Characterization of N-phosphoryl oligopeptide libraries by ESI-MS and HPLC-MS

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Summary

N-diisopropoxyphosphoryl oligopeptide libraries were constructed through the transformation from the homo-oligopeptide libraries, which were synthesized with the assistance of phosphorus oxychloride. The molecular mass distributions of N-phosphoryl oligopeptide libraries were monitored by ESI-MS, and the components identification and quantitative analysis were carried out by HPLC-MS.

Abbreviations: ESI-MS – electrospray ionization mass spectrometry; HPLC-MS – high performances liquid chromatography mass spectrometry; CE – capillary electrophoresis; FTIR – fourier transform infrared spectrometry; NMR – nuclear magnetic resonance spectroscopy; GFC – gel filtration chromatography; CID – collision-induced dissociation; DIPPH – diisopropylphosphite; POC₁₃ – phosphorus oxychloride; DIPP-AA – N-diisopropyloxyphosphoryl amino acid; ESI-MSⁿ – electrospray ionization tandem mass spectrometry.

Introduction

With the advances in solution phase combinatorial chemistry, more and more libraries of compounds are being synthesized for screening in the drug discovery process [1, 2]. Peptide libraries have been broadly recognized as useful sources for screening bioactive compounds [3, 4]. Several analytical methods have been developed for the separation and the structural characterization of combinatorial mixtures and parallel compound libraries, including HPLC [5], CE [6], FTIR [7], NMR [8] and mass spectrometry [9–12]. In the analysis of combinatorial mixtures, the use of mass spectrometry is the most widespread and its application is well described in recent reviews and research papers [13–15]. For peptide libraries, ESI-MS has been found to be the method of choice, providing rapid, sensitive, and informative analyses, especially when coupled to chromatographic separation [16, 17]. HPLC-MS has also been demonstrated to be an efficient method for analysis of peptide libraries [18].

Molecular diversity provides a source of novel lead compounds for drug discovery. To expand the diversity of compounds, the design principle of switchover was used to change a library to another library by transformation of a functional group [19, 20]. In our previous work, it was discovered that α-amino acids could be assembled into homo-peptide libraries with the assistance of phosphorus oxychloride [21]. In the present study, first, the construction of N-diisopropoxyphosphoryl peptide libraries by the transformation from homo-peptide libraries was undertaken. This post-modification strategy has the potential to be extended to other types of reaction for altering peptides and may allow still greater chemical diversity. ESI-MS was used to monitor the construction of N-diisopropoxyphosphoryl peptide libraries. Then, the products were purified by GFC and analyzed by HPLC-MS. N-diisopropoxyphosphoryl peptides...
were characterized based on molecular weight and their structural features acquired from fragmentation patterns by MS\textsuperscript{n} experiments in the positive and negative mode.

**Material and methods**

**Reagents and chemicals**

L-amino acids were purchased from Baitai Biochemical Co. (Sigma agency). Acetonitrile (HPLC grade) was purchased from Merck KGaA (Darmstadt, Germany). Deionized water was generated from Milli-Q water purifying system purchased from Millipore (MA, U.S.A.). Other common chemicals used were of the highest purity commercially available. Ammonium acetate solution (0.005 M) was prepared by dissolving 0.385 g of ammonium acetate in 1.0 L deionized water. Sephadex LH 20 was used as packing for the GFC column.

**HPLC conditions**

The HPLC system used was an Agilent 1100 series (CA, U.S.A.) high-performance liquid chromatograph, consisting of a degasser, binary pump, manual injector and a UV detector. Analyses were performed by UV absorbance at a wavelength of 214 nm and at a flow-rate of 0.8 mL min\textsuperscript{−1}. Chromatographic separations were performed with a Microsorb C18 column (250 × 4.6 mm i.d., particle size 5 µm). Solvent A was distilled water containing 5 mM ammonium acetate and 5% acetonitrile, and solvent B was acetonitrile containing 5% 5 mM ammonium acetate. Gradient runs were programmed as follows: linear gradient increase from 5% solvent B to 30% solvent B for 20 min. The effluent from the HPLC system was connected to mass spectrometer through a split value (split ratio = 9:1).

**MS conditions**

A Bruker Esquire 3000 (Bruker Daltonik, Bremen, Germany) ion trap mass spectrometer interfaced to an electrospray ionization (ESI) source was used for mass analysis and detection. Ionization of analytes was carried out using the following setting of the ESI: nebulizer gas flow 7 psi, dry gas 4 L min\textsuperscript{−1}, dry temperature 300 °C, capillary voltage 4000 V. Calibration of \( m/z \) was performed using a standard ES-tuning-mix. Scan range was 50–3000 \( m/z \) and scan resolution was normal (13 000 \( m/z \) s\textsuperscript{−1}). MS\textsuperscript{n} spectra were obtained by CID experiments with helium after isolation of the appropriate precursor ions. For HPLC-MS analysis, ionization of analytes was carried out using the following settings of the ESI: nebulizer gas flow 30 psi, dry gas 12 L min\textsuperscript{−1}, dry temperature 350 °C, capillary voltage 4000 V.

**Synthetic procedure**

Homo-oligopeptide libraries and DIPPH were prepared according to the literatures [21, 22] respectively. After four days the reactions, in which \( \alpha \)-amino acids (0.01 mol) were assembled into homo-oligopeptides with the assistance of phosphorus oxychloride, were quenched with water. Then, the homo-oligopeptide crude products were acquired by elimination of the solvents and salts. Homo-oligopeptide crude products were identified by ESI-MS and DIPPH was identified by \( ^{1} \)H-NMR, \( ^{31} \)P-NMR. The crude products above were dissolved in 10 mL water, 10 mL triethylamine, 5 mL ethanol and cooled in ice-salt bath to 0 °C. 0.005 mol DIPPH, 10 mL tetrachloromethane were added into the mixture and stirred for 3 h. The mixture was adjusted to pH = 3–4 with 1 N hydrochloric acid in ice-salt bath and then fully extracted with ethyl acetate(5× 10 mL). Oily liquid was acquired after a rotary evaporator had removed solvents. The GFC column loaded with N-diisopropoxyphosphoryl peptides was eluted with methanol to remove the low molecular weight impurities out of the column. All fractions containing library compounds were collected to get more pure library products for the analysis by HPLC-MS and MS\textsuperscript{n} experiments.

**Result and discussion**

**Monitoring the products of the N-phosphoryl oligopeptide libraries by ESI-MS**

In the process of the formation of homo-oligopeptide libraries as literature [21], ESI-MS was used to monitor the reaction. Analysis of the reaction solution by ESI-MS and ESI-MS\textsuperscript{n} showed that after L-amino acids had reacted with POCI\textsubscript{3} for only 1 h and quenched with water, a series of mass peaks corresponding to oligopeptides were already observed. The length of peptide slowly increased as reaction time prolonged. The ESI-MS\textsuperscript{n} experiments of the products were investigated to confirm the structure. The \( b \) type ions were the main fragment ions of these protonated