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ORIGINAL ARTICLE

An optimized Fmoc synthesis of human defensin 5

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Abstract Human α-defensin 5 (DEF5), expressed by the Paneth cells of human small intestine, plays an important role in host defense against microbial infections. DEF5, a 32-residue peptide adopting a three-stranded β-sheet fold stabilized by three internal disulfide bonds, is not efficiently produced by recombinant expression techniques and is, therefore, an interesting goal for chemical synthesis. While DEF5 production by Boc-based solid-phase synthesis has been described, to date no synthetic account by the more convenient Fmoc method has been published. Herein, we report an optimized solid-phase synthesis of DEF5 using the Fmoc strategy. Starting from a rather problematic initial synthesis using standard Wang resin and coupling protocols, the sequence elongation process has been monitored by mini-cleavage and MS analysis at strategic points, to identify problematic spots and act accordingly. For expediency, some of the optimization rounds have been run on defensin 5 amide. Main modifications have included the ChemMatrix® resin, known to decrease chain aggregation, and the use of pseudoproline dipeptide units at selected positions. Combination of some of these improvements results in a significantly purer product, to the extent that it can undergo in situ anaerobic oxidative folding to the native form without the need of an intermediate purification step. A typical synthesis run yielded about 15 mg of >95 % pure material. This approach should facilitate production of DEF5 and of selected analogs for structure–activity studies and other applications.

Keywords Human defensin 5 · Fmoc solid-phase peptide synthesis · ChemMatrix resin · Pseudoproline dipeptides

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Introduction

Defensins are antimicrobial peptides (AMPs) that play an important role in host defense as components of the innate immune system (Ganz et al. 1985; Lehrer et al. 1991; Lehrer and Ganz 2002). Human defensins are divided into two subgroups: α- and β-defensins, both consisting of a triple-stranded β-sheet structure, stabilized by three intramolecular disulfide bonds. Despite different Cys connectivities in α-defensins (1–6/2–4/3–5) and β-defensins (1–5/2–4/3–6) (Ganz 2003; Szyk et al. 2006), the fact that Cys-5 and -6 are in adjacent positions results in a similar general shape ("defensin fold") for both types of peptides. Up to now, six different human α-defensins are known, four (DEF1–DEF4) made by neutrophils (Ganz et al. 1985; Wilde et al. 1989) and two (DEF5 and DEF6) by the Paneth cells of the small intestine (Porter et al. 1998; Ganz 2003). Human α-defensin 5 (DEF5), the focus of this work, is a 32-residue AMP (Fig. 1) largely responsible for antimicrobial activity in the crypt lumen (Salzman et al. 2003). It is stored in Paneth cell secretory vesicles as a 75-amino acid propeptide which, in response to microbial invasion, is converted into a 43-aa propeptide by the metalloproteinase matrilysin (Schneider et al. 2005), then by trypsin

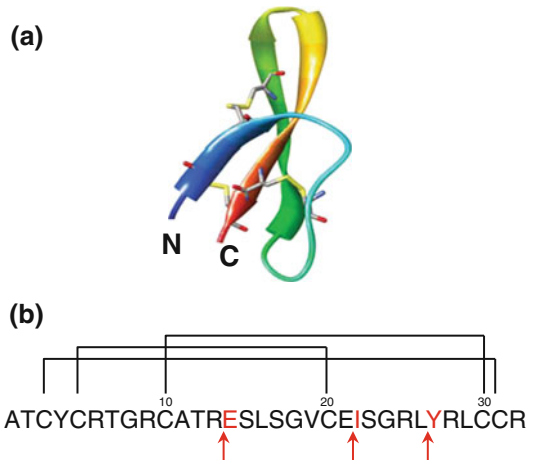

Fig. 1 **a** Three-dimensional structure of DEF5 showing the triple-strand β-sheet and the disulfide bridges (Wanniarachchi et al. 2011); **b** amino acid sequence and disulfide connectivity of DEF5. Residues in *red* refer to points at which peptide–resin samples were taken and a mini-cleavage (*arrow*) performed; see text for more details

proteolysis into the 32-residue mature form that is released into the intestinal lumen (Ghosh et al. 2002). DEF5 has been reported to play an important role in Crohn's disease, where loss of endogenous DEF5 is observed in the small intestine, while other Paneth cell products remain unchanged (Ishikawa et al. 2010); in addition, a single R13H point mutation in DEF5 has been observed in patients with inflammatory bowel disease (Shi 2007).

A growing interest on defensin structural and functional studies has placed increasing pressure on the sources of these peptides. Most studies to date have used defensins directly purified from cell extracts and tissues, but low yield makes studies costly and highly time-consuming. The alternative of overexpressing DEF5-coding genes was for long complicated by the toxicity of the peptide toward host cells, its susceptibility to proteolytic degradation and its small size; only recently these difficulties have been partly avoided and expression of mature DEF5 achieved in moderate efficiencies in *Pichia pastoris* or *Escherichia coli* expression systems (Wang et al. 2009; Wanniarachchi et al. 2011; Wommack et al. 2012). Even so, a peptide of DEF5's size and complexity would ideally appear to be well within the scope of chemical synthesis methods, with their inherent expediency and flexibility. Surprisingly, however, while various synthetic accounts of "defensin like" peptides—i.e., around 30-residue long and with three disulfide bridges—came up in a thorough revision of the literature, only one synthesis of DEF5 (Wu et al. 2004) has been hitherto reported, and that by a synthetic (Boc-based) methodology that is unfeasible at many labs due to hazard restrictions to the use of anhydrous HF. It seemed worthwhile, therefore, to explore whether DEF5 could be efficiently made by the more convenient Fmoc methodology that we have successfully applied to other peptides of similar size and complexity (Vila-Perelló et al. 2003; Rodrigues et al. 2012). Indeed, the goal proved considerably more challenging than originally expected: a trial DEF5 synthesis run using standard Fmoc methods turned up a very unpromising product in very low yields. What follows is an account of the various synthetic improvements that have finally enabled a reasonably efficient Fmoc synthesis of DEF5. This has involved identifying problematic stretches during the sequence assembly process and introducing corrective modifications, particularly regarding the solid support and the use of Ψ−Pro dipeptide units at identified trouble spots. These improvements can be combined to produce an hexathiol DEF5 precursor clean enough to be oxidized as a crude product, with no need of purifying the intermediate, thus increasing the efficiency of the synthetic approach.

Materials and methods

Chemicals

Fmoc-protected amino acids, HBTU and Fmoc-L-Arg(Pbf)-Wang resin were from Iris Biotech (Marktredwitz, Germany). Fmoc-Rink-amide ChemMatrix resin was from PCAS BioMatrix, Inc. (Saint-Jean-sur-Richelieu, Quebec, Canada). Fmoc-Rink-amide (MBHA) resin, Fmoc-Glu(OtBu)-Ser($\psi^{Me,Me}$pro)-OH and Fmoc-Ile-Ser($\psi^{Me,Me}$-pro)-OH were from Novabiochem (Laüfelfingen, Switzerland). An authentic sample of DEF5 was purchased from Innovagen (Sweden). HPLC-grade CH_3CN and peptide synthesis-grade DMF, CH_2Cl_2, DIEA and TFA were from Carlo Erba-SdS (Sabadell, Spain). All other reagents were of the highest quality commercially available from Sigma-Aldrich (Madrid, Spain).

General peptide synthesis procedures

Peptides were assembled in an ABI433 peptide synthesizer (Applied Biosystems, Foster City, CA) running Fmoc (FastMoc) SPPS protocols at 0.1-mmol scale on either Fmoc-L-Arg(Pbf)-Wang resin, Fmoc-Rink-amide ChemMatrix resin, Fmoc-Rink-amide (MBHA) resin or aminomethyl ChemMatrix resin. Side-chain functionalities were protected with tert-butyl (Glu, Ser, Thr, Tyr), N^G-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Arg), trityl (Cys) groups. Eightfold excess of Fmoc-L-amino acids and HBTU, in the presence of a double molar amount of DIEA, were used for the coupling steps, with DMF as solvent. After chain assembly, full deprotection and cleavage were carried out with TFA/H_2O/EDT/TIS (94:2.5:2.5:1 v/v, 90 min, rt). Peptide isolation was initially done by the standard procedure in Fmoc chemistry,

namely, precipitation with cold diethyl ether and centrifugation. However, as this practice was shown to be inadequate for DEF5 (see "Results and discussion"), the cleavage suspension was passed through a syringe fitted with a polyethylene porous disk to filter off the resin, which was rinsed with an additional 1 mL of TFA; the filtrate was then poured onto cold diethyl ether to give a white precipitate that was collected by centrifugation, redissolved in 0.1 M acetic acid and lyophilized.

Analysis and purification

Analytical reversed-phase HPLC was performed on C18 columns (4.6 × 50 mm, 3 μm, Phenomenex, Torrance, CA) in a model LC-2010A system (Shimadzu, Kyoto, Japan). Solvent A was 0.1 % TFA in water and solvent B was 0.1 % TFA in CH_3CN. Elution was done with linear 20–50 % gradients of solvent B into A over 15 min at 1 mL/min flow rate, with UV detection at 220 nm for the linear peptide and with 5–40 % linear gradient of B into A for oxidized peptide. LC–MS was performed in a LC–MS 2010EV instrument (Shimadzu) fitted with an XBridge column (4.6 × 150 mm, 3.5 μm, Waters, Cerdanyola del Vallès, Spain) eluted with a 20–50 % linear gradient of B into A for the unfolded (reduced) peptide and with a 5–40 % linear gradient of B into A for the oxidized peptide ($A = 0.1$ % formic acid in water; $B = 0.08$ % formic acid in acetonitrile) over 15 min at a flow rate of 1 mL/min, with UV detection at 220 nm. Preparative HPLC runs were performed on a Luna C18 column (21.2 × 250 mm, 10 μm; Phenomenex), using linear gradients of solvent B (0.1 % in ACN) into A (0.1 % TFA in water), as required, with a flow rate of 25 mL/min. Fractions of high (>95 %) HPLC homogeneity and with the expected mass were combined, lyophilized, and used in subsequent experiments.

Oxidative folding

The reduced (hexathiol) forms of DEF5 or DEF5[a] were oxidized at 5-μM concentration in 0.1 M NH_4OAc, pH 7.8, 25 °C (Vila-Perelló et al. 2003). The peptide, with or without previous HPLC purification (see "Results and discussion"), was dissolved at the specified concentration in the above buffer containing both reduced (GSH) and oxidized (GSSG) glutathione to give a 1:100:10 peptide:GSH:GSSG ratio. The solution was then placed under inert (N_2) atmosphere and stirred overnight at 25 °C. Progress of the folding reaction was monitored by analytical HPLC and, once completed, the target products were purified to homogeneity by preparative RP-HPLC and subsequently lyophilized. The synthetic DEF5, thus folded and purified, coeluted in analytical HPLC with an authentic DEF5 sample.

Results and discussion

The goal of this research was to find a strategy for producing DEF5, and eventually analogs thereof, by an efficient solid-phase synthesis method using Fmoc chemistry. The only reports of DEF5 and DEF5 analog synthesis published so far (Wu et al. 2004; De Leeuw et al. 2007) rely on Boc chemistry for chain assembly and anhydrous HF for final cleavage and deprotection, the last step being impractical for many laboratories given the safety hazards and consequent restrictions on HF use. In the search for a robust Fmoc-based synthetic route to DEF5 we have encountered a variety of hurdles that have been overcome, with reasonable success, as summarized in the workflow diagram shown in Fig. 2.

A first attempt to prepare DEF5 by a standard Fmoc procedure using Fmoc-L-Arg(Pbf)-Wang resin as starting material clearly showed that optimization would be required. UV monitoring of the sequence assembly process indicated several non-quantitative couplings and, accordingly, while LC–MS analysis of the crude product showed the expected hexathiol (MW 3588) as the main peak (Fig. 3a), the overall quality of the material was rather low. In view of this, in a second trial ChemMatrix® resin, a PEG-based support known to minimize the aggregation phenomena (De la Torre et al. 2007; García-Ramos et al. 2010) suspected largely responsible for the poor results of the first synthesis, was used instead of polystyrene-based Wang resin. Assuming also that the synthetic problems were unrelated to the (carboxyl or amide) nature of the C-terminus, a Rink amide-functionalized ChemMatrix® resin, eventually furnishing DEF5 amide (DEF5[a]), was chosen to simplify the C-terminal attachment step. In addition, the chain elongation process was monitored by mini-cleavage of peptide–resin aliquots (N-deprotected beforehand) and LC–MS analysis of the product at three pre-selected positions: (1) Tyr^{27}, early in the synthetic process, to ensure that chain assembly was proceeding adequately or, if not, try to solve the problem by decreasing resin loading; (2) Ile^{22} and (3) Glu^{14}, two positions preceded each by a Ser residue, hence amenable to pseudoproline (Ψ-Pro) dipeptide replacement, a tactic to deliberately disrupt β-sheet structures causing interchain aggregation and sluggish chain growth (Wöhr et al. 1996; Abedini and Raleigh 2005). MS confirmed that synthesis was proceeding smoothly at steps (1) and (2); in both cases, the mini-cleavage afforded a highly homogeneous product with the mass expected at that stage. Mini-cleavage at (3), however, showed the (14–32) sequence to be present only in minor amounts and accompanied by various deletion peptides indicating low coupling efficiencies in the 14–23 (ESLSGVCEIS) stretch. Elongation up to the N-terminus, deprotection and cleavage gave a crude (Fig. 3b) of very similar complexity to that of the

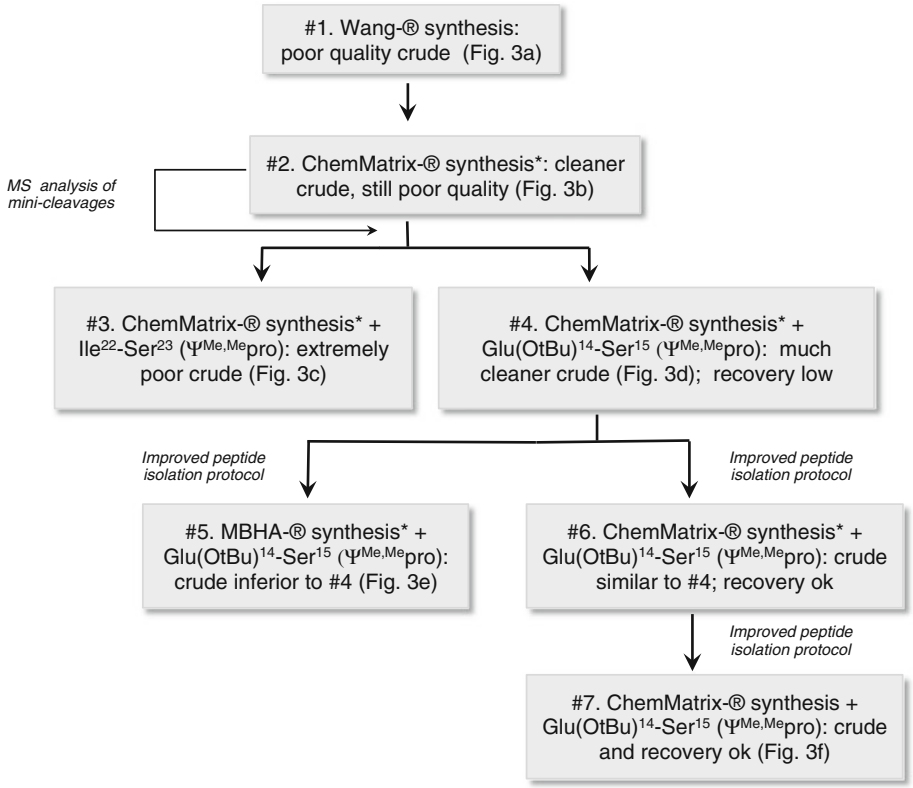

Fig. 2 Workflow in the optimization of DEF5 synthesis. *Asterisks* in runs #2–#6 refer to DEF5[a] instead of DEF5 being used as synthetic target, for expediency reasons

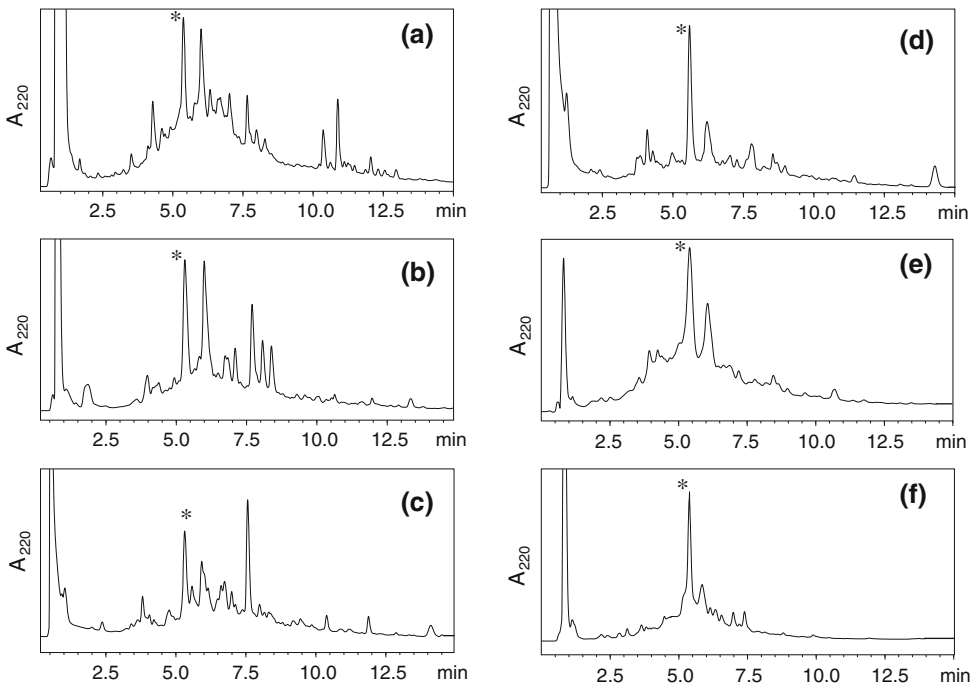

Fig. 3 Optimization of DEF 5 synthesis; see workflow chart in Fig. 2 for details. HPLC profiles of crude products (**a**) and (**f**) correspond to DEF5 syntheses; **b**–**e** to DEF5[a] syntheses. In all instances, the *asterisk*-marked peak eluting at ca. 5.3 min corresponds to the hexathiol precursor of DEF5 or DEF5a. Elution was with a linear 20–50 % gradient of B into A over 15 min, at 1 mL/min flow rate; solvents A and B as described in the experimental

mini-cleavage, only marginally cleaner than the first synthesis (Fig. 3a). In view of this, two new syntheses of DEF5 amide were planned, again on ChemMatrix® resin, and with Ψ-Pro replacements at the above-mentioned positions. The synthesis with the Ile-Ser($\psi^{Me,Me}$pro)-OH dipeptide replacement at positions 22–23 brought no improvement over previous runs; indeed, the main peak of the extremely poor crude (Fig. 3c) could not be matched by LC–MS to the target 3,587 Da mass. In contrast, replacement at positions 14–15 with Glu(OtBu)-Ser($\psi^{Me,Me}$pro)-OH dipeptide

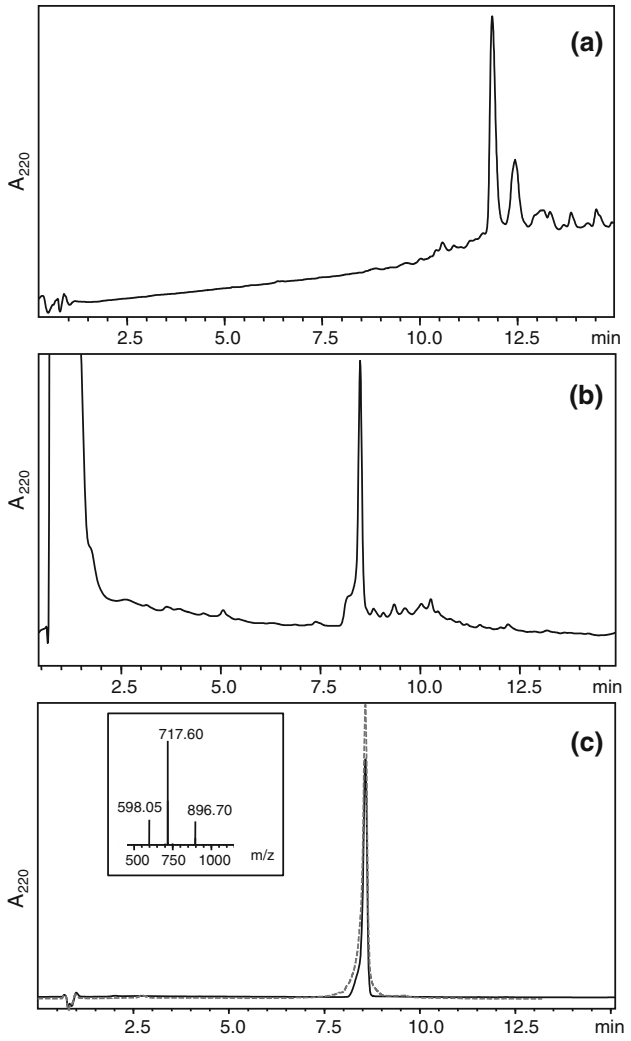

Fig. 4 **a** Hexathiol precursor peptide (run #7, Fig. 2) is efficiently converted to native-folded DEF5 (**b**); for conditions see text. **c** HPLC-purified DEF5 (*full line*) coelutes with an authentic sample (*broken line*); inset: ESI–MS spectrum of DEF5: $[M+6H]^{6+}$ (m/z 598.05), $[M+5H]^{5+}$ (m/z 717.60) and $[M+4H]^{4+}$ (m/z 896.70) peaks are shown. In all cases, HPLC elution was with a linear 5–40 % gradient of B into A over 15 min, at 1 mL/min flow rate; solvents A and B as described in the experimental

resulted in a product with and HPLC profile (Fig. 3d) significantly cleaner than any of the earlier attempts, and with the expected mass of 3,587 Da for the main peak. Purification of this crude product by preparative HPLC, however, afforded a disappointingly low recovery of DEF5[a] hexathiol precursor. Suspecting that the standard workup of the TFA cleavage solution, i.e., adding cold diethyl ether and centrifuging, was in this case inefficient, hence the peptide remained stuck to the resin, we resorted to a more thorough peptide isolation procedure, namely, filtering-off the resin first (see "Materials and methods") and then treating the filtrate with chilled ether to induce peptide precipitation in the absence of resin. After centrifugation and drying, the amount of solid crude product was ca. five times higher than with the standard procedure. This improved isolation protocol was applied systematically henceforth (Fig. 2). At this point it was clear that Ψ-Pro replacement of the Glu^{14}-Ser^{15} dipeptide combined with ChemMatrix® resin usage had meant a significant difference in DEF5[a] synthesis. To elucidate the specific contribution of the resin to the improved result, another DEF5[a] synthetic run was done, this time using Rink-amide-MBHA resin, the Glu(OtBu)-Ser($\psi^{Me,Me}$pro)-OH dipeptide at positions 14–15, and the optimized peptide isolation protocol described above. The crude product (Fig. 3e) was slightly cleaner than in the first—also polystyrene-based—synthesis (Fig. 3a), hence confirming the favorable effect of the ψ-Pro replacement, but nonetheless of poorer quality than when ψ-Pro and ChemMatrix® resin were simultaneously used, proving that only by combined usage of both modifications a substantial improvement was achieved. These advantages were again verified in the synthesis of the carboxyl (natural) sequence of DEF5, for which an aminomethyl ChemMatrix® resin was functionalized with the HMPP linker, loaded in manual mode with Fmoc-Arg(Pbf)-OH, then elongated as above in the synthesizer. The resulting crude (Fig. 3f) was of comparable quality to the previous DEF5[a] material.

For the final, oxidative folding step of DEF5 synthesis, an HPLC-purified hexathiol precursor was initially used. Disulfide formation in 0.1 M NH_4OAc buffer containing both reduced (GSH) and oxidized (GSSG) glutathione under N_2 atmosphere was complete after overnight reaction and led to a single-folded product with the expected 3,582 Da mass. In addition, taking cue from the good results obtained by Wu et al. (2004) in the oxidation of the hexathiol precursor in unpurified form, we proceeded accordingly and confirmed that unpurified crude (Fig. 4a) could be similarly converted to the oxidized/folded form using the above anaerobic conditions (Fig. 4b). In this way, a typical synthesis run (0.1 mmol) required only a single HPLC purification step and led to highly homogeneous (>95 %) folded product in amounts of ca. 15 mg. The correct folding (Cys^1–Cys^6, Cys^2–Cys^4, Cys^3–Cys^5) of our synthetic material was verified by comparison with an authentic (commercial) DEF5 sample by analytical HPLC, with both peptides found to coelute (Fig. 4c).

Conclusion

Results presented in this work illustrate how concomitant use of ChemMatrix® resin and ψ-Pro dipeptide replacement at the strategic Glu^{14}-Ser^{15} position transform the initially impractical production of DEF5 into a satisfactory stepwise Fmoc synthesis method. The two critical improvements in the sequence assembly process,

complemented by optimized workup after cleavage, enabled oxidative folding directly on the synthetic crude, avoiding one intermediate purification step and, thus, making the global process quite efficient. This optimized synthesis paves the way to obtaining DEF5 and its analogs in sufficient quantities for structural and biological studies.

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Conflict of interest The authors declare to have no conflict of interest.

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