Journal of Peptide Science
J. Peptide Sci. 2006; **12**: 361–368
Published online 14 November 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.735

JOURNAL OF PEPTIDE SCIENCE

Revisited and large-scale synthesis and purification of the mutated and wild type neu/erbB-2 membrane-spanning segment

LUCIE KHEMTÉMOURIAN, SÉBASTIEN LAVIELLE, KATELL BATHANY, JEAN-MARIE SCHMITTER and ERICK J. DUFOURC*

UMR 5144 MOBIOS, CNRS-University Bordeaux 1, Bordeaux-Pessac, France

Received 20 September 2005; Accepted 26 September 2005

Abstract: Solid-phase syntheses of the hydrophobic peptides Neu_{TM35} (^{1}EQRASPVTFIIATVVGVLLFLILVVVVGILIKRRR^{35}) and Neu^{*}_{TM35} (^{1}EQRASPVTFIIATVEGVLLFLILVVVVGILIKRRR^{35}), corresponding to the native and mutated (V15E) transmembrane domain of the neu/erbB-2 tyrosine kinase receptor, respectively, were accomplished using Fmoc chemistry. The use of a new resin and cleavage and purification conditions led to large increases in yields and peptide purity. Two ^{15}N-labelled versions of both wild type and mutated peptides were also synthesized. Approximately 20–40 mg of peptide was obtained using a small-scale synthesis, whereas *ca* 100 mg of pure peptide was collected on a medium scale. Peptide purity, as monitored by HPLC and mass spectrometry, ranged from 95 to 98% for the six peptides synthesized. Secondary structure as determined by UV circular dichroism (CD) in trifluoroethanol (TFE) showed *ca* 74% α-helical content for the native peptide and *ca* 63% for that bearing the mutation. Secondary structure of Neu_{TM35} was retained in DMPC (dimyristoylphosphatidylcholine)/DCPC (dicaproylphosphatidylcholine) membrane bicelles, and evidences for dimers/oligomers in the lipid bilayer were found. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: solid-phase synthesis; hydrophobic peptide; reversed-phase HPLC; MALDI-ToF mass spectrometry; ^{15}N-labelled amino acids; circular dichroism; α-helices

INTRODUCTION

Growth factor receptors of the tyrosine kinase family consist of two large, separately folded domains, one external and one cytosolic, connected by a single transmembrane segment. They are involved in a variety of cellular processes including receptor dimerization and signal propagation, which may sometimes dysfunction and lead to several major diseases such as cancer or Alzheimer disease. The sequence of the membrane-spanning peptides appears to be important for their function. For example, a single-point mutation in the proto-oncogene neu/erbB-2 resulting in a substitution of a valine residue to glutamic acid at position 664 within the transmembrane region may transform it into an oncogene [1–3]. The mutant receptor then has constitutive tyrosine kinase activity in the absence of ligand, apparently as a result of greatly enhanced receptor dimerization [4]. Recent methods in the field of 'native-like' membrane environments have now made it possible to structurally characterize peptides derived from these domains using biophysical techniques [5]. However, because of their extreme hydrophobic character, physicochemical studies on these peptides have been hindered by the difficulty to synthesize them in large amounts. Strong interchain associations within the peptide–resin matrix have to be overcome [6]. Also, high quality purification of hydrophobic membrane-spanning peptides remains a challenging problem. Certain membrane-spanning peptides are insoluble in traditional solvents such as acetonitrile or isopropanol, and it is necessary to find new solvent systems for their solubilization and purification [7].

Reliable methods to prepare large quantities of highly purified transmembrane peptides are therefore clearly needed. The peptide corresponding to residues 621–654 of neu/erbB-2 was prepared by Jones and coworkers and by Rigby and coworkers [8,9] by solid-phase synthesis. This sequence includes a part of the putative transmembrane region and a 10-residue stretch of the cytoplasmic domain with residue Thr_{654}, which is phosphorylated during (Epidermial Growth Factor) EGF-mediated signal transduction. The sequence was confirmed by mass spectrometry as having a purity of 90–95%. No information about purification and yield was given, and the complete transmembrane domain that contains the mutation, i.e. residues 650–684, was missing. The 36-aa peptides comprising residues adjacent to and including the putative transmembrane domain of proto-oncogenic and mutant Neu were synthesized by Houliston *et al.* [10] using the Fmoc strategy and purified by reversed-phase chromatography. The purity of the peptides was checked by mass spectrometry. Again no information about synthesis, purification and yield was given, and no HPLC or matrix assisted laser desorption and

*Correspondence to: Erick J. Dufourc, UMR 5144 MOBIOS, CNRS-Université Bordeaux 1, IECB, 2 rue Robert Escarpit, 33607 Pessac, France; e-mail: e.dufourc@iecb.u-bordeaux.fr

ionization time of flight (MALDI-ToF) spectra were shown. The synthesis and the purification of the Neu_{TM35} domain corresponding to residues 651–684 were also reported by Goetz *et al.* [11]; however, they did not obtain good yields (15% for the synthesis and 5–10% for the purification, so a total yield of 0.7–1.5%). During the synthesis, and in order to minimize expected difficulties in peptide chain assembly, they allowed longer coupling times, up to 50 min per residue, and added the aprotic polar solvent dimethylsulfoxide during the last 20 min for optimal solubilization and growth of peptide chains within the resin matrix. With this technique, the synthesis lasted 4–5 days. The method of purification was based on a two-step strategy; gel permeation and then reversed-phase HPLC. Smaller and truncated peptide sequences resulting from the acetylation blocking of unreacted amino groups during the synthesis were eliminated in the first step. The second purification, reversed-phase HPLC with a Waters DeltaPak C4 column (15 μ, 100 Å, 7.8 × 300 mm), led to a large improvement in synthetic peptide purity. Elution was performed with 0.05% aqueous trifluoroacetic acid (TFA) for eluent A and a solvent mixture with high eluotropic strength for eluent B: 0.05% TFA in acetonitrile–isopropanol (80 : 20 v/v). In this procedure, elution conditions were found optimal when eluent B varied in a 20-min linear gradient from 70 to 100%. They were able to obtain 2–5 mg of purified peptide working on a small-scale (0.1 mmole) synthesis.

We decided to improve the method reported by Goetz and coworkers with the aim of obtaining larger quantities of the peptide in a faster way. In order to increase the yield, it appeared judicious to use a NovaSynTGA resin, which is chemically more robust, less loaded and compatible with a wide range of reaction conditions, rather than a Wang resin. Moreover, the solid-phase synthesis was carried out using the Fmoc strategy in a simple coupling mode (20 min) and the method of purification was based on a one-step-only strategy, using reversed-phase HPLC.

In the present work, the peptides will be numbered 1–35; the point mutation that may occur at Val664, i.e. replacement with Glu, will then correspond to V15E in our sequence. In this paper, we report the challenging solid-phase chemical synthesis and purification of the wild type Neu_{TM35} (^{1}EQRASPVTFIIATV**V**GVLLFLILVVVVGILIKRRR^{35}) and mutated Neu^{*}_{TM35} (^{1}EQRASPVTFIIATV**E***GVLLFLILVV VVGILIKRRR^{35}) peptides. ^{15}N-labelled versions of these two peptides were also prepared, extending the number of 35-aa hydrophobic peptides synthesized to six. We also describe the structural investigation of these peptides by circular dichroism (CD) while dissolved in the membrane-mimicking solvent trifluoroethanol (TFE) and embedded in phospholipid membranes (bicelles).

MATERIALS AND METHODS

Chemicals

Fmoc-Arg(Pbf) NovasynTGA resin, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate (HBTU), *N*-hydroxybenzotriazole (HOBt) and *N*-α-Fmoc-amino acids were purchased from VWR-NovaBiochem (Läufelfingen, Switzerland). The amino acids were protected as follows: *t*-butyl (tBu) for threonine, glutamic acid, serine; *t*-butoxycarbonyl (Boc) for lysine, glutamine; and 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (pbf) for arginines. ^{15}N-labelled amino acids (valine and leucine) were obtained from Euriso-top, groupe CEA (Gif-sur-Yvette, France).

Piperidine, dimethylformamide (DMF), diisopropylethylamine (DIEA) and anhydride acetic acid were purchased from SDS (Peypin, France); TFA was obtained from Applied Biosystems (Courtaboeuf, France); triisopropylsilane (TIS), *N*-methylpyrrolidone (NMP) from ACROS organics (Geel, Belgium); 1,2-ethanedithiol (EDT) from Aldrich (Saint Quentin Fallavier, France). Dichloromethane (DCM) and solvents for HPLC (acetonitrile and isopropanol) were of Grade A and obtained from Fischer (Geel, Belgium).

Peptide Synthesis

The syntheses were performed on an Applied Biosystems 433A Peptide Synthesizer (PE Biosystem, Courtaboeuf, France) using Fmoc strategy. The polyethylene glycol–polystyrene (PEG–PS) resin was preloaded with an unprotected arginine substituted at 0.21 mmol g^{-1}. Fmoc chemistry was carried out in four major steps per cycle: (i) deprotection of Fmoc groups by piperidine, (ii) activation of added amino acid with HBTU/HOBt (37.9 g/13.6 g) in 200 ml of DMF, (iii) coupling by amide link formation with a solution of 35% DIEA in NMP and (iv) capping to prevent truncated peptide elongation with acetic anhydride/DIEA/HOBt (19 ml/9 ml/0.8 g) in 400 ml of NMP. Each deprotection step was monitored by conductivity.

Cleavage from the Resin

The final peptide mixture was cleaved from its resin, and removal of protecting groups was accomplished by treatment with TFA/EDT/TIS/thioanisole/crystalline phenol (84/4/2/4/6, v/v/v/v/g). The solution was prepared at 0 °C, and typically, 10 ml was mixed with 0.6 g of peptide-containing resin. Total deprotection and cleavage were achieved after 120 min in a covered Erlenmeyer. After the reaction time elapsed, the reaction mixture was filtered through a medium-porosity frit-glass funnel into another round bottom flask to separate the resin support from the peptide solution. One milliliter TFA was added to the reaction flask to wash out any remaining resin. The flask was then rinsed with 5 ml DCM. The crude peptide was precipitated by adding *ca* 100 ml of cold diethyl ether. The peptide was collected by filtering the mixture through a fine-porosity frit-glass funnel and further dissolved in an aqueous solvent (with 10% acetonitrile and 0.1% TFA) for lyophilization.

Purification and Analysis

The crude peptides were dissolved in a mixture of 25% acetonitrile in milli-Q water, both containing 0.1% TFA,

and purified by reversed-phase HPLC (Waters Alliance 2695 with a photodiode array detector) using a semipreparative Vydac (Hesperia, USA) C4 column (5 μm, 300 Å, 250 × 10 mn). The peptides were eluted with various gradient mixtures composed of solvent A (milli-Q water) and solvent B (solution of acetonitrile and isopropanol 3 : 2 v/v). Both solutions (A) and (B) contained 0.1% TFA. The column was equilibrated in 100% of A at a flow rate of 3 ml/min. Absorption was monitored at 215 nm. Four milligrams of crude peptide were dissolved in 1 ml of a mixture of 25% solvent B and 75% solvent A. The sample was loaded into a 2-ml loop, injected immediately into the column at room temperature and eluted over 40 or 45 min going from 100 to 0% of solvent A. For each peptide, the major peak was collected. The purity of the peptides was not less than 95% as estimated by UV and MALDI-ToF mass spectrometry.

Peptide-containing Membrane Bicelles

DMPC/DCPC bicelles membranes were prepared as reported earlier [12,13]. The appropriate amounts of phospholipids were weighed to obtain a mole content, $X = [\text{DMPC}]/[\text{total lipid}]$, of 80% with a total lipid hydration of 90% (w/w) in H_20, 100 mM KCl. Hydrated samples were centrifuged at 6500 rpm for 5 min, vigorously stirred in a vortex mixer and centrifuged again at 6500 rpm for 5 min. The samples were then frozen in liquid nitrogen for 30 sec, heated at 50 °C for 10 min in a water bath, vigorously stirred again in a vortex and finally centrifuged at 6500 rpm for 5 min. This cycle of bicelles formation was repeated three times until a viscous, translucent suspension was obtained at 30–40 °C. In order to prepare the peptide embedded in membrane bicelles, peptides and lipids were cosolubilized in TFE (molar ratio 1/900). The organic solvent was evaporated and water added. The system was vigorously mixed and lyophilized. This step was repeated once to ensure complete removal of traces of organic solvents. A buffer was finally added, and several cycles of centrifugation, cooling and heating were performed to ensure bicelles formation (*vide supra*).

Circular Dichroism

CD spectra were run on a Mark VI Jobin Yvon dichrograph at 0.2 nm intervals over the wavelength range 184–270 nm. The dichrograph was calibrated using isoandrosterone (Roussel-Uclaf, France) in dioxane and camphorsulfonic acid in water. The optical rotation was checked with cytochrome *c* (horse heart) and calmodulin, lysozyme (chicken egg white), all from Sigma-Chimie (St Quentin, France). Measurements were carried out in cells of 0.1 mm path length at room temperature in TFE and at 35 °C in DMPC/DCPC bicelles (1,2-dimyristoyl-/dicaproyl-*sn*-glycero-3-phosphocholine). This corresponds to the center of the composition–temperature bicelle domain as reported elsewhere in [12]. The dichroic signals of pure DMPC/DCPC bicelles and of TFE gave little background [14], but they were nonetheless recorded for subtraction. Peptide concentrations in the different media were 500 μM in TFE and 600 μM in bicelles (peptide/lipid ratio 1/900).

Secondary-structure content was estimated from CD spectra using the deconvolution program CDFriend (S. Buchoux, unpublished). This program, developed in the laboratory, uses standard curves obtained for each canonical structure (α-helix, β-sheet, helix-II and random coil). L_iK_j peptides of known length, secondary structure [15,16] and CD spectrum were used. The program uses a simulated annealing algorithm to get the best combination of α-helix, β-sheet, helix-II and random coil that exhibits the lowest normalized root mean square deviation (NRMSD) with respect to the experimental spectrum. The procedure appeared to be robust, simple to use (no initial guesses) and very adaptable for the secondary-structure characterization of hydrophobic peptides. The procedure will be described elsewhere. Accuracy is estimated to be $\leq$5%. Before deconvolution, experimental spectra were generally smoothed with a 5-point FFT Filter using the Origin Microcal software.

LC-Q Mass Spectrometry

The mass spectrometer used has a source with electrospray ionization (ESI) and an analyzer with an ion trap. At the time of injections, a split was installed at the exit of the LC so that 1/10 of the injection went in the spectrometer for the analysis and 9/10 was collected. Optimization of the choice of the columns and various gradients enabled us to obtain pure products with yields in the range of 15–22%.

MALDI-ToF Mass Spectrometry

MALDI-ToF mass spectrometry was performed on a Bruker REFLEX III instrument in the reflectron mode with a 20-kV acceleration voltage and a 23-kV reflector voltage. α-Cyano-4-hydroxy-cinnamic acid (Sigma) was used as a matrix, prepared as a saturated solution of 50% acetonitrile/0.1% TFA in water. Peptide was mixed in the ratio 1 : 1 (v/v) with the matrix solution. Samples were prepared with the dried-droplet method on a stainless steel target with 26 spots [17]. External mass calibration was achieved with a mixture of eight peptides having masses ranging from 961 Da (fragment 4–10 of adrenocorticotropic hormone) to 3495 Da (β-chain of oxidized bovine insulin).

RESULTS

Chemical Syntheses

The solid-phase synthesis was carried out using the Fmoc strategy in a simple coupling mode, with 0.1 mmol of resin and tenfold excess of amino acids, for the small-scale synthesis of Neu_{TM35} and Neu^*_{TM35}. As the structural studies require large amounts of ^{15}N-labelled peptides, the syntheses of ^{15}N-labelled Neu_{TM35} and Neu^*_{TM35} were carried out on a medium-range scale: 0.25 mmol of resin and with a fourfold excess of amino acids. The cleavages of all the peptides were performed with the mixture TFA/TIS/thioanisole/EDT/crystalline phenol during for two h.

Three different synthesis pathways were tested with the aim of providing sufficient amounts of pure Neu_{TM35}, Neu^*_{TM35} and ^{15}N-labelled peptides for future biophysical studies. All syntheses were performed with a NovasynTGA arginine preloaded resin. The first synthesis was carried out to obtain the peptide Neu_{TM35}.

From 476 mg resin, only 135 mg of crude peptide was obtained after cleavage (Table 1). The MALDI-ToF spectrum of the crude product (data not shown) indicates a major peak of molecular mass 3888.22 g mol^{-1}, in agreement with the theoretical molecular weight of the 35-amino acid peptide of 3888.44 g mol^{-1}. In addition, there were several other peaks that correspond to truncated peptides acetylated with acetic anhydride during the capping steps. The second synthesis was performed to obtain the mutated Neu$^{*}_{TM35}$ peptide. The same resin was used as before, and 201 mg of crude peptide was obtained after cleavage (Table 1). The main peak in the mass spectrum occurs at a molecular mass of 3918.80 g mol^{-1}, which corresponds to the theoretical molecular weight of 3918.42 g mol^{-1}. The MALDI-ToF spectrum, (Figure 1), shows that the end capping segments are clearly seen and no corresponding adducts are detected. The last syntheses were performed with the aim of incorporating ^{15}N-labelled amino acids (valine and leucine) for NMR studies. These syntheses were carried out in the same way as for Neu$_{TM35}$ and Neu$^{*}_{TM35}$ and four new peptides were thus obtained and characterized by MALDI-ToF (data not shown). From 1.136 mg resin, 486 mg of the crude peptide $^{15}N_2$-Neu$_{TM35}$ was obtained after cleavage. The main MALDI-ToF peak indicates a molecular mass of 3890.10 g mol^{-1} (theoretical m.w. = 3890.44 g mol^{-1}). Another synthesis was performed for the peptide with four labels, $^{15}N_4$-Neu$_{TM35}$: 398 mg of crude peptide was recovered with a main MS peak of molecular mass 3892.23 g mol^{-1} (theoretical m.w. = 3892.44 g mol^{-1}). For the labeled versions of the mutated peptide, 425 mg of crude $^{15}N_2$-Neu$^{*}_{TM35}$ was obtained. The major MS peak shows a molecular mass of 3920.25 g mol^{-1} (theoretical m.w. = 3920.42 g mol^{-1}). Also, 541 mg of crude $^{15}N_4$-Neu$^{*}_{TM35}$ was recovered, with the MALDI-ToF spectrum showing a molecular mass of 3922.26 g mol^{-1} (theoretical m.w. = 3922.42 g mol^{-1}). All the results are compiled in Table 1, in which synthesis yields are also indicated. The synthesis yield is calculated from the mass of collected crude peptide relative to the theoretical crude peptide mass. The latter is calculated from the theoretical yield, which depends on the number of coupling steps under the assumption of a 99% yield per step [18,19]. For example, for a 35-residue peptide, the theoretical yield would be $0.99^{34} \times 100 = 71\%$. Synthesis yields vary from 35 to 55%. This is a clear improvement with respect to published procedures. The mutated peptide appears to be slightly easier to synthesize.

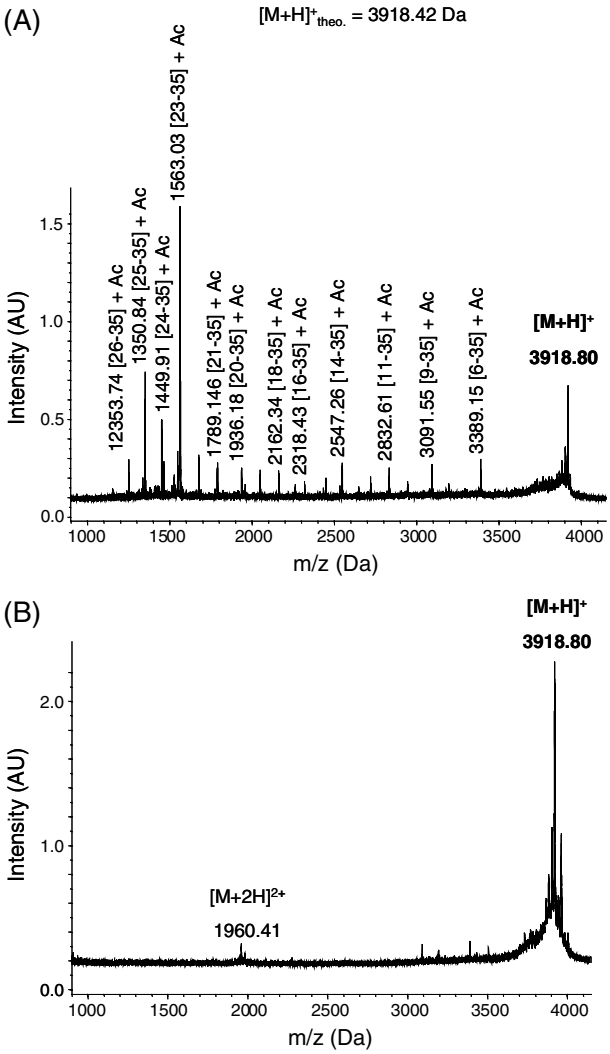

Figure 1 (A) MALDI-ToF spectrum of (A) the crude reaction mixture containing the 35-amino acid long Neu$^{*}_{TM35}$ domain. The numbering in brackets [x-35] corresponds to truncated peptide segments. (B) Spectrum of Neu$^{*}_{TM35}$ after purification by reversed-phase HPLC. The second ionization product is observed at $(M + nH)/(n)$, with m/z of 3918.80 Da. The mass if before the main peak comes from degradation by the MALDI laser.

Purification of Peptides

All purifications were performed by HPLC using a semipreparative column Vydac C4 (5 µm, 300 Å, 250 × 10 mn) with different gradients, depending on whether the peptide is mutated or not. For Neu$_{TM35}$, the best purification was achieved using a linear gradient with the following time intervals: eluent A varied for 18 min from 100 to 20%, then for 14 min from 20 to 0% and a final stage with 100% B applied for 5 min. Neu$_{TM35}$ was eluted at a high percentage of acetonitrile/isopropanol (93%). The retention time was approximately 26 min, and 20 mg of peptide, characterized by MALDI-ToF (data not shown), was recovered (Table 1). For Neu$^{*}_{TM35}$, the time intervals used were as follows: eluent A varying from 100 to 50% for 15 min, then from 50 to 10% for 11 min, then from 10 to 0% for 10 min and a final stage with 100% B applied for 5 min. The retention time was approximately 29 min at a high percentage of acetonitrile/isopropanol (91%). A typical elution profile

Table 1 Successive masses and yields along the reaction steps (synthesis and purification)

Peptides	Crude peptide[a] (mg)	Synthesis yield[b] (%)	Pure peptide[c] (mg)	Purification yield[d] (%)	Final yield[e] (%)	Purity[f] (%)
Neu_{TM35} EQRASPVTFIIATVVGVLLFLILVVVVGILIKRRR	135	35	20	15	5	95
$^{15}N_2$-Neu_{TM35} EQRASPVTFIIATVVGVLLFLILVVVVGILIKRRR	486	50	83	15	8	96
$^{15}N_4$-Neu_{TM35} EQRASPVTFIIATVVGVLLFLILVVVVGILIKRRR	398	41	55	15	6	95
Neu_{TM35}* EQRASPVTFIIATVEGVLLFLILVVVVGILIKRRR	201	52	40	20	10.5	97
$^{15}N_2$-Neu_{TM35}* EQRASPVTFIIATVEGVLLFLILVVVVGILIKRRR	425	44	94	22	10	98
$^{15}N_4$-Neu_{TM35}* EQRASPVTFIIATVEGVLLFLILVVVVGILIKRRR	541	55	98	18	10	98

The synthesis of the Neu_{TM35} and the Neu^*_{TM35} ^{15}N-labelled were carried out on a medium scale (0.25 mmol of resin with a fourfold excess of amino acids). Singly underlined amino acids indicate mutation, doubly underlined show positions of ^{15}N-labelling.
[a] Crude peptide mass obtained after synthesis and cleavage.
[b] Synthesis yield (experimental mass of crude peptide/theoretical mass of crude peptide) see text.
[c] Pure peptide mass obtained after purification.
[d] Purification yield (pure peptide/crude peptide) (w/w) in %.
[e] Total yield (synthesis yield × purification yield).
[f] Degree of purity of the pure peptides.

is shown in Figure 2, with the major peak indicating the pure Neu^*_{TM35} peptide. Total mass after purification was 40 mg. Purification and characterization of the ^{15}N-labelled peptides were carried out in the same way as for Neu_{TM35} and Neu^*_{TM35}. We collected 83 mg of $^{15}N_2$-Neu_{TM35}, 55 mg of $^{15}N_4$-Neu_{TM35}, 94 mg of $^{15}N_2$-Neu^*_{TM35} and 98 mg of $^{15}N_4$-Neu^*_{TM35}. Table 1 sums up the results together with purification yields and total yields. Purifications yields are calculated from the peptide masses obtained after purification relative to the masses before purification. Purification yields range from 15 to 22%, which is a large improvement compared to those from published procedures. Total yields (product of synthesis and purification yields) range between 5 and 8% for Neu_{TM35} and it is 10% for the Neu^*_{TM35}. The final purity of all peptides varies from 95 to 98%, as is required for structural analysis. It was determined by HPLC using the software Millenium (Waters) and mass spectrometry (Figures 1 and 2). No by-adducts were detected after purifications.

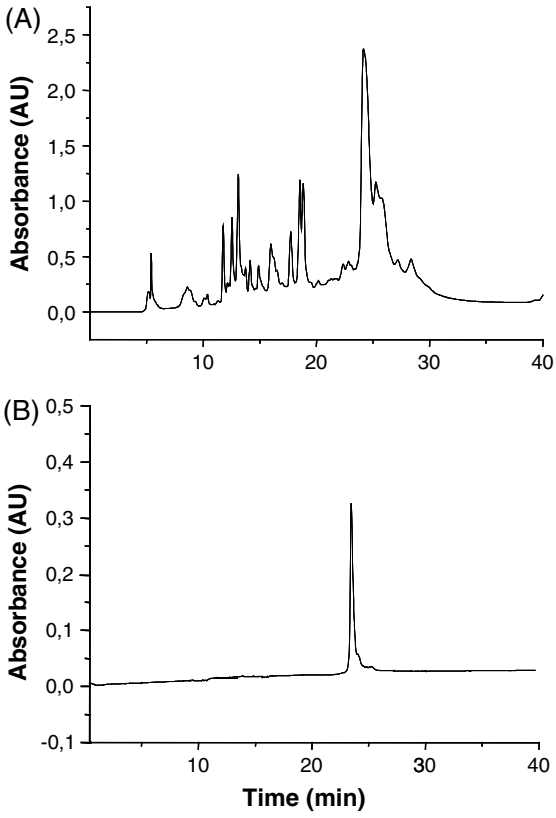

Figure 2 Photodiode UV chromatograms at 215 nm of (A) the crude peptide Neu^*_{TM35} and of (B) the purified peptide Neu^*_{TM35} with reversed-phase chromatography on a semipreparative C4 column at a flow rate of 3 ml/min. The major fraction was eluted after treating for 29 min with 91% of eluent B (see text).

Secondary-structure Analysis in TFE and Membranes by Circular Dichroism

Because the synthesized 35-residue peptides are membrane-spanning segments composed of *ca* 70% nonpolar amino acids, the secondary structure was first studied in a membrane 'mimicking' solvent TFE. Figure 3(A) shows the mean residue-weighted ellipticity of Neu_{TM35} and Neu^*_{TM35} in TFE. The spectrum of a standard α-helix is also shown as a dash–dot line. Although there are differences in the curves, all

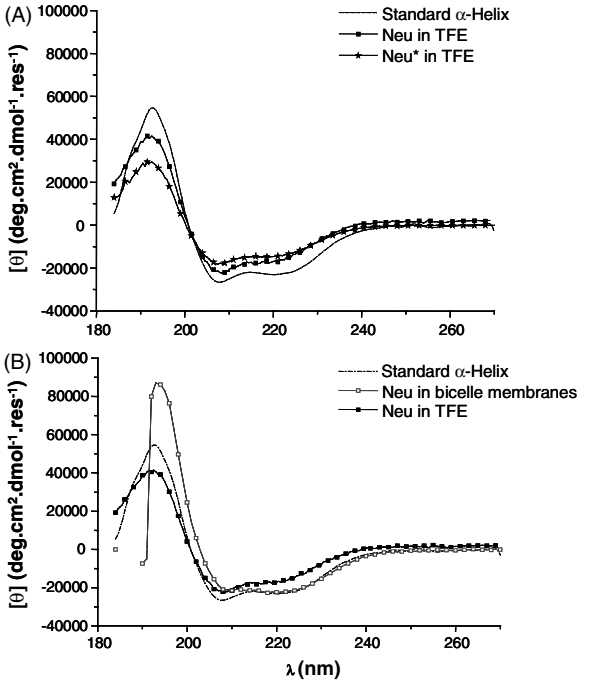

Figure 3 (A) Far ultraviolet CD spectrum in TFE for Neu_{TM35} (filled square) and Neu^*_{TM35} (star). (B) Far ultraviolet CD spectrum for Neu_{TM35} in TFE (filled squares) and in membranes bicelles (empty squares). Standard α-helix trace (dash–dot) is also shown.

show the pronounced positive (190 nm) and negative (208 nm and 220 nm) absorption bands characteristic of α-helices. The curve corresponding to Neu_{TM35} is closer to that of the standard suggesting a higher α-helix content than Neu^*_{TM35}. The curves were submitted to spectral deconvolution that uses a base of standard curves for random coil, α-helix, β-sheet and type II helix (*vide supra*). For Neu_{TM35}, 76% α-helical structural elements was found but only 63% for Neu^*_{TM35}. The same experiments and deconvolutions were performed for the ^{15}N-labelled peptides. We found 73 and 72% α-helical contents for $^{15}N_2$-Neu_{TM35} and $^{15}N_4$-Neu_{TM35}, respectively, whereas 65 and 61% were found for $^{15}N_2$-Neu^*_{TM35} and $^{15}N_4$-Neu^*_{TM35}.

DMPC/DCPC membrane bicelles are known to give little background in optical spectroscopy [14]. Therefore CD data of Neu_{TM35} embedded in membranes were collected, Figure 3(B), for a better modeling of the natural medium. In this figure, the CD traces of this peptide in TFE and of the standard α-helix are also shown. The presence of maxima and minima, characteristic of α-helices, are also detected for Neu in bicelles indicating that the secondary structure seen in TFE is retained in membranes. However, an important change in the intensity maximum is noticed, $[\theta]_{190} = 86\,000$ deg cm^2/dmol per residue compared to $[\theta]_{190} = 35\,000$ deg cm^2/dmol per residue in TFE and the minimum (208 and 220 nm) intensities are approximately equal.

DISCUSSION

In this work, three main results appear: (i) large improvement of both synthesis yields and purification of the 35-aa hydrophobic peptide Neu_{TM35} and its ^{15}N-labelled versions, (ii) the synthesis and purification of the mutated version, Neu^*_{TM35}, and its ^{15}N-labelled versions with better yields than for the wild type and (iii) the secondary-structure determination for both peptides in TFE and in phospholipid model membranes. These different points will be discussed below.

Improvement of Neu_{TM35} Synthesis and Purification

In order to increase the yields of the synthesis and purification, it was decided to test two different kinds of resins, three different cleavage pathways, two different HPLC columns and several gradients by LC-Q to optimize the purification.

First of all, it appeared judicious to use a NovaSynTGA resin instead of the Wang resin classically used for long peptides (Novabiochem, France). The NovasynTGA is chemically more robust, less loaded and compatible with a wide range of reaction conditions. This resin can be well swollen, the percentage of substitution is lower compared to polystyrene resins (such as Wang) and the distribution of the reactive groups is more homogeneous. All these factors contribute in reducing peptide aggregation and secondary-structure formation in the course of synthesis. In earlier syntheses developed by Goetz and coworkers, coupling times were prolonged up to 50 min per residue and dimethylsulfoxide was added during the last 20 min for optimal solubilization of the growing peptide in the resin matrix. This step was removed in this procedure because it did not bring extra yield and increased the total synthesis time. We ended up with two days for an entire small-scale synthesis instead of 4–5 days as reported by the above authors. To improve the synthesis yield, HBTU/HOBt activators were used, which were recently developed in solid-phase synthesis and gave good results [19–21].

Improving cleavage was pursued with the aim of removing both the entire protecting groups and the resin from the peptide. Tests were carried out on small quantities of resin/peptides, between 10 and 15 mg. The standard cleavage is in principle performed with TFA/TIS/H_2O (95 : 2.5 : 2.5 v/v/v), but when the peptide has an *N*-terminal glutamic acid, water must be omitted to prevent the conversion of the *N*-terminal glutamic acid to a pyroglutamyl residue [21], and hence all tests were performed without water. In cases where problems occur, the addition of EDT or reagent K to the mixture is generally known to provide a satisfactory solution because it is an extremely good scavenger for tBu cations. Our first trial was carried out with TFA/TIS (95/5,v/v), the second with TFA/TIS/EDT and the final one with TFA/TIS/thioanisole/EDT/crystalline

phenol. In parallel, a few tests were carried out in order to determine the time for adequate cleavage. TFA/TIS was used with a cleavage time ranging from 2 to 12 h. This mixture was not effective enough because we detected on the MS spectrum one peak corresponding to the peptide with two tBu groups; these peaks were still present after 12 h of cleavage. With the solution TFA/TIS/EDT, we also observed a peak corresponding to one tBu, but with less intensity than in the preceding experiment. This peak was unfortunately still present after 12 h of cleavage. In the last trial, TFA/TIS/thioanisole/EDT/crystalline phenol was used, and no tBu peak was observed in the MS spectra after 2 h of cleavage. These improvements led to synthesis yields (after cleavage) that were two to three times better than those published earlier.

In general, the low yields obtained after purification are related to the dominant hydrophobic character of the peptide sequence: Neu_{TM35} contains in majority alanine, isoleucine, leucine, phenylalanine and valine. Tests carried out on the LC-Q enabled us to optimize the gradient and the column for purification. The column Vydac C4 (5 μm, 300 Å, 250 × 10 mn) appeared to be up-to-date, the most adapted for these hydrophobic peptides: the combination of 300 Å, high purity silica with thorough and stable bonding chemistry makes it a good candidate for peptide separation. After testing a C18 column for which no peptide elution could be attained, a C4 column that has less affinity for hydrophobic peptides was chosen. The best gradient was divided into two slopes, small peptides being eluted at the beginning, whereas the desired peptides at the end. We noticed that the use of the isopropanol was of much benefit for solubilization and peptide desorption from the column. Although the peptide is very hydrophobic, purification yields of 15%, which represents a marked increase compared to the 5–10% reported earlier were obtained. The entire procedure (synthesis and purification) which is faster and less tiresome enabled us to recover larger quantities of peptides (20–80 mg, Table 1).

Synthesis and Purification of Mutated Peptides

Because the single-point mutation at position 15 (Val → Glu) confers to the peptide a less hydrophobic character, the procedure for synthesis and cleavage was kept the same but the purification was slightly modified. The synthesis yield for the peptide Neu^*_{TM35} is 44–55%, which is quite good compared to the theoretical yield (71%). The synthesis yields for Neu_{TM35} and Neu^*_{TM35} are approximately the same (35–50% for Neu_{TM35}). The method for purification was slightly changed, since Neu^*_{TM35} is less hydrophobic than Neu_{TM35}. The best purification was obtained with three gradient slopes, the first to elute small, truncated peptides at the beginning of the run, and the other two to recover the desired peptides. The results show that Neu^*_{TM35} was eluted with a slightly lower percentage of acetonitrile/isopropanol (91% for Neu^*_{TM35} and 93% for Neu_{TM35}). The purification yield increased to 18–22%. It therefore appears that modifying the peptide sequence by only one amino acid may lead to marked differences in total synthesis yield. We believe that the position of the substitution, a glutamic acid replacing a valine in the middle of a hydrophobic stretch, may have a great importance in peptide affinity for solvent or column.

Secondary-structure Analysis

CD spectroscopy was used to investigate the nature and amount of secondary-structure elements for all the peptides synthesized. Experiments were performed in pure TFE and in phospholipid membranes that give reduced light scattering. Both peptide structures mainly adopt α-helical structures in TFE, but the wild type Neu_{TM35} has an average content of ≈74% whereas that of the mutated peptide ≈63%. The difference is beyond experimental error and our study clearly indicates that less α-helix content is found in Neu^*_{TM35}, a result that could be attributed to the presence of glutamic acid in the middle of a hydrophobic stretch that might perturb the α-helix stability. Goetz and coworkers [22] solved the 3D structure on Neu_{TM35} in TFE and found in it 74% α-helix, 17% π-helix and 9% random coil. The π-bulge was observed for the sequence ^{22}ILVVVVG^{28}. Our CD results agree very well with the NMR findings concerning the α-helix content. However, they differ in the π-helix finding, primarily because there are no standard curves for pure π-helices in CD deconvolution procedures (ours and others), its contents appearing as random coils. Membrane proteins require an amphiphilic environment for their proper folding and function. The suitable environment is of course the phospholipid membrane. The CD trace of Neu_{TM35} in bicelles shows that the main α-helical secondary structure found in TFE is retained in membranes, thereby validating studies in this membrane 'mimicking' solvent. Interestingly, the intensity ratio of bands occurring at 220 and 208 nm, $\theta_{220}/\theta_{208}$, is about unity in membranes. This ratio has been used by other groups as an indicator of two-stranded, α-helical coiled-coil structure [23,24]. In relation to our study of Neu_{TM35} in membranes, it appears that the flat membrane interface would favor the existence of dimers/oligomers.

More CD and NMR experiments are in progress in our laboratory to further investigate the structure and dynamics of the mutated and wild type transmembrane fragments of the neu/erbB-2 receptor.

CONCLUSION

The peptide corresponding to the wild type and mutated tyrosine kinase transmembrane receptor has been

synthesized in appreciable amounts with very high purity (95–98%). Compared with already published procedures this method presents improvements in synthesis, cleavage and purification steps. Use of a new resin, cleavage reagents, HPLC columns and appropriate elution solvents allowed optimization of the preparation procedures.

These conditions could be generalized for the synthesis and purification of other protein sequences containing large hydrophobic domains by keeping the same resin, the same HPLC column and isopropanol in elution mixtures. Other parameters such as cleavage reagents and ramp for gradients may be adapted, depending on specific sequences.

Acknowledgements

We thank Dr Fabrice Cornille (Senn Flamma Marketing, Cachan, France), Dr. Loic Martin (CEA, dsvidf, Saclay, France) and Dr Claude Manigand (UBS, Bordeaux, France) for assistance and help in the purification of the peptides. This work was supported by the Association pour la recherche sur le cancer, France (ARC-4261), the Fondation pour la recherche médicale (France) and the Ligue Nationale contre le cancer (France).

REFERENCES

1. Cao H, Bangalore L, Bormann BJ, Stern DF. A subdomain in the transmembrane domain is necessary for p185neu* activation. *EMBO J.* 1992; **11**: 923–932.
2. Chen LI, Webster MK, Meyer AN, Donoghue DJ. Transmembrane domain sequence requirements for activation of the p185c-neu receptor tyrosine kinase. *J. Cell. Biol.* 1997; **137**: 619–631.
3. Smith SO, Aschheim K, Groesbeek M. Magic angle spinning NMR spectroscopy of membrane proteins. *Q. Rev. Biophys.* 1996; **29**: 395–449.
4. Weiner DB, Liu J, Cohen JA, Williams WV, Greene MI. A point mutation in the neu oncogene mimics ligand induction of receptor aggregation. *Nature* 1989; **339**: 230–231.
5. Sizun C, Aussenac F, Grelard A, Dufourc EJ. NMR methods for studying the structure and dynamics of oncogenic and antihistaminic peptides in biomembranes. *Magn. Reson. Chem.* 2004; **42**: 180–186.
6. Goetz M, Schmitter JM, Geoffre S, Dufourc EJ. Chemical synthesis of yeast mitochondrial ATP synthase membranous subunit 8. *J. Pept. Sci.* 1999; **5**: 245–250.
7. Glover KJ, Martini PM, Vold RR, Komives EA. Preparation of insoluble transmembrane peptides: Glycophorin-A prion, and FGFR. *Anal. Biochem.* 1999; **272**: 270–274.
8. Jones DH, Rigby AC, Barber KR, Grant CWM. Oligomerization of the EGF receptor transmembrane domain: a ^{2}H NMR study in lipid bilayers. *Biochemistry* 1997; **36**: 12 616–12 624.
9. Rigby AC, Barber KR, Shaw GS, Grant CWM. Transmembrane region of the epidermal growth factor receptor. Behaviour and interactions via 2H-NMR. *Biochemistry* 1996; **35**: 12 591–12 601.
10. Houliston RS, Hodges RS, Sharom FJ, Davis JH. Comparison of proto-oncogenic and mutant forms of the transmembrane region of the neu receptor in TFE. *FEBS Lett.* 2003; **535**: 39–43.
11. Goetz M, Rusconi F, Belghazi M, Schmitter JM, Dufourc EJ. Purification of the c-erbB2/neu membrane-spanning segment: a hydrophobic challenge. *J. Chromatogr. B* 2000; **737**: 55–61.
12. Raffard G, Steinbruckner S, Arnold A, Davis JH, Dufourc EJ. Temperature-composition diagram of Dimyristoyl-Dicaproyl Phosphatidylcholine "Bicelles" self-orienting in the magnetic field. A solid state 2H and 31P-NMR study. *Langmuir* 2000; **16**: 7655–7662.
13. Arnold A, Labrot T, Oda R, Dufourc EJ. Cation modulation of "bicelle" size and magnetic alignment as revealed by solid state NMR and electron microscopy. *Biophys. J.* 2002; **83**: 2667–2680.
14. Loudet C, Khemtemourian L, Aussenac F, Gineste S, Achard MF, Dufourc EJ. Bicelle membranes and their use for hydrophobic peptide studies by circular dichroism and solid state NMR. *Biochim. Biophys. Acta* 2005; **1724**: 315–323.
15. Castano S, Desbat B, Dufourcq J. Ideally amphipathic b-sheeted peptides at interfaces: structure, orientation, affinities for lipids and hemolytic activity of $(KL)_mK$ peptides. *Biochim. Biophys. Acta* 2000; **1463**: 65–80.
16. Castano S, Cornut I, Büttner K, Dasseux JL, Dufourcq J. The amphipatic helix concept: length effects on ideally amphipathic $L_iK_j(i = 2j)$ peptides to acquire optimal hemolytic activity. *Biochim. Biophys. Acta* 1999; **1416**: 161–175.
17. Rusconi F, Schmitter JM. Préparation des échantillons en vue d'une analyse par spectrométrie de masse MALDI-ToF. *Analusis* 1998; **26**: M13–M17.
18. Chan WC, White PD. *Fmoc Solid Phase Peptide Synthesis. A Practical Approach.* Oxford University Press: London, 2000; 9–40.
19. Fields CG, Lloyd DH, MacDonald RL, Otteson KM, Noble RL. HBTU activation for automated Fmoc solid-phase peptide synthesis. *Pept. Res.* 1991; **4**: 95–101.
20. Nilsson MRNL, Raleigh DP. Synthesis and purification of amyloidogenic peptides. *Anal. Biochem.* 2001; **288**: 76–82.
21. Grant GA. *Synthetic peptides: A User's Guide.* WH Freeman and Company: New York, 1992.
22. Goetz M, Carlotti C, Bontems F, Dufourc EJ. Evidence for an α-helix ->π-bulge helicity modulation for the *neu/erbB-2* membrane-spanning segment. A ^{1}H NMR and circular dichroism study. *Biochemistry* 2001; **40**: 6534–6540.
23. Lau SY, Taneja AK, Hodges RS. Synthesis of a model protein of defined secondary and quaternary structure. Effect of chain length on the stabilization and formation of two-stranded alpha-helical coiled-coils. *J. Biol. Chem.* 1984; **259**: 13 253–13 261.
24. Sourgen F, Maroun RG, Frère V, Bouziane M, Auclair C, Troalen F, Fermandjian S. A synthetic peptide from the human immunodeficiency virus type-1 integrase exhibits coiled-coil properties and interferes with the *in vitro* integration activity of the enzyme. Correlated biochemical and spectroscopic results. *Eur. J. Biochem.* 1996; **240**: 765–773.

