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Uranyl Photocleavage of Phosphopeptides Yields Truncated C-Terminally Amidated Peptide Products

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The uranyl ion (UO_2^{2+}) binds phosphopeptides with high affinity, and when irradiated with UV-light, it can cleave the peptide backbone. In this study, high-accuracy tandem mass spectrometry and enzymatic assays were used to characterise the photocleavage products resulting from the uranyl photocleavage reaction of a tetraphosphorylated β-casein model peptide. We show that the primary photocleavage products of the uranyl-catalysed reaction are C-terminally amidated. This could be of great interest to the pharmaceutical industry, as efficient peptide amidation reactions are one of the top challenges in green pharmaceutical chemistry.

Introduction

Every third protein is associated with metal ions, and in the case of enzymes the metal ions are often found in the active site where they serve an essential role for the enzymatically catalysed chemical reaction.[1] The ability of metalloproteins to coordinate specific metal ions has been exploited to engineer a super uranyl-binding protein that binds uranyl (UO_2^{2+}) with extremely high affinity and selectivity.[2] This protein enables the possibility to sequester uranyl directly from, for example, sea water, where the uranyl concentration is 3 mg ton^{-1}, thus representing a possible source of uranium.[3] Uranyl is the most stable and common ionic form of uranium in the aerobic environment. Several recent studies have characterised the interactions between uranyl and targeted proteins in vivo[4] and peptide ligands.[5] The preferred uranyl-binding sites in proteins and peptides consists predominantly of hard oxygen donors such as carboxylate, phenolate and phosphate groups.[6]

Uranyl has excellent photocatalytic properties, as its excited state is strongly oxidising.[7] Uranyl photocleavage has been used for nearly three decades to investigate the conformation of DNA and RNA structures and their interactions.[8] More recently, uranyl has been used to photocleave the polypeptide backbone in a number of uranyl-binding proteins.[9] Interestingly, the photocleavage reaction is highly efficient, particularly for phosphorylated uranyl-binding proteins, with backbone cleavage occurring in the vicinity of the phosphorylated residues.[9b] This unique property has been exploited to use uranyl as a two-pronged tool in a one-pot procedure, for purification of C-terminally tagged recombinant proteins and photolytic tag removal.[10] In this approach, the targeted protein is expressed with a C-terminal tag that is readily phosphorylated by a kinase. By virtue of the high affinity of uranyl towards the phosphorylated tag, the tagged protein is efficiently enriched by immobilised uranyl. Subsequently, the tag is removed through uranyl-induced photocleavage by irradiation with UV light. Although uranyl has been demonstrated to function as an artificial photochemical "protease", the peptide products resulting from uranyl photocleavage of proteins and peptides (as well as the mechanism) have not been elucidated. It has been suggested that uranyl photocleavage of a tetraphosphorylated peptide obtained from β-casein yields peptide products with a free C terminus (i.e. -COOH, similar to cleavage by proteolytic enzymes).[9b] However, in this study we show that the peptide products resulting from uranyl photocleavage of the β-casein peptide do not have free C termini; instead the photocleavage products are C-terminally amidated (i.e. -CONH₂). We arrive at this conclusion after high-accuracy MS and MS/MS measurements as well as enzymatic assays.

Our findings are potentially very interesting from a pharmaceutical perspective, as more than 50 % of all biologically active peptides in humans are amidated at the C terminus, and this amidation is often directly linked to bioactivity.[11] Consequently, there is great interest in the pharmaceutical industry for cost-effective ways of generating C-terminally amidated peptides. Accordingly, in 2007 amide formation was voted one of the top challenges in green pharmaceutical chemistry by the American Chemical Society.[12] Currently, most amidated peptides are made by either standard chemical synthesis or enzyme-catalysed reactions. The benefit of the enzymatic approach is that it avoids the relatively harsh chemicals, solvents

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and waste associated with traditional chemical synthesis.[12] However, the enzymatic approach is limited by the substrate specificity of the amidating enzyme, primarily peptidyl-glycine α-amidating monoxygenase (PAM), which catalyses amidation of peptides containing a C-terminal glycine.[13] In fact, amidation of a series of pharmaceutically important peptides is highly inefficient if not impossible by PAM.[13] Hence, increased knowledge of uranyl-catalysed photocleavage might pave a new way to efficiently generate C-terminally amidated peptides.

Results and Discussion

Uranyl-induced photocleavage occurs with surprisingly high efficiency for a number of phosphorylated uranyl-binding proteins.[9b] For example, uranyl-bound β-casein is quantitatively cleaved into a few specific peptides upon UV irradiation. Similarly, uranyl efficiently photocleaves the tetraphosphorylated tryptic peptide derived from β-casein (residues 1–25: RELE-ELNVPGEIVE(pS)L(pS)(pS)(pS)EESITR).[9b] Here we show that the main products formed by uranyl photocleavage are C-terminally amidated peptides.

Briefly, β-casein peptide solution was mixed with a fivefold molar excess of uranyl, as this ratio has previously been shown to be optimal for the photocleavage reaction,[9b] and incubated for 1 h at room temperature. The coordination of uranyl to the β-casein peptide was confirmed by MS (Figure S1 in the Supporting Information). The samples were placed on ice and irradiated with UV light ($\lambda = 365$ nm) for 15 min in solution. The peptides were purified and desalted on reversed-phase micro columns (this step also removes uranyl) followed by MS or MS/MS analysis. Figure 1A shows the control MALDI TOF mass spectrum of the intact β-casein peptide before UV irradiation. The peak at m/z 3122.2 corresponds to the singly protonated β-casein peptide (theoretical mass $[M+H]^+$ 3122.2 Da), with four peaks arising from sequential neutral losses of 98 Da (H_3PO_4) from the phosphorylated serine residues. This neutral loss is a well-known gas phase reaction that occurs in the MALDI process.[14] The peak at m/z 1672.9 ("Int. Std.") corresponds to singly protonated neurotensin, which was added as an internal mass calibrant.

Figure 1B shows the mass spectrum of the peptide products formed by UV irradiation of uranyl-bound β-casein peptide. Uranyl-induced photocleavage gives rise to three products: m/z 1097.6 (peptide (1–9)), 1624.8 (1–14) and 1904.9, (1–16). Two of the three cleavage sites are close to the phosphorylation motifs at amino acid residues 15 and 17, as expected. The last cleavage product might be a result of intramolecular folding of the peptide in solution, but this is speculative as the specificity of this photocleavage reaction is not fully understood. Interestingly, the observed masses do not correspond to the theoretical masses of products formed by peptide bond hydrolysis. The expected fragments generated by hydrolysis would be m/z 1098.6, 1625.8 and 1905.9. These theoretical masses are 1 Da higher, thus suggesting that the photocleavage reaction does not results in peptide products with a free C-terminal -COOH. Rather, the observed masses strongly suggest that the pep-
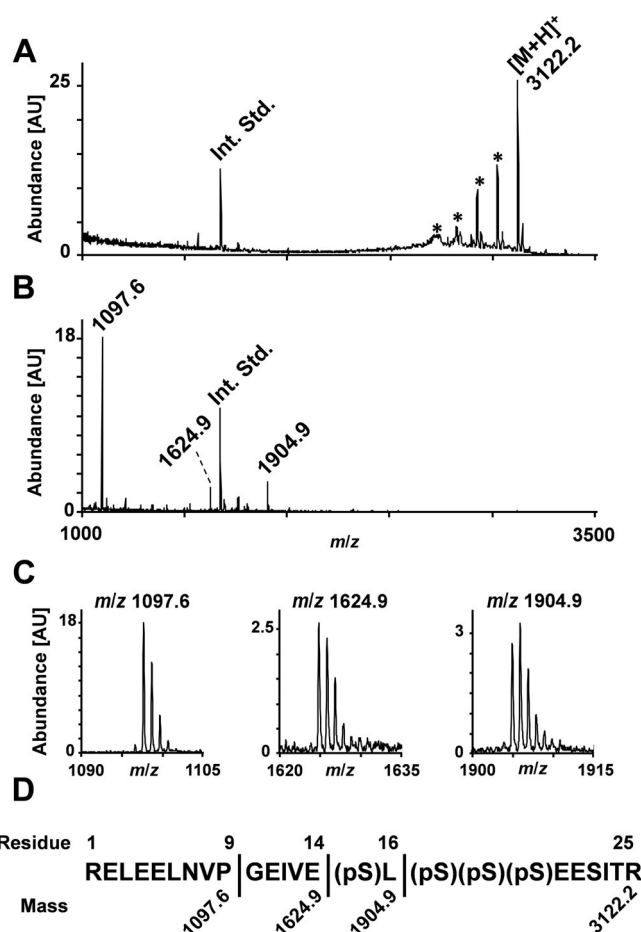

Figure 1. Mass spectra of the tetraphosphorylated β-casein peptide in the presence of uranyl, A) without UV and B) after UV irradiation. Asterisked peaks are MALDI-induced neutral losses of H_3PO_4 from the intact peptide. C) Detail of mass spectra displaying the isotope distributions of the three major photocleavage products. D) Sequence of the tetraphosphorylated β-casein peptide. The observed cleavages are indicated with solid lines, with residue number (above) and mass (below). Int. Std. = internal standard (neurotensin).

tides formed by uranyl photocleavage are C-terminally amidated (-CONH$_2$). In order to obtain their masses with high accuracy, they were analysed with an ESI hybrid ion trap Orbitrap mass spectrometer (Table 1). The masses of the truncated pep-

Table 1. Comparison between theoretical and observed masses of the photocleavage products. Theoretical masses were calculated in GPMAW (www.gpmaw.com).

Fragment	Theoretical mass $[M+H]^+$		Observed mass $[M+H]^+$		Deviation: α-amidation vs. orbitrap mass
	Hydrolysis (free C-term.)	α-amidation (C-term. amidation)	MALDI-TOF	Orbitrap	
1–9	1098.5790	1097.5950	1097.63	1097.5961	1.0 ppm
1–14	1625.8381	1624.8541	1624.82	1624.8565	1.5 ppm
1–16	1905.9205	1904.9365	1904.90	1904.9393	1.5 ppm
1–25	–	–	3122.21	–	–

tides are in excellent agreement with the theoretical masses of C-terminally amidated sequences (mass deviations < 1.5 ppm).

To verify that their C termini are indeed amidated, the peptides were subjected to collision induced dissociation (CID) experiments. Figure 2A shows the CID spectrum for peptide (1–9). The b_8-fragment ion comprises the first eight residues (1–8),
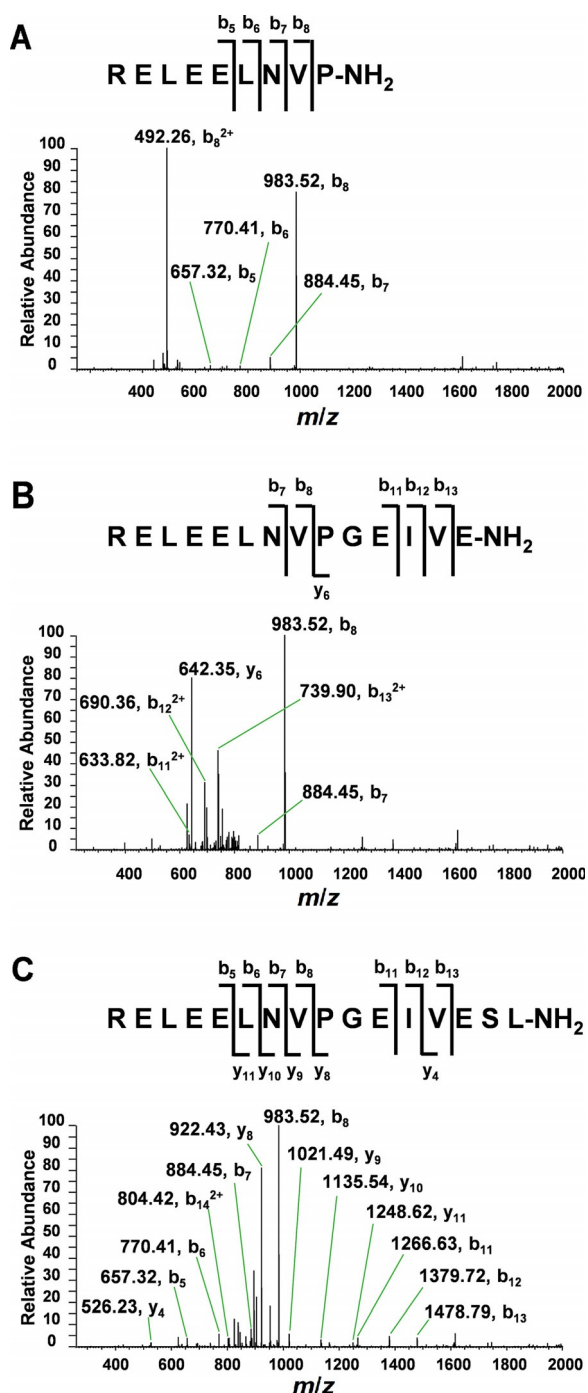

Figure 2. CID spectra of peptides (1–9), (1–14) and (1–16) formed by uranyl photocleavage of the β-casein peptide. Mass spectra of photocleavage products with precursor masses: A) m/z 549.30, 2+ (1097.6 Da), B) m/z 812.93, 2+ (1624.9 Da), and C) m/z 953.47, 2+ (1904.9 Da). Sequences of the respective photocleavage products are shown below each spectrum, indicating the identified b- and y-ions.

and its mass agrees well with the unmodified sequence (mass deviation 0.5 ppm). The mass of the C-terminal residue was readily obtained by subtracting the mass of the b_8-fragment ion from the mass of the peptide ion (1–9) (i.e. precursor ion mass). The experimental mass of the C-terminal proline residue clearly shows that it was amidated, as proved by the excellent agreement with the theoretical mass for an amidated proline amino acid (mass deviation 1.9 ppm). Consequently, the CID spectrum of peptide (1–9) confirms unambiguously that its C terminus was amidated. Similarly, the CID spectrum of peptide (1–14) (Figure 2B) demonstrates that residues 1–13 are unmodified, as the mass of the b_{13}-fragment ion fits well with the theoretical mass of the unmodified sequence (mass deviation 1.3 ppm). The C-terminal residue is contained within the y_6-fragment ion of residues 9–14 (Figure 2B), and the mass of this fragment ion is in excellent agreement with the theoretical mass of the amidated sequence (mass deviation 1.2 ppm). The masses of the two fragment ions (b_{13}^{2+} and y_6^+) thus provide conclusive evidence for the amidation of C terminus in peptide (1–14). The CID product spectrum of peptide (1–16) contains a rich set of b- and y-fragment ions (Figure 2C). Again, all b-fragment ions corresponded to unmodified sequences, whereas the masses of the y-fragment ions corresponded to sequences with an amidated C-terminal Leu residue (y_4^+, mass deviation 0.6 ppm). It should be noted that the absence of b_{14}- and b_{15}-fragment ions means that the amidation is localised to the last three residues of peptide (1–16), that is, -Glu-pSer-Leu. However, as the amidation was located at the C terminus in peptide (1–9) and peptide (1–14), we consider it is most likely that this is also the case for peptide (1–16).

To further validate the presence of an amidated C terminus in the peptide products of uranyl photocleavage, we incubated the photocleaved sample with an exopeptidase (carboxypeptidase Y) that enzymatically cleaves peptides from their C-terminal ends, but requires a free C terminus (-COOH) for full enzymatic activity.[15] An amidated C terminus will thus protect a peptide from enzymatic degradation. When the photocleaved sample was incubated with carboxypeptidase Y, there was no sign of degradation of any of the peptides formed by uranyl photocleavage. However, the peak corresponding to the internal standard (neurotensin) disappeared, and several peaks corresponding to partial degradation products of the internal standard appeared (Figure S2). Together with the CID data, this orthogonal enzymatic assay provides strong evidence that peptides (1–9), (1–14) and (1–16) formed by uranyl photocleavage are C-terminally amidated. Our results highlight the importance of using high-accuracy mass spectrometers for unambiguous characterisation of peptides.

In order to confirm the requirement of uranyl for the photocleavage reaction, the β-casein peptide was UV irradiated in the absence of uranyl. As expected, no photocleavage was observed without uranyl (Figure S3). In addition, the importance of the phosphate groups in the β-casein peptide was investigated. A peptide with the same sequence but without phosphorylation (RELEELNVPGEIVESLSSSEESITR) was UV irradiated in the presence of uranyl. No photocleavage was observed, thus testifying that phosphate groups are required for the cleavage
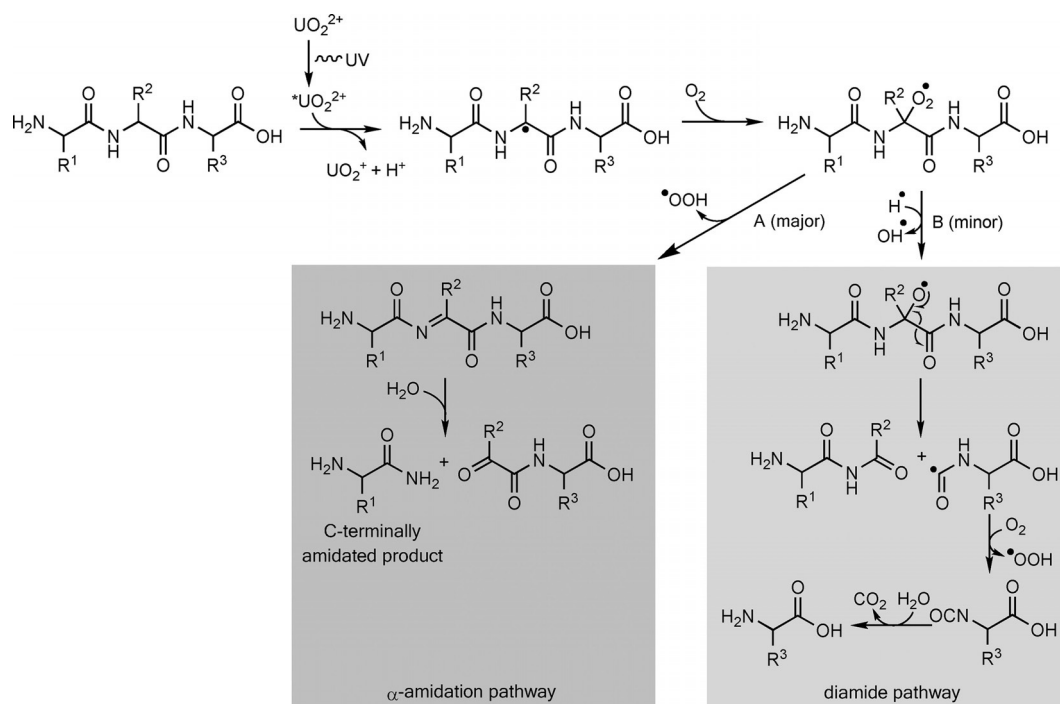
Scheme 1. Proposed mechanism for uranyl-dependent photocleavage. The two suggested pathways are A) the α-amidation pathway and B) the diamide pathway. R groups = amino acid side chains. Adapted from refs. [17a] and [17b].

reaction, most likely because they present efficient uranyl-binding sites in the peptide (Figure S4).

The discovery that the photolytic cleavage yields C-terminally amidated peptides suggests that the backbone cleavage mechanism could follow an α-amidation-like pathway (Scheme 1 A).[16] This pathway requires oxygen (Scheme 1).

In order to investigate whether the uranyl photocleavage reaction exhibits a similar dependence on oxygen, we conducted the experiment under essentially oxygen-free conditions. Oxygen was efficiently removed by bubbling xenon gas through the solution in a glovebox containing an inert nitrogen atmosphere (Figure S5). Figure 3 shows the mass spectrum obtained from UV irradiation of uranyl-bound β-casein peptide in an oxygen-free environment. Interestingly, in the absence of oxygen there was no formation of photocleavage products (Figure 3 B). In contrast, uranyl photocleavage of DNA has been reported to occur also under oxygen-free conditions.[18] The strong oxygen dependence of uranyl-induced photocleavage thus appears to be unique for polypeptide backbone cleavage.

There are several lines of evidence to support the involvement of a radical-mediated mechanism, akin to the α-amidation pathway (Scheme 1 A) for polypeptide backbone cleavage by UV-activated uranyl. Firstly, uranyl is strongly oxidising in the excited state, and it has been reported to efficiently photo-oxidise organic compounds by hydrogen abstraction upon UV irradiation.[7, 17a] Secondly, the first step for radical-mediated oxidative cleavage of a polypeptide backbone (by, for example, hydroxyl radical attack) is hydrogen abstraction from typically the α-carbon, β-carbon (C-3) or γ-(C-4) sites on the amino acid side-chains.[16] In the following step, the α-carbon-centred radical reacts with molecular oxygen to give an intermediate

peroxy radical, which forms an imine species upon loss of OOH•; this in turn is readily hydrolysed to form a C-terminally amidated peptide and a ketoacyl peptide (α-amidation pathway; cleavage of the N—C_α bond; Scheme 1). It should be noted that the peroxy radical can also follow the "diamide
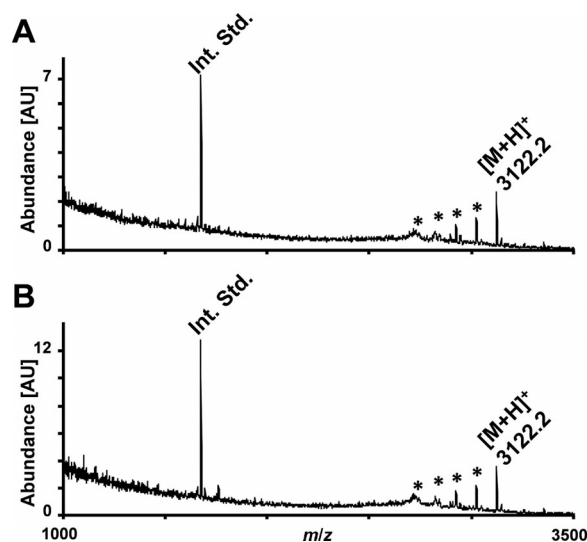

Figure 3. Mass spectra obtained from the β-casein peptide incubated with uranyl in an essentially oxygen-free environment, A) without UV and B) after UV irradiation. Asterisked peaks are MALDI-induced neutral losses of H_3PO_4 from the intact peptide. The experiment was carried out in a glovebox (oxygen < 1 ppm). Prior to UV irradiation the samples were degassed by bubbling xenon through the samples for 5 min. Finally the samples were desalted and the mass was analysed by MALDI-MS. Int. Std. = internal standard (neurotensin).

pathway" for backbone cleavage, where the C_α–CO bond is cleaved to form an imide (N-terminal fragment) and a peptide isocyanate (C-terminal fragment, which is further hydrolysed to yield CO_2 and a peptide; Scheme 1B). For hydroxyl radical-mediated oxidative backbone cleavage, the products from both pathways are typically observed.[16a] For example, metal-catalysed oxidation of proteins or peptides typically leads to products formed by both the α-amidation and diamide pathways.[19]

At first glance, the MALDI mass spectra suggest that uranyl photocleavage proceeds solely by an α-amidation-like pathway to yield exclusively C-terminally amidated peptides. However, closer inspection of the MALDI spectrum (Figure 1) reveals a minor peak at m/z 1125.6. We attribute this to the diamide product formed by a similar α-carbon-centred radical, as that leading to the α-amidation product (1–9) at m/z 1097.6. However, there are no other peaks in the MALDI spectrum corresponding to diamide products (m/z 1762.8 and m/z 2042.8) formed by a similar α-carbon-centred radical as that leading to the α-amidation products (1–14) and (1–16). Thus, the reaction studied here, as measured by MALDI MS, seems to favour the α-amidation pathway over the diamide pathway.

The high preference of uranyl photocleavage to follow an α-amidation-like pathway is highly important for its potential to synthesise pharmaceutical relevant C-terminally amidated peptides. Importantly, in contrast to metal-catalysed oxidation, uranyl photo-oxidation seems to strongly favour backbone cleavage over other types of oxidative modification (e.g. side-chain oxidation, carbonylation, side-chain cleavage).[19, 20] In order to put this into perspective, bovine serum albumin was found to undergo oxidative damage at 106 different residues in in vitro metal-catalysed oxidation.[21] In this respect, uranyl appears to present a novel approach to achieve site-selective cleavage to yield C-terminally amidated peptides with limited byproducts. In a pharmaceutical setting, purity of the final product is a concern, as would be uranyl toxicity. The uranyl salt is catalytic and is not incorporated into the photocleavage products. However, proper immobilisation or chromatographic purification of excess uranyl is of absolute importance in further development.

Conclusion

By using high-accuracy mass spectrometry, collision-induced dissociation experiments and an enzymatic assay, we have unambiguously demonstrated that the peptides formed by uranyl photocleavage are C-terminally amidated. The uranyl photocleavage reaction depends on the presence of oxygen; in the absence of oxygen, no cleavage products were observed. We hypothesise that the photocleavage reaction preferentially follows an α-amidation-like pathway. The efficient cleavage suggests that uranyl photocleavage could be used as an alternative way of producing pharmaceutically relevant C-terminally amidated peptides. In this light, studying the mechanism and cleavage pattern of uranyl photocleavage in even more detail holds great potential. Using the β-casein model peptide, we observe three distinct photocleavage products,

which would require an additional chromatographic purification step in a pharmaceutical setting. Thus, understanding what determines the cleavage specificity is important and should be addressed in future studies. This might give rise to the identification of specific peptide sequences that result only in one photocleavage product and therefore would be more suitable for pharmaceutical production.

Experimental Section

Sample preparation: $UO_2(NO_3)_2$ (catalogue no. 94270, Sigma–Aldrich) was dissolved in ultrapure Milli-Q water at RT to give a 100 mM stock solution. Concentrations above 1 mM favour the nitrato complex $UO_2(NO_3)_2$ in solution over hydrolysis of uranyl.[22] Immediately before use, the uranyl solution was diluted (500 μM) in Tris·HCl (20 mM, pH 8.0). The reactions were carried out in low-binding Eppendorf tubes containing synthetic β-casein peptide (10 μM >95% purity; AnaSpec), $UO_2(NO_3)_2$ (50 μM) and neurotensin (0.5 μM; internal standard, >90% purity; Sigma–Aldrich) in Tris·HCl (20 mM, pH 8.0). For the experiment with the nonphosphorylated peptide a synthetic nonphosphorylated β-casein peptide was used (>93.8% purity; GenScript, Nanjing, China).

UV irradiation/photocleavage: Samples (20 μL) were transferred to the lids of 1.5 mL low-binding Eppendorf tubes, and the lids were placed on a freezer block (0°C). The UV lamp (6 W; UVP, Upland, CA) was placed 1.0 cm directly above the lids, then the samples were irradiated (365 nm) for 15 min, transferred to low-binding tubes and stored at −20°C.

Experiments under oxygen-free conditions: All samples and equipment were transferred through an airlock into a glovebox with an oxygen concentration below 1 ppm. Samples were degassed by bubbling xenon (Air Liquide Taastrup, Denmark) through the solution for 5 min. Samples were transferred to lids from Eppendorf tubes, and UV irradiation was performed in the glovebox as described above.

Oxygraph measurements: The oxygen level was monitored with an oxygraph+ (Hansatech Instruments, Kings Lynn, UK) to determine the time required to remove oxygen from Tris·HCL buffer. Tris·HCl (500 μL, 20 mM, pH 8) was placed in the oxygraph chamber with a stirring magnet in the bottom, and the chamber was sealed. When a stable oxygen level was observed, xenon was bubbled continuously into the solution through the chamber inlet, while observing the oxygen concentration until a stable reading of zero oxygen was achieved.

Desalting: Samples were dried in a Concentrator Plus vacuum centrifuge (Eppendorf) and redissolved in formic acid (0.1%). Samples were desalted on reversed-phase microcolumns packed in stage tips by using a C8 plug and Oligo R3 resin (Applied Biosystems). The columns were equilibrated with formic acid (0.1%), and the sample (also in 0.1% formic acid) was loaded. The column was washed with formic acid (0.1%), and the sample was eluted using acetonitrile/water (20 μL, 1:1 v/v) containing formic acid (0.1%).

Carboxypeptidase Y treatment: Samples were prepared as described above (i.e., 10 μM β-casein peptide, 50 μM $UO_2(NO_3)_2$ and 0.5 μM neurotensin in Tris·HCl). The UV-irradiated samples (not desalted) were mixed with citrate buffer (18 μL, 0.1 M, pH 6) and carboxypeptidase Y (2 μL, 5 U mL^{-1} in citrate buffer; Sigma–Aldrich), and incubated over night at 25°C. Samples were desalted before MALDI-MS analysis.

Acknowledgements

Conflict of Interest

The authors declare no conflict of interest.

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