obtained in different runs when various acetonitrile concentrations had been used.

The colored pentapeptide library was prepared using the PM technology [4]. The starting Boc-Gaba-*EPAB*-[Lys(Z)]$_5$-resin (**VI**) (1.75 g; 0.42 mmol) was swollen in 90 ml of a DCM/DMF mixture (2:1 v/v). The slurry was thoroughly shaken, then divided into 17 equal portions by pipetting (5–5 ml); the remainder (*ca.* 4 ml) was diluted by the same solvent mixture (15 ml) and 1 ml of the thin suspension was pipetted into each of 17 thicker slurries. The resin portions were coupled separately with a different Boc-amino acid then mixed. This three-step operation cycle (portioning, coupling and mixing) was repeated three times. In the last (fifth) cycle the mixing step was omitted. Cleavage was carried out as it was described in the case of the coloured peptides. The samples were precipitated with ether, then filtered, washed with the same solvent, dried over KOH, dissolved in water and freeze dried.

FAB-MS experiments were performed with a Fisons VG ZAB-2SEQ hybrid tandem mass spectrometer of BEQQ configuration coupled to an OPUS 2000 data system and equipped with a liquid secondary ion mass spectrometer source (Cs^+ ion gun used at 30 keV). The samples dissolved in DMSO were mixed with 3-nitrobenzyl alcohol matrix for the coloured FAVLG derivatives and with glycerol for FAVLG before subjected to FAB-MS analysis. The electrospray ionization mass spectrometric experiments were carried out using a Finnigan MAT 95SQ hybrid tandem mass spectrometer. The solvent applied was a mixture of MeOH and water (1:1, v/v) with a small percentage of acetic acid.

The biological experiments were performed using longitudinal muscle strips of male guinea pig (300–350 g) ileum [20,24]. The animals were stunned and bled, and longitudinal muscle strips (60–120 mg) of ileum were prepared, mounted under a resting tension of 0.5 g in 4 ml Krebs solution and kept at 37°C. A constant stream of 95% oxygen and 5% carbon dioxide was bubbled through the fluid. The strips were equilibrated for 20–30 min before being exposed to the peptides. Contractions of the strips were elicited by the peptide solutions and the ED_{50} values were determined.

Acknowledgements

The authors thank the Hungarian Scientific Research Foundation (OTKA, No. T15718, T19306 and T19858) for financial support, and Zsuzsanna Gémes and Judit Őszi for their skilful technical assistance.

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