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Synthesis of peptides containing 5-hydroxytryptophan, oxindolylalanine, *N*-formylkynurenine and kynurenine

Toni Todorovski,[a] Maria Fedorova,[a] Lothar Hennig[b] and Ralf Hoffmann[a]*

ROS, continuously produced in cells, can reversibly or irreversibly oxidize proteins, lipids, and DNA. At the protein level, cysteine, methionine, tryptophan, and tyrosine residues are particularly prone to oxidation. Here, we describe the solid phase synthesis of peptides containing four different oxidation products of tryptophan residues that can be formed by oxidation in proteins *in vitro* and *in vivo*: 5-HTP, Oia, Kyn, and NFK. First, we synthesized Oia and NFK by selective oxidation of tryptophan and then protected the α-amino group of both amino acids, and the commercially available 5-HTP, with Fmoc-succinimide. High yields of Fmoc-Kyn were obtained by acid hydrolysis of Fmoc-NFK. All four Fmoc derivatives were successfully incorporated, at high yields, into three different peptide sequences from skeletal muscle actin, creatin kinase (M-type), and β-enolase. The correct structure of all modified peptides was confirmed by tandem mass spectrometry. Interestingly, isobaric peptides containing 5-HTP and Oia were always well separated in an acetonitrile gradient with TFA as the ion-pair reagent on a C_{18}-phase. Such synthetic peptides should prove useful in future studies to distinguish isobaric oxidation products of tryptophan. Copyright © **2011 European Peptide Society and John Wiley & Sons, Ltd.**

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Keywords: ROS; oxidative stress; tryptophan; oxidation; solid phase peptide synthesis

Introduction

ROS can be produced by many different chemical reactions. These reactions can originate from either cellular events, e.g. metal-catalyzed reactions, mitochondrial electron transport reactions, and neutrophil or macrophage activation during inflammation, or external events, e.g. UV light, X-rays, or pollutants. ROS as natural by-products of the oxygen metabolism play important roles in cellular signaling [1,2], but can also modify and often functionally deactivate other biomolecules, such as lipids, DNA, and proteins. For this reason, elevated ROS levels are prevented by effective scavenging systems based on enzymes (e.g. superoxide dismutase, catalase, glutathione peroxidase, thioredoxin, thioredoxin reductase, and peroxiredoxin) or small antioxidants (e.g. ascorbic acid (vitamin C), carotenes, glutathione, lipoic acid, tocopherol (vitamin E), ubiquinol, or uric acid (2,6,8-trioxypurine). The potential damage from ROS depends on several factors, including the following: cell type, absolute level and duration of ROS production, ROS species generated, site of generation, and proximity of the oxidants to a possible target. Usually, proteins as major components in most environments represent the major targets [3–5]. Among the 20 proteinogenic amino acid residues, methionine, cysteine, tyrosine, tryptophan, histidine, and phenylalanine are especially prone to oxidation [6].

In tryptophan residues, the indole ring is easily oxidized by different ROS, forming several well-known tryptophan oxidation products: 5-HTP, Oia, DiOia, NFK, Kyn, OH-Kyn, and OH-NFK [7,8]. As these oxidation products cannot be reduced by the cellular machinery, and often change the protein structure and function, they are usually degraded by enzymes or the proteasome [9,10], but they can also accumulate and aggregate in the cells [11]. These processes may link oxidation processes directly to the pathogenesis of several age-related diseases, such as Alzheimer's disease, Parkinson's disease, atherosclerosis, and diabetes mellitus [12–16]. Kyn derivatives and Oia appear also to play a key role in cataractogenesis [17–19]. Furthermore, 5-HTP, DiOia, Oia, and Kyn are formed in the brains of patients with eosinophilia-myalgia syndrome [20]. More recently, elevated levels of oxidized tryptophan residues in plasma proteins were reported for diabetes [21] and for skeletal muscles in an *in vivo* model of acute oxidative stress [22,23].

Despite the importance and often studied effects of tryptophan oxidation in proteins by ROS, general strategies to specifically synthesize suitably modified peptide models for functional and structural studies, or to improve analytical strategies, have, to the best of our knowledge, not yet been reported. In this study, we

* *Correspondence to: Ralf Hoffmann, Institute of Bioanalytical Chemistry, Biotechnologisch-Biomedizinisches Zentrum, BBZ, Universität Leipzig, Deutscher Platz 5, 04103 Leipzig, Germany.*
E-mail: Hoffmann@chemie.uni-leipzig.de

a *Institute of Bioanalytical Chemistry, Center for Biotechnology and Biomedicine (BBZ), Faculty of Chemistry and Mineralogy, Universität Leipzig, Leipzig, Germany*

b *Institute of Organic Chemistry, Faculty of Chemistry and Mineralogy, Universität Leipzig, Leipzig, Germany*

Abbreviations used: *CID, collision-induced dissociation; DiOia, dioxindolylalanine; Fmoc-OSu, N-(9-fluorenylmethoxycarbonyl)-succinimide; Kyn, kynurenine; MSTFA, N-methyl-N-(trimethylsilyl)trifluoroacetamide; NFK, N-formylkynurenine; 5-HTP, 5-hydroxytryptophan; OH-Kyn, hydroxykynurenine; OH-NFK, hydroxy-N-formylkynurenine; Oia, oxindolylalanine; PSD, post-source decay; ROS, reactive oxygen species; RP-HPLC, reversed phase high-performance liquid chromatography; SPE, solid phase extraction.*

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report the synthesis of Fmoc-protected 5-HTP, Oia, Kyn and NFK derivatives. These building blocks were successfully incorporated into three peptide sequences, recently shown to be oxidized *in vivo* [22,23], with standard procedures on solid phase. The correct structures were confirmed for all peptides by tandem mass spectrometry. Interestingly, all isobaric peptides containing 5-HTP and Oia were well separated by RP-HPLC.

Materials and Methods

Reagents

DMSO (99.9%, water free), acetic anhydride (>99%), formic acid (98–100%), TFA (spectrophotometric grade, >99%), and sodium hydroxide (NaOH, >99%) were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany), while potassium chloride (laboratory reagent grade, >99% purity) was obtained from Fisher Scientific GmbH (Schwerte, Germany). Hydrochloric acid (37%), acetic acid (100%), and methanol (>99.9%) were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Citric acid (analytical grade) was purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany). Tryptophan (>99%) and 5-HTP (>99%) were obtained from Fluka (Sigma-Aldrich Chemie GmbH). DMF (peptide synthesis grade), DCM (99.9%), and acetonitrile (HPLC-S gradient grade) were obtained from Biosolve (Valkenswaard, Netherlands). Standard Fmoc-amino acid derivatives were purchased either from MultiSynTech GmbH (Witten, Germany) or from ORPEGEN Pharma (Heidelberg, Germany), containing *tert*-butyl-based protecting groups at serine, threonine, tyrosine aspartic acid, and glutamic acid; the triphenylmethyl protecting group at asparagine, glutamine, and histidine; and the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) protecting group for arginine. Water was purified in-house with a PURELAB ultra analytic system (ELGA, Berkefeld GmbH, Ransbach, Germany).

Synthesis of Oia

The synthesis of Oia relied on a published strategy of Trp oxidation by DMSO (yield 65%) [24]. The crude product was dissolved in 3% (v/v) aqueous MeCN containing 0.1% (v/v) TFA at a concentration of 20 g/l. Aliquots of 800 mg were loaded on a Strata C18-E cartridge (internal diameter 27 mm, length 135 mm, particle size 50 µm, pore size 6.5 nm, Phenomenex Inc., Aschaffenburg, Germany). The column was washed with the same eluent composition (80 ml) before Oia was eluted [50 ml, 12% (v/v) aqueous MeCN containing 0.1% (v/v) TFA].

Synthesis of Kyn and NFK

Kyn was synthesized by oxidation of Oia [20]. Oia (1 g, 4.54 mmol) was dissolved in NaOH solution (0.15 mol/l, 100 ml) containing KCl (0.05 mol/l, pH 12.75). While air was continuously bubbled through the solution, the reaction was monitored by RP-HPLC. The highest yield (>90%) of Kyn was obtained after 2 h, and was contaminated by only a small amount (≈10%) of DiOia. For longer reaction times the content of DiOia did not increase further, but several, as yet uncharacterized, by-products appeared. The reaction mixture was neutralized by addition of hydrochloric acid (1 mol/l) and the solvent was removed on a rotary evaporator. The solid product was purified by SPE using the same conditions described above for Oia. The overall yield was 55%. NFK was synthesized from purified Kyn by selective formylation (yield 60%) and used without further purification [20].

Table 1. Reaction conditions used to couple the Fmoc-group to 5-HTP, Oia, and NFK and the yields obtained for the purified compounds

Compound	MSTFA (eq.)	Reaction time of silylation (min)	Reaction time for Fmoc coupling (h)	Yield (%)
Fmoc-5-HTP	3	30	24	40
Fmoc-Oia	3	30	48	35
Fmoc-NFK	3	210	26	30

Fmoc-Protection of Tryptophan Derivatives

5-HTP, Oia, Kyn, and NFK were protected at the α-amino group with Fmoc by a standard procedure [25]. Briefly, each amino acid derivative (1 g) was suspended in methylene chloride (10 ml) and treated with three equivalents of MSTFA at room temperature (RT). The mixture was refluxed (50 °C) until a clear solution was obtained. The solution was then cooled to RT, one equivalent of Fmoc-OSu was added, and the resulting mixture was stirred at RT until the reaction was complete (Table 1), as monitored by TLC. After completion of the reaction, the carboxyl group was deprotected with methanol (2.25 ml, RT, 0.5 h) and the solvent evaporated on a rotary evaporator. Water (30 ml) was added to the crude product, stirred for 30 min, and filtered. The residue was then washed three times with a mixture of aqueous citric acid (10%) and methanol (1 : 1 by volume, 5 ml) and water until the filtrate was neutral. The combined filtrates were dried under vacuum (40 °C) and then lyophilized [25]. The obtained Fmoc-amino acids were finally purified by RP-HPLC on a C_{18}-column (internal diameter 21.2 mm, length 250 mm, particle size 10 µm, pore size 30 nm, Phenomenex) using a linear gradient from 40.5 to 42.3% aqueous acetonitrile (0.1% TFA) over 1 min, followed by a linear gradient from 42.3 to 69.3% over 30 min. The flow rate was 10 ml/min. The correct syntheses were confirmed by NMR on a Varian Mercury plus (Darmstadt, Germany) (Supporting information, Table S1). The monoisotopic masses were determined by MALDI-TOF-MS in positive-ion mode using a 4700 proteomic analyzer (MALDI-TOF/TOF-MS, Applied Biosystems GmbH, Darmstadt, Germany). The matrix was α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics GmbH, Bremen, Germany). The recorded monoisotopic masses for Fmoc-5-HTP (yield 40%), Fmoc-Oia (yield 35%), and Fmoc-NFK (yield 30%) were *m/z* 443.18 (theoretical 443.16), 443.16 (theoretical 443.16), and 459.12 (theoretical 459.15), respectively.

Fmoc-Kyn was released from Fmoc-NFK (20 mg) by hydrolysis (10% aqueous TFA, 10 ml, 8 h, RT) using the conditions described for the free amino acid [26]. On the basis of the peak areas obtained on RP-HPLC, more than 90% of Fmoc-NFK was converted to Fmoc-Kyn. TFA was removed (rotary evaporator, 50 °C), and the solid product was then washed several times with water and dried under vacuum. The obtained product (yield 85%) was characterized by NMR (Supporting information, Table S1) and MALDI-MS (*m/z* 431.15, theoretical 431.16).

Solid Phase Peptide Synthesis

Peptides were synthesized by Fmoc/^{t}Bu-chemistry on a Syro2000 multiple peptide synthesizer (MultiSynTech GmbH) using eight equivalents of the amino acid derivatives activated with 1,3-DIC in the presence of DIC/HOBt in DMF [27] at the 26-µmol scale. The modified tryptophan derivative was incorporated manually by activating the corresponding Fmoc derivative (5 eq.) with HATU

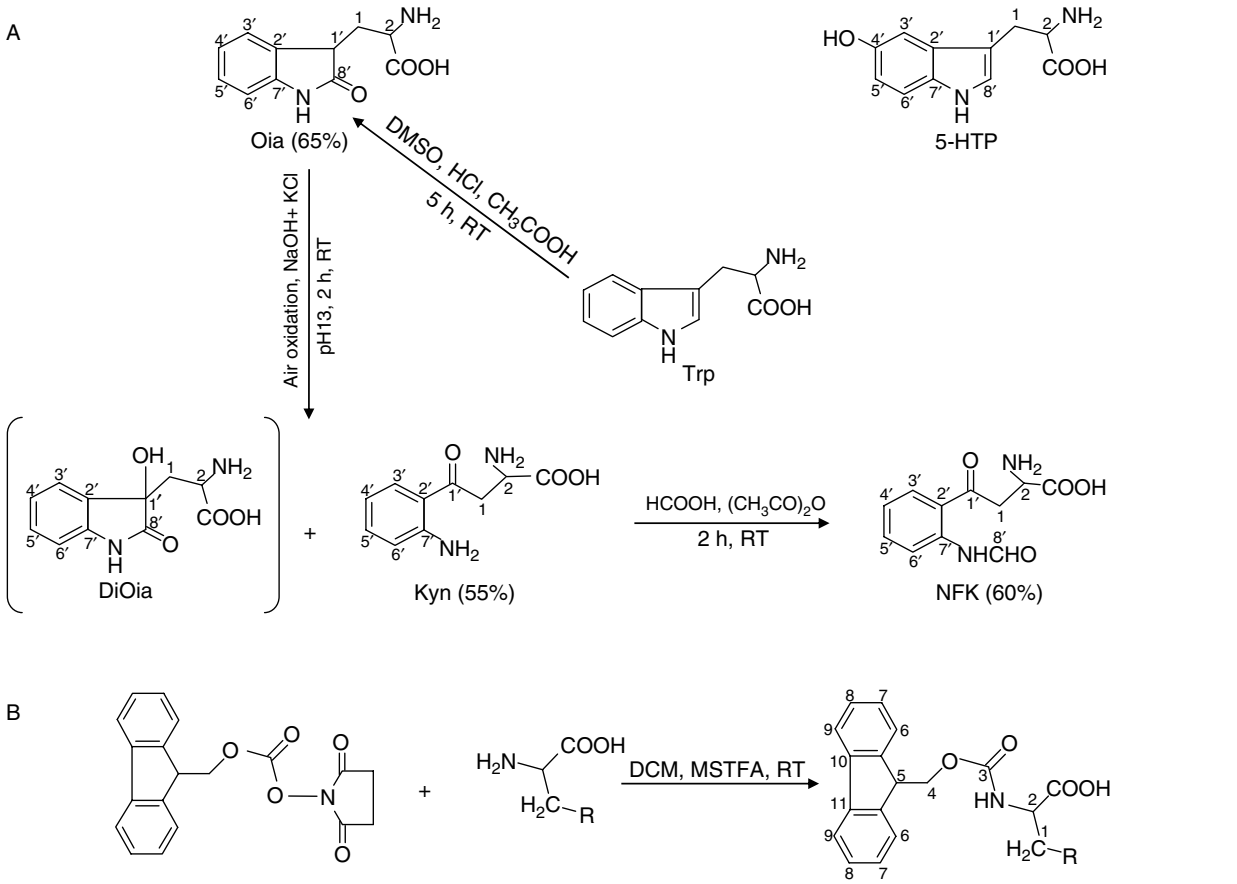

Figure 1. Reaction schemes showing the synthetic strategies used to obtain the different oxidation products of tryptophan (A) and the corresponding Fmoc-protected derivatives (B). Tryptophan was oxidized to Oia with DMSO in the presence of hydrochloric acid and glacial acetic acid [24] and then further to Kyn, which was then formylated to obtain NFK. The numbering of the C-atoms in each structure indicates the assigned signals of the NMR spectra, as presented in Materials and Methods. R indicates the side chain of Trp and its analogs, e.g. indole for Trp and oxindole for Oia.

(4.9 eq.) in the presence of DIPEA (10 eq.) [28]. The efficiency of the manual coupling was verified by a Kaiser test [29] and RP-HPLC.

The resin-bound peptides were cleaved and deprotected with TFA containing a scavenger mixture (12.5%, v/v) of water, thioanisol, *m*-cresol, and ethandithiol (2 : 2 : 2 : 1 by vol.) at RT for 2 h. The peptides were then precipitated in cold diethyl ether and centrifuged (1620 *g*). The pellet was washed twice with cold ether, dried under air, dissolved in 3% aqueous acetonitrile containing 0.1% TFA, and stored at −20 °C.

Peptide Purification

Crude peptides were initially analyzed on a Jupiter C_{18}-column (internal diameter 2 mm, length 150 mm, particle size 5 µm, pore size 30 nm, Phenomenex) using a linear gradient from 3 to 57% aqueous acetonitrile (0.1% TFA) for 30 min at a flow rate of 0.2 ml/min using an Äkta HPLC System (GE Healthcare GmbH, Munich, Germany). All peptides were purified on a Jupiter C_{18}-column (internal diameter 10 mm, length 250 mm, particle size 5 µm, pore size 30 nm, Phenomenex) by a linear gradient starting at 3% aqueous acetonitrile (slope of 1% acetonitrile per minute) and containing 0.1% TFA as the ion-pair reagent at a flow rate of 5 ml/min. The lyophilized fractions were characterized on the microbore Jupiter C_{18}-column using a linear gradient from 3 to 27% aqueous acetonitrile (0.1% TFA) over 10 min, followed by a gradient of 27–48% over the next 35 min with a flow rate of 0.2 ml/min. The monoisotopic masses of all peptides were recorded in positive ion reflector TOF (re-TOF) mode with the matrix α-cyano-4-hydroxy-cinnamic acid, as described above. In addition, the peptide sequences, including the modified positions, were confirmed in TOF/TOF mode in the absence (PSD mode) or presence of air in the collision cell (CID mode).

Results and Discussion

Synthesis of Fmoc-5-HTP, Fmoc-Kyn, Fmoc-NFK, and Fmoc-Oia

Oia and NFK were obtained in reasonable yields by oxidation of tryptophan and in high purities after SPE. The NMR data confirmed the expected structures, including the L-configuration of the chiral center at the α-C-atom (Figure 1). The Fmoc-group was incorporated into both compounds at high levels of around 70%. Following purification with RP-HPLC, the overall yields obtained were between 30 and 40% (Table 1) and purity levels were above 95%. Fmoc-Kyn-OH was obtained by acid hydrolysis from Fmoc-NFK incorporated without side chain protection.

Peptide Synthesis

On the basis of our previous work on the analysis of tryptophan oxidation of skeletal muscle proteins in a rat model for oxidative

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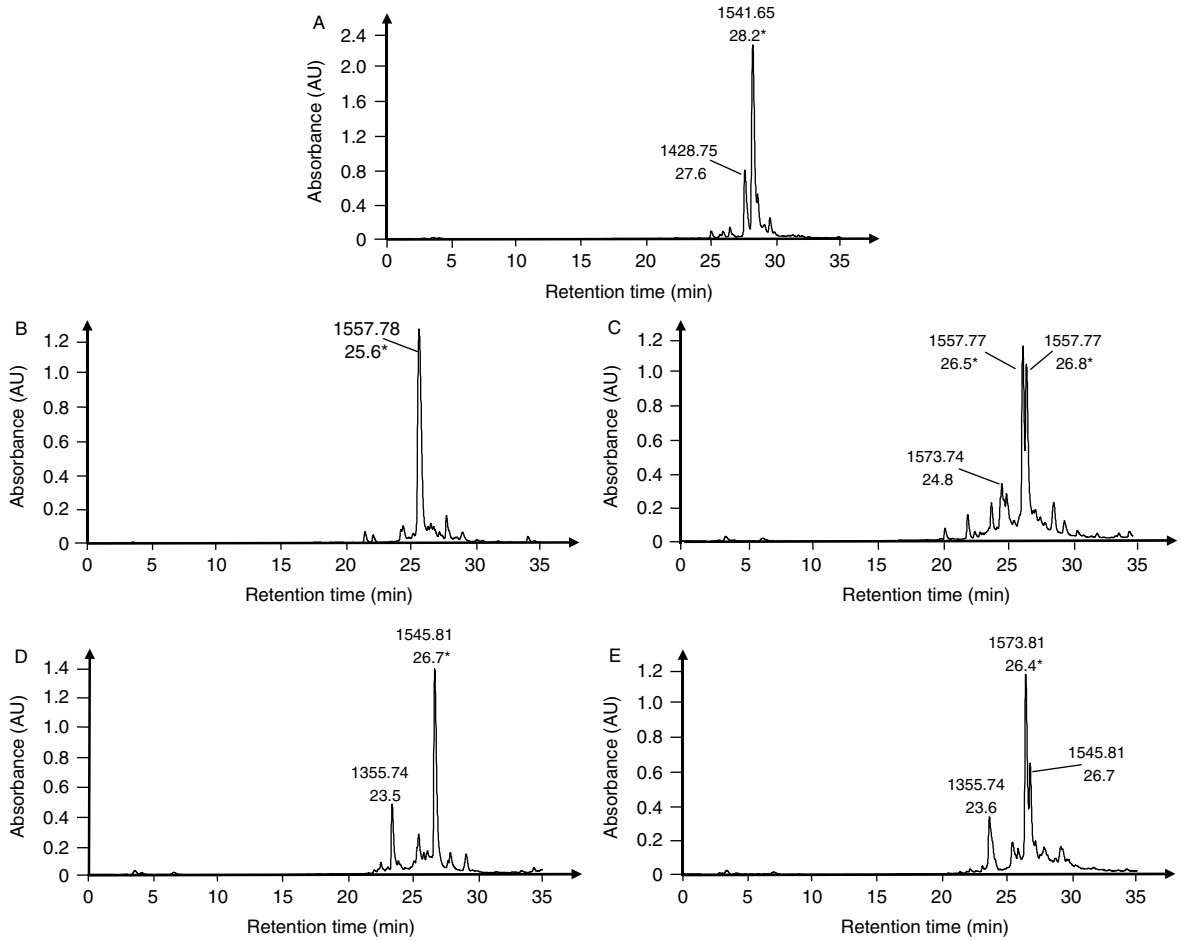

Figure 2. Reversed-phase chromatogram of the crude H-LAQSNGXGVMVSHR-OH peptides containing Trp (A), 5-HTP (B), Oia (C), Kyn (D), and NFK (E) in position seven of the sequence. Peaks marked with an asterisk contain the correct sequence. The peptides were analyzed on the microbore Jupiter C_{18}-column. Numbers on top of the peaks indicate the retention time (lower) and *m/z* values (upper) recorded by MALDI-MS.

stress, we selected three sequences containing a single tryptophan residue prone to oxidation [23]. Peptides IWHHTFYNELR, SFLVWVNEEDHLR, and LAQSNGWGVMVSHR, which correspond to skeletal muscle actin (residues 87–97), creatin kinase M-type (residues 224–236), and β-enolase (residues 359–372), respectively, were all identified, along with the oxidized Trp-residues, in a tryptic digest of the rat skeletal protein extract using tandem mass spectrometry. These 11- to 14-residue-long peptides should also provide a good separation of potential by-products by RP-HPLC, and an easier characterization of the targeted structure and potential by-products by MALDI-MS. All modified peptides containing 5-HTP, NFK, Kyn, or Oia were obtained in high yields and with high purities, without any indication of reduced coupling or Fmoc-cleavage efficiencies at the Trp-positions. The crude peptides typically contained the desired product at the following levels: 35% (NFK), 50% (Kyn), 55% (Oia), and 65% (5-HTP), as judged from the RP-chromatograms (Figure 2 and Table 2), independent of the Trp derivative. Thus, all four oxidation products were successfully incorporated by standard protocols, which should also allow the synthesis of longer peptides. Importantly, all derivatives appeared to be stable during Fmoc deprotection with piperidine and the final cleavage with TFA. Only the formamide of NFK was partially hydrolyzed during TFA cleavage. At prolonged cleavage times (200 min), the hydrolysis rate was approximately 15%, as judged by RP-HPLC. The resulting Kyn-containing peptides eluted 0.2–1.0 min later (0.1–0.6% acetonitrile), which allowed reliable purification. Shorter cleavage times or lower temperatures should further reduce this side reaction. At first view, it appears surprising that the NFK-containing peptide, with its formylated amino group, elutes earlier than the corresponding Kyn-peptide, with the free amino group. The relatively strong retention of the Kyn-containing peptides is probably attributable to the formation of ion-pair adducts between the TFA anion and the positively charged amino group of the Kyn-residues.

Besides the targeted peptides, the RP-chromatograms contained only minor by-products with deleted or partially protected sequences. This indicated that neither of the Trp derivatives was irreversibly modified by reactive intermediates formed during the TFA cleavage and that the regular scavenger mixtures can effectively protect them. Moreover, the next *N*-terminal amino acid derivatives were efficiently coupled. Importantly, the aromatic amino group in the side chain of Kyn was not acylated during the solid phase synthesis within the detection limits, indicating that it is not necessary to protect it for HATU- and DIC-activation. For longer peptides containing Kyn at the *C*-terminal end, it might still be useful to incorporate fully protected Kyn derivatives. The same might also be true for syntheses performed at elevated temperatures, such as those that are microwave assisted. In conclusion, all peptides were easily purified by RP-HPLC and displayed the correct

Table 2. Peptide sequences used in this study, their monoisotopic masses (MALDI-TOF-MS), retention times (RP-HPLC), and corresponding yields of purified peptides

Peptide sequence	Theoritical *m/z*	Experimental *m/z*	Retention time (min)	Yield (%)
SFLVWVNEEDHLR	1643.81	1643.76	28.5	49
SFLV[5-HTP]VNEEDHLR	1659.81	1659.84	26.4	30
SFLV[Oia]VNEEDHLR	1659.81	1659.82	27.1/27.7	29
SFLV[NFK]VNEEDHLR	1675.81	1675.82	27.0	16
SFLV[Kyn]VNEEDHLR	1647.81	1647.84	28.0	26
LAQSNGWGVMVSHR	1541.76	1541.65	23.2	52
LAQSNG[5-HTP]GVMVSHR	1557.76	1557.78	21.1	38
LAQSNG[Oia]GVMVSHR	1557.76	1557.77	21.7/21.9	32
LAQSNG[NFK]GVMVSHR	1573.76	1573.81	21.8	20
LAQSNG[Kyn]GVMVSHR	1545.76	1545.81	22.1	30
IWHHTFYNELR	1515.74	1515.70	24.0	53
I[5-HTP]HHTFYNELR	1531.74	1531.78	22.9	39
I[Oia]HHTFYNELR	1531.74	1531.78	23.3/23.4	38
I[NFK]HHTFYNELR	1547.74	1547.78	23.6	16
I[Kyn]HHTFYNELR	1519.74	1519.59	23.8	31

All Oia-containing peptides yielded two signals in the chromatograms with identical masses, which are most likely caused by tautomerization of the Oia-residue.

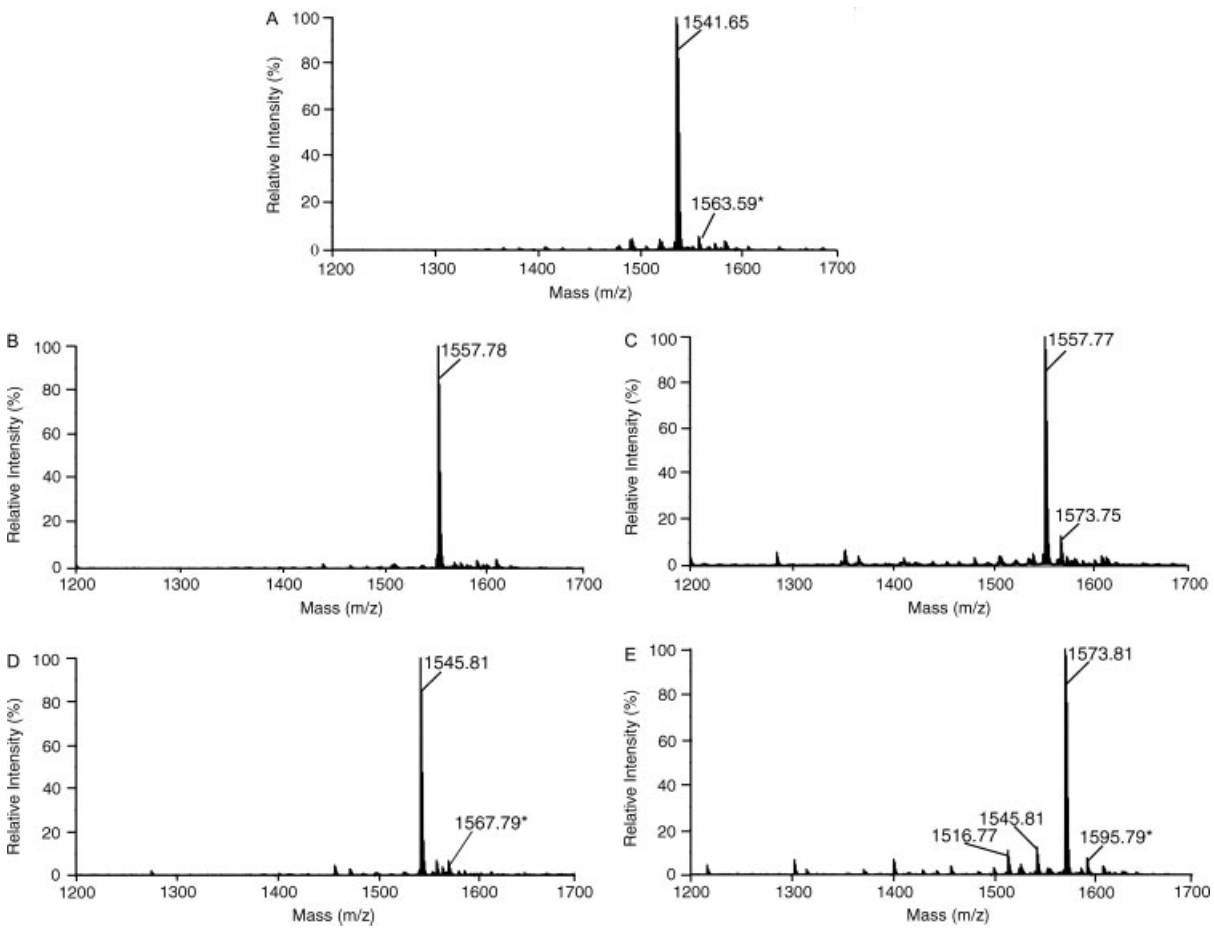

Figure 3. MALDI reflector TOF mass spectra of the purified H-LAQSNGXGVMVSHR-OH peptides containing Trp (A) 5-HTP (B), Oia (C), Kyn (D), and NFK (E) in position seven of the sequence. The main signals at *m/z* 1541.65, 1557.78, 1557.77, 1573.81, and 1545.81 correspond to the monoisotopic signals of the expected $[M+H]^+$ ions. Sodiated ions are labeled with an asterisk. The mass spectra were recorded in positive-ion mode with the matrix, α-cyano-4-hydroxy-cinnamic acid.

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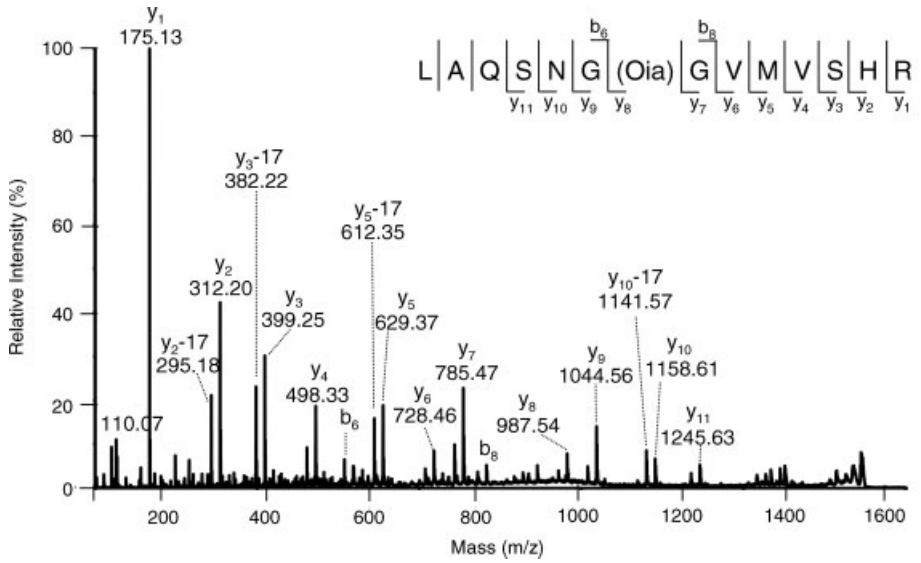

Figure 4. MALDI-PSD mass spectrum of the purified H-LAQSNG-Oia-GVMVSHR-OH peptide. The mass spectrum was recorded in positive-ion mode with the matrix, α-cyano-4-hydroxy-cinnamic acid.

masses in the MALDI mass spectra, as shown for the β-enolase sequence (Figure 3).

All Oia-containing peptides eluted in two peaks with similar retention times (Figure 2 and Table 2). Both fractions always showed the correct peptide mass in MALDI-MS and identical fragmentation patterns in MALDI-PSD and CID mode (Figure 4). Reinjection of either fraction again yielded the same two peaks with identical retention times and area ratios (data not shown). Most likely, these peaks represented the lactam–lactim tautomers of the Oia residue [20,30], although a sterically hindered *cis–trans* configuration of the peptide backbone cannot be ruled out.

Owing to the isomeric nature of Oia and 5-HTP, it is not possible to distinguish the corresponding isobaric peptides by MS, not even after fragmentation, as both derivatives can form the same fragment ions with respect to the *m/z* values. Thus, it is necessary to separate them first, e.g. by liquid chromatography (LC), such as RPC, which can be favorably coupled on-line to a mass spectrometer. For the three sequences studied here, the isobaric compounds were separated with the 5-HTP-containing peptides always eluting about 0.5–1 min (0.3–0.6% acetonitrile) earlier (Table 2 and Figure 2).

Conclusions

The oxidized tryptophan derivatives were incorporated in different peptide sequences by the Fmoc/^{t}Bu-strategy using either HATU or DIC activation in high yields without major side reactions. Only the formyl group of NFK was slowly released during the final TFA-cleavage step, but the Kyn-containing peptides obtained were separated by RP-HPLC and therefore did not contaminate the purified peptides. With this study, we have identified widely applicable strategies for the synthesis of 5-HTP, Kyn, NFK, and Oia peptides that will be useful for the detailed study of their fragmentation behavior in tandem mass spectrometry, and which could even enable the distinction of isobaric derivatives like 5-HTP and Oia by characteristic fragmentation patterns. Moreover, such peptides could serve as standards to confirm suggested oxidative modifications by their retention times and fragmentation pattern in LC-MS techniques. Finally, isotope-labeled synthetic peptides (e.g. ^{13}C or ^{15}N) will enable the quantification of all four oxidation products relative to the Trp content. Thus, the oxidation levels of Trp-containing proteins can be quantified for *in vivo* studies.

Supporting information

Supporting information may be found in the online version of this article.

References

1 D'Autreaux B, Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat. Rev. Mol. Cell Biol.* 2007; **8**: 813–824.
2 Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2000; **279**: L1005–L1028.
3 Davies MJ. Singlet oxygen-mediated damage to proteins and its consequences. *Biochem. Biophys. Res. Commun.* 2003; **305**: 761–770.
4 Davies KJA, Delsignore ME, Lyn SW. Protein damage and degradation by oxygen radicals. II. Modification of amino acids. *J. Biol. Chem.* 1987; **262**: 9902–9907.
5 Stadtman ER, Levine RL. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* 2003; **25**: 207–218.
6 Berlett BS, Stadtman ER. Protein oxidation in aging, disease and oxidative stress. *J. Biol. Chem.* 1997; **272**: 20313–20316.
7 Simat TJ, Steinhart H. Oxidation of free tryptophan and tryptophan residues in peptides and proteins. *J. Agric. Food Chem.* 1998; **46**: 490–498.
8 Garrison WM. Reaction mechanism in the radiolysis of peptides, polypeptides and proteins. *Chem. Rev.* 1987; **87**: 381–398.
9 Chang TC, Chou WY, Chang GG. Protein oxidation and turnover. *J. Biomed. Sci.* 2000; **7**: 357–363.
10 Louie JL, Kapphahn RJ, Ferrington DA. Proteasome function and protein oxidation in the aged retina. *Exp. Eye. Res.* 2002; **75**: 271–284.
11 Squier TC. Oxidative stress and protein aggregation during biological aging. *Exp. Gerontol. Mol. Aging* 2001; **36**: 1539–1550.
12 Butterfield DA. Oxidative stress in neurodegenerative disorders. *Antioxid. Redox. Signal.* 2006; **8**: 1971–1973.
13 Beckman KB, Ames BN. The free radical theory of aging matures. *Physiol. Rev.* 1998; **78**: 547–581.
14 Hsiai T, Berliner JA. Oxidative stress as a regulator of murine atherosclerosis. *Curr. Drug Targets* 2007; **8**: 1222–1229.

15 Hamblin M, Friedman DB, Hill S, Caprioli RM, Smith HM, Hill MF. Alterations in the diabetic myocardial proteome coupled with increased myocardial oxidative stress underlies diabetic cardiomyopathy. *J. Mol. Cell Cardiol.* 2007; **42**: 884–895.
16 Norris EH, Giasson BI. Role of oxidative damage in protein aggregation associated with Parkinson's disease and related disorders. *Antioxid. Redox Signal.* 2005; **7**: 672–684.
17 Rousseva LA, Gaillard ER, Paik DC, Merriam JC, Ryzhov V, Garland DL, Dillon JP. Oxindolealanine in age-related human cataracts. *Exp. Eye Res.* 2007; **85**: 861–868.
18 Vazquez S, Aquillina JA, Jamie JF, Sheil MM, Truscott RJW. Novel protein modification by kynurenine in human lenses. *J. Biol. Chem.* 2002; **277**: 4867–4873.
19 Snytnikova OA, Kopylova LV, Chernyak EI, Morozov SV, Kolosova NG, Tsentalovich YP. Tryptophan and kynurenine levels in lenses of Wistar and accelerated senescence OXYS rats. *Mol. Vis.* 2009; **15**: 2780–2788.
20 Simat T, Meyer K, Steinhart H. Synthesis and analysis of oxidation and carbonyl condensation compounds of tryptophan. *J. Chromatogr., A* 1994; **661**: 93–99.
21 Jain SK. Can tryptophan oxidation lead to lower tryptophan level in diabetes? A commentary on ''Propagation of protein glycation damage involves modification of tryptophan residues via reactive oxygen species: Inhibition by pyridoxamine.'' *Free Radical Biol. Med.* 2008; **44**: 1273–1275.
22 Fedorova M, Kuleva N, Hoffmann R. Identification of cysteine, methionine and tryptophan residues of actin oxidized in vivo during oxidative stress. *J. Proteome Res.* 2010; **9**: 1598–1609.
23 Fedorova M, Todorovski T, Kuleva N, Hoffmann R. Quantitative evaluation of tryptophan oxidation in actin and troponin I from skeletal muscles using a rat model of acute oxidative stress. *Proteomics* 2010; **10**: 2692–2700.
24 Labroo RB, Cohen LA. Preparative separation of the diastereoisomers of dioxindolyl-L-alanine and assignment of stereochemistry at C-3. *J. Org. Chem.* 1990; **55**: 4901–4904.
25 Raillard SP, Mann AD, Baer TA. An efficient procedure for the preparation of Fmoc-amino acids. *Org. Prep. Proced. Int.* 1998; **30**: 183–186.
26 Savige WE. New oxidation products of tryptophan. *Aust. J. Chem.* 1975; **28**: 2275–2287.
27 Frolov A, Singer D, Hoffmann R. Solid-phase synthesis of glucose-derived Amadori peptides. *J. Pept. Sci.* 2007; **13**: 862–867.
28 Chan WC, White PD. *Fmoc Solid Phase Peptide Synthesis*. Oxford University press: New York, 2003.
29 Kaiser E, Colescott RL, Bossinger CD, Cook PI. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* 1970; **34**: 595–598.
30 Van de Weert M, Lagerwerf FM, Haverkamp J, Heerma W. Mass spectrometric analysis of oxidized tryptophan. *J. Mass Spectrom.* 1998; **33**: 884–891.

