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Mass Spectrometric Analysis of Oxidized Tryptophan

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Oxindolylalanine and oxindolylalanine-containing peptides were prepared by treatment of tryptophan and tryptophan-containing peptides with mixtures of dimethyl sulfoxide and hydrochloric acid in acetic acid (DMSO–HCl–HAc). The reaction between tryptophan and DMSO–HCl–HAc was monitored by fast atom bombardment mass spectrometry (FAB-MS) and the proposed chlorotryptophan intermediate in the reaction was observed. Almost complete conversion of tryptophan to oxindolylalanine was obtained in reaction mixtures containing 3.75 M HCl when the reaction was performed in an open tube. A higher HCl concentration (5.5 M) and a closed reaction tube promoted the formation of by-products, such as dioxindolylalanine and 3-chlorooxindolylalanine. Extensive hydrolysis *C*-terminal of tryptophan was observed when tryptophan-containing peptides were treated with DMSO–HCl–HAc containing 5.5 M HCl, during which the tryptophan residue was modified to dioxindolylalanine lactone. Hydrolysis was not observed in mixtures containing 3.75 M HCl. The presence of oxindolylalanine in peptides could be demonstrated by characteristic peaks in FAB collision-induced dissociation tandem mass spectra. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: tryptophan; tryptophan-containing peptides; oxidation; oxindolylalanine; fast atom bombardment; collision-induced dissociation tandem mass spectrometry

INTRODUCTION

Oxidation of peptides and proteins is a frequently occurring phenomenon *in vitro* during various analytical and synthetic procedures, during storage and under physiological conditions,[1–8] but may also occur *in vivo*.[1] Modification of amino acids alters the physico-chemical and functional properties of peptides and proteins, and may even result in toxic products.[1,9] For example, accumulation of oxidized proteins *in vivo* has been related to diseases such as cataract, emphysema and rheumatoid arthritis.[1,10–12] Furthermore, oxidation is known to decrease the nutritional value of food and feedstuffs.[9,13]

Detection of possibly oxidized products is usually performed by reversed-phase high-performance liquid chromatography (RP-HPLC)[14], UV spectrometry[11,15–17] or mass spectrometry.[2,4,18,19] In most cases, detection is based on the different behaviour of the oxidized product compared with the (known) non-oxidized species, *e.g.* a shortened retention time in RP-HPLC analysis.[14] In some cases the method of analysis also allows characterization of the product, *e.g.* oxidation products of tryptophan (Trp) yield specific changes in UV absorption spectra.[16] However, the detection and characterization of oxidation in peptides and proteins of unknown sequence are much more difficult, since changes indicative of modification cannot be detected using these methods. Recently, a tandem mass spectrometric method was developed which allows simultaneous detection and characterization of oxidized methionine (Met) residues in peptides of known and unknown sequence.[19] The tandem mass spectra of peptides containing oxidized Met residues show characteristic product ions. This mass spectrometric procedure may also be used for the analysis of peptides containing oxidized Trp residues. In contrast to Met, oxidation of Trp can yield various products, several of which are isomers.[2,15,17,20–24] A number of these products are shown in Scheme 1.

A common oxidation product of Trp is oxindolylalanine (Oia), which is observed when Trp is treated with, e.g., hydrogen peroxide,[9] trichloromethylperoxy radicals ($CCl_3OO^{\cdot}$)[24] or iron(III) cyanide.[5] Generally, the yield of Oia is low, whereas several other products may also be produced, including the isomeric 5-hydroxytryptophan (5-OH-Trp). High yields of Oia (80%) can be obtained within 1 h by treatment of Trp with a mixture of dimethyl sulfoxide and hydrochloric acid in acetic acid (DMSO–HCl–HAc).[15,16,18] This mixture also oxidizes cysteine (Cys) to cystine and Met to methionine sulfoxide, while slight overoxidation of Trp to dioxindolylalanine (Dia) may be observed. The

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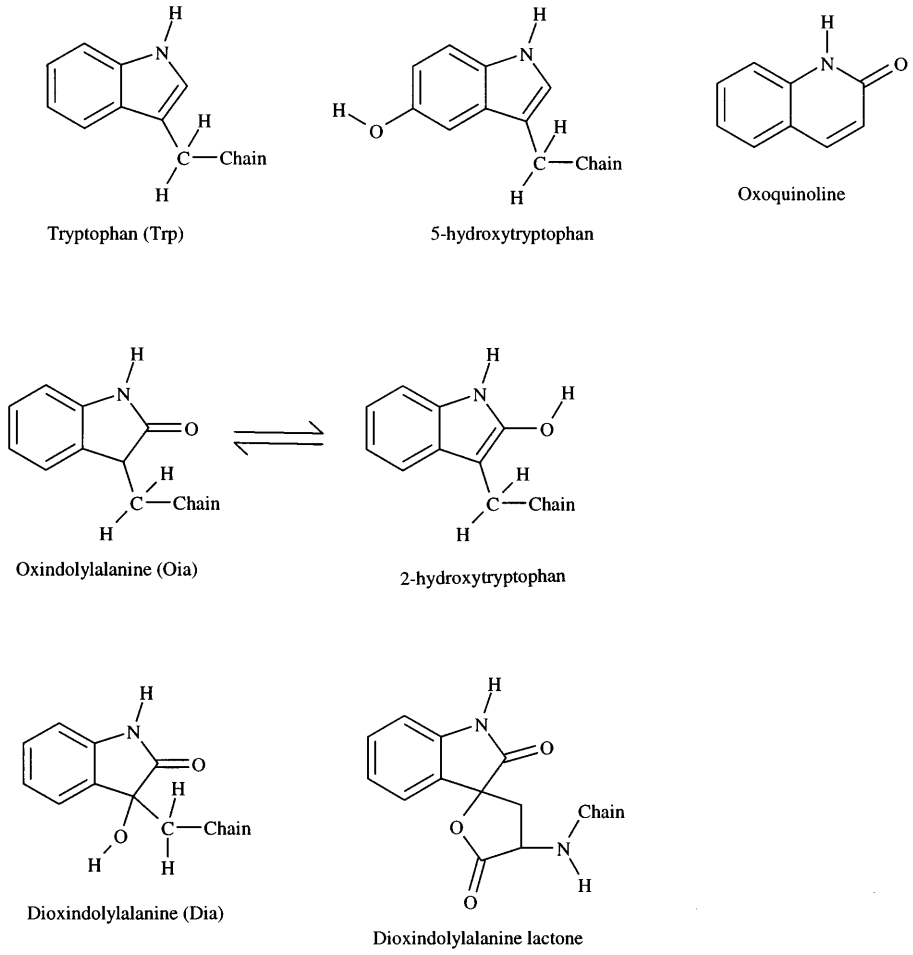
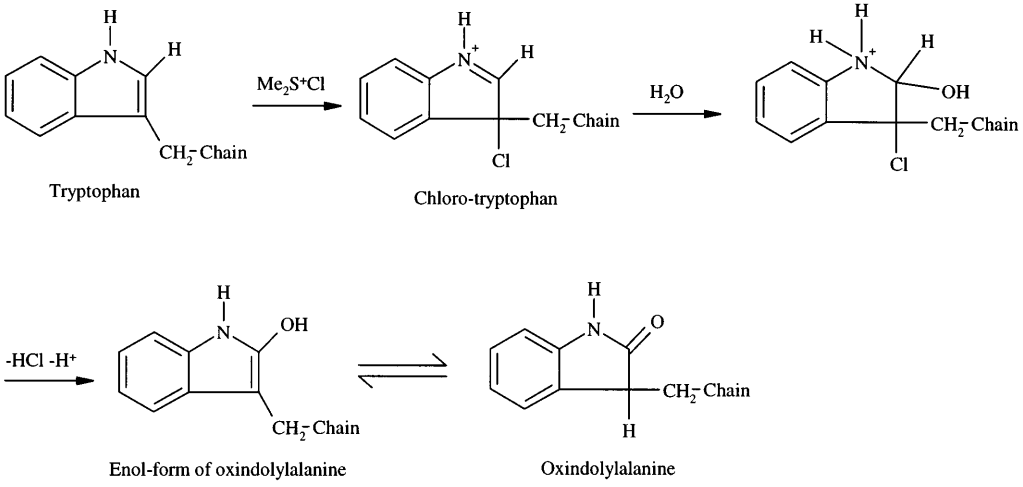


Scheme 1. Tryptophan and related (oxidation) products.

proposed reaction mechanism is shown in Scheme 2.[15] It has been proposed that in DMSO–HCl–HAc mixtures, chlorosulfonium ions are formed,[25] which then react with Trp to yield a 3-chlorotryptophan ion.[15] Addition of water to the chlorinated tryptophan, followed by deprotonation and elimination of HCl, result in the generation of 2-hydroxytryptophan (2-OH-Trp). This enol species is in equilibrium with the keto species, Oia. Electrochemical experiments have shown the existence of the enol species,[23] but in other experiments the keto form better explains the behaviour, *e.g.* diastereoisomers are observed in RP-HPLC.[20]

The aim of this work was to investigate the proposed mechanism of the oxidation of Trp by DMSO–HCl–HAc and to characterize intermediate and reaction products using fast atom bombardment mass spectrometry (FAB-MS) and collision-induced dissociation tandem mass spectrometry (CID-MS/MS). A second objective was the identification of fragment ions which are characteristic of oxidized Trp residues in peptides.



Scheme 2. Reaction scheme of tryptophan oxidation in DMSO–HCl–HAc (cf. Ref. 15).

EXPERIMENTAL

Reagents

Glacial acetic acid and 14 M hydrochloric acid were purchased from Lamers en Pleuger ('s Hertogenbosch, The Netherlands), dimethyl sulfoxide was purchased from Aldrich (Milwaukee, WI, USA). D,L-Tryptophan was of unknown origin; impurities were not observed in its FAB mass spectrum. δ-Sleep-inducing peptide (W–A–G–G–D–A–S–G–E), luteinizing hormone-releasing hormone (pyroE–H–W–S–Y–G–L–R–P–G–NH_2), angiotensin-converting enzyme inhibitor (P–T–H–I–K–W–G–D), adrenocorticotropic hormone (4–9) (M–E–H–F–R–W) and [D-Pro^2,D-Phe^7,D-Trp^9]-substance P (R–P–K–P–Q–Q–F–F–W–L–M–NH_2) were purchased from Saxon Biochemicals (Hannover, Germany) and used without further purification.

Preparation of DMSO–HCl–HAc mixtures

Two DMSO–HCl–HAc mixtures were prepared by slowly adding 2 ml of 14 M HCl to either 2 or 4 ml of HAc. To both mixtures 400 µl of DMSO were then added while maintaining room temperature. The compositions of the two solutions were thus 1:5:5 (v/v) DMSO–HCl–HAc (5.5 M HCl) and 1:5:10 (v/v) DMSO–HCl–HAc (3.75 M HCl). The solutions were stored in a tightly closed tube and used within a few days.

Oxidation of Trp and Trp-containing peptides with DMSO–HCl–HAc

Trp and the Trp-containing peptides were treated with either of the DMSO–HCl–HAc mixtures at room temperature by adding 100–200 µl of the mixture to *ca.* 200 µg of dry compound in an Eppendorf microtube. The reaction mixtures were left at room temperature for periods from 15 min to several days in the case of Trp, and from 1 to 24 h for the peptides, after which they were analyzed mass spectrometrically.

FAB-MS and FAB-CID-MS/MS

Positive-ion FAB mass spectra were recorded on a JEOL JMS-SX/SX102A four sector instrument of B_1E_1–B_2E_2 geometry, operating at an accelerating voltage of 10 kV. The xenon FAB gun was operated at 6 kV and 5 mA emission current. The scan rate was 30 s for the m/z 0–2400 range and the resolution was 1000. About 2 µl of the sample solution, corresponding to *ca.* 10 nmol of the starting material, directly taken from the reaction mixture, were added to *ca.* 1 µl of glycerol (OPG Farma, Utrecht, The Netherlands). FAB mass spectra were obtained by scanning the magnet of MS-1. FAB-CID mass spectra were acquired by selecting the desired precursor ion with MS-1 and pressurizing the collision cell in the third field-free region with nitrogen until the main beam from MS-1 was reduced to *ca.* 50% of its original intensity. The resulting high-energy CID spectra were obtained by *B*/*E* linked scanning of MS-2. Data acquisition and processing were performed using an HP-9000 data system. Average profile spectra (4–8 scans) are given.

RESULTS AND DISCUSSION

Oxidation of Trp by DMSO–HCl–HAc, reaction kinetics and products

The reaction between Trp and DMSO–HCl–HAc was monitored using FAB-MS. Apart from the ions which can be assigned to glycerol, DMSO and clusters thereof, a number of ions, at m/z 205, 221, 237, 239, 241, 255, 257 and 267, were observed in the spectra. These ions probably correspond to the starting product, intermediate product(s) and final products. We presumed the ions below m/z 200 to be fragment ions of the various reaction products. Both m/z 239 and 241 ions, and also m/z 255 and 257 ions, occur in a 3:1 abundance ratio in every spectrum, indicating the presence of a chlorine atom in these species. This was confirmed by comparison of the FAB-CID mass spectra of these ions (data not shown). The FAB-CID mass spectra of the ions at m/z 205, 221, 237, 255 and 267 are shown in Fig. 1. In addition, the relative abundances of these ions are shown in Table 1 as a function of reaction time.

The ion at m/z 205 [Fig. 1(a)] corresponds to protonated Trp, and its mass spectrum is identical with that of untreated Trp. The ion at m/z 221 corresponds to protonated Oia, and its CID spectrum [Fig. 1(b)] is similar to that of Trp, exhibiting only apparent intensity differences and mass increases of 16 u of some fragment ions. As in the spectrum of Trp, side-chain fragment ions are observed in the spectrum of Oia. Owing to the inherent stability of C—C bonds connected to conjugated systems, in the spectrum of Trp the abundance of the m/z 130 ion (C_α—C_β bond cleavage) is much higher than that of m/z 117 (C_β—C_γ cleavage). In contrast, the side-chain fragment ions of Oia at m/z 146 and 133 are of equal abundance, indicating the absence of a conjugated substituent at C_γ. This is in agreement with expected absence of aromaticity in the oxindolyl ring.

The relatively low abundance and the abundance changes with time of m/z 239 (Table 1) indicate that this formally corresponds to the $[MH]^+$ ion of a chlorine-containing intermediate (Scheme 2). The presence of a chlorine atom was confirmed by FAB-CID analysis of m/z 239 and 241 ions, as both spectra contained characteristic fragment ions formed by elimination of HCl from $[MH]^+$ (data not shown). However, owing to the low abundance and interference from matrix ions, full structural assignment was not possible.

The ions at m/z 237, 255/257 and 267 originate from by-products in the reaction. The species at m/z 237 probably represents protonated Dia, a known by-product [Fig. 1(c)].[15,16] The FAB-CID mass spectra of m/z 255 [Fig. 1(d)] and 267 [Fig. 1(e)] show many characteristics similar to those of protonated Dia. The fragment ion at m/z 219 is probably produced by elimination of water, HCl and CH_3SH from the $[MH]^+$ species at m/z 237, 255 and 267, respectively. Hence the ions at m/z 255 and 267 are proposed to correspond to

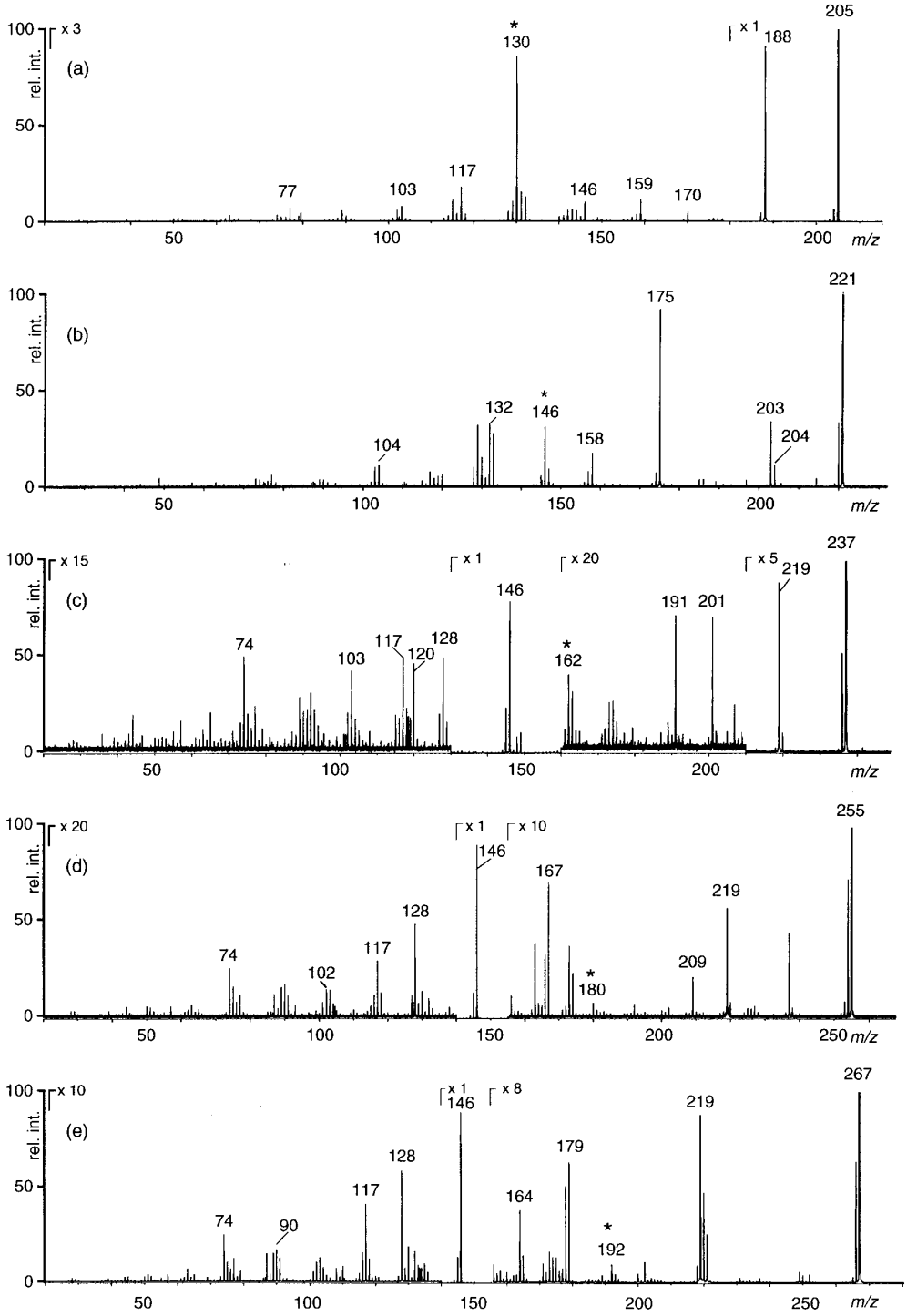


Figure 1. FAB-CID mass spectra of ions observed when Trp is treated with DMSO–HCl–HAc mixtures. (a) *m/z* 205; (b) *m/z* 221; (c) *m/z* 237; (d) *m/z* 255; (e) *m/z* 267. Complete side-chain ions are indicated by asterisks.

protonated 3-chloro-Oia and 3-thiomethoxy-Oia, respectively. 3-Chloro-Oia is not an unexpected by-product, since it is known that 3-bromo-Oia can be isolated when Trp is treated with DMSO–HBr–HAc.[17] 3-Bromo-Oia is unstable in the presence of water and can react further to give Dia.[17] A similar conversion may also occur with 3-chloro-Oia in the presence of water. The thiomethoxy species is only observed after prolonged exposure of Trp to DMSO–HCl–HAc in a closed tube. It is unclear what reaction causes the formation of this by-product.

The very abundant fragment ion at *m/z* 146 in Fig. 1(c)–(e) has been noted earlier in the FAB mass spectrum of a dioxindolylmoiety-containing dipeptide.[26] Östin *et al.*[26] proposed this to be the oxoquinolonium ion (protonated oxoquinoline, Scheme 1), which is the same ion as proposed for *m/z* 146 in Oia. However, as shown in Fig. 2, the FAB-CID mass spectra of these two ions are different. The most abundant ion (*m/z* 118) arising from fragmentation of *m/z* 146 from Oia probably corresponds to elimination of CO (28 u), which is consistent with the structure of the oxoquinolonium

Table 1. Relative abundances of selected ions as a function of reaction time in the FAB mass spectra of Trp treated with DMSO–HCl–HAc[a]

	5.5 M HCl								3.75 M HCl					
	Open tube				Closed tube				Open tube			Closed tube		
m/z	15 min	100 min	185 min	2 days	15 min	100 min	185 min	2 days	25 min	145 min	2 days	25 min	145 min	2 days
205	21	4	1.6	1.5	9.5	1.2	3.6	1.2	27	20	1.9	15	1.7	2
221	75	92	95	93	84	91	88	48	70	79	95	79	93	93
239	4	0	0	0	4.7	0	0	0	2.5	0.6	0	4.9	0	0
237	0	1.4	1	5.5	0	1	2.3	31	0	0	2.8	0	0.8	2.5
255	0.8	2.2	2.1	0	1.8	6.5	6.4	15	0	0.6	0	0.9	4.2	2.2
267	0	0	0	0	0	0	0	3.9	0	0	0	0	0	0.7

[a] Relative abundances (%) are presented as a fraction of the total abundance all selected ions.

ion. In contrast, the main ion (*m/z* 128) from fragmentation of *m/z* 146 of Dia corresponds to elimination of water (18 u). The latter could represent the presence of an alcohol or aldehyde group in this species. However, the structural information which could be derived from the CID spectrum [Fig. 2(b)] appeared to be insufficient to propose a reliable structure.

In Table 1 the effect of two reaction variables on reaction kinetics is shown. Apparently, a higher HCl concentration and the use of a closed reaction tube

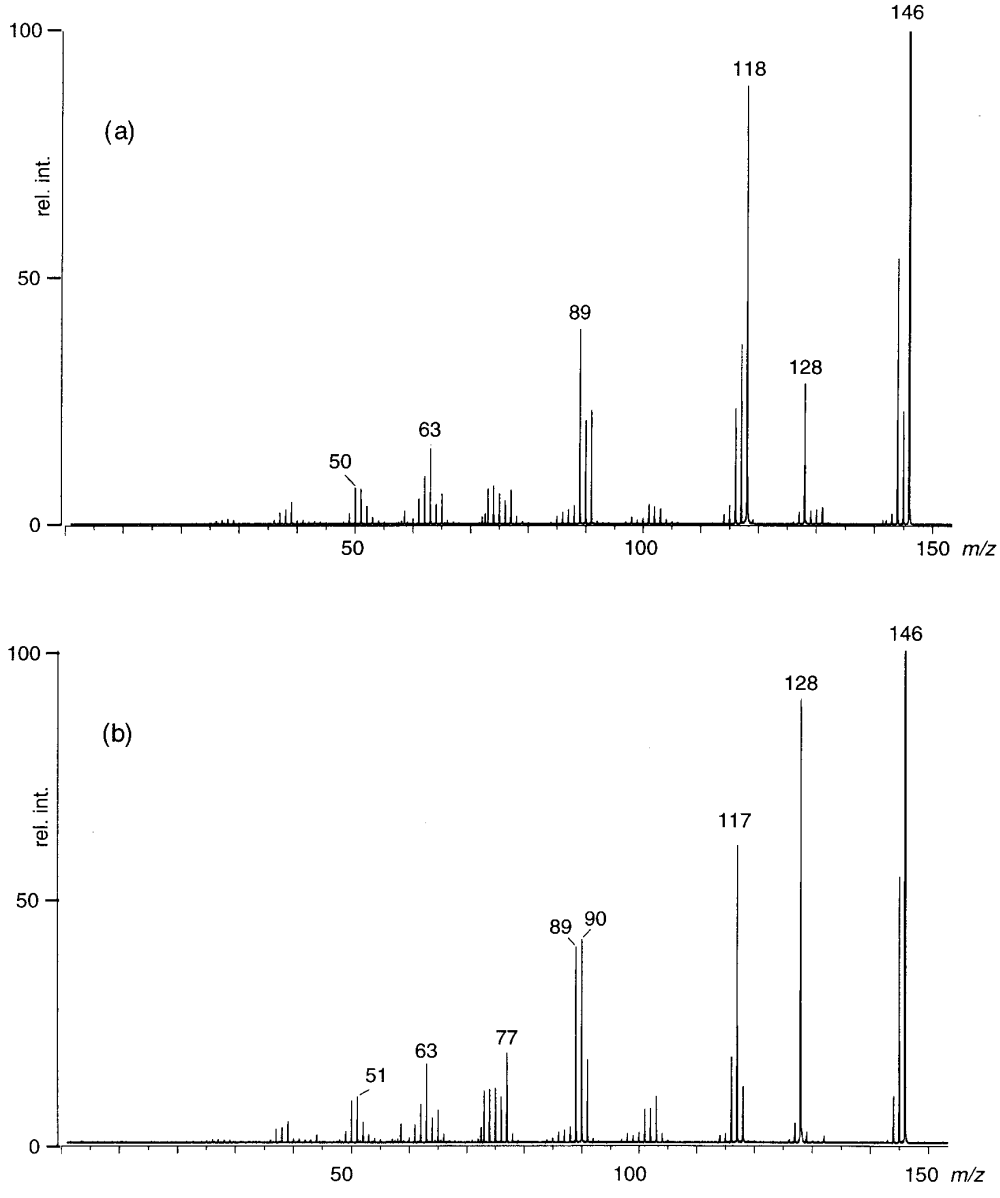
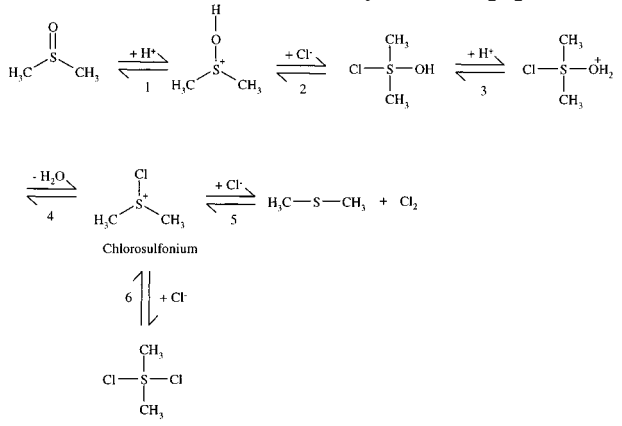


Figure 2. FAB-CID mass spectra of the fragment ion at *m/z* 146 of (a) Oia and (b) Dia.

increase the oxidation rate. This can be rationalized by the effect of both variables on the proposed equilibria in DMSO–HCl–HAc mixtures, shown in Scheme 3.[15,25,27] Increasing the HCl concentration and closing the reaction tube will influence equilibria (1)–(3) and equilibrium (5), respectively, yielding a higher concentration of the principal reactive species, the chlorosulfonium ion. The combination of a high HCl concentration and closing the reaction tube apparently yields relatively large amounts of by-products. This may also be a consequence of accumulation of the chlorosulfonium ion in the reaction tube.

Oxidation of Trp-containing peptides

Trp-containing peptides were treated with DMSO–HCl–HAc in an open tube to minimize by-product formation. Nonetheless, the by-products observed earlier, Dia, 3-chloro-Oia and 3-thiomethoxy-Oia, may still be observed, in addition to other products. The latter include oxidation of methionine residues to methionine sulfoxide,[15,18] acid-induced acetylation of peptide residues, *e.g.* at threonine (Thr) and serine (Ser) residues, and acid-induced hydrolysis. In Fig. 3 the FAB mass spectra of angiotensin-converting enzyme inhibitor (ACEI, *M* 952.5) treated with DMSO–HCl–HAc containing (a) 3.75 and (b) 5.5 M HCl are shown. In Fig. 3(a) the main product is observed at *m/z* 969.5, which corresponds to mono-oxidized ACEI. CID sequence analysis of this compound (data not shown) showed that the Trp residue was oxidized. Another product is observed at *m/z* 1011.5, corresponding to acetylated [Oia^6]-ACEI. CID sequence analysis of this compound pointed to acetylation of the Thr residue (data not shown).



Scheme 3. Equilibria in water-containing DMSO–HCl mixtures (cf. Refs 25 and 27).

[Oia^6]-ACEI and acetylated [Oia^6]-ACEI are also observed in Fig. 3(b). In addition, Dia- and 3-thiomethoxy-Oia-containing ACEI are observed, including their acetylated analogues. In Fig. 3(b), a very abundant ion is observed at *m/z* 795.5, with its acetylated analogue at *m/z* 837.6. This mass does not correspond to a common fragment ion (A, B or Y″ ions) of one of the reaction products. Moreover, its intensity is much higher than those of the other ions, which further indicates that we are dealing with a new reaction product, rather than a fragment ion. CID sequence analysis of this compound (data not shown) yielded the amino acid sequence PTHIKX, in which X is an unidentified amino acid. This corresponds to the *N*-terminal part of ACEI up to the Trp residue, which apparently has been altered to yield a new amino acid residue with a residue mass of 200 u. It is known that peptides can be hydrolyzed by DMSO–HBr–HAc mixtures at the *C*-terminal amide bond of the Trp residue, during which the Trp residue is converted into Dia lactone (Scheme 1).[17] Apparently, the same hydrolysis also occurs when peptides are treated with DMSO–HCl–HAc mixtures containing 5.5 M HCl, but not 3.75 M HCl. In fact, all peptides used in this study yielded this type of hydrolysis in mixtures of 5.5 M HCl. Additionally, the *C*-terminal Trp of adrenocorticotropic hormone (4–9) was partly altered to Dia lactone. Closer

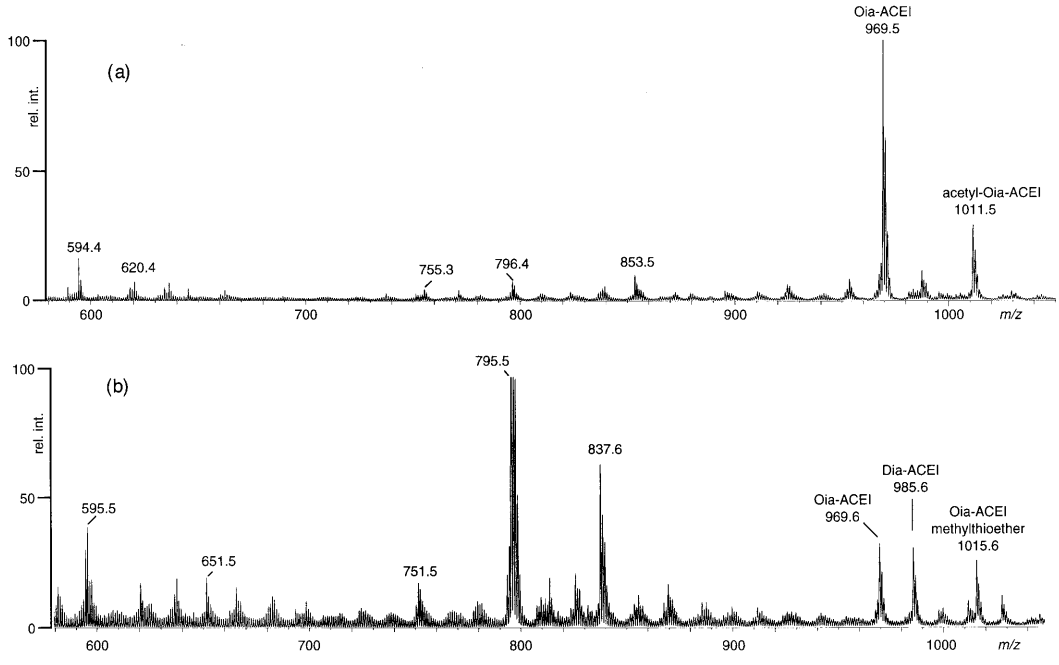


Figure 3. FAB mass spectra of ACEI treated in an open tube for 1 h with DMSO–HCl–HAc mixtures containing (a) 3.75 and (b) 5.5 M HCl.

analysis of Fig. 3(b) also reveals an ion at *m/z* 797.5, the abundance of which is too high to be an isotope peak of *m/z* 795.5. CID sequence analysis of this compound indicated this to be the peptide PTHIKX, in which the unknown amino acid was Oia.

By adjustment of the reaction conditions, *i.e.* HCl concentration of 3.75 M and 1 h reaction time, hydrolysis could be prevented and the Trp residues in all peptides selectively converted into Oia. In Fig. 4 the FAB-CID mass spectrum of protonated Oia-containing luteinizing hormone-releasing hormone ([Oia^3]-LH-RH) is shown. The spectrum of this compound is characteristic for Oia-containing peptides. The presence of Oia is reflected in its immonium ion at *m/z* 175, a mass difference of 202 u between subsequent sequence ions and $[MH]^+ - 145$ u at *m/z* 1053.5 (Fig. 4). The eliminated neutral species of 145 u probably corresponds to oxoquinoline (Scheme 1). In the high-energy CID spectra of peptides, loss of side-chains as radicals from $[MH]^+$ is frequently observed, *e.g.* $[MH]^+ - 130$ u from Trp-containing peptides. Apparently, the side-chain of Oia is not lost as a radical, but rather eliminated as a molecule with concomitant hydrogen transfer to the backbone of the peptide. However, in adrenocorticotropic hormone (4–9), which contains a *C*-terminal Oia, the side-chain was lost as a radical, indicating the influence of the position of Oia in the peptide backbone on its fragmentation behavior.

Another characteristic product ion was only observed in the spectrum of [Oia^3]-LH-RH, at *m/z* 802.4. Owing to the specific amino acid sequence of LH-RH, with an arginine residue near the *C*-terminus, the side-chain specific V and W ions are produced. Generally, the aromatic amino acids yield V ions[28] and, in agreement, a V ion was observed at *m/z* 803.4 in the FAB-CID mass spectrum of LH-RH (data not shown). In [Oia^3]-LH-RH, however, a W ion was observed (*m/z* 802.4). Since the formation of a W ion requires cleavage of the C_β—C_γ-bond,[28] this indicates that C_β—C_γ is no longer attached to a conjugated system, as expected for Oia. Both the elimination of oxoquinoline and the presence and identity of a corresponding W ion may allow distinction between Oia and 5-, 6- or 7-hydroxy-Trp. The last species will probably show loss of the side-chain from $[MH]^+$ as a radical and the formation of a V ion, analogous to Trp.

CONCLUSIONS

In order to investigate the mass spectrometric characteristics of oxidized Trp and oxidized Trp-containing peptides, it was necessary first to prepare these compounds. It was demonstrated that Oia and Oia-containing peptides can be obtained by treatment of Trp and Trp-containing peptides with DMSO–HCl–HAc mixtures. However, the HCl concentration must not exceed 4 M and the reaction tube should not be closed in order to prevent the formation of by-products and hydrolysis of the peptide at the Trp residue.

In general, oxidation of peptides of known sequence can be detected by their different behavior in various analytical procedures compared with the non-oxidized species, such as a mass increase in mass spectrometric analysis or shortened retention times in RP-HPLC analysis. Characterization of the product(s) then requires further analysis, such as by FAB-CID to determine site(s) of modification and the resulting product. However, in FAB-CID analysis of peptides of known and unknown sequence, characteristic product ions may be present, which allow the simultaneous detection and characterization of the oxidation product(s). We have shown here that oxidation of Trp residues to Oia can be recognized by several characteristic product ions in the FAB-CID mass spectra of oxidized species. The most important marker ion results from elimination of oxoquinoline from $[MH]^+$, yielding an abundant fragment

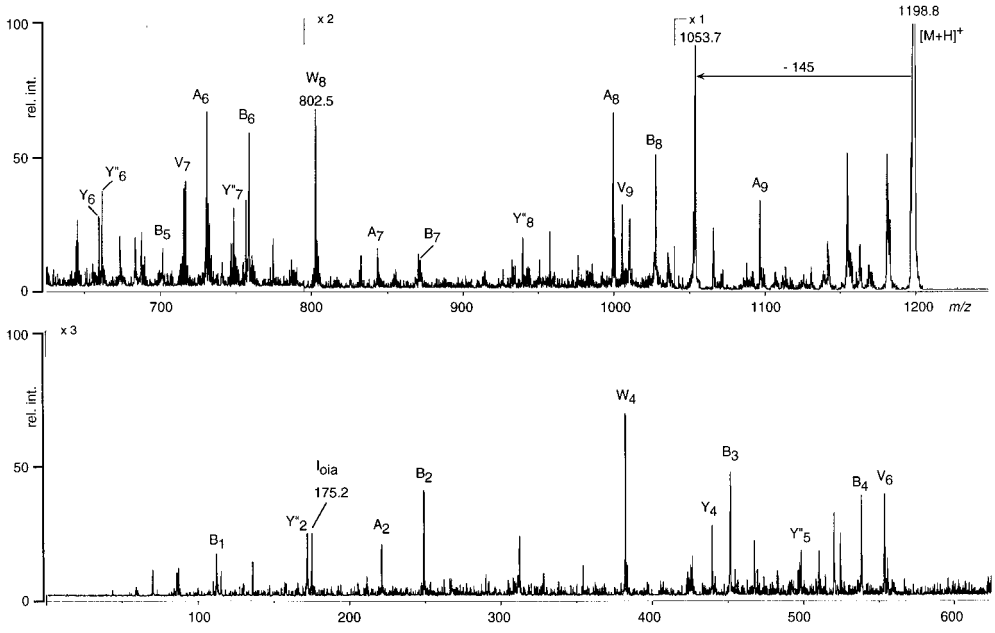


Figure 4. FAB-CID mass spectrum of Oia-containing luteinizing hormone-releasing hormone (pyroE–H–X–S–Y–G–L–R–P–G–NH_2, in which X is Oia). Characteristic mass differences of Oia-containing peptides are indicated.

ion at $[MH]^+ - 145$ u. However, peptides containing Oia at the *C*-terminal position show preferential loss of the complete side-chain as a radical (146 u). Further evidence for the presence of Oia is the Oia immonium ion at m/z 175. A mass difference of 202 u between subsequent sequence ions then indicates the position of the Oia residue in the sequence. Finally, in the FAB-CID spectra of peptides containing arginine at or near the *C*-terminus, a W ion may be observed at the position of Oia.

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