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Polyethyleneglycol-Based Resins as Solid Supports for the Synthesis of Difficult or Long Peptides†

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An evaluation of the polyethyleneglycol-based ChemMatrix® resin as solid support for the synthesis of challenging peptide sequences is presented. Comparison with conventional polystyrene and polyethyleneglycol-polystyrene resins in several instances of typically difficult solid phase syntheses shows a consistently better performance of the ChemMatrix® resin in terms of end product purity. Representative test sequences include a 15-residue antibiotic, a gp41 ectodomain hybrid sequence, a calcipressin fragment with an N-terminal Arg_{11} extension, and two chemokines of 69- and 64-amino acid residues. Interestingly, a difference in only five amino-acids between the two chemokine sequences had a remarkable impact on synthetic results, which in the case of the 69-residue peptide required additional refinements (β-sheet-breaking pseudoproline dipeptides) for success.

KEY WORDS: cecropin A-melittin hybrid; gp41 ectodomain; cell-penetrating peptide; cytokines.

INTRODUCTION

Despite the substantial improvement in solid phase synthetic methodology over the last decades (Albericio and Kates, 2000), problems subsist such as the incorporation of sterically (e.g., *N*-alkyl or α,α-disubstituted) taxing units, or the poor solvation of peptide-resins, resulting in aggregation and defective chain growth (deletions, truncations, etc). These snags invariably decrease the purity of the synthetic product and, with a large size peptide target, may pose unsurmountable challenges to purification. Different solutions to these problems have been proposed, such as more effective coupling agents (uronium, aminium, acyl fluoride-like, etc) (Carpino 1993), improved solvation through the use of DMSO, NMP, hexafluoroisopropanol or other favorable solvent mixtures, or the use of bis-Fmoc,Hmb-protected amino acids (Johnson et al., 1995) or pseudoproline dipeptide units (Mutter et al., 1995; White et al., 2004) to prevent aggregation.

The polymer support obviously plays an often decisive role in the outcome of any solid phase synthesis (Forns and Fields, 2000), and resins providing better hydrophilicity/hydrophobicity balances (Kempe and Barany, 1996; Meldal, 1997) have been reported to provide improved results. Among the different types of supports available, polyethyleneglycol (PEG)-based materials are finding increasing application for peptide synthesis. In particular, demonstrable advantages over classic polystyrene (PS) have been reported for amphiphilic resins such as PEG-PS (Meldal, 1997; Forns and Fields, 2000). More recently, cross-linked resins entirely based on PEG, such as VersaBeads™ and ChemMatrix® (Côte 2005; García-Martín 2006a, b)

† This paper is dedicated to the memory of Bruce Merrifield, a dear teacher, mentor and friend.

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have been introduced. This communication describes a comparative study of the ChemMatrix® resin vs. PS or PEG-PS resins using as test models several sequences which in our experience pose considerable synthetic difficulties when made on the latter supports.

MATERIALS AND METHODS

Chemicals

Fmoc-protected amino acids were obtained from Senn Chemicals (Dielsdorf, Switzerland); Fmoc-Ser(tBu)-Ser((Me,Me-pro)OH, Fmoc-Rink-amide (MBHA) resin and Rink amide linker were from Novabiochem (Läufelfingen, Switzerland). Aminomethyl and Fmoc-L-Asn(Trt)-Wang-ChemMatrix® resins, and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*-hydroxybenzotriazole (HOBt) were from Matrix Innovation (Montreal, Canada). *O*-(7-azabenzotriazole-1-yl)-*N*,*N*,*N*′N′-3-tetramethyluronium hexafluorophosphate (HATU) was from GenScript Corporation (Piscataway, NJ). HPLC-grade acetonitrile, and peptide synthesis-grade *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), *N*,*N*-diisopropylethylamine (DIEA) and trifluoroacetic acid (TFA) were from SDS (Peypin, France).

Solid phase synthesis

Automated synthesis was performed in an Applied Biosystems model 433 peptide syntesizer employing standard Fmoc chemistry and 0.1 mmol FastMoc protocols on either 0.05 (ChemMatrix® resins) or 0.1 mmol scale (Fmoc-Rink-amide (MBHA) resin), with 10-fold excess of Fmoc-protected-L-amino acids and HBTU/HOBt or HATU coupling chemistries. For the C-terminal carboxamide sequences (Table I) made on ChemMatrix® resins, the Rink amide linker was coupled to aminomethyl ChemMatrix® resin using conventional HBTU activation.

Manual synthesis was carried out in polypropylene syringes fitted with a polyethylene porous disk. Fmoc-amino acids and Fmoc-Ser(tBu)-Ser(($\Psi^{Me,Me}$pro)OH were coupled by means of HATU using a 4-fold excess.

Protected peptide resins were N-deblocked prior to full deprotection and cleavage with trifluoroacetic acid-water-triisopropylsilane (95:2.5:2.5 v/v, 90 min, rt); for Cys- or Met-containing sequences, the cleavage mixture was trifluoroacetic acid-water-ethanedithiol-triisopropylsilane (94:2.5:2.5:1 v/v, 90 min, rt). Peptides were precipitated by addition of chilled methyl tert-butyl ether, taken up in aqueous HOAc (10% v/v) and lyophilized.

Analysis, purification and folding

Analytical reversed-phase HPLC was performed on a C_{18} column (4.6 × 50 mm, 3 μm), solvent A 0.045% TFA in H_2O, solvent B 0.036% in ACN, flow rate 1 mL/min, UV detection at 220 nm. Preparative HPLC was performed on a C_{18} column (21.2 × 250 mm, 10 μm), solvent A 0.045% TFA in H_2O, solvent B 0.036% in ACN, flow rate 25 mL/min. Linear gradients of solvent B into A were used for elution (see Figure captions for details). Fractions of adequate purity (HPLC) and with the expected mass (MALDI-TOF, Voyager DE-STR, Applied Biosystems, Foster City, CA, sinapinic or α-hydroxycinnamic acid matrices; spectra obtained in the linear mode) were combined and lyophilized. MALDI-TOF mass spectra were acquired in a Voyager DE-STR system (Applied Biosystems, Foster City, CA) using sinapinic or α-hydroxycinnamic acid matrices.

For the two CCL4 chemokines, the tetrathiol precursor (30 μM, 30 mL) obtained as above was folded by air oxidation in 0.1 M Tris, 2 M Gn–HCl buffer, pH 8.5, rt. HPLC monitoring of the reaction showed no further evolution after 4 h. The oxidized peptide mixture was desalted by direct loading to a Diaion column (0.25 g dry gel, pre-conditioned in water), washed with 4–5 volumes of 1% trifluoroacetic acid in water, then eluted with 1% trifluoroacetic acid-acetonitrile (1:1 v/v). The two folding isomers were subsequently separated by preparative HPLC. Mass analysis of these peptides was done in the linear TOF modes.

RESULTS AND DISCUSSION

The performance of the ChemMatrix® resin has been studied on several peptide models which we report and discuss in order of increasing difficulty.

Cecropin A-melittin hybrid peptide KWKLFKKIGAVLKVL-amide

This 15-residue cecropin A-mel1992ittin hybrid peptide antibiotic is active against Gram-positive and Gram-negative bacteria (Andreu et al.,1992), fungi (Cavallarin et al., 1998) and protozoa (Andreu and Rivas, 2001). Regularly made at our laboratory, this

Table I. Sequences of synthesized peptides

Cecropin A(1–7)-melittin(2–9) hybrid	KWKLFKKIGAVLKVL-amide
gp41 ectodomain peptide	NEQELLELDKWASLWNWFNITNWLWYIK-amide
Arg_{11}-extended peptide	RRRRRRRRRRRKYELHAATDTTPSVVVHVCES-amide
Arg_{11}-extended peptide(scrambled)	RRRRRRRRRRRPETASVADVSVEYHTLKTHVC-amide
CCL4-L1 chemokine	APMGSDPPTA$_{10}$**CC**FSYTARKL$_{20}$PRNFVVDYYE$_{30}$TSSL**C**SQPAV$_{40}$ V**FQTKR**GKQV$_{50}$**C**ADPSESWVQ$_{60}$EYVYDLELN-acid (*)
CCL4-L2 chemokine	APMGSDPPTA$_{10}$**CC**FSYTARKL$_{20}$PRNFVVDYYE$_{30}$TSSL**C**SQPAV$_{40}$ V· · · ·GKQV$_{50}$**C**ADPSESWVQ$_{60}$EYVYDLELN-acid (**)

*$Cys^{11,35}$ and $Cys^{12,51}$ disulfides; **disulfide pattern and numbering as for CCL4-L1. The five dots denote missing residues

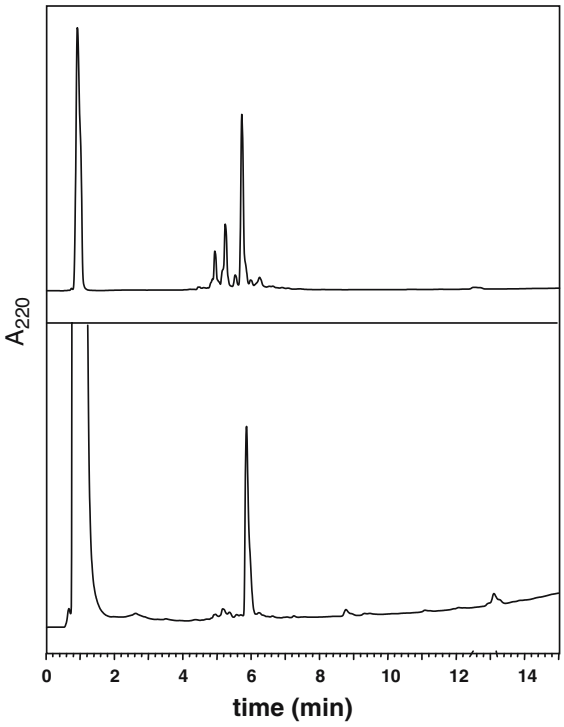

Fig. 1. Effect of the solid support on the quality of cecropin A(1–7)-melittin(2–9) (synthetic crude shown). Fmoc synthesis on Rink amide-functionalized polystyrene (upper panel) and ChemMatrix® (lower panel). HPLC conditions: linear gradient of B (5%–95%) into A over 15 min.

peptide tends to give surprisingly complex crude products even under manufacturer's "optimized" (Fmoc) chemistry. Thus, in a typical synthesis on PS, with recouplings at selected positions (Figure 1, upper panel), the main peak corresponding to the target sequence typically amounted to only 50% of the HPLC area. In contrast, the same peptide assembled on Rink amide-ChemMatrix® resin, without any recouplings, led to almost homogeneous material (>93% by HPLC) (Figure 1, lower panel).

gp41 ectodomain peptide: NEQELLELDKW ASLWNWFNITNWLWYIK-amide

We next examined a 28-residue peptide spanning two hydrophobic immunogenic regions (residues 656–683, gp160 numbering) of the HIV-1 gp41 ectodomain. This peptide is relevant to understanding the interplay between the two membrane-transferring domains that promote HIV-1 fusion (Lorizate et al., 2006). A first synthesis on PS using single couplings by manufacturer's recommended (FastMoc) protocols led to an intractable mixture (Figure 2, upper panel). Some improvement was obtained by resorting to PEG-PS and introducing recouplings at (Peptide Companion®-predicted) difficult positions; even so, a crude with less than 30% of the target material was obtained (Figure 2, middle panel). The ChemMatrix® synthesis, using only single couplings, remarkably improved on the above results, affording a very satisfactory crude (>85% by HPLC) from which the target sequence was easily purified (Figure 2, lower panel).

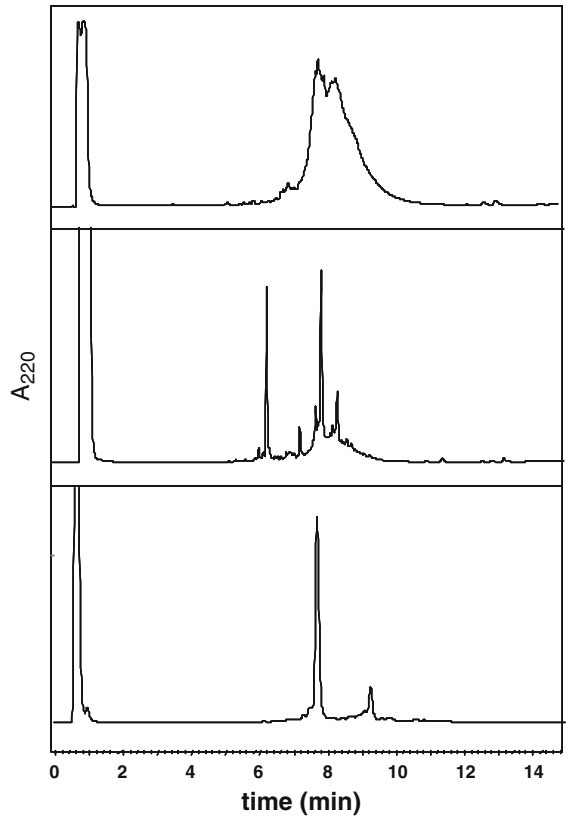

Fig. 2. Effect of the solid support on the quality of HIV-1 gp41 ectodomain peptide. Fmoc synthesis on Rink amide-functionalized polystyrene (upper panel, single HBTU/HOBt couplings), PEG-PS (middle panel, 9 -out of 28- recoupling steps) or ChemMatrix® (lower panel, single couplings throughout). HPLC conditions: linear gradient of B (30%–60%) into A over 15 min.

Arg_{11} – containing cell penentrating sequence

Our next test synthesis involved a calcipressin-based 21-residue sequence (Table I), N-terminally extended by 11 Arg residues, to facilitate cell internalization (Rothbard et al., 2000). It is known that sequences with long repeats of a single amino acid tend to pose synthetic difficulties (Merrifield et al.,

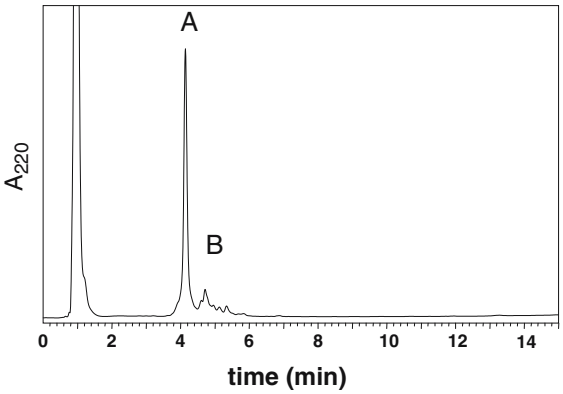

Fig. 3. Synthesis of a calcipressin-derived sequence with an N-terminal Arg_{11} extension on Rink amide-ChemMatrix® resin. Byproduct B corresponds to the target sequence (A) with one Pbf group. HPLC conditions: linear gradient of B (5%–95%) into A over 15 min.

1988); for Arg_n-containing peptides, complex crudes for $n > 8$ or higher are frequent, usually including single and multiple Arg deletions. This can be attributed to steric conflict among bulky, consecutive Pbf side chain protecting groups. In this particular instance, however, the synthesis on ChemMatrix® yielded crude products of remarkable quality (> 85%) (Figure 3). A control synthesis with a scrambled calcipressin sequence gave an essentially superimposable chromatogram (not shown). The only significant products detected consisted on Pbf adducts that could be minimized by longer cleavage times.

Chemokines CCL4-L1 and CCL4-L2

The touchstone of the present study were two 64- and 69-residue chemokines from the CCL4 family. Human CCL4, also known as MIP-1β (macrophage inflammatory protein), is an chemokine with important polymorphism in HIV^+ patients (Colobran et al., 2005). Two variants of CCL4 with clinical relevance to HIV susceptibility have been described, CCL4-L1 (S47G mutant) and CCL4-L2, an allele variant lacking five amino acids. This shorter variant was initially chosen as synthetic target, with a strategy that included the use of preloaded Fmoc-L-Asn(Trt)-Wang-ChemMatrix® and recoupling at 16 positions of predicted (Peptide Companion®) difficulty. A peptide-resin aliquot extracted after 30 coupling cycles was processed (cleavage, HPLC and MS analysis) and showed a satisfactory progress (Figure 4, upper panel). After assembly of the full 64-residue sequence, the synthetic crude (Figure 4, lower panel) contained a fairly well resolved main peak with a mass consistent with the expected linear (tetrathiol) precursor of CCL4-L2 [m/z (MH^+) 7128.76; theory 7129.00]. Following HPLC preparative purification, this precursor was air-oxidized (see Materials and Methods for details) to give a mixture of two folding (disulfide pairing) isomers, from which the main component, bioactive CCL4-L2, was readily purified (MALDI-TOF MS, m/z (MH^+) 7121.60; theory, 7124.97).

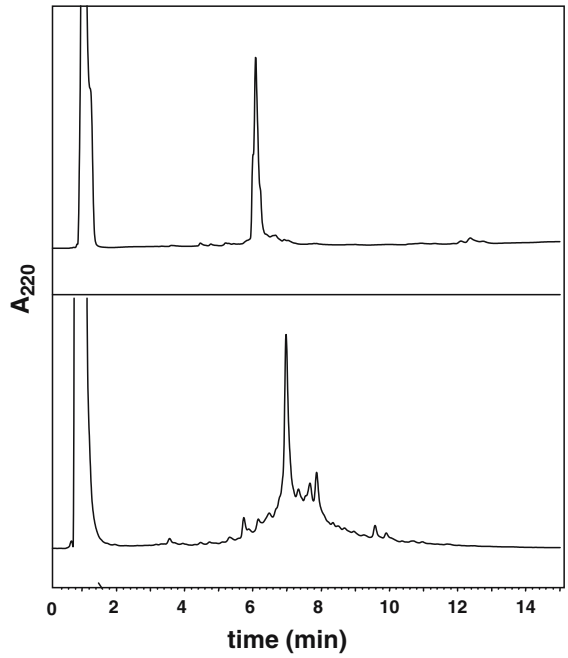

Fig. 4. Synthesis of chemokine CCL4-L2 on Rink amide-ChemMatrix® resin. Upper panel: synthetic product after Ser^{36} incorporation (30 couplings); lower panel: synthetic crude after full sequence assembly. HPLC conditions: linear gradient of B (25%–45%) into A over 15 min.

In view of this successful result, a similar synthesis of CCL4-L1 using exactly the same strategy was launched. For comparison purposes, a parallel synthesis on Rink amide-polystyrene was run. As for CCL4-L2, resin aliquots halfway through each synthesis (35 couplings, Ser^{36}) were taken and processed (cleavage, HPLC and MS analysis), showing substantially different results (Figure 5). The ChemMatrix®-synthesized intermediate gave an essentially homogeneous product (Figure 5, lower panel; compare with Figure 4, upper panel) of the expected mass, allowing for a good prognosis of the total synthesis, while the polystyrene-synthesized intermediate (Figure 5, upper panel) was of such poor quality that synthesis was abandoned at that point.

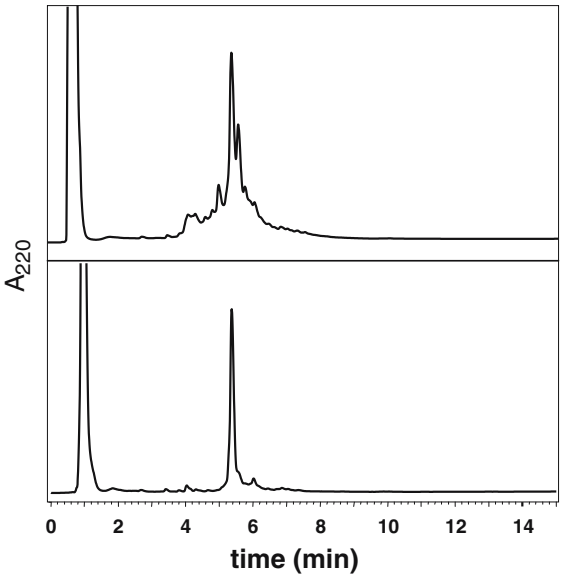

Fig. 5. Effect of the solid support on the quality of chemokine CCL4-L1. Synthesis product after 35 Fmoc cycles (Ser^{36}) on Rink amide-polystyrene (upper panel) or ChemMatrix® (lower panel). See text for further details. HPLC conditions: linear gradient of B (15%–75%) into A over 15 min.

Interestingly, despite the similar progress of the two ChemMatrix®-based syntheses up to the halfway mark, the mere five residue difference between CCL4-L1 and CCL4-L2 translated into strikingly different outcomes: the final crude product for CCL4-L1 gave a mountainous HPLC (not shown) from which the expected 69-residue (tetrathiol) precursor was totally absent, while several peaks corresponding to single and multiple Ser, Thr and Glu deletions were identified, clearly suggesting serious coupling difficulties in the Glu^{30}-Thr-Ser-Ser^{33} stretch. Suspecting aggregation as the origin of defective chain growth, a synthesis with additional precautions was undertaken: (i) recouplings were programmed for 19 positions with (Peptide Companion®) predicted difficulties; (ii) automated synthesis was used for the Leu^{34}-C-terminus and N-terminus-Tyr^{29} sections, while (iii) the Glu^{30}-····-Ser^{33} was assembled in the manual mode and, in addition, (iv) a Ser(tBu)-Ser(-$\Psi^{Me,Me}$pro) dipeptide (Mutter et al., 1995; White et al., 2004), expected to act as β-sheet-breaking unit, was introduced instead of the two regular, consecutive Ser(tBu) residues at positions 32 and 33. These improvements, particularly the latter one, turned out to be critical for the success of this second CCL4-L1 synthesis. Indeed, HPLC of the final crude (Figure 6, upper panel) clearly showed a major component from which the tetrathiol precursor

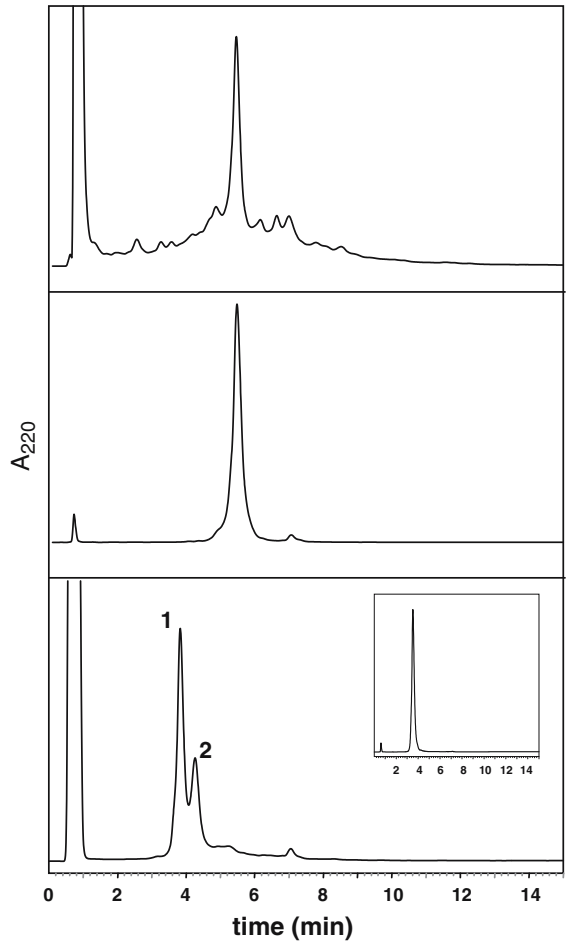

Fig. 6. Optimized synthesis of chemokine CCL4-L1 on Rink amide-ChemMatrix® resin, using a Ψ-Pro dipeptide at $Ser^{32,33}$. See text for further details. Upper panel: crude synthetic product; middle planel: HPLC purified (tetrathiol) precursor; lower panel: folding reaction after 4 h air oxidation. Inset: purified folding isomer 1 (bioactive). HPLC conditions: linear gradient of B (27%–40%) into A over 15 min.

[MALDI-TOF MS, m/z (MH^+) 7788.56; theory 7789.78] was readily purified (Figure 6, middle panel). Similarly to CCL4-L2, air-oxidation of this material at mildly basic pH (see Materials and Methods for details) led to a mixture of two folding isomers (Figure 6, lower panel) from which the main component, bioactive CCL4-L1 (MALDI-TOF MS, m/z (MH^+) 7784.42; theory 7785.75) was efficiently recovered after desalting on Diaion resin and preparative HPLC.

CONCLUDING REMARKS

The different synthetic accounts presented in this work provide compelling evidence of the superior

performance of ChemMatrix® over PEG-PS or PS resins in a number of cases representative of either long or potentially difficult synthetic jobs. The improved hydrophilicity/hydrophobicity properties claimed for ChemMatrix® appear to be fulfilled consistently enough for this resin to be considered the support of choice for the solid phase synthesis of predictably complex peptides. Even so, the CCL4-L1 results once again illustrate how synthetic success is the result of a multifactorial strategy to which the resin is a crucial though not unique contributor.

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