Use of the Same Polymer for Synthesis and Purification of Peptides

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This work reveals an uncommon but valuable biotechnological approach regarding the use of a same polymer (benzhydrylamine-resin, BHAR) for synthesis and anion exchange purification of peptides. Initially, the octapeptide DRVYIHPF-NH₂ was synthesized in 1% and 3% cross-linked BHAR, attaching 2.5 mmol g⁻¹ ammonium groups. Due certainly to its less rigid polymeric backbone, higher synthesis yield (about 80%) was achieved with the former resin. Next, the negatively charged peptides DEVYEHPF-NH₂ and DEVYESPF-NH₂ (−1 and −3 in neutral pH, respectively), both synthesized in 1% BHAR were submitted to chromatographic separation test in this same type of resin (1% and 3%). Concordant with results of peptide synthesis and swelling data of resin beads obtained by microscopy, an improved separation of both peptides occurred with 1% BHAR batch. These findings demonstrated that BHAR, applied so far for peptide synthesis, when containing high amount of positively charged ammonium groups, can be also used alternatively as a solid support for chromatographic purification of this type of biological molecule.

Keywords: peptide synthesis, resin, anion exchange chromatography, polymer, solvent polarity

Introduction

The inception of solid phase peptide synthesis (SPPS)¹ almost four decades ago² represented a landmark in the use of cross-linked beaded polymers, which were, until that time, restricted to stationary phases in column chromatography. Since then, various studies have been conducted in order to develop more varied applications for such polymeric materials. In addition to the peptide synthesis method itself, the concept of performing chemical reactions on an insoluble polymeric structure has, for instance, been successfully extended to oligonucleotides³ and polysaccharides.⁴ In recent years, a process denoted solid phase organic synthesis,⁵,⁶ which employs a combinatorial chemistry approach, has proven fruitful in the generation of peptide libraries⁷,⁸ and new therapeutic drugs.⁹

Since that time, a large number of different resins have been developed.¹⁰,¹¹ One of the first used for application in SPPS,¹²,¹³ was the benzhydrylamine-resin (BHAR), a phenylmethylamine group-bearing copoly (styrene-divinylbenzene)-type polymer for the synthesis of α-carboxamide peptides.¹⁴ Due to the dominant hydrophobic character of the styrene component of the resin, improved solvation of its matrix, containing usually low 0.2-0.5 mmol g⁻¹ amine groups (degree of substitution) for peptide chain growth, occurs preferably in apolar...
organic solvents. However, increasing the degree of substitution may drastically alter this BHAR solvation profile. We have previously demonstrated that very highly ammonium substituted BHAR batches (>1.5-2.0 mmol g⁻¹) actually display improved swelling in polar solvents, including water. These findings prompted us to initiate the evaluation of its use as ion exchange supports for purification of negatively charged disaccharides and gangliosides in aqueous solution. Some physicochemical characteristics of this type of cationic resin have also been described.

As a continuation of these studies, the aim of the present report was to demonstrate a different biotechnological application, i.e., the possibility of synthesizing and purifying (by ion exchange procedure) negatively charged peptides with the same polymer (highly substituted BHAR). Furthermore, in order to evaluate the effect of the degree of divinylbenzene cross-linking of the resin, the synthesis and purification of model negatively charged peptides were both carried out in highly substituted (2.5 mmol g⁻¹) BHAR containing 1% and 3% cross-linking degrees. As a preliminary step, BHAR batches with protonated (for anion-exchange chromatography) and deprotonated (for coupling reaction during peptide synthesis) amine functions were submitted to solvation studies through microscopy for measurement of the diameters of the dry and swollen resin beads. This approach correlates the swelling properties of each resin with a novel solvent polarity scale previously proposed by this laboratory in order to facilitate the choice of the most appropriate solvent system for optimized solvation of each solid support. In fact, the present study represents an inverted strategy recently proposed, in which classical anion-exchange resins such as DEAE-MacroPrep (BioRad Laboratories, Hercules, CA, USA) and DEAE-Sephadex A50 (Amersham Biosciences, Piscataway, NJ, USA) were used for peptide synthesis and for further dual-affinity anion-exchange purification of antibody molecules correlated with malaria transmission.

**Experimental**

All amino acid derivatives were purchased from Bachem, Torrance, CA, USA. Solvents and reagents were acquired from Aldrich-Sigma and Advanced ChemTech Inc. The DEAE-MacroPrep resin and copoly (styrene-1% and 3% divinylbenzene) resins (BioBeads SX-1⁰ and SX-3⁰) were bought from BioRad Laboratories, Hercules, CA, USA.

**Peptide synthesis**

The peptides were synthesized manually accordingly to the standard Nα-Boc/Bzl protecting group strategy. The following Boc-amino acid derivatives were used: cyclohexyl for Asp and Glu; tosyl for Arg and His and 2-Br-carbobenzoxy for Tyr. After the coupling of the C-terminal amino acid to the resin, the successive α-amino group deprotection and neutralization steps were performed in 30% TFA/DCM (30 min) and 10% TEA/DCM (10 min), respectively. In most cases, the amino acids were coupled at 3-fold excess using DIC/HOBt in DCM/DMF (1:1, v/v) and, if necessary, HBTU/HOBt/DIEA in 20% DMSO/NMP for recoupling steps. After a two-hour coupling time, the ninhydrin test was performed to estimate the completeness of the reaction. Anhydrous HF was used for cleavage of the peptide from the resin. To enhance the yield of peptide removal from the resin when the hydrophobic amino acid residues are located at the C-terminal position attached to BHAR, a 4 h reaction at 0 °C was used. The peptides were extracted into 5% aqueous HOAc and lyophilized.

**Analytical HPLC**

Analysis was performed in a Waters system consisting of two 510 HPLC pumps, an automated gradient controller, a Rheodyne manual injector, a 486 detector and a 746 data module. Unless otherwise stated, peptides were analyzed in a C18 Vydac column (4.6 x 150 mm, 300 Å pore size, 5 mm particle size) with the solvent systems A: H2O containing 0.1% TFA; and B: 90% MeCN in H2O containing 0.08% TFA. A linear gradient of 10-90% B in 54 min was applied at a flow rate of 2.0 mL min⁻¹ and with detection at 210 nm.

**Preparative HPLC**

Purification of peptides was carried out as follows: solvent A: H2O containing 0.1% TFA; solvent B: 90% MeCN in H2O containing 0.08% TFA. Retention time-dependent linear gradients were determined through analytical HPLC of the peptide with the same solvent systems. The flow rate was 6.0 mL min⁻¹ and peak detection was at 210 nm.

**Synthesis of highly substituted benzhydrylamine-resins**

Synthesis of the highly substituted BHAR batches was controlled through specific forcing of the benzylation step, as has been previously described. The proportion of acylating agents, benzoyl chloride and aluminum chloride for the 2.5 mmol g⁻¹ BHAR batches (1% and 3% cross-linkage) was, typically, 4 mmol g⁻¹ of styrene-1%...
divinylbenzene copolymer in a concentration of 0.8 mol L\(^{-1}\) at 35°C for 15 h. For both batches, the reductive amination step (Leuckart reaction) was carried out with an excessive amount of reactant (60 mmol per gram of benzoylated copolymer) for 30 h at 170°C. In order to generate the free ammonium group in the resin, hydrolysis of the formyl group-bearing copolymer was performed by refluxing with a mixture of 12 N HCl:EtOH (1:1, v/v) at 90°C for 5 h.

**Swelling measurement of resin beads**

Prior to their use in chromatography and microscopic measurement of bead sizes, the amino protonated (Cl\(^{-}\) form) or deprotonated (treated with 10% TEA/DCM) BHAR batches were dried in vacuo using an Abderhalden-type apparatus with MeOH reflux. They were then exhaustively sized, a process which entailed suspending them in DCM and EtOH and sifting them through pore metal sieves to lower the standard deviations of resin diameters to approximately 4-5%. After being allowed to equilibrate overnight, 150 to 200 dry and swollen beads of each resin were spread onto a microscope slide and measured directly at low magnification in an Olympus, model SZ 11 microscope linked to the software program Image Plus, version 3.0.01.00. Since the sizes in a sample of beads are log-normally (rather than normally) distributed, the central value and the distribution of the particle diameters were estimated using more accurate geometric mean values and geometric standard deviations.\(^{25}\) The average amount of solvent (in percentage) absorbed by the resin beads was calculated by the equation:

\[
\frac{\text{swollen volume} - \text{dry volume}}{\text{swollen volume}} \times 100
\]

where bead volumes were calculated from their diameters.

**Mass Spectrometry**

The LC/ESI-MS experiments were performed on a system consisting of a Waters Alliance model 2690 separations module and model 996 photodiode array detector (Waters, Eschborn, Germany) controlled with a Compaq AP200 workstation coupled to a Micromass model ZMD mass detector (Micromass, Altrincham, Cheshire, UK). The samples were automatically injected on a Waters narrow bore Nova-Pak column C\(_18\) (2.1 x 150 mm, 60 Å pore size, 3.5 μm particle size). The elution was carried out with solvents A (0.1% TFA/H\(_2\)O) and B (60% acetonitrile/0.1% TFA/H\(_2\)O) at a flow rate of 0.4 mL min\(^{-1}\) using a linear gradient from 5% to 95% B in 30 min. The condition used for mass spectrometry measurements was a positive ESI.

**Amino acid analyzer**

Peptide composition was controlled by amino acid analysis (AAA) which was performed on Biochrom 20 Plus amino acid analyzer (Pharmacia LKB Biochrom Ltd., Cambridge, England) equipped with an analytical cation-exchange column. The peptides were hydrolyzed with HCl 6 mol L\(^{-1}\) in sealed tubes under nitrogen atmosphere at 110°C for 72 h. The samples were concentrated in high vacuum, suspended in 0.2 mol L\(^{-1}\) sodium citrate buffer, adjusted to pH 2.2 and automatically injected into the analyzer.

**Anion-exchanger chromatography**

The 1% and 3% BHAR batches were pre-treated with 20% EtOH in water and washed thoroughly with the initial buffer before being packed into the column. The linear pH-gradients used for elution were from 0.02 mol L\(^{-1}\) NH\(_4\)Ac, pH 5 to 10% HOAc, pH 2.3, or from 0 to 2 mol L\(^{-1}\) NaCl linear salt-gradient in NH\(_4\)Ac, pH 5, using a GM-1 Mixer (Amersham Pharmarcia Biotech) and a Foxy 200 collector (Isco, Lincoln, NE, USA). The amount of positively charged groups per column was fixed at 4 mmol, including (for comparison) the commercial DEAE-MacroPrep\(^{\text{®}}\) resin.

**Results and Discussion**

**Swelling degree of BHAR**

To identify the solvent systems which swell the 1% and 3% BHAR to a greater degree, the two 2.5 mmol g\(^{-1}\) substituted batches were initially studied with their amine function in protonated (chloride) and deprotonated forms. Table 1 displays the 25 solvent systems (single and mixed) used for swelling studies of dry and swollen beads under microscopy. These solvent systems encompass a broad section of the polarity scale represented by the (AN+DN) constant.\(^{21,22}\) The AN and DN terms represent Gutmann’s electron acceptor and electron donor numbers,\(^{26}\) respectively. Due to the coexistence of two opposite conceptual physicochemical terms, this novel polarity scale was recently designated amphoteric.\(^{27}\) The volume of solvated bead (in percentage) occupied by each solvent system was selected as swelling parameter to monitor solvation capacity of resin batches in ionized and non-ionized forms.

By examining the swelling values shown in Table 1, it can be seen that the swelling percentages of the resins ranged from a minimum of approximately 30%
Table 1. Swelling of 1 and 3% BHAR with amine groups in deprotonated and protonated forms

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Polarity (AN+DN)</th>
<th>BHAR (% cross-linking)</th>
<th>1(%)</th>
<th>3(%)</th>
<th>1(%)</th>
<th>3(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Toluene</td>
<td>3.4</td>
<td>78</td>
<td>67</td>
<td>46</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>2 DCM</td>
<td>21.4</td>
<td>81</td>
<td>82</td>
<td>59</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>3 Chloroform</td>
<td>27.1</td>
<td>80</td>
<td>80</td>
<td>65</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>4 NMP</td>
<td>40.6</td>
<td>79</td>
<td>73</td>
<td>82</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>5 DMF</td>
<td>42.6</td>
<td>79</td>
<td>68</td>
<td>88</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>6 DMSO</td>
<td>49.1</td>
<td>56</td>
<td>69</td>
<td>91</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>7 TFE</td>
<td>53.5</td>
<td>67</td>
<td>64</td>
<td>84</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>8 EtOH</td>
<td>69.1</td>
<td>30</td>
<td>36</td>
<td>88</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>9 MeOH</td>
<td>71.3</td>
<td>28</td>
<td>37</td>
<td>86</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>10 Formamide</td>
<td>63.8</td>
<td>31</td>
<td>47</td>
<td>82</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>11 50% TFE/TOL</td>
<td>28.5</td>
<td>81</td>
<td>82</td>
<td>80</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>12 20% TFE/DCM</td>
<td>27.5</td>
<td>85</td>
<td>81</td>
<td>73</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>13 50% TFE/DCM</td>
<td>37.5</td>
<td>69</td>
<td>74</td>
<td>79</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>14 80% TFE/DCM</td>
<td>47.4</td>
<td>66</td>
<td>59</td>
<td>77</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>15 20% DMSO/NMP</td>
<td>42.3</td>
<td>71</td>
<td>65</td>
<td>83</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>16 50% DMSO/THF</td>
<td>38.6</td>
<td>74</td>
<td>75</td>
<td>88</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>17 65% NMP/THF</td>
<td>36.1</td>
<td>78</td>
<td>80</td>
<td>82</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>18 50% DCM/DMF</td>
<td>32.0</td>
<td>70</td>
<td>72</td>
<td>74</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>19 50% DCM/DMSO</td>
<td>35.3</td>
<td>73</td>
<td>68</td>
<td>86</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>20 50% MeOH/DMSO</td>
<td>60.2</td>
<td>58</td>
<td>59</td>
<td>89</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>21 50% TFE/DMF</td>
<td>48.1</td>
<td>64</td>
<td>69</td>
<td>83</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>22 50% TFE/DMSO</td>
<td>51.3</td>
<td>57</td>
<td>54</td>
<td>91</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>23 10% TEA/DCM</td>
<td>25.1</td>
<td>85</td>
<td>85</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>24 10% TEA/DMF</td>
<td>44.5</td>
<td>80</td>
<td>78</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>25 10% TEA/DMSO</td>
<td>50.4</td>
<td>73</td>
<td>73</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>

*swollen volume – dry volume) / swollen volume x 100; using the following diameters of dry beads: BHAR: 1% (43 µm), 3% (45 µm); nd = not determined; ^b (Cl - form).

Figure 1. Correlation between swelling of different cross-linked BHAR as a function of the (AN+DN) polarity parameter. A (1%) and C (3%), amine groups in protonated form. B (1%) and D (3%), amine groups in deprotonated form.
It is of note, and antithetical to the results obtained with the amine deprotonated resins, that the protonated 1% BHAR showed a slightly greater degree of swelling (approximately 90%) in the maximum solvation region in comparison to the 80% swelling observed in its 3% counterpart (Figure 1). This finding is likely due to the fact that, when the cross-linking percentage is higher, the resin matrix structure is more rigid and therefore hinders expansion of the polymer backbone.

Moreover, all four contour plots of resin swelling clearly show the maximum solvation region of each resin (Figure 1), thus reconfirming the accuracy of the (AN+DN) term in reflecting polarity of the medium. The results of previous studies of more traditional polarity scales such as Hildebrand's $\delta$ term, Dimroth-Reichardt's $\delta$ or even the dielectric constant support our findings. In summary, the swelling displayed by the BHAR batches confirmed our hypothesis that batches containing high amounts of protonated amine forms, due to the positively charged groups attached to their structure, swelled to a greater degree in more polar solvents. As evidence of this, the 1% and 3% BHAR protonated batches displayed good swelling capacity (70% and 50%, respectively) when measured in the 0.02 mol L$^{-1}$ aqueous ammonium acetate buffer (pH 5.0) commonly used for ion exchange chromatography at the initial equilibrium and solute loading steps.

Comparative peptide synthesis

Following the solvation studies, both 1% and 3% BHAR batches were compared as to their capacity for peptide synthesis applications. The vasoactive angiotensin II (DRVYIHPF, AII) was selected as the model peptide for comparative synthesis yielding its C$\alpha$-amide analogue. The synthesis efficiency was clearly superior when the 1% BHAR batch was used. No difficulties in assembling the entire peptide chain were observed, and there was no need for any recoupling process during the progressive attachment of amino acid residues of the sequence. Conversely, when the 3% BHAR was used, many more difficulties occurred, including multiple recoupling reactions throughout the synthesis.

The composite Figure 2 displays the analytical HPLC profiles of crude peptides synthesized with both BHAR batches. The AII synthesis yield was based upon the area of the AII peak in the HPLC chromatogram and indicated values of approximately 80% and 35%, respectively, when 1% and 3% BHAR batches were used for the synthesis, respectively. These results are again likely due to the more

![Figure 2](image-url)
rigid and sterically hindered polymer backbone of the latter BHAR batch, which seems to severely impede diffusion of reactants and side products during the peptide synthesis.

Use of BHAR as an anion-exchange resin

To compare the anion-exchange capacity of 1% and 3% protonated BHAR batches, the negatively charged peptides DEVYEHPF-NH₂ (P2) and DEVYEDPF-NH₂ (P3) – with net charges of about −1 and −3 at pH 5 – were previously synthesized in the 1% BHAR. As expected, the synthesis yield was approximately 80-85%, yielding 71 and 79 mg of the desired peptides, respectively (on a 0.1 mmol synthesis scale). The HPLC profiles of these two crude peptides resembled closely those shown in Figure 2 (AII-NH₂). To complete the set of peptides necessary for the comparative anion-exchange chromatography with BHAR, the model AII-NH₂ (P1) – previously synthesized in the 1% versus 3% BHAR comparative study – was used as a control for the void volume determination of columns due to the positive charge of this sequence at pH 5 (+2). These three crude peptide sequences were purified until homogeneity in conventional HPLC purification prior to use for separation test with 1% and 3% BHAR batches.

Columns for chromatographic experiments and containing 4 mmol of ammonium groups of 1% BHAR, 3% BHAR and also the commercial weak anion-exchange DEAE-MacroPrep® resins were previously equilibrated in 0.02 mol L⁻¹ ammonium acetate buffer, pH 5. Through a swelling versus pH of the medium approach, we have previously demonstrated that, at this and lower pH levels, the amine groups of BHAR are totally protonated, since an average pKa value of approximately 7 was determined for this basic function.³² A pH-gradient ranging from pH 5 to 2.3 (10% HOAc) was applied. Figure 3 displays comparative chromatograms showing the observed anion-exchange of peptides P1, P2 and P3 in 1% BHAR, 3% BHAR and DEAE-MacroPrep® resin. More succeeded separation of the negatively charged P2 and P3 peptides occurred mainly with 1% BHAR, indicating that for the 3% BHAR batch, variation in the chromatographic

Figure 3. Anion exchange chromatography of P1: DRVYIHPF-NH₂ (+2); P2: DEVYEHFP-NH₂ (-1) and P3: DEVYEDPF-NH₂ (-3) on: (A) 1% BHAR; (B) 3% BHAR; and (C) DEAE-MacroPrep®. Conditions: 4.0 mmol of ammonium groups per column and linear pH-gradient from 0.02 mol L⁻¹ ammonium acetate, pH 5 to 10% HOAc, pH 2.3 (200 mL each). Absorbance, detection at 254 nm.
condition should be necessary for achievement of improved resolution and elution of solutes. The worse performance of the 3% BHAR in terms of anion-exchange capacity seems to be due to its more compact and sterically hindered matrix. Otherwise, the commercial DEAE-MacroPrep® resin also succeeded in the separation of components, but the elution of the two negative peptides was more rapid than it was with the 1% BHAR.

Finally, Figure 4 shows the same comparative experiments, but, in this case, the salt gradient (0 to 2 mol L⁻¹ NaCl gradient) was applied. Poorer fractionation results were obtained with both BHAR batches, and retention of the two negative peptides was stronger than that observed during the pH gradient. The 1% BHAR only partially eluted the injected peptides, whereas the remaining quantity of solutes could only be removed with strong 20% HOAc aqueous solution. Again, the worst result was observed with 3% BHAR, which completely retained both negatively charged peptides in the column and eluted with the 20% HOAc aqueous solution wash. In this salt-gradient protocol, the best results were obtained with the commercial DEAE-MacroPrep® resin. The poorer chromatographic behavior of BHAR when the salt gradient was applied might be due to the well-known fact that its beads shrink in aqueous solution of high ionic strength.¹⁹

In conclusion, similar to the results obtained when 1% and 3% BHAR were used for peptide synthesis, the best results in terms of application for anion-exchange chromatography were observed with the former batch. Collectively, these findings are in close accordance with the improved solvation in organic (for peptide synthesis) or aqueous (for column chromatography) media of the 1% resin over the 3% resin. Most relevantly, the results herein obtained thus proved the feasibility of using BHAR for synthesis and purification of the same type of compound (negatively charged peptides). However one must stress that these two types of technological applications are much more effective when low cross-linked and highly amine substituted BHAR batches are to be used.

Figure 4. Anion exchange chromatography of P1: DRVYIHPF-NH₂ (+2); P2: DEVYEHPF-NH₂ (-1) and P3: DEVYEDPF-NH₂ (-3) on: (A) 1% BHAR; (B) 3% BHAR; and (C) DEAE-MacroPrep®. Conditions: 4.0 mmol of ammonium groups per column and linear pH-gradient from 0 to 2 mol L⁻¹ NaCl gradient in 0.02 mol L⁻¹ ammonium acetate, pH 5 (200 mL each). Absorbance, detection at 254 nm.
Acknowledgments

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References

1. Abbreviation and Notes used: Abbreviations for amino acids and nomenclature of peptide structure follow the recommendations of IUPAC-IUB (Commission on Biochemical Nomenclature, *J. Biol. Chem.* 1971, 247, 997). Other abbreviations are as follows: AAA, amino acid analysis; AII, angiotensin II; BzI, benzyl; BHAR, benzhydrylamine-resin; t-Boc, tert-butyloxycarbonyl; DCM, dichloromethane; C18, octadecyl; DEAE, diethylaminoethyl; DIC, diisopropylcarbodiimide; DIA, diisopropylethylamine; DMF, N,N’-dimethylformamide; DMSO, dimethylsulfoxide; ESI, electron spray ionization; EtOH, ethanol; HBTU, N-[(1H-benzotriazol-1-yl)-dimethylamino]methylene-N,N’-methylmethanaminium hexafluorophosphate-N-oxide; HOAc, acetic acid; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MeCN, acetonitrile; MeOH, methanol; NMP, N-methylpyrrolidinone; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; tosyl, p-toluenesulfonyl; TOL, toluene.

32. Etchegaray, A.; Carvalho, R. S. H.; Boschov, P.; Nakaie, C. R.; *Chromatographia* 1996, 43, 82.

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