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Synthesis of heptapeptides and analysis of sequence by tandem ion trap mass spectrometry

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Abstract: Two heptapeptides have been prepared by Fmoc methodology using Wang resin as solid support. For attachment of the first amino acid, several coupling systems were evaluated, and DIC/DMAP system could give yields of >99 % and low levels of racemization. The selection of scavenger combination to deprotect side chains revealed that H_2O/p-cresol was good at scavenging trityl and 1,2-ethanedithiol was highly efficient for scavenging t-butyl. Through shortening the preactivation time to 5 min, the racemization which occurred during formation of amide bonds coupled by HBTU was minimized. The crude peptides were characterized by RP-HPLC and MS, and sequenced by MS/MS to acquire reliable amino acid sequence information.

Keywords: Peptide synthesis, mass spectrometry, peptide sequencing, racemization, RP-HPLC, scavenger

1 Introduction

New biologically active peptides continue to be discovered at a rapid rate, which exhibit remarkable diverse functionalities and include antibiotics, toxins, immunosuppressants, ion transport regulators, inhibitors of protein binding, enzyme inhibitors, and so on [1]. Thus the accompanying demands for peptides also considerably increase. Solid phase peptide synthesis, as developed by R.B. Merifield [2], has proved to be a powerful tool for producing peptides. However, many problems involving cost, racemization, purity and yield still prohibit its industrialization.

Attachment of the first amino acid to resin is yet a serious problem [3]. For ma-

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jority of amino acids, amide bond formation during solid phase synthesis is very highly efficient and accompanied by very low levels of racemization, whereas anchoring of the first amino acid to the Wang resin linker requires ester bond formation with the issues of low efficiency and high levels of racemization. Although some groups have reported that appropriate coupling system can produce acceptable yields [4, 5], there never has been a general strategy. Hence, a specific coupling system should be set up aiming at different resin linkers and amino acids. In addition, another puzzling problem concerning racemization should also be minimized through optimizing experimental conditions. The factors leading to racemization are variable, which mainly contain coupling reagents [6], solvent polarity [7], reaction temperature [8], base strength [9] and coupling protocols [10]. Recently a novel concept about the effect of preactivation step on racemization brings an effective outcome for avoiding racemization [11–15]. Meanwhile, to solve these issues, the analytical protocols are also very important.

Reversed-phase high-performance liquid chromatography (RP-HPLC) has become the predominant analytical technique for peptides due to its versatility and high resolving power [16]. Surprisingly, it may even separate two diastereoisomer peptides with the only difference of L or D isomers at one amino acid residue [17]. Nevertheless, HPLC with UV detection alone is not always sufficient to determine diastereoisomer peptides. In contrast, through incorporation of RP-HPLC and mass spectrometry (MS), diastereoisomer peptides can be differentiated by retention time, m/z and sequencing information. Therefore, by this means, racemization may be monitored in synthesis process [8, 15, 18]. Besides, elucidation of peptide sequence is more informative than determination of molecular weight.

Perfect combination of collisionally activated decomposition (CAD) technique and electrospray-ionization (ESI) in ion trap mass spectrometers accelerates development of *de novo* sequencing, which is a highly efficient tool for determination of amino acid sequence of synthetic or natural peptides [19]. In comparison with Edman degradation or nuclear-Overhauser-effect techniques of NMR, sequencing peptides by tandem mass spectrometry (MS/MS) has some distinct advantages [20]: (1) a peptide mixture without further purification can be sequenced by mass-selection function of the instrument; (2) the sample amount necessary for analysis usually is less than 1 pmol to acquire a high quality mass spectrum, which provides the molecular weight as well as the purity; (3) the experiment operation is noticeably simplified. As a result, sequence elucidation by MS/MS is very suitable for analyzing synthetically crude peptide.

We report here two peptides (heptapeptide **1**, H-Pro-Phe-Asn-Ser-Leu-Ala-Ile-OH; heptapeptide **2**, H-Pro-Leu-Ile-Phe-Ser-Pro-Ile-OH), which were synthesized by the Fmoc methodology [3] using Wang resin. Some problems involving substitution degree tests, attachment of the first amino acid, scavenger and racemization were discussed, all of which affect the yields and purities of expected peptides. To sum up, a systematical method has been established in this study for fast acquiring and analyzing linear peptides from Wang resin and Fmoc-amino acid.

2 Experimental

2.1 Materials

Wang-resin, Fmoc-amino acids and coupling reagents for peptide synthesis were purchased from GL Biochem (Shanghai, China). All solvents for peptide synthesis were commercially analytical grade and were redistilled before used. For mass spectrometry and HPLC, HPLC-grade formic acid, TFA and acetonitrile were supplied by Merck (Germany), and the HPLC-grade water was redistilled and subjected to ion-exchange process in a Milli-Q unit (Millipore).

2.2 Synthesis of heptapeptides

The peptides were prepared manually by the classic Fmoc methodology using the Wang resin as solid support in a glass reaction vessel (30 ml) fitted with a sintered glass frit. Side chain protecting groups included t-butyl (^{t}Bu) for Ser and trityl (Trt) for Asn. Solvent and soluble reagents were removed by suction. Washings of resin was performed with dichloromethane (DCM), ethanol (EtOH) and N,N-dimethylformamide (DMF) (5 ml, 3×2 min), respectively.

2.2.1 Heptapeptide 1, H-Pro-Phe-Asn-Ser-Leu-Ala-Ile-OH

2.2.1.1 Formation of ester bonds. The first Fmoc-amino acid, Fmoc-Ile-OH, was anchored on the Wang resin using N,N'-diisopropylcarbodiimide (DIC) as the coupling reagent. A solution of Fmoc-Ile-OH (1.58 g) and *p*-dimethylaminopyridine (DMAP) (0.13 g) in DMF (10 ml) was poured into the glass reaction vessel containing Wang resin (1.12 g, 1.005 mmol/g). After activation for 20 min, DIC (0.56 g) was added to the vessel. The coupling reaction was maintained for 6 h. Then the resin was filtered in vacuum and washed with DCM, EtOH and DMF. The resin was added with 50:50 acetic anhydride/pyridine (5 ml, 1×10 min and 2×20 min) to cap the unreactive hydroxyl. Removal of the Fmoc protecting group was performed by incubation of the resin in 50:50 piperidine/DCM (v/v) (5 ml, 1×5 min and 2×10 min). Finally the total resin was weighed (1.10 g) and the substitution degree (1.0138 mmol/g) was determined by the salicylaldehyde test [21].

2.2.1.2 Formation of amide bonds. The other Fmoc-amino acids, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Phe-OH and Fmoc-Pro-OH, were attached to resin one by one using HBTU as the coupling reagent. After Fmoc-Ala-OH (0.7 g) was preactivated using 1-hydroxybenzotriazole (HOBt) (0.30 g), 2,5-dihydroxybenzoic acid (DIEA) (0.43 g) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium (HBTU) (0.85 g) in DMF (10 ml) for 5 min, this reaction mixture was poured into the resin. Then the coupling reaction was maintained for 4 h, and their completion was verified by the ninhydrin (Kaiser) test [3]. The coupling process was

repeated under the same conditions in case of positive ninhydrin test. After washing of the resin, the Fmoc group was also removed by piperidine/DCM as above. Then the coupling procedures of the other Fmoc-amino acids were the same as that of Fmoc-Ala-OH.

2.2.1.3 Cleavage of peptides from resin. After the final step of deprotection of Fmoc group, the obtained peptide resin was dried in vacuum and treated with trifluoroacetic acid (TFA)/H_2O/1,2-ethanedithiol (EDT)/ p-cresol (92.5/2.5/2.5/2.5, v/v, 3 ml, 1×30 min and 2×60 min) to cleave the peptide from the resin and remove the side chain protecting groups i.e. ^{t}Bu for Ser and Trt for Asn. Then the resin was filtered in vacuum and washed with TFA and DCM several times. The filtrate was collected. Then the solvent was evaporated and the obtained solid products were suspended in cold ether (20 ml) and precipitated by centrifugation. Eventually, after removal of ether in vacuum, heptapeptide **1** was obtained as a white powder (0.836 g, yield 99 %, purity > 92%).

2.2.2 Heptapeptide 2, H-Pro-Leu-Ile-Phe-Ser-Pro-Ile-OH

Operating as the method of synthesis of heptapeptide **1,** from Wang resin (1.12 g, 1.005 mmol/g), Fmoc-Ile-OH, Fmoc-Pro-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Phe-OH, and Fmoc-Leu-OH, we obtained heptapeptide **2** as a white powder (0.858 g, yield 98 %, purity > 95 %).

2.3 Chromatography

The peptides were characterized on an Elite P200-II HPLC system (Daliang, China) using a Hewllett-Packed Lichrosorb C_{18} reversed-phase analytic column (10μ, 100 Å, 200×4.6mm, USA). The peptides (1 mg) were dissolved in water/acetonitrile 50:50 (v/v, 1 ml), filtered and manually injected through a Rheodyne injector with 20 μl sample loop. The mobile phase used was A: 0.1 % aqueous TFA and B: 0.1% TFA in acetonitrile, with a flow-rate of 0.7 ml/min and a linear gradient of 0-7 % B over 10 min, then 7-30 % B over 4 min, and 30-50 % B over 40 min. Elution of products were detected at 214 nm by an Elite UV-200II detector. The pump system and data collection were controlled by an Elite Echrom98 operation system.

2.4 Mass spectrometry

Experiments were performed using a Finnigan LCQ ion-trap mass spectrometer (San Jose, CA, USA) equipped with an ESI source operated in positive mode. The spray needle was set at a potential of 4.3 kV, and a nitrogen sheath gas flow of 30 (arbitrary units) was used to stabilize the spray. The capillary temperature was maintained at 200 °C. The tube-lens offset was 20 V and the electron multiplier voltage was 800 V. Helium gas was introduced into the center of the ion trap at a pressure of 1mTorr to improve the trapping efficiency of the analyte ions introduced into the ion trap. The

background helium gas also served as the collision gas during the CAD event. A typical experimental protocol consisted of infusing a 5 pmol/μl solution of peptide in 50:50 (v/v) acetonitrile/H_2O containing 1 % formic acid into the mass spectrometer at a flow-rate of 2 μl /min by the syringe pump.

2.4.1 Full scan and MS/MS

Full scan experiments were performed within mass range from 200 to 1000 Da, and all spectra were acquired in the mass-centroid mode. Subsequently, the sequencing spectra were obtained by CAD of molecular ions $[M+H]^+$. For experimental involving CAD, success relied on determining an optimum combination of the number of scans, isolation width, Activation Q value and relative collision energy. Eventually, the data of CAD fragment ions were analyzed by Bioworks 3.1R software.

2.4.2 RP-HPLC/MS

Reversed-phase high-performance liquid chromatography/mass spectrometry (RP-HPLC/MS) was also carried out in the same mass spectrometer. Using PEEK tubing, the Finnigan Surveyor plus HPLC system was connected directly to the ESI probe; a flow-rate of 80 μl/min to the ESI probe was obtained by splitting a primary flow of 0.7 ml/min by means of a T-piece to monitor the peptides under the condition of baseline separation. The elution gradient was as above (2.3).

3 Results and discussion

3.1 Determination of substitution degree of resin

The required amount of amino acid for every coupling reaction depends precisely on quantitative test of amine content on resin (substitution degree, mmol/g, S). So it is the key on saving cost and improving yield. We chose the salicylaldehyde test, which has the distinctive advantages of speed and sensitivity, compared with other tests [21]. Thus we made the standard curve of quantitative test (Fig. 1), and found that it was linear in a given region (R^2=0.9996) to agree with the Lambert-Beer law. Meanwhile, the molar extinction coefficient was 4.2065. Therefore, the substitution degree could be calculated via equation:

$$S = 3.0 \cdot OD_{315}/(4.2065 \cdot R) \quad (1)$$

where OD_{315} was the ultraviolet absorbance at 315 nm and R was the measured resin weight.

3.2 Characterization of heptapeptides by RP-HPLC and MS

The crude heptapeptides **1** and **2** without further purification respectively showed more than 92 % and 95 % purity on RP-HPLC (Fig. 2 and Fig. 3). Then the desired $[M+H]^+$ =

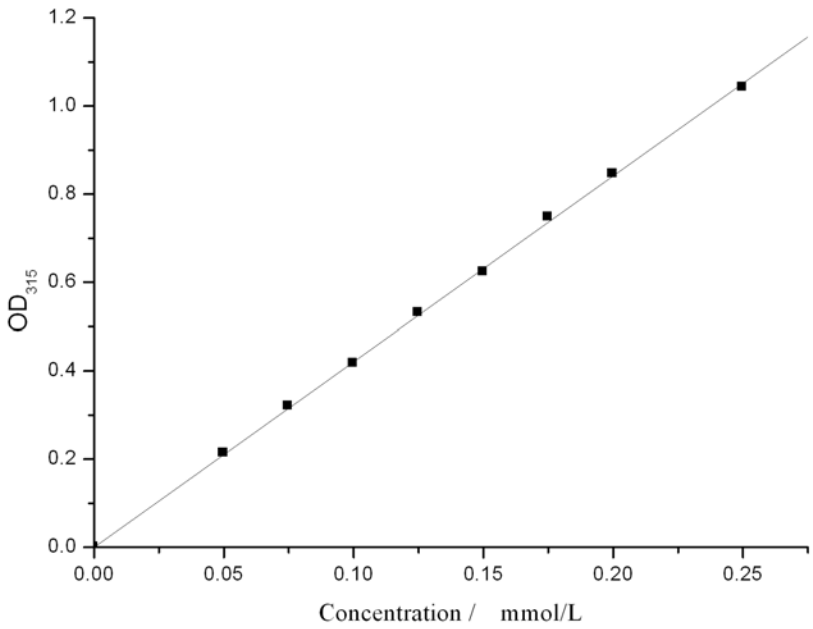

Fig. 1 Standard curve of alicylaldehyde test.

761.5 ion of crude heptapeptide **1** with a high relative abundance was detected by full scan of ESI-MS (Fig. 4). It was interesting that a typical ESI-produced spectrum of crude heptapeptide **2** was observed containing the ions of $[M+H]^+$=786.5, $[M+Na]^+$=808.4, $[M+K]^+$=824.6, $[M+H+Na]^{2+}$=405.1 and $[M+H+K]^{2+}$=413.0 (Fig. 5). In summary, both quantification by RP-HPLC and MS indicated that the purities of desired peptides were considerably high. Based on the substitution degrees and purities, the overall yields of the heptapeptide **1** and **2** were respectively calculated at 91 and 93 %.

3.3 *De novo* sequencing of heptapeptides by MS/MS

To acquire precise information of amino acid sequence, we utilized the CAD technique to sequence the two synthetic heptapeptides on an ESI ion trap mass spectrometer. Through the CAD of the molecular ion $[M+H]^+$ obtained by full scan, two sets of fragment ions y_n and b_n (n=2-6) were clearly detected (Fig. 6 and Fig. 7). Nevertheless, for ion trap mass spectrometer, the so-called low mass cut-off feature [19] limited the lowest mass fragment ion that could be detected in a CAD experiment to approximately 1/3 of the parent ion. For example, if a parent ion was detected at m/z 600, the lowest fragment ion that could be stabilized and detected was at m/z 200. In this experiment, although the $b_1$and y_1 ions (m/z< 200) in low mass range were observed by means of lowering the Activation Q value to 0.16, it was still hard to observe integral sets of b_n and y_n in the same spectrum, due to arbitrary loss of ions in the high mass range. For instance, when y_1 ion was observed, y_6 ion disappeared in the same spectrum. In order

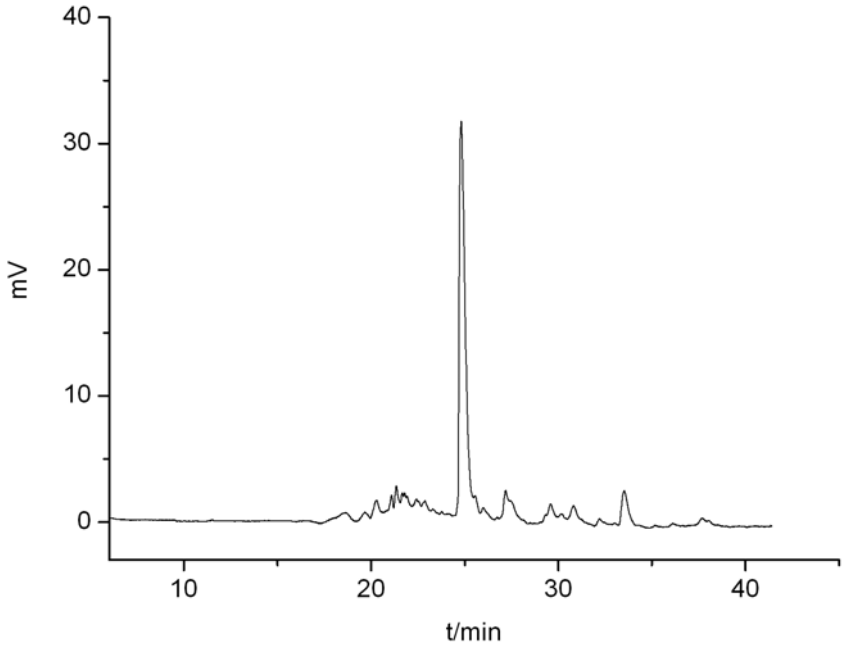

Fig. 2 RP-HPLC profile of crude heptapeptide **1**(t_R 24.80 min).

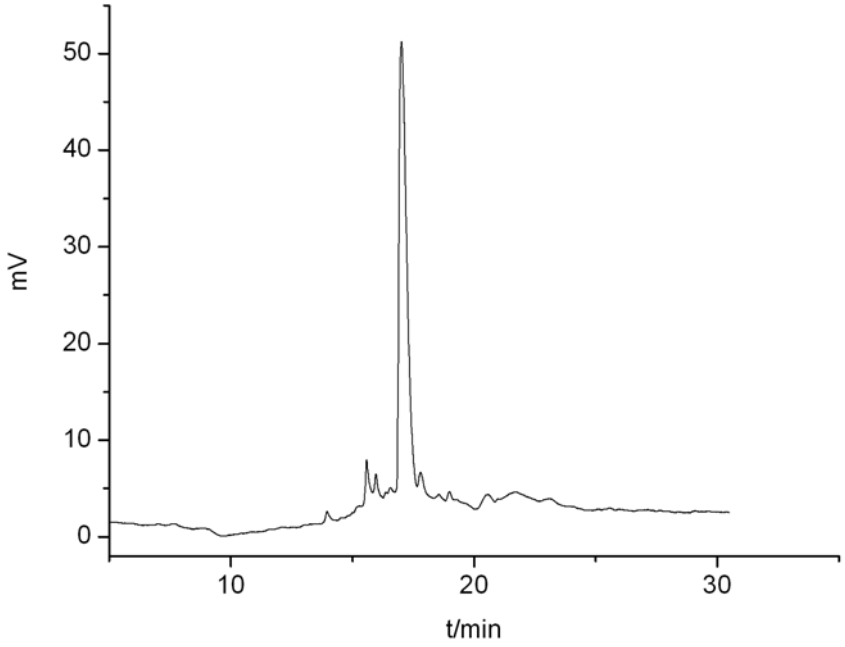

Fig. 3 RP-HPLC profile of crude heptapeptide **2** (t_R 17.02 min).

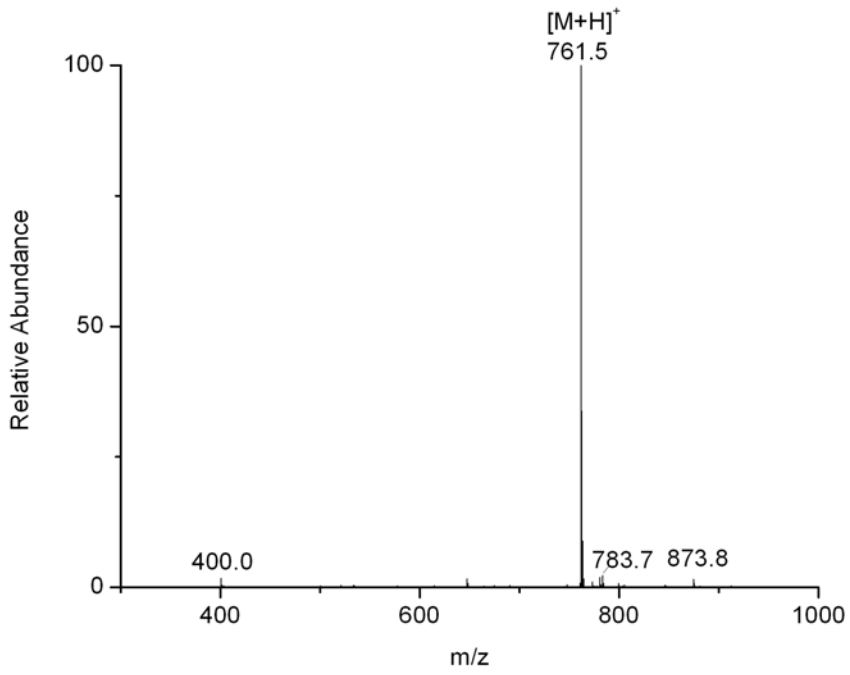

Fig. 4 ESI full scan spectrum of crude heptapeptide **1**.

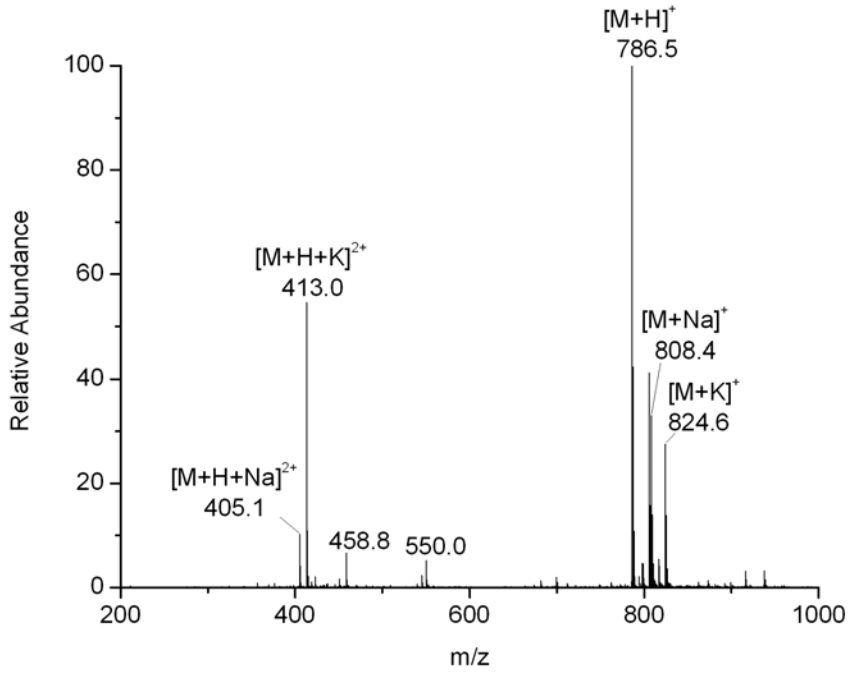

Fig. 5 ESI full scan spectrum of crude heptapeptide **2**.

to offset this drawback, it was necessary to detect both sets of b_n and y_n (n=2-6) in the same spectrum so that the connectivity of b_n and y_n ions could be verified to promote reliability of sequencing results. In conclusion, the sequencing results of heptapeptide **1** and **2** by MS/MS reasonably proved the correctness of their sequences.

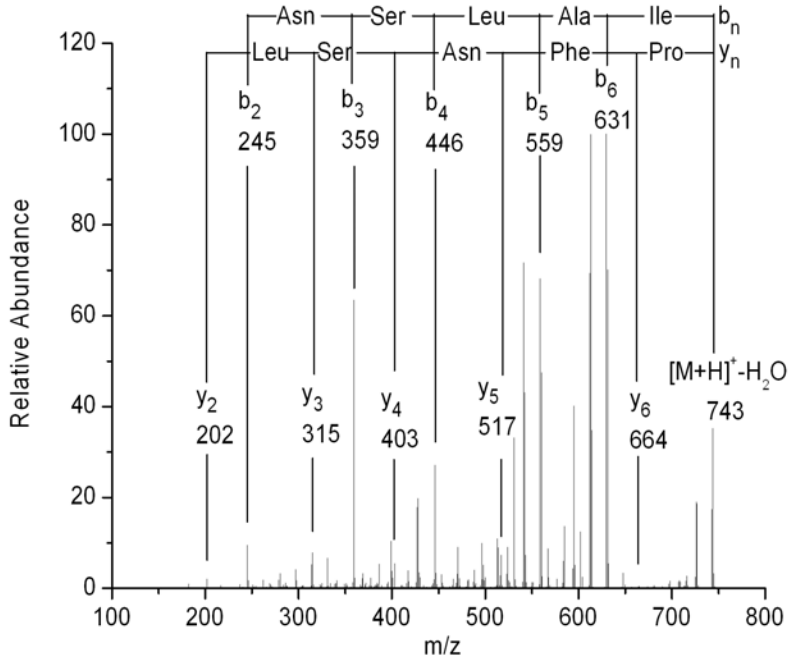

Fig. 6 CAD spectrum of crude heptapeptide **1** from $[M+H]^+$ ion of m/z 761.5. The x, z, a, and c ions were not labeled for clarity.

3.4 Attachment of the first Amino acid

In order to avoid racemization and increase substitution degree, we tried to select an appropriate coupling system for load the first amino acid to Wang resin. Grandas et al. [22] have reported anchoring of Fmoc-amino acids to hydroxymethyl resin using the system of N,N'-dicyclohexylcarbodiimide (DCC)/HOBt in good yield without racemization. Following this method, we loaded the Fmoc-Ile-OH to Wang resin, but found the precipitated urea from this method were hard to remove from the resin by washings, because of its poor solubility. So we turned to utilizing another carbodiimide coupling reagent DIC whose urea could be easily removed. However, using DIC/HOBt system the yield was only 26 % after 6 h, and was not obviously improved through prolonging the reaction time. Finally we attempted to take advantage of a never reported DIC/DMAP system, which gave a high substitution degree of 1.0138 mmol/g and an excellent yield of > 99 %. What is more, the chromatographic analysis of heptapeptide **1** and **2** indicated that the levels of racemization were also quite low (Fig. 2 and Fig. 3). Compared with

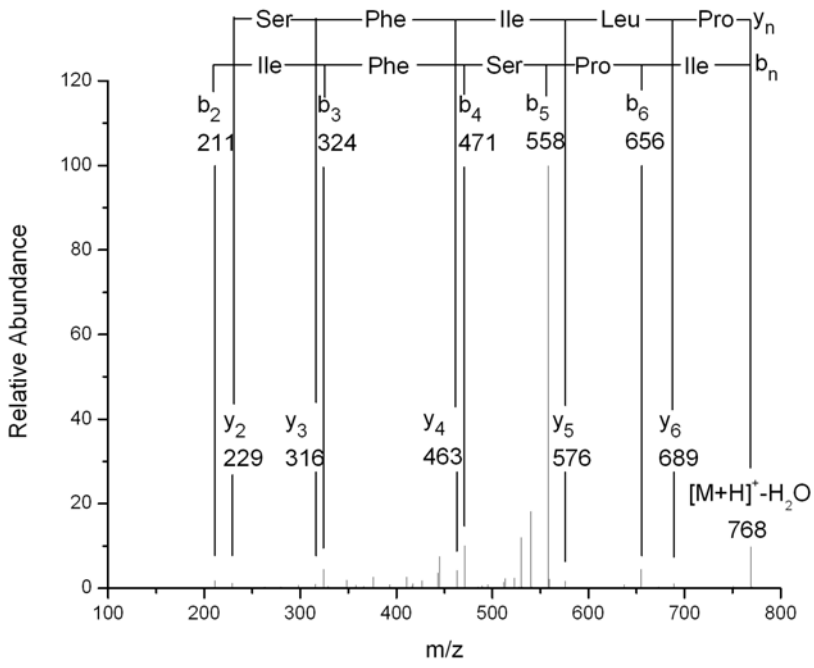

Fig. 7 CAD spectrum of crude heptapeptide **2** from $[M+H]^+$ ion of m/z 786.5. The x, z, a, and c ions were not labeled for clarity.

the reported yield of 69 % [22], this method was very successful. Furthermore, in theory, it could be further applicable to loading of other amino acids to Wang resin linker.

3.5 The choice of scavengers for deprotection of side chains

Initially, the scavenger combination of TFA/H_2O/p-cresol (95/2.5/2.5, v/v) was utilized to cleave the heptapeptide **1** from resin support and remove the protecting groups of side chains Trt and ^{t}Bu. The obtained crude peptides were dissolved in acetonitrile/H_2O and injected into ESI mass spectrometer, and the full scan spectrum indicated that the sample had two main molecular ions of $[M+H]^+$=761.3 and $[M(^t\text{Bu})+H]^+$ = 817.3 (Fig. 8). This analytical result illustrated that H_2O/p-cresol was good at scavenging Trt, while only scavenging ^{t}Bu of 28 % (calculated by relative abundance). Therefore, we employed another combination of TFA/H_2O/EDT/ p-cresol (92.5/2.5/2.5/2.5, v/v), and the obtained crude peptides were analyzed in the same way. Finally, the complete disappearance of $[M(^t\text{Bu})+H]^+$ in the ESI spectrum (Fig. 4) illustrated that EDT was highly efficient for scavenging ^{t}Bu. In addition, this scavenger combination was also available for heptapeptide **2**.

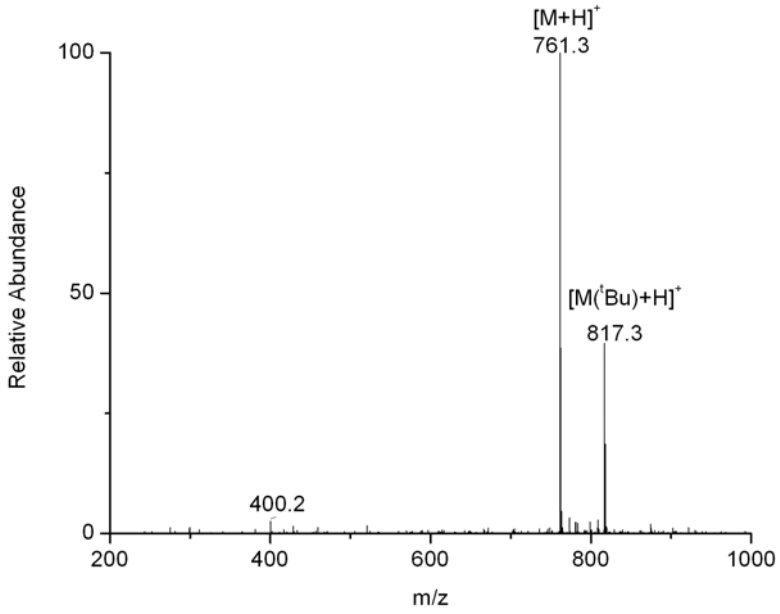

Fig. 8 ESI full scan spectrum of crude heptapeptide **1** without complete deprotection of ^{t}Bu.

3.6 Effect of preactivation step on racemization

Racemization has been a major concern for synthetic chemists, since it is directly relative with the biological activation of synthetic peptides. During the process of synthesis of heptapeptide **1,** we encountered substantial levels of racemization, as the HPLC profile (Fig. 9) of crude peptides revealed that the 4 chromatographic peaks respectively represented 4 diastereoisomer peptides, which had the same m/z 761.5 and the same amino acid sequence assayed by RP-HPLC/MS/MS. Analyzing the reasons of racemization, we supposed that if racemization only occurred at Ile residue during anchoring of the first amino acid to resin, there, in regard to existence of LD-Ile, should be 2 diastereoisomer peptides in the HPLC profile, instead of 4. In this case, we deduced that racemization should occur during the formation of the amide bond coupled by HBTU/HOBt. Moreover, Han et al. [15] reported that racemization levels of cysteine was in general reduced by a factor of 6- or 7-fold by avoiding the preactivation step, and other literature [11–14] also reported the same effect of preactivation step on racemization. Indeed, we originally used a preactivation procedure over 30 min so as to enhance the coupling speed with HBTU/HOBt, and found that a long time of preactivation often led to the changing of resin color from white to brown and generating of some insoluble substances. Eventually we achieved desired peptides with minimal racemization (Fig. 2) through shortening the preactivation time to 5 min (see 2.2.1.2). However, in view of coupling speed, we did not try to abandon the preactivation step. To sum up, the case indicated that appropriate preactivation time can generally lower levels of racemization.

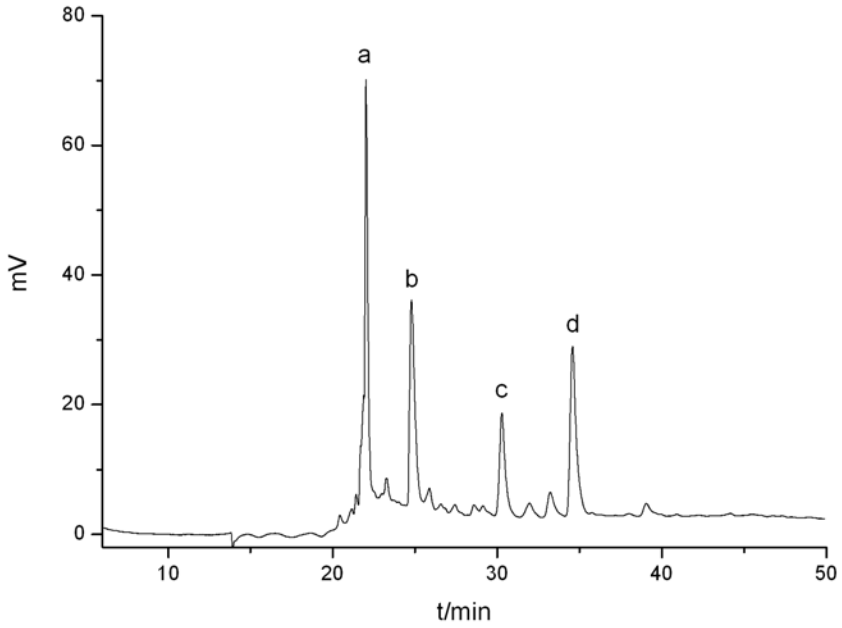

Fig. 9 RP-HPLC profile of crude heptapeptide **1** with serious racemization (**a** t_R 22.02 min, **b** t_R 24.78 min, **c** t_R 30.27 min, **d** t_R 34.58 min). According to the t_R 24.80 min of heptapeptide **1** in Fig. 2, b was the desired heptapeptide **1,** and **a, c** and **d** were its diastereoisomers.

4 Conclusions

In this investigation, two heptapeptides were prepared by stepwise Fmoc methodology using the Wang resin as solid support. Substitution degree tests, attachment of the first amino acid, scavenger selection and racemization issue were discussed. Finally, optimization of experimental conditions resulted in high overall yield, and sequencing peptide by MS/MS revealed precise sequence information. In summary, the systematical protocols described in this manuscript may be applied to fast synthesis of linear peptides with high purity, low levels of racemization and cheap cost, as well as reliable elucidation of synthetic peptide sequence.

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References

[1] S.A. Kates and F. Albericio: *Solid-Phase Synthesis*, Marcel Dekker, Inc., New York, 2000.

[2] R.B. Merrifield: "Solid phase peptide synthesis. I. The synthesis of a tetrapeptide", *J. Am. Chem. Soc.*, Vol. 85, (1963), pp. 2149–2154.

[3] W.C. Chan and P.D. White: *Fmoc Solid Phase Peptide Synthesis. A Practical Approach,* Oxford University Press, Inc., New York, 2000.

[4] Z.G. Zhang, H.B. Cai, L. Bai and S.L. He: "A comparison between the yield of the attachment of Boc-AA-O^-Cs^+ to Merrifeild resin and of Fmoc-AA-OH to Wang resin", *Chinese J. Org. Chem.*, Vol. 20, (2000), pp. 419–423.

[5] A. Eric, R.C. Linda and C.S. Robert: "Peptide synthesis: Part 10. Use of pentafluorophenyl esters of fluorenylmethoxycarbonylamino acids in solid phase peptide synthesis", *Tetrahedron*, Vol 44, (1988), pp. 843–857.

[6] Y.H. Ye, T.H. Li and X.H. Jiang: "DEPBT as an efficient coupling reagent for amide bond formation with remarkable resistance to racemization", *Biopolymers*, Vol. 80, (2005), pp. 172–178.

[7] M. Kunishima, A. Kitao, C. Kawachi, Y. Watanabe, S. Iguchi, K. Hioki and S. Tani: "A racemization test in peptide synthesis using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM)", *Chem. Pharm. Bull.*, Vol. 50, (2002), pp. 549–550.

[8] M.P. Souza, M.F.M. Tavares and M.T.M. Miranda: "Racemization in stepwise solid-phase peptide synthesis at elevated temperatures", *Tetrahedron*, Vol. 60, (2004), pp. 4671–4681 .

[9] Y.M. Angell, J. Alsina, F. Albericio and G. Barany: "Practical protocols for stepwise solid-phase synthesis of cysteine-containing peptides", *J. Peptide Res.,* Vol. 60, (2002), pp. 292–299.

[10] A.D. Fenza, M. Tancredi, C. Galoppini and P. Rovero: "Racemization studies of Fmoc-Ser(^{t}Bu)-OH during stepwise continuous-flow solid-phase peptide synthesis", *Tetrahedron Lett.* Vol. 39, (1998), pp. 8529–8532.

[11] T. Tedeschi, R. Corradini, R. Marchelli, A. Pushl and P.E. Nielsen: "Racemization of chiral PNAs during solid-phase synthesis: effect of the coupling conditions on enantiomeric purity", *Tetrahedron: Asymmetry*, Vol. 13, (2002), pp. 1629–1636.

[12] R. Corradini, S. Sforza, A. Dossena, G. Palla, R. Rocchi, F. Filira, F. Nastri and R. Marchelli: "Epimerization of peptide nucleic acids analogs during solid-phase synthesis: optimization of the coupling conditions for increasing the optical purity", *J. Chem. Soc., Perkin Trans.*, Vol. 20, (2001), pp. 2690–2696.

[13] D.N.W. Van, S. Yuval and F. Albericio: "$Cu(OBt)_2$ and $Cu(OAt)_2$, copper(II)-based racemization suppressors ready for use in fully automated solid-phase peptide synthesis", *J. Peptide Sci.* Vol. 7, (2001), pp. 115–120.

[14] L.A. Carpino, A. El-Faham and F. Albericio: "Racemization studies during solid-phase peptide synthesis using azabenzotriazole-based coupling reagents", *Tetrahedron*

Lett., Vol. 35, (1994), pp. 2279–2282.

[15] Y.X. Han, F. Albericio and G. Barany: "Occurrence and minimization of cysteine racemization during stepwise solid-phase peptide synthesis", *J. Org. Chem.,* Vol. 62, (1997), pp. 4307–4312

[16] M.T.W. Hiern: *HPLC of Proteins, Peptides and polynucleotides*, VCR, New York, 1991.

[17] A.D. Fenza and P. Rovero: "Assessment of new 6-Cl-HOBt based coupling reagents for peptide synthesis. Part 2: Racemization studies", *Lett. Peptide Sci.*, Vol. 9, (2002), pp. 125–129

[18] W. Qi, C.X. Jia, Z.M. He and B. Qiao: "Analysis of racemization products of synthetic heptapeptide by reversed phase high performance liquid chromatography/mass spectrometry", *Chinese J. Anal. Chem.*, submitted for publication.

[19] V.H. Wysocki, K.A. Resing, Q.F. Zhang and G.L. Cheng: "Mass spectrometry of peptides and proteins", *Methods*, Vol 35, (2005), pp. 211–222.

[20] B.M. Souza, M.R. Marques, D.M. Tomazela, M.N. Eberlin, M.A. Mendes and M.S. Palma: "Mass spectrometric characterization of two novel inflammatory peptides from the venom of the social wasp *Polybia paulista*", *Rapid Commun. Mass Specrom.*, Vol. 18, (2004), pp. 1095–1102.

[21] Y.S. Zou and X.Z. Qian: "Test of the amino group of amino acid and polypeptides on solid phase", *Prog. Biochem. Biophys.*, Vol. 14, (1978), pp. 12–14.

[22] A. Grandas, X. Jorba, E. Giralt and E. Pedroso: "Anchoring of Fmoc-amino acids to hydroxylmethyl resins", *Int. J. Peptide Protein Res.*, Vol. 33, (1989), pp. 386–390.