Research Article

Journal of PeptideScience

Received: 26 July 2010 Revised: 19 October 2010 Accepted: 11 November 2010 Published online in Wiley Online Library: 24 February 2011

(wileyonlinelibrary.com) DOI 10.1002/psc.1342

Derivatization of peptides as quaternary ammonium salts for sensitive detection by ESI-MS

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A series of model peptides in the form of quaternary ammonium salts at the *N*-terminus was efficiently prepared by the solid-phase synthesis. Tandem mass spectrometric analysis of the peptide quaternary ammonium derivatives was shown to provide sequence confirmation and enhanced detection. We designed the 2-(1,4-diazabicyclo[2.2.2] octylammonium)acetyl quaternary ammonium group which does not suffer from neutral losses during MS/MS experiments. The presented quaternization of 1,4-diazabicyclo[2.2.2]octane (DABCO) by iodoacetylated peptides is relatively easy and compatible with standard solid-phase peptide synthesis. This methodology offers a novel sensitive approach to analyze peptides and other compounds. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: quaternary ammonium salts; derivatization of peptides; peptide fragmentation; sequencing

Introduction

ESI-MS has become an essential tool for protein identification in proteomics [1,2] and for analysis of large combinatorial libraries of peptides [3]. One of the common problems during analysis of trace amounts of peptide mixtures, obtained from the enzymatic protein digestion as well as during synthesis of combinatorial peptide libraries, is a limited sensitivity caused by the insufficient ionization efficiency of some peptides. This may result in the incomplete sequence coverage in the analysis of protein hydrolyzates, which makes peptide mass fingerprinting searches problematical. Therefore, an enhanced detectability is an important goal of mass spectrometric analysis.

Analytes are typically detectable by the ESI-MS only if they are ionic in solution. Therefore, amines or carboxylic acids could be ionized in solution with a proper adjustment of pH. The more basic Arg-containing peptides have a higher proton affinity than the peptides containing only amino groups, resulting in a higher MS response factor of Arg-containing peptides. One of the known methods to increase ionization efficiency of peptides is their derivatization to form fixed charge ionic species [4].

The usefulness of such a fixed charge group depends on its stability in solution and during MS analysis. Moreover, the introduced group should allow the unambiguous peptide sequencing using current fragmentation techniques [5]. There are several derivatization strategies for peptides, employing quaternary ammonium [6–8], sulphonium [9–12], and phosphonium [13–15] salt formation. Up to now, the peptide conjugates containing fixed charge salts were usually synthesized in solution; this derivatization method was scarcely explored on the solid support.

Solid-phase synthesis is the most common method of synthesis of peptides [16] as well as many other biopolymers [17]. The solid-phase synthesis technique is a method of choice in preparation of one-bead-one-compound (OBOC) libraries of peptides including those prepared by the split-and-mix method [18,19]. Because of the limited amount of substance on a single bead, the analysis of peptide beads requires very sensitive analytical methods. It may be concluded that a solid-phase derivatization procedure enhancing ionization efficiency might be useful in the combinatorial chemistry.

In this article, we present the solid-phase synthesis of model peptides conjugated with various linear and bicyclic quaternary ammonium salts (QAS). On the basis of fragmentation pathways of the synthesized conjugates, we discuss the efficiency of QAS for enhancement of the sensitivity of peptide analysis by the electrospray ionization tandem MS.

Materials and Methods

Reagents

All solvents and reagents were used as supplied. Fmoc amino acid derivatives (Fmoc-Asp(OBu^t)-OH, Fmoc-Val-OH, Fmoc-Tyr(Bu^t)-OH, Fmoc-Thr(Bu^t)-OH, Fmoc-Ala-OH), and PyBOP were purchased from Novabiochem. *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide (TBTU), 1-hydroxybenzotriazole (HOBt), the MBHA-Rink amide resin (0.69 mmol/g), and TFA were obtained from IrisBiotech. Tertiary amines used for the synthesis of QAS: betaine, triethylamine (Et_3N), tripropylamine (Pr_3N), tributylamine (Bu_3N), 1,4-diazabicyclo[2.2.2]octane (DABCO) and solvents for peptide synthesis (*N*,*N*-DMF, DCM), and (*N*-DIEA) were obtained from Aldrich; iodoacetic acid from Merck; *N*,*N*′-diisopropylcarbodiimide (DIC) and triisopropylsilane (TIS) from Fluka.

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Peptide Synthesis

Synthesis of model tetrapeptides on the MBHA-rink amide resin: H-Asp(OBu^t)-Val-Tyr(Bu^t)-Thr(Bu^t)-Resin (peptidyl resin 1), H-Asp(OBu^t)-Ala-Ala-Ala-Resin (peptidyl resin 2), H-Ala-Ala-Tyr(Bu^t)-Ala-Resin (peptidyl resin 3), H-Ala-Ala-Ala-Thr(Bu^t)-Resin (peptidyl resin 4), and H-Ala-Ala-Ala-Ala-Resin (peptidyl resin 5) was performed manually in polypropylene syringe reactors (Intavis AG) equipped with polyethylene filters, according to a standard Fmoc (9-fluorenylmethoxycarbonyl) synthesis procedure [20].

Quaternary Ammonium Salts (QAS) Formation

All QAS groups were introduced to the peptides on the solid support (peptidyl resins 1–5). Peptide derivatives of 2-(triethylammonium)acetyl (Teaa), 2-(tripropylammonium)acetyl (Tpaa), 2-(tributylammonium)acetyl (Tbaa), and 2-(1,4-diazabicyclo[2.2.2]octylammonium)acetyl (Dbaa) were obtained according to the procedure A. 2-(Trimethylammonium)acetyl (Tmaa) derivatives were obtained according to the procedure B.

Procedure A

Amino groups of peptides (peptidyl resins 1–5) were first iodoacetylated according to the modified procedure described previously by Cebrat *et al*. [21]. The mixture of iodoacetic acid (3.2 mg, 17 μmol) and DIC (2.2 mg, 17 μmol) dissolved in DMF (0.5 ml) was added to the peptidyl resin (5 mg, 3.5 μmol) suspended in DMF (0.5 ml) and the reaction was allowed to proceed for 3 h. The completeness of derivatization of the *N*-terminal amino groups was confirmed by the Kaiser test [22]. Then the appropriate amine: Et_3N (7.0 mg, 69 μmol), Pr_3N (9.9 mg, 69 μmol), Bu_3N (12.8 mg, 69 μmol), or DABCO (7.7 mg, 69 μmol) in DMF (0.5 ml) was added to the reaction vessel and mixed for 24 h.

Procedure B

The mixture of betaine (8.1 mg, 69 μmol), PyBOP (35.9 mg, 69 μmol) HOBt (9.3 mg, 69 μmol), and DIEA (8.9 mg, 69 μmol) was dissolved in DMF (1 ml) and added to the peptidyl resin (5 mg, 3.5 μmol) suspended in DMF (0.5 ml). The reaction was allowed to proceed for 24 h.

All derivatized peptides were cleaved from the resin simultaneously with the side chain deprotection using a solution of TFA/H_2O/TIS (95/2.5/2.5, v/v/v) at room temperature for 2 h. Peptides were precipitated from the cleavage mixture with ice-cold diethyl ether (Et_2O). Crude compounds were dissolved in water, lyophilized, and purified by RP-HPLC. All analytical data are presented in Table 1.

General Methods

Purification

All QAS were purified by the analytical HPLC using a thermo separation HPLC system with UV detection (210 nm) on a Vydac protein RP C18 column (4.6 × 250 mm, 5 μm), with a gradient elution of 0–40% *B* in *A* (*A* = 0.1% TFA in water; *B* = 0.1% TFA in acetonitrile/H_2O, 4:1) over 30 min (flow rate 1 ml/min). Main peak, corresponding to the QAS-peptide derivative, was collected and the fraction was lyophilized.

Mass spectrometry

All MS experiments were performed on an FT-ICR (ion cyclotron resonance) MS Apex-Qe Ultra 7T instrument (Bruker Daltonics, Germany) equipped with an ESI source. Spectra were recorded for samples dissolved in acetonitrile (MeCN), methanol (MeOH), water, water with formic acid (0.1%), and a combination of these solvents. Analyte solutions were introduced at a flow rate of 3 μl/min. The instrument was operated in the positive ion mode and calibrated before each analysis with the Tunemix™ mixture (Bruker Daltonics, Germany) in quadratic method. The instrument parameters were as follows: scan range: 100–1600 *m/z*; drying gas: nitrogen; temperature: 200 °C; potential between the spray needle and the orifice: 4.2 kV. In the MS/MS experiments, the singly charged $[M]^+$ precursor ions were selected on the quadrupole and subsequently fragmented in the hexapole collision cell. Argon was used as a collision gas. The obtained fragments were directed to the ICR mass analyzer and registered as an MS/MS spectrum. The collision voltage (18–29 V) was optimized for the best fragmentation. For MS spectra analysis, a Bruker Compass DataAnalysis 4.0 software was used. A sophisticated numerical annotation procedure (SNAP) algorithm was used for finding peaks. All obtained signals had a mass accuracy error in the range of 2 ppm. The signal-to-noise (S/N) ratio was in the range of 1000.

Results and Discussion

Synthesis

We present two different strategies of quaternary ammonium peptide derivative synthesis on the solid support at the 3 μmol (5 mg of peptidyl resin) scale. The series of tetrapeptides containing five different QAS was synthesized on the solid phase. The immunosuppressive fragment of HLA-DQ molecule [23] containing the DVYT sequence (compounds **1a–e**) was selected as a model peptide. To analyze effect of the carboxyl and/or hydroxyl group on the fragmentation pattern we synthesized a series of its analogs, where each hydrophilic amino acid residue was replaced by alanine (compounds **2a–e** to **4a–e**). We also synthesized the tetra-alanine QAS analogs (compounds **5a–e**).

To obtain peptides containing 2-(triethylammonium)acetyl (Teaa), 2-(tripropylammonium)acetyl (Tpaa), 2-(tributylammonium)acetyl (Tbaa), and 2-(1,4-diazabicyclo[2.2.2]octylammonium) acetyl (Dbaa) residue, the *N*-terminal amino group of peptides attached to the resin was first iodoacetylated in the presence of DIC as a coupling reagent for 3 h at room temperature. We also tested other coupling reagents, including TBTU, TCTU (*N*-[(6-chloro-1*H*-benzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide), and HBTU for the synthesis, but the reactions were not complete even after 24 h, as judged from the Kaiser test. The obtained iodoacetylated peptides were treated on the solid support with an excess of tertiary amine (Et_3N, Pr_3N, Bu_3N, and 1,4-diazabicyclo[2.2.2]octane). The reaction resulted in nucleophilic substitution of iodine by a tertiary amine (Figure 1(A)) after 24 h at room temperature. The derivatized peptides were cleaved from the resin simultaneously with the side chain deprotection using a solution of TFA/H_2O/TIS (95/2.5/2.5, v/v/v) at room temperature for 2 h. The prolongation of the cleavage time did not affect the reaction yield. Quaternary ammonium groups were stable at these conditions. The QAS peptides were obtained with a relatively good yield (over 60%, Table 1), as judged from the RP-HPLC analysis of

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Table 1. Synthesis of QAS-peptide derivatives – analytical data

Nr	Sequence of QAS peptide	Yield[a] (%)	RT[b] (min)	$[M]^+_{found}$ m/z[c]	$[M]^+_{calculated\ for\ formula}$ m/z[c]
1a	Tmaa-DVYT-NH_2	28	14.6	595.3092	595.3086; $C_{27}H_{43}N_6O_9$
1b	Teaa-DVYT-NH_2	78	16.0	637.3556	637.3556; $C_{30}H_{49}N_6O_9$
1c	Tpaa-DVYT-NH_2	65	19.5	679.4035	679.4025; $C_{33}H_{55}N_6O_9$
1d	Tbaa-DVYT-NH_2	68	24.7	721.4498	721.4495; $C_{36}H_{61}N_6O_9$
1e	Dbaa-DVYT-NH_2	75	14.8	648.3352	648.3352; $C_{30}H_{46}N_7O_9$
2a	Tmaa-DAAA-NH_2	20	11.4	445.2400	445.2405; $C_{18}H_{33}N_6O_7$
2b	Teaa-DAAA-NH_2	80	12.4	487.2874	487.2875; $C_{21}H_{39}N_6O_7$
2c	Tpaa-DAAA-NH_2	63	16.7	529.3340	529.3344; $C_{24}H_{45}N_6O_7$
2d	Tbaa-DAAA-NH_2	65	22.2	571.3806	571.3814; $C_{27}H_{51}N_6O_7$
2e	Dbaa-DAAA-NH_2	62	11.3	498.2668	498.2671; $C_{21}H_{36}N_7O_7$
3a	Tmaa-AAYA-NH_2	17	14.8	493.2762	493.2769; $C_{23}H_{37}N_6O_6$
3b	Teaa-AAYA-NH_2	68	15.5	535.3235	535.3239; $C_{26}H_{43}N_6O_6$
3c	Tpaa-AAYA-NH_2	64	18.4	577.3697	577.3708; $C_{29}H_{49}N_6O_6$
3d	Tbaa-AAYA-NH_2	63	24.7	619.4157	619.4178; $C_{32}H_{55}N_6O_6$
3e	Dbaa-AAYA-NH_2	62	14.3	546.3032	546.3035; $C_{26}H_{40}N_7O_6$
4a	Tmaa-AAAT-NH_2	28	11.3	431.2608	431.2613; $C_{18}H_{35}N_6O_6$
4b	Teaa-AAAT-NH_2	78	12.6	473.3080	473.3082; $C_{21}H_{41}N_6O_6$
4c	Tpaa-AAAT-NH_2	71	16.6	515.3547	515.3552; $C_{24}H_{47}N_6O_6$
4d	Tbaa-AAAT-NH_2	69	21.8	557.4015	557.4021; $C_{27}H_{53}N_6O_6$
4e	Dbaa-AAAT-NH_2	51	10.6	484.2876	484.2878; $C_{21}H_{38}N_7O_6$
5a	Tmaa-AAAA-NH_2	33	11.4	401.2503	401.2507; $C_{17}H_{33}N_6O_5$
5b	Teaa-AAAA-NH_2	68	12.3	443.2973	443.2976; $C_{20}H_{39}N_6O_5$
5c	Tpaa-AAAA-NH_2	61	17.0	485.3443	485.3446; $C_{23}H_{45}N_6O_5$
5d	Tbaa-AAAA-NH_2	60	21.4	527.3913	527.3915; $C_{26}H_{51}N_6O_5$
5e	Dbaa-AAAA-NH_2	71	11.4	454.2769	454.2772; $C_{20}H_{36}N_7O_5$

[a] The yield of synthesis was calculated on the basis of integration of a chromatogram.
[b] HPLC Vydac protein RP C18 column (4.6 × 250 mm, 5 µm), with a gradient elution of 0–40% *B* in *A* (*A* = 0.1% TFA in water; *B* = 0.1% TFA in acetonitrile/H_2O, 4 : 1) over 30 min (flow rate 1 ml/min) RT = retention time.
[c] *m/z* ratios are presented for the monoisotopic ions.

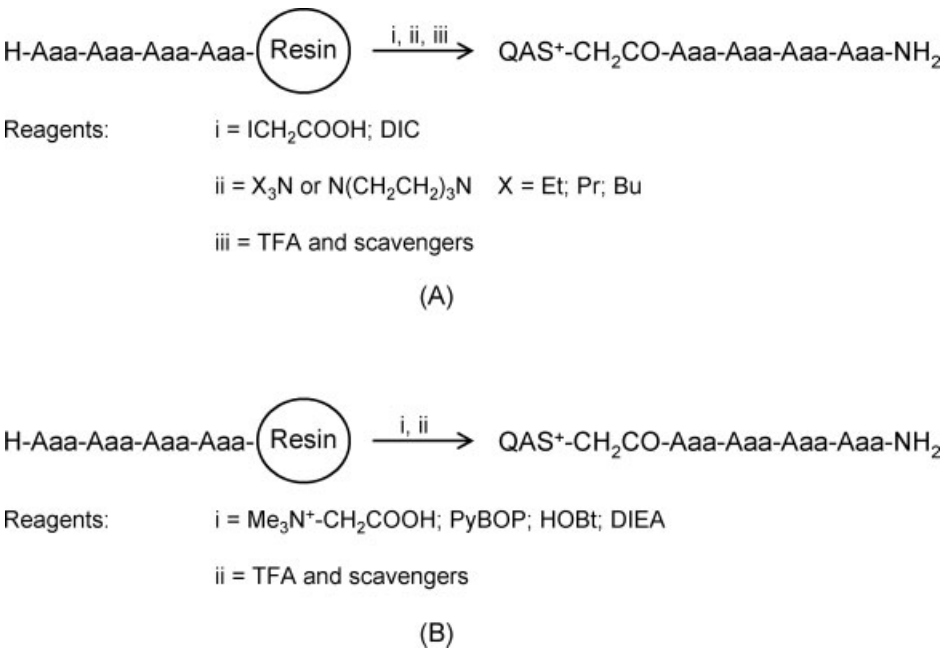

Figure 1. Two strategies of solid-phase synthesis of QAS of peptides. (A) Iodoacetylation of peptides followed by reaction with an appropriate tertiary amine. (B) Direct reaction of peptides with betaine. All QAS-peptide derivatives were cleaved from the resin using standard methods. Aaa = amino acid residue.

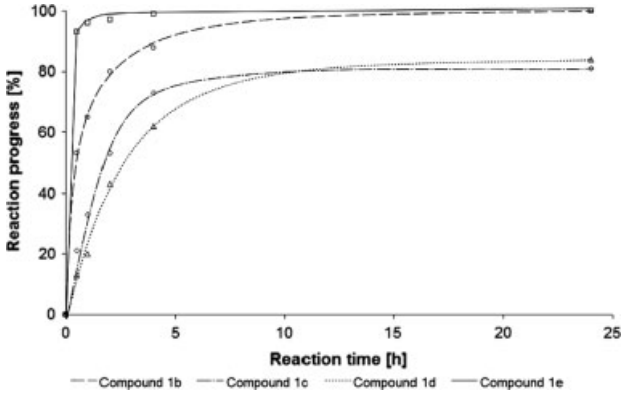

Figure 2. Progress of derivatization reaction as a function of time monitored by HPLC on the basis of the ratio of the product signal intensity: Et_3N^+-CH_2CO-DVYT-NH_2 (compound **1b**); Pr_3N^+-CH_2CO-DVYT-NH_2 (compound **1c**); Bu_3N^+-CH_2CO-DVYT-NH_2 (compound **1d**); $DABCO^+$-CH_2CO-DVYT-NH_2 (compound **1e**) *versus* the signal intensity of the unreacted peptide ICH_2-CO-DVYT-NH_2.

the crude products. All the QAS model peptides were purified by the analytical RP-HPLC. As can be expected, the retention time is related to the hydrophobic character of a QAS peptide (Table 1).

The substitution of the iodine atom in peptide *N*-iodoacetamide with the tertiary amines was investigated by monitoring the formation of the QAS peptides by RP-HPLC. In Figure 2, the increasing ratio of signal intensity of the product *versus* the unreacted peptide in the HPLC profile was plotted against the reaction time. The signals corresponding to the substrate and to the product were identified by the MS analysis. In general, the derivatization reactions were completed after 24 h. However, the signal corresponding to the derivative started to appear after 30 min. The same tendency of QAS formation was observed for other peptides.

We decided to check the applicability of a direct introduction of the (carboxymethyl)trimethylammonium inner salt (betaine) to the *N*-terminal amino group of a peptide attached to the solid support. The reaction was performed in DMF using PyBOP as a coupling reagent (Figure 1(B)). The coupling reaction yield was relatively low (overall yield of the Tmaa-peptides synthesis was 17–33%). The prolongation of the reaction time, repetition of the coupling reaction, as well as application of other coupling reagents (TBTU, TCTU, and HBTU) for this synthesis did not improve the final yield of the reaction. This may be explained by a limited solubility of betaine in DMF and the electrostatic repulsion between the positive charge of the quaternary nitrogen atom and the partial positive charge of the carbon atom of the activated carboxyl group in betaine-PyBOP ester. This method seems to be inferior as compared to the formation of QAS on the solid support described previously. However, taking into account a relatively low price of betaine and the handling requirements of trimethylamine, it could be treated as an acceptable alternative for the solid-phase synthesis of short peptides containing the Tmaa salt.

The crude peptides containing QAS groups were identified by a high-resolution ESI-MS in the positive ion mode. The spectra were recorded for samples dissolved in aqueous solution of acetonitrile (50%) with formic acid (0.1%). The quaternary ammonium moiety introduces the fixed charge to a peptide molecule. In contrast, for ESI-MS analysis of regular peptides the protonation of amino groups is necessary. The representative MS spectra for crude compounds **2a** and **4a** are shown in Figure 3. The analyte signal at $m/z = 445.24$ and at $m/z = 431.26$, respectively, was assigned to the $[M]^+$ ions, providing a very high signal-to-background ratio and a high ionization efficiency. The mass spectra also show weak signals at $m/z = 346.17$ and $m/z = 332.19$ that correspond to protonated H-DAAA-NH_2 and H-AAAT-NH_2 peptides, respectively. However, the HPLC analysis shows that the amount of unmodified peptide is 40% for compounds **2a** and **4a**.

We analyzed the effect of the solvent, used for ESI-MS analysis, on the intensity of the signals corresponding to a peptide and its quaternary ammonium derivative. A sample of the equimolar mixture of peptide H-AAYA-NH_2 and one of its QAS derivatives: Me_3N^+-CH_2CO-AAYA-NH_2 (**3a**), Pr_3N^+-CH_2CO-AAYA-NH_2 (**3c**), or Bu_3N^+-CH_2CO-AAYA-NH_2 (**3d**) was dissolved in various solvents (MeOH, MeCN, H_2O, H_2O:HCOOH (100:0.1), and H_2O:MeCN:HCOOH (50:50:0.1)). The quaternary ammonium peptide derivatives dissolved in acetonitrile and in methanol give a very intensive MS signal. The peak corresponding to compound **3d** in the mass spectrum recorded for MeOH solution was a hundred times as intensive as that of H-AAYA-NH_2. For the mixture dissolved in acetonitrile only the signal corresponding to QAS was observed. This phenomenon confirms that the permanent positive charge of QAS allows detection without the proton transfer from the solvents used in the electrospray ionization. Addition of water or acids, necessary for protonation of amines, does not significantly affect the intensity of a signal corresponding to a singly charged QAS peptide, but increases the intensity of a signal assigned to peptide H-AAYA-NH_2 (Figure 4).

In a further experiment, the influence of the QAS-peptide derivative concentration on the detection limit was examined by HR-ESI-MS (high resolution electrospray ionization mass spectrometry). Figure 5 shows the signal intensity assigned to the molecular ion peak of compound **5d** plotted *versus* its concentration. The detection limit was estimated to be 5 fmol, which means that femtomolar levels of peptides could be detected by the proposed labeling method. For the 2.5 fmol concentration of compound **5d**, the signal can still be observed; however, an identification of the isotopic pattern was problematic. It is important to note that the experiment was conducted as a regular ESI-MS analysis, using a standard flow rate of 3 µl/min. It may be expected that application of a nano-ESI-MS ionization method may further increase sensitivity of the analysis of the QAS peptides [24].

ESI-CID-MS/MS Analysis of QAS-Peptide Derivatives

There are two known distinct fragmentation pathways of protonated peptides: charge-directed (Ch-D) and charge-remote (Ch-R). Ch-D reactions occur for peptides containing a proton attached to a free amino group. The proton is mobile and can migrate from one amide group to another [25]. The Ch-R fragmentation was observed for peptides with a fixed charge [26]. As the Arg side chain strongly favors protonation, the fragment ions are mostly produced by the Ch-R fragmentation [27,28]. The effect of an Arg residue can be mimicked by derivatizing a peptide with a fixed positive charge-carrying molecule, including QAS [14]. The Ch-R fragmentation does not depend on the incoming proton but is due rather to the intramolecular hydrogen shifting within the precursor ion molecule. As far as we know, the co-existence of both fragmentation pathways during the ESI-MS/MS fragmentation of the non-protonated $[M]^+$ molecular ion of QAS-peptide derivatives has not been reported.

We performed the ESI-MS/MS experiments on $[M]^+$ ions of all the synthesized QAS-peptide derivatives. The collision energy

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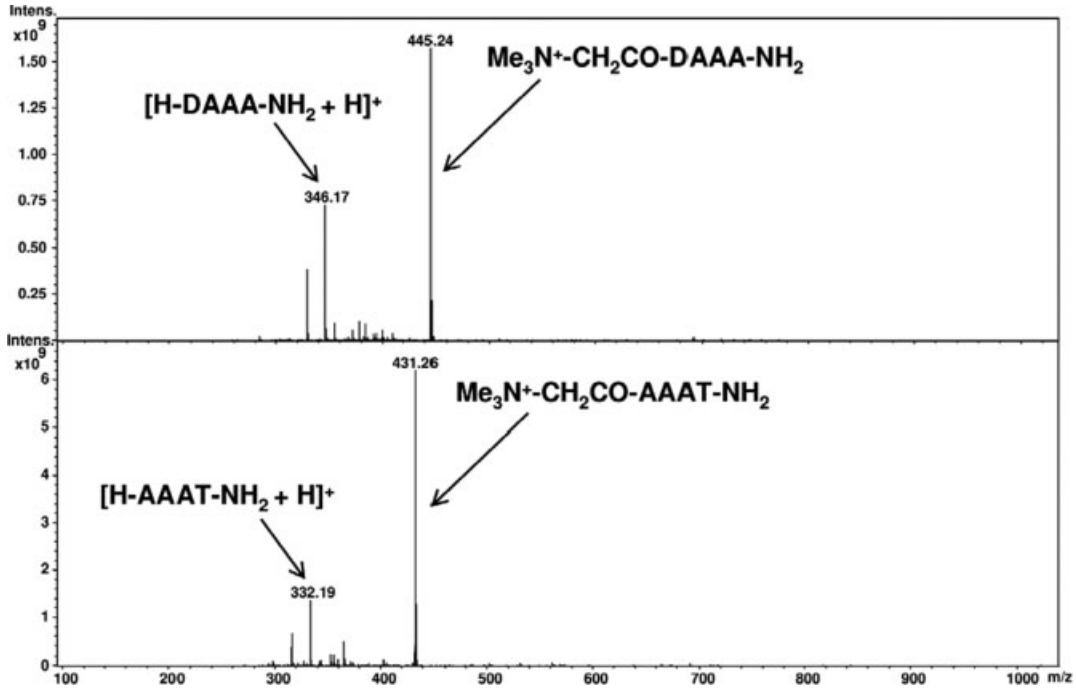

Figure 3. ESI-MS spectra of crude compounds **2a** and **4a**, respectively. The signals corresponding to molecular ions $[M]^+$ of QAS-peptide cations, and $[M+H]^+$ ions of unmodified peptide are presented.

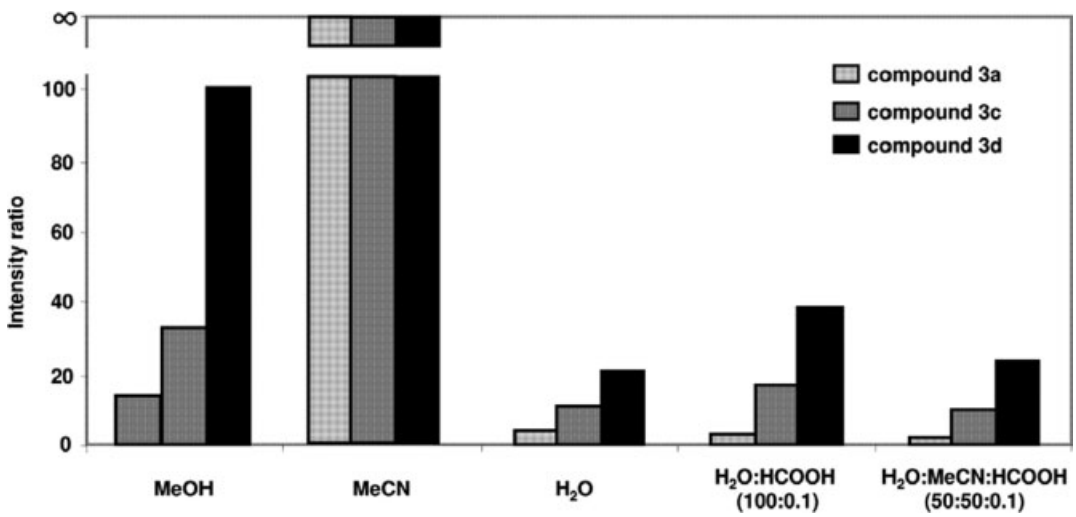

Figure 4. The effect of solvent on the intensity of signals corresponding to peptide (H-AAYA-NH_2) and its quaternary ammonium derivatives (QAS^+-CH_2CO-AAYA-NH_2) based on the ratio of the monoisotopic signal intensity corresponding to QAS^+-CH_2CO-AAYA-NH_2 (**3a, c**, and **d**) *versus* the monoisotopic signal intensity corresponding to peptide H-AAYA-NH_2.

was optimized to obtain a good quality fragmentation mass spectrum. QAS derivatives of hydrophobic peptides (compounds **5a–e**) and peptides containing only one hydrophilic amino acid residue (compounds **2a–e** to **4a–e**) required lowering the collision energy (18–22 V) as compared to QAS of the hydrophilic peptides (compounds **1a–e**; 22–29 V). All signals had a mass accuracy error in the range of 2 ppm, which unambiguously confirmed the proposed formulas of the detected fragments.

The representative MS/MS spectra of the parent ion $[M]^+$ for compounds **1a–d** are presented in Figure 6. The analysis of these spectra reveals a series of a and b type ions. The QAS-acetic acid residue was considered as the first amino acid in the peptide sequence. A series of b ions, ranging from b_2 to b_4, was accompanied by corresponding ions of type a. The fragmentation of compounds **2a–d** to **5a–d** produced the same series of fragment ions. Similar fragmentation pathways were previously observed for ESI-MS/MS of other QAS peptides [6] as well as peptides containing trimethoxyphenylphosphonium salts [29]. Beside neutral losses of water [M-18.0106] and CH_3CHO [M-44.0262] molecules from the side chain of Thr [30], multiple ions resulting from decomposition of the QAS group were observed.

The signals corresponding to the fragments where QAS was eliminated as a tertiary amine were observed for Tmaa and Teaa peptide derivatives (compounds **1a** and **1b**, respectively).

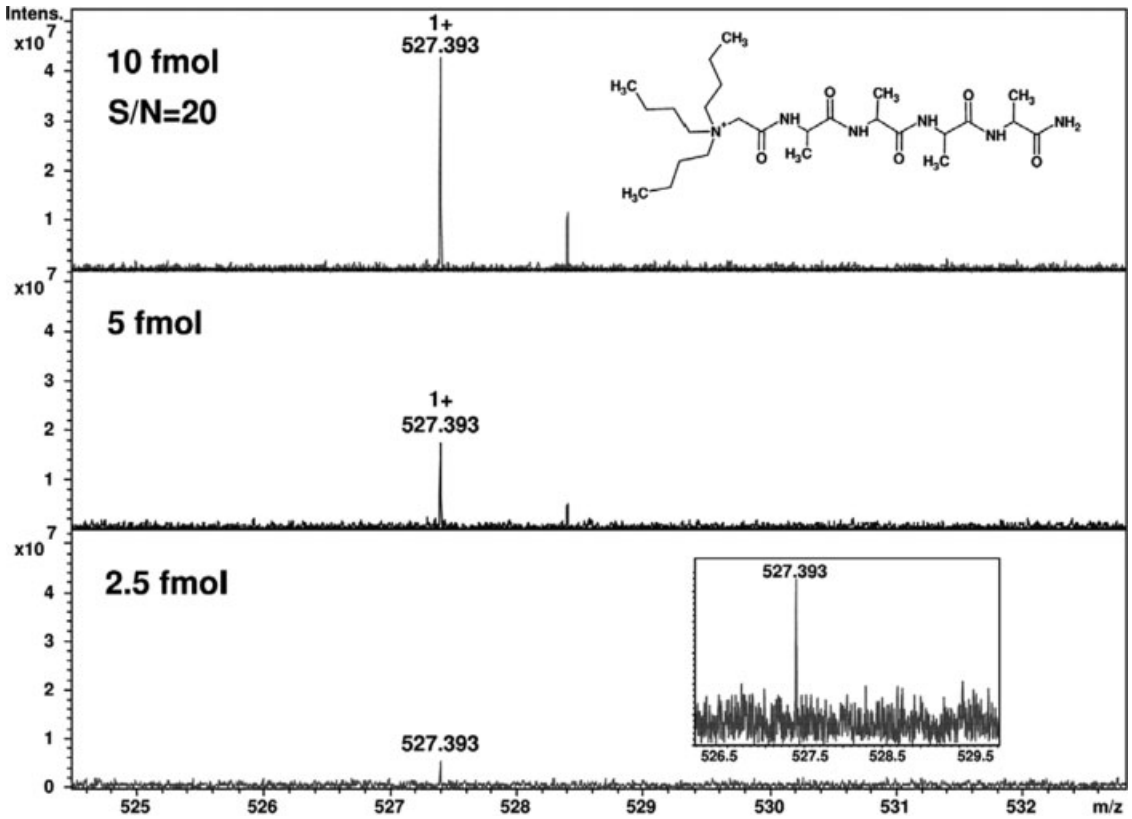

Figure 5. The influence of Bu_3N^+-CH_2CO-AAAA-NH_2 (compound **5d**) concentration on the HR-ESI-MS detection limit.

The neutral losses (59.0735 for trimethylamine or 101.1204 for triethylamine) were observed for a_4 nad b_4 ions for compounds **1a–b** to **5a–b**. A series of fragment ions at [a-28.0313] and [b-28.0313] for Teaa, at [a-42.0469] and [b-42.0469] for Tpaa, and at [a-56.0626] and [b-56.0626] for Tbaa peptide derivatives was observed as the consequence of a partial fragmentation of the QAS group by Hofmann elimination. The neutral loss of the alkene molecule (C_nH_{2n}) is accompanied by retention of the mobile proton on nitrogen of ammonium salt (Figure 7). This mobile proton can migrate to the amide groups and, therefore, may contribute to the Ch-D fragmentation pathways.

The MS/MS analysis of peptide conjugates may be a complex task because of intensive fragmentations of the non-peptide components. Some modifications of peptides, including glycation and *N*-phosphorylation, are known to cause multiple and complicated neutral losses during the collision induced dissociation (CID) process [31,32]. It has been recently found that electron capture dissociation (ECD) [32,33] or electron transfer dissociation (ETD) [34] methods offer an important advantage in peptide sequence analysis because of a significant reduction in the neutral losses related to the derivatization. Another approach used to reduce the possibility of neutral losses in MS/MS analysis of glycated peptides is based on formation of cyclic complexes with borates. This modification results in stabilization of the sugar moiety and therefore the simplification of the fragmentation pattern [35].

Taking into account the loss of alkene molecules from the above described QAS derivatives, a new type of the QAS group which would not undergo the Hofmann elimination seems to be desirable. We used bicyclic tertiary amine such as DABCO to form a new QAS-peptide derivative, containing 2-(1,4-diazabicyclo[2.2.2]octylammonium)acetyl (Dbaa) group. It should be noted that the quaternization of DABCO by iodoacetylated peptides was found to be relatively easy and fully compatible with the standard solid-phase peptide synthesis. We observed that the Dbaa formation is faster than that of Teaa, Tpaa, and Tbaa and takes less than 4 h (Figure 2). HRMS-CID mass spectra of peptides containing DABCO (**1e, 2e, 3e, 4e**, and **5e**) represent mainly the a and b ions; however, no peaks corresponding to fragmentation of DABCO were observed.

Our results indicate that the DABCO group is a promising new moiety that may be used for sensitive detection by ESI-MS. A high stability of the DABCO group in the collision cell of tandem mass spectrometers makes this derivatization the most suitable for routine MS/MS analysis. Another advantage of the DABCO group as a reagent for peptide derivatization is its relatively high reactivity, which may be explained by entropic effect, as there are two equivalent nitrogen atoms located at the opposite sides of the rigid molecule. The MS analysis revealed that only one of two nitrogen atoms participates in the formation of the QAS, which can be explained by the small distance between nitrogens and therefore a strong electrostatic repulsion. The high nucleophilicity is responsible for the fast and quantitative reaction of DABCO with the resin-bound iodoacetamide moiety [36]. To the best of our knowledge, this group was not applied previously as a charge-derivatizing agent for biomolecules.

Conclusions

We designed and synthesized on the solid support a series of peptide conjugates containing QAS, which do not require a further ionization for detection by an electrospray MS. The

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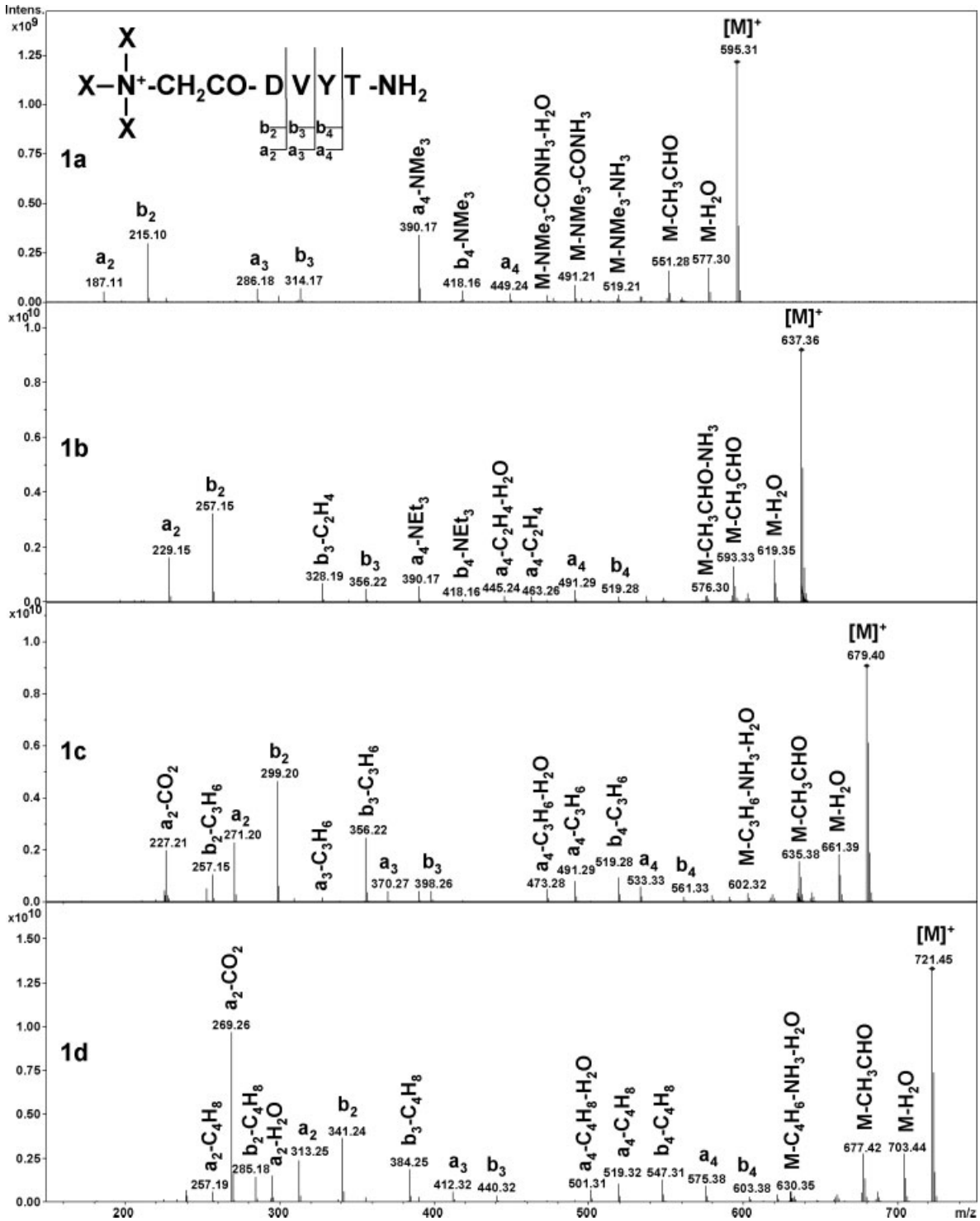

Figure 6. ESI-MS/MS spectra of the $[M]^+$ molecular ion of compounds **1a**–**d**. The neutral loss of the alkene molecule (C_nH_{2n}) occurs for compounds **1b**–**d**. *X* = alkyl group: Me, Et, Pr, or Bu, respectively.

methodology presented here offers a sensitive means to analyze minute amounts of peptides or other compounds, even dissolved in aprotic solvents.

Tandem mass spectrometric analysis of the peptides containing DABCO QAS provides not only an enhanced detection level but also a convenient sequence analysis, as only the series of a and b ions are formed. The method may be particularly useful in analysis of the OBOC peptide libraries prepared by the split-and-mix method.

Acknowledgements

This work was supported by a grant No. N N401 222734 from the Ministry of Science and Higher Education of Poland. Valuable suggestions by Dr Alicja Kluczyk are gratefully acknowledged.

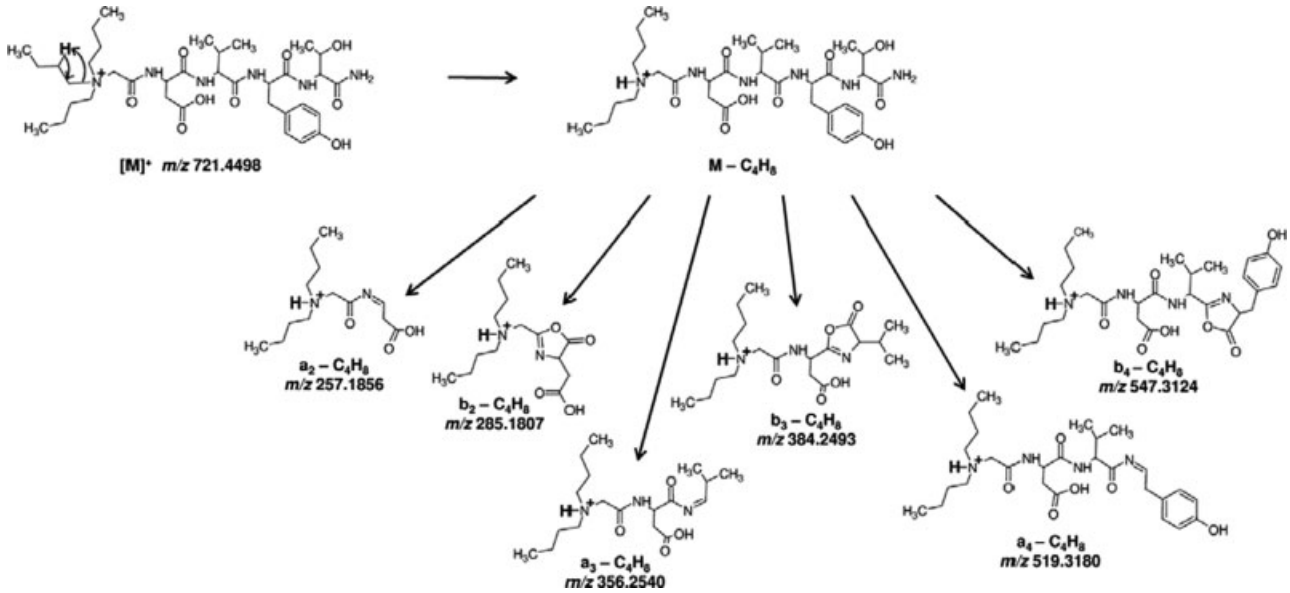

Figure 7. Neutral losses of the alkene molecule from Bu_3N^+-CH_2CO-DVYT-NH_2 and its a and b fragment ions resulting from the Hofmann elimination. The *m/z* values from experiment are given. A similar elimination pattern was observed for compounds **1b**, **1c**, **2b–d** to **5b–d**.

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