Protein Nitration Hot Paper

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Light-Controlled Tyrosine Nitration of Proteins

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Abstract: *Tyrosine nitration of proteins is one of the most important oxidative post-translational modifications in vivo. A major obstacle for its biochemical and physiological studies is the lack of efficient and chemoselective protein tyrosine nitration reagents. Herein, we report a generalizable strategy for light-controlled protein tyrosine nitration by employing biocompatible dinitroimidazole reagents. Upon 390 nm irradiation, dinitroimidazoles efficiently convert tyrosine residues into 3-nitrotyrosine residues in peptides and proteins with fast kinetics and high chemoselectivity under neutral aqueous buffer conditions. The incorporation of 3-nitrotyrosine residues enhances the thermostability of lasso peptide natural products and endows murine tumor necrosis factor-α with strong immunogenicity to break self-tolerance. The light-controlled time resolution of this method allows the investigation of the impact of tyrosine nitration on the self-assembly behavior of α-synuclein.*

Introduction

Bioactive nitroarene natural products are widely distributed in all life domains including plants, fungi, bacteria and mammals.[1] For example, cyclic peptide natural product ilamycin B2 contains a 3-nitrotyrosine (3-NT) residue which contributes to its antibiotic bioactivity against human and bovine tubercular bacteria (Figure 1 A).[2] In addition to small molecule metabolites, nitration of cellular biomolecules, including oligonucleotides, lipids and proteins, are common biological events in living systems as a consequence of elevated oxidative stress.[3] Tyrosine (Tyr) nitration represents one of the most important oxidative protein posttranslational modifications (PTMs) in many organisms and is closely related to human physiology, pathology and aging.[3a,4] Tyrosine residues are converted to 3-NT residues by nitric oxide-derived oxidants in vivo, and the accumulation of 3-NT in cellular proteins is usually considered as a biomarker for inflammation-associated conditions and various diseases including cancer and neurological disorders.[3b,5]

Nitration of tyrosine dramatically alters its physicochemical properties, including the pK_a value of the phenol moiety, the hydrophobicity, redox potential and steric bulkyness, thereby impacting the structure and function of proteins.[3a] For example, enzymatic inactivation by tyrosine nitration is well-established for human manganese superoxide dismutase (MnSOD), where the bulky side chain of 3-NT close to the active site shuts down the entrance of reactive oxygen species.[6] Protein–protein recognition and self-assembly behavior of proteins could also be affected by tyrosine nitration, as demonstrated by the altered aggregation tendency of α-synuclein, β-amyloid and the microtubule-associated tau protein upon nitration.[7]

The unique property of 3-NT allows its application as chemical probe in biochemical research. 3-NT can be employed as a fluorescence quenching unit for investigating protein folding, conformational change and ligand-protein interactions.[8] Furthermore, 3-NT residues are highly immunogenic and implicated in the pathology of autoimmune diseases.[9] As an immunogenic epitope, the incorporation of 3-NT in proteins is applied to trigger autoimmune T cell responses that can break self-tolerance.[10] Schultz and coworkers demonstrated that a murine TNF-α mutant containing a single 3-NT is able to induce a strong antibody response and breaks the self-tolerance toward wild-type mTNF-α.[11] Overall, tyrosine nitration as an oxidative PTM is of high relavence to human biology, and the resulting 3-NT residue is a useful molecular tool for biochemical research.

Despite the biological importance of tyrosine nitration in proteins, related studies have been impeded by the lack of convenient methods to incorporate 3-NT residues into proteins. Genetic-code expansion platforms provide a powerful tool to generate proteins with 3-NT at a specific position in vivo.[11,12] However, such methods often suffer from low protein yields, especially when multiple 3-NT incorporation is desired. Furthermore, reductive modification of 3-NT to 3-aminotyrosine residues in heterologous expression hosts, such

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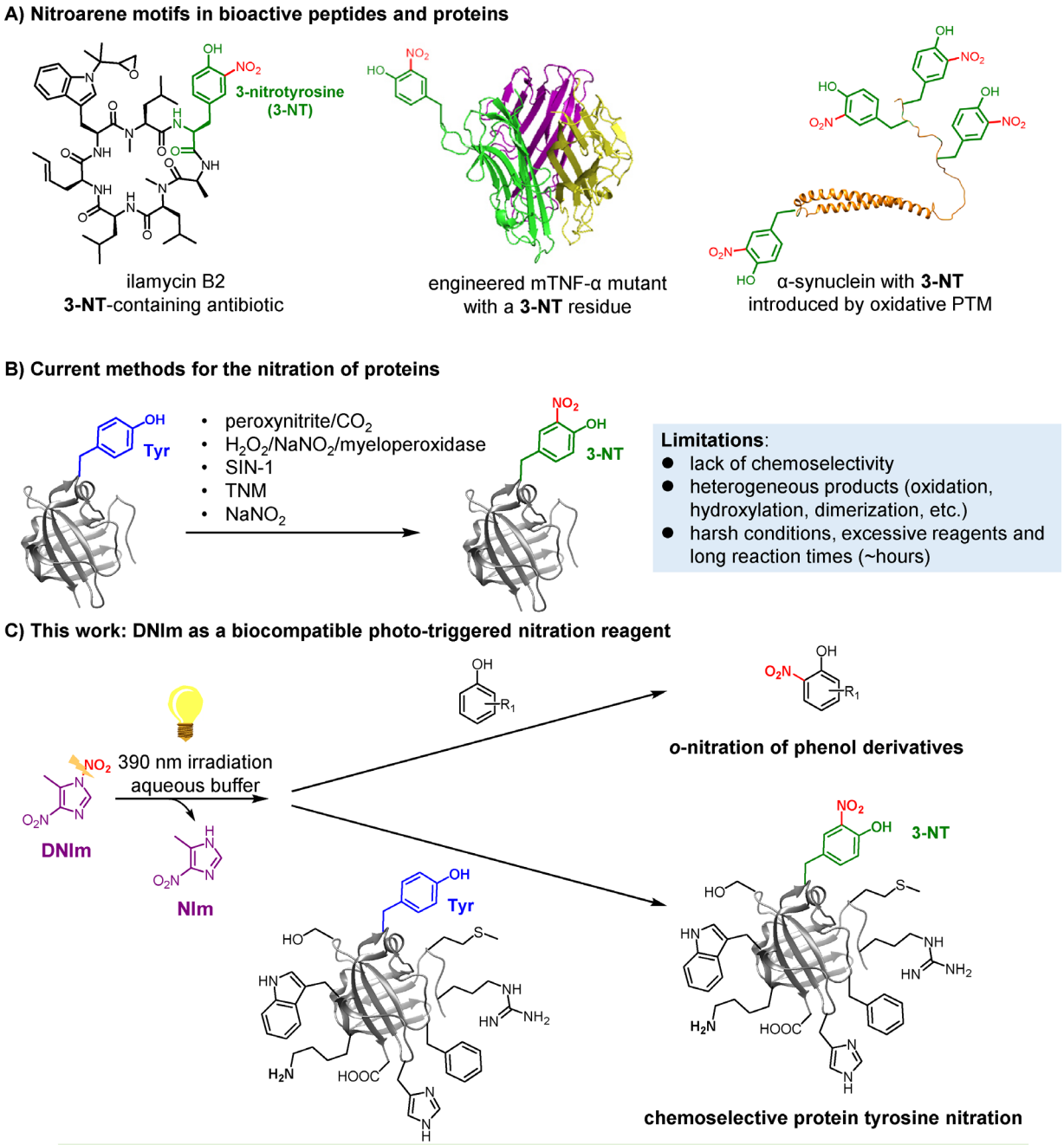

Figure 1. Light-induced nitration of phenol derivatives and tyrosine residues in proteins by 5-methyl-1,4-dinitroimidazole (DNIm). A) 3-NT residues in bioactive peptides, engineering therapeutic and cellular proteins. The structures of mTNF-α and α-synuclein are modified from their crystal structures (PDB codes 2TNF and 2KKW). B) Current methods for tyrosine nitration of proteins. C) Light-induced nitration of phenol derivatives and tyrosine residues proteins by DNIm.

as *E. coli*, poses additional challenges to its application.[13] Chemical nitration of proteins serves as an important alternative. Currently used reagents for protein nitration include peroxynitrite/CO_2,[14] H_2O_2/sodium nitrite/myeloperoxidase,[14c] 3-morpholinosydnoimine (SIN-1),[15] tetranitromethane (TNM)[16] and $NaNO_2$ (Figure 1B).[17,22] However, these chemical methods usually suffer from the requirement of excess amount of nitration reagents, prolonged reaction time (usually hours), and low-to-moderate modification yields.[13] More importantly, these protein nitration reagents usually lack chemoselectivity toward tyrosine, leading to undesired side reactions such as hydroxylation of tyrosine, oxidation of cysteine/methionine/tryptophan residues and protein oligomerization via 3,3′-dityrosine crosslinking.[13] Herein, we

report the development of 5-methyl-1,4-dinitroimidazole (DNIm) as a light-controlled and biocompatible protein nitration reagent. Upon 390 nm irridiation, DNIm efficiently converts tyrosine residues into 3-NT residues in peptides and proteins with fast kinetics and high chemoselectivity under aqueous buffer conditions. We demonstrate that the incorporation of 3-NT residues enhances the thermostability of lasso peptide natural products and endows murine tumor necrosis factor-α with strong immunogenicity to break self-tolerance. Furthermore, the light-controlled time-resolution of this method allows the investigation of the impact of tyrosine nitration on the self-assembly behavior of α-synuclein.

Results and Discussion

Nitroarene compounds, such as 2-nitrophenyl and 2-nitroveratrole, are often photo-responsive and utilized as photo-cleavable structural units.[18] We therefore explored the light-responsive property of DNIm, which is stable toward biological nucleophiles such as lysine. DNIm has an absorbance spectrum up to 400 nm, and thus 390 nm LED was selected as a light source (Supporting Information, Figure S1). Upon light irradiation in acetonitrile, DNIm was fully converted into 4-nitro-5-methyl-imidazole (NIm) and 5-methyl-(2,4)-dinitroimidazole (2,4-DNIm) in 6 min (Figure S2). Treatments with heat (50 °C) under dark conditions failed to induce the decomposition of DNIm, indicating that this reaction is triggered specifically by light (Figure S3). When *p*-methylphenol (**1a**) and tyrosine derivative **1b** were supplied as substrates, the *ortho*-positions of **1a** and **1b** were readily nitrated by DNIm with concomitant products **2a** and **2b** in about 70% isolated yield (Figure 2A, Figure S4). Radical trapping reagents, including 2,2,6,6-tetramethylpiperidinyloxy (TEMPO), butylated hydroxytoluene (BHT) and 1,1-diphenylethylene, significantly inhibited the nitration of **1a**, suggesting that the reaction follows a radical mechanism (Figure 2A, Figure S4). Indeed, when DNIm was irradiated by 390 nm LED in the presence of 1,1-diphenylethylene, adducts of 1,1-diphenylethylene conjugated with a nitro group or a NIm moiety were observed by LC-MS and GC-MS analysis (Figure S5), indicating the generation of nitro and NIm radical species. When compound **1b** was reacted with DNIm in the presence of BHT, nitro-BHT **3b** and tyrosine-BHT conjugate **4b** were detected by both GC-MS and LC-MS analysis, indicating the presence of nitro and tyrosine radical species during the reaction (Figure S6). Kinetic analysis of the DNIm-mediated nitration reaction revealed that the light-induced cleavage of $N(1)$-NO_2 bond is the rate-limiting step, as the initiate rate of DNIm consumption and nitrophenol product **2a** formation were both independent of the concentration of substrate **1a** (Figure S7). Thus, we propose that light irradiation cleaves the $N(1)$-NO_2 bond in DNIm by generating a NIm radical (**1**) and a nitryl radical (Figure 2B). The NIm radical (**1**) subsequently abstracts a hydrogen atom from the phenol substrate (**2**) to generate a radical species (**3**), which could couple with a nitro radical to generate the desired *ortho*-nitrophenol product (**4**) (Figure 2B). In the absence of phenol derivatives, the NIm radical (**1**) could recombine with a nitro radical at the C2 position to generate a stable and light-insensitive product 2,4-DNIm (Figure 2B, Figure S2).

Next, we explored the applicability of DNIm in the nitration of small molecules, peptides and proteins. Phenol derivatives bearing electron-donating and electron-withdrawing groups at *para*- and *ortho*-positions were well accepted for the reaction, yielding corresponding nitration products in high yields (Figure 2C, entries **2a**–**2i**). Naphthol and 7-hydroxy-2*H*-chromen-2-one derivatives were also nitrated by DNIm in 47–63% yields (Figure 2C, entries **2j**–**2m**). Bioactive phenol derivative naringenin was smoothly nitrated in 63% yield as well (Figure 2C, entry **2n**). Tyrosine nitration of peptides is efficiently accomplished by DNIm under aqueous buffer conditions. Tripeptide **P-1** was nitrated by DNIm under light in PB buffer at pH 6.0 with more than 95% conversion (Figure 3A). No dimerization of **P-1** by tyrosine crosslinking was detected by HPLC and ESI-MS (Figure S8). The nitration of peptides by DNIm was chemoselective toward tyrosine residues under aqueous conditions, as tripeptides **P-2**–**P-4** containing Phe, His and Met were not modified by DNIm (Figure 3B, Figure S9–S11). As an exception, Cys-containing tripeptide **P-5** was quantitatively converted into disulfide-linked product **Di-(P-5)** (Figure S12). Peptides **P6**–**P8**, as well as endomorphin and Leu-enkephalin, were all nitrated specifically at tyrosine residues, demonstrating the utility of this reaction in generating 3-NT-containing bioactive peptides (Figure 3B, Figure S13–18).

We further challenged DNIm to modify complex tyrosine-containing lasso peptide natural products, which are ribosomally synthesized and posttranslationally modified peptides with unique lariat knot topologies.[19] The thermostability of lasso peptides mainly depends on the bulky residues (the plug residues) above and below the ring structures to prevent the unthreading of the C-terminal tails (Figure 3C).[20] Using microcin J25 as a lasso peptide model substrate, DNIm efficiently introduced two 3-NT residues in this molecule, including the sterically hindered Y20 residue (Figure 3C, Figure S19). Caulonodin IV was selected as an example of heat-sensitive lasso peptide, which contains two tyrosine residues (Y15 and Y16) as the upper plug residues and one phenylalanine as the lower plug residue (Figure 3D).[21] These results show that DNIm is capable of nitrating Tyr residues in crowded local environments. While it is known that the thermal stability of lasso peptides is often primarily dictated by characteristics of their lower plug residues, the alteration of other residues can also affect the thermal stability of these compounds. We envisioned that nitration of Y15 and/or Y16 residues would improve the thermostability of caulonodin IV by increasing the bulkiness of the upper plug residues (Figure 3D). Under standard nitration conditions, DNIm successfully converted Y15 and Y16 into 3-NT residues, as determined by LC-MS/MS analysis (Figure S20). The resulting $(3\text{-NT})_2$-caulonodin IV exhibited significantly enhanced resistance against thermally induced unthreading than wild-type caulonodin IV under various temperatures (Figure 3E, Figure S21). For example, after incubation in aqueous buffer at 95 °C for 10 min, 56% of $(3\text{-NT})_2$-caulonodin IV remained in the lasso fold, whereas only 29% wild-type caulonodin IV

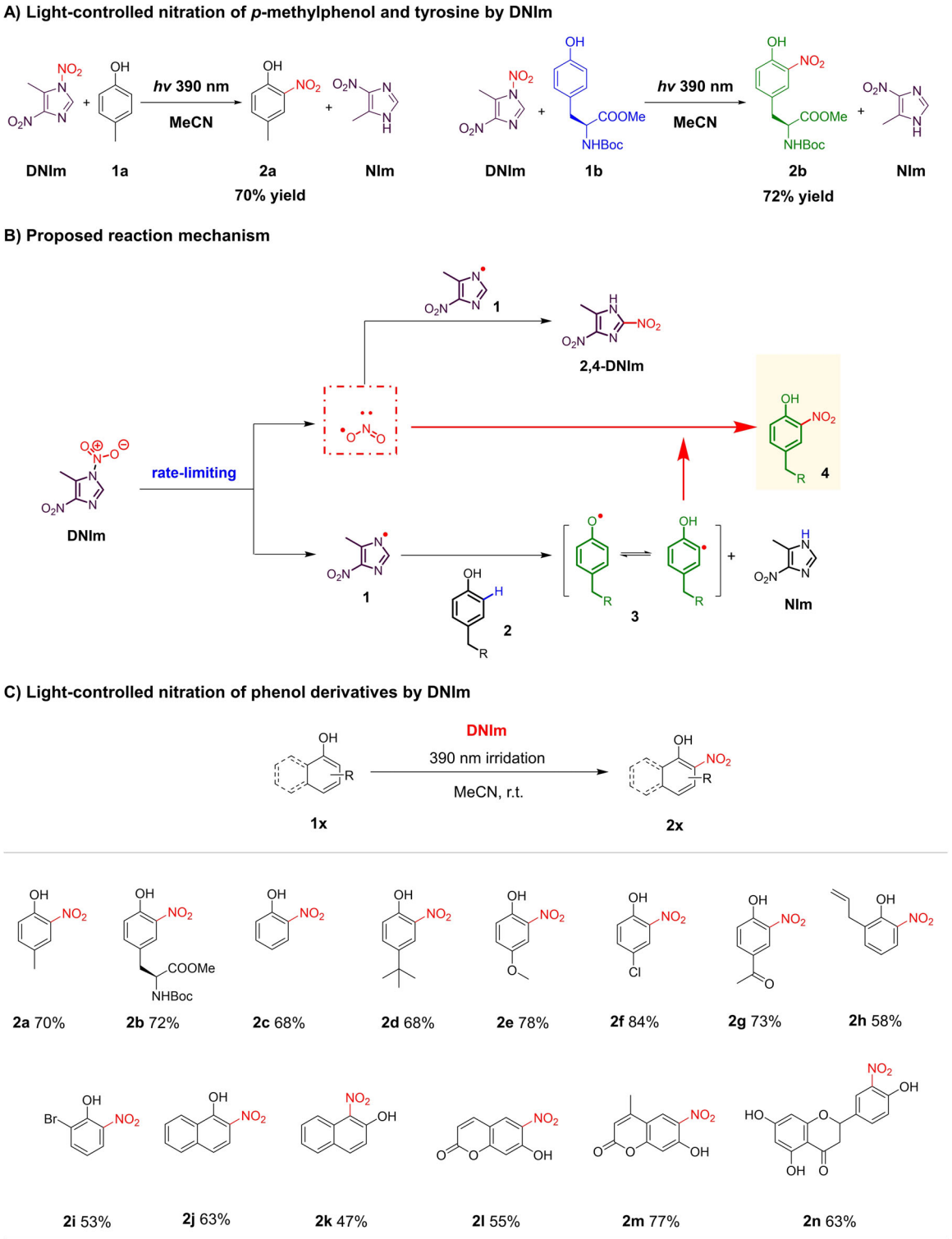

Figure 2. Light-induced nitration of phenol derivatives by DNIm. A) Nitration of *p*-methylphenol and tyrosine by DNIm. Reaction conditions: substrate (**1a**) or (**1b**) (2.0 mM) and DNIm (2.4 mM) are reacted in acetonitrile under 390 nm LED irradiation for 1 h. B) Proposed reaction mechanism of DNIm-mediated nitration of phenol derivatives. C) Substrate scope of DNIm-mediated nitration of phenol derivatives.

retained their lasso topology (Figure 3E, right panel). Thus, by simple late-stage nitration of lasso peptides by DNIm, we can generate these unique interlocked structural units with enhanced thermostability. Together, these data demonstrate that DNIm is a biocompatible and robust nitrating reagent highly specific towards tyrosine residues in complex peptides.

To examine the ability of DNIm to introduce 3-NT into proteins, lysozyme was selected as a model protein with three

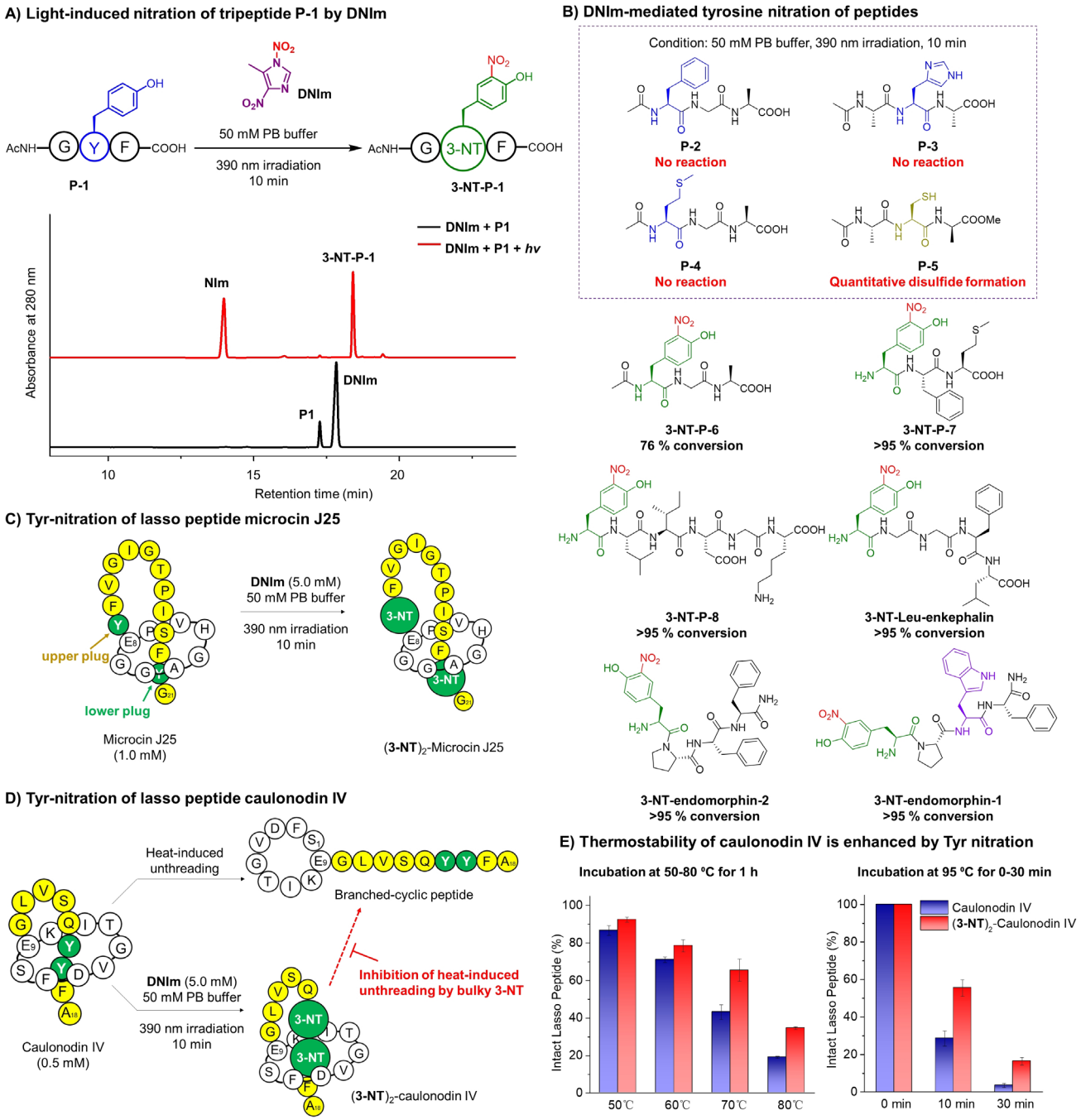

Figure 3. DNIm-mediated nitration of tyrosine-containing peptides. A) Light-induced nitration of tripeptide (**P-1**) by DNIm. The conversion was >95% based on three independent assays, as determined by HPLC analysis. B) DNIm-mediated tyrosine nitration of peptides. C) DNIm-mediated tyrosine nitration of microcin J25; D) DNIm-mediated tyrosine nitration of caulonodin IV; E) Thermostability of caulonodin IV and (3-NT)$_2$-caulonodin IV in aqueous conditions.

Tyr residues. Incubation of lysozyme with 14 equiv. of DNIm (to the total protein) in PB buffer at pH 6.0 under 390 nm irradiation generated lysozyme with a single nitration, (3-NT)-lysozyme, as the major product in 10 min (Figure 4). Further MS analysis of trypsin digested (3-NT)-lysozyme indicated that Tyr23 was the dominant nitration site, which is in agreement with previous studies that Tyr23 is the most accessible tyrosine residue of lysozyme (Figure S22, Table S4).[22] In addition, RNase A and histone-H2A were also nitrated with high tyrosine specificity (Figure S22, Table S5). Together, these data demonstrate that DNIm is a highly efficient and chemoselective tyrosine nitration reagent for proteins under both native and denaturing conditions.

The high chemoselectivity and fast reaction rate of DNIm-mediated tyrosine nitration prompted us to explore its utility in investigating the impact of this oxidative PTM on the biophysical properties of proteins. α-Synuclein (ASN) is of particular interest to study because its tyrosine nitration is

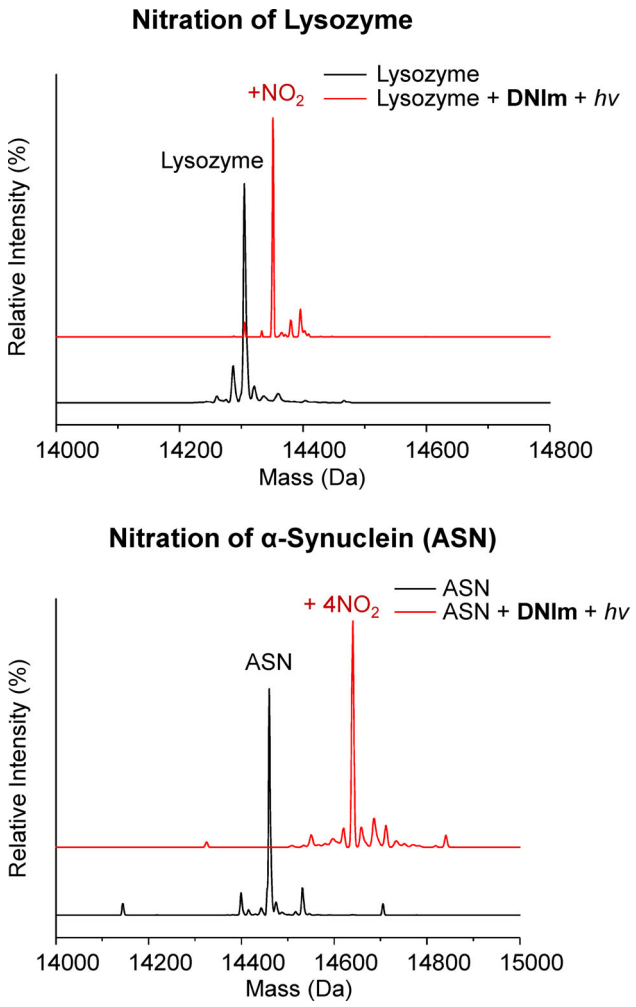

Figure 4. Light-triggered tyrosine nitration of proteins by DNIm. Modification conditions: protein (50 μM) and DNIm of indicated concentrations are incubated in 50 mM phosphate buffer at pH 6.0 under 390 nm for 10 min.

associated with neurodegenerative diseases, including Parkinson's disease.[3a,5] DNIm exhibits superior capability in nitrating ASN proteins, compared to $NaNO_2$ and peroxynitrite solutions, by cleanly converting them into $(3\text{-NT})_4$-ASN with tetra-nitration in only 10 min, as determined by LC-MS/MS analysis (Figure 4, Figure S23–S27). The ASN_{Y_F} mutant protein with all four Tyr residues mutated into Phe was not modified by DNIm, further demonstrating the high Tyr-specificity of this method (Figure S28). TNM is one of the commonly used tyrosine nitration reagents, and treatment of ASN proteins by TNM resulted in $>80\,\%$ proteins as Tyr-Tyr crosslinked oligomers instead of Tyr-nitrated proteins.[7c] In sharp contrast, SDS-PAGE analysis of a Tyr-nitrated ASN sample generated by DNIm indicated that only less than 5 % ASN dimer was generated, further demonstrating the superiority of this Tyr-nitration reagent (Figure S22). $(3\text{-NT})_4$-ASN generated by our method retains the same secondary structure as wild-type ASN, as shown by CD spectroscopy (Figure S29). However, $(3\text{-NT})_4$-ASN completely lost the tendency to aggregate through in vitro agitation, as indicated by a ThT fluorescent assay (Figure S30). Furthermore, addition of $(3\text{-NT})_4$-ASN into wild-type ASN samples resulted in delayed aggregation in a dose-dependent manner and yielded overall lower levels of ThT fluorescence (Figure S31).

The aggregation of ASN in vivo is a highly complex process, which is further complicated by Tyr-nitration of these proteins. At any given time point, ASN proteins in the forms of monomers, oligomers, protofibrils and fibrils, along with a mixture of their Tyr-nitration products, all participate and impact the dynamic balance of aggregation and disassembly. Previous studies usually utilize synthetic ASN proteins bearing 3-NT residues at specific positions or nitrated ASN proteins generated by conventional protein nitrating reagents,[7b–d] which must be purified as monomers to remove any crosslinked byproducts before subjected to in vitro agitation assays to monitor their self-assembly behavior. Such over-simplified systems failed to mimic the in vivo scenario and hardly provide any information regarding the impact of tyrosine nitration on ASN's biophysical behavior in real time. Due to the high Tyr-specificity of DNIm-mediated ASN nitration, the purification step is not necessary, and all oligomerization forms of ASN proteins can be well reserved. Furthermore, this method is light-controlled and fast, allowing us to monitor the impact of tyrosine nitration on the self-assembly behavior of ASN during the course of aggregation with time-resolution. We designed an assay where wild-type ASN was first agitated in vitro for a given period of time to allow the progressive formation of β-sheet-rich ASN oligomers, protofibrils or fibrils (Figure 5 A). At selected time points (0, 1, 2 and 5^{th} day), ASN samples were treated with 2.0 or 10.0 equiv. DNIm for 10 min under 390 nm irradiation, which yielded partial or full tyrosine nitration of ASN proteins, respectively. The resulting ASN samples were then subjected to further agitation, and the aggregation was monitored by the ThT fluorescence assay every 24 hours until the 8th day. Results showed that, without the DNIm treatment, the aggregation of ASN reached the maximum level in 5 days (Figure 5 B and C). When the samples were treated with DNIm at the early stage of agitation (day-0 and day-1), nitrated ASN proteins completely lost its tendency to assemble into β-sheet-rich oligomers via agitation afterwards (Figure 5 B and C, day-0 and day-1 nitration samples). Tyrosine nitration on day-2, when significant amount of β-sheet-rich ASN oligomers have accumulated, decreased the intensity of ThT fluorescence by 26–42 % instantly upon the DNIm treatment, and inhibited further agitation-induced increase of ThT fluorescence (Figure 5 B and C, Figure S32). These results indicate that ASN nitration at this stage induced partial dissociation of ASN oligomers and more importantly, halts the progress of further ASN assembly. In contrast, fibrillous constructs of ASN (samples of day-5) were highly resistant to the DNIm treatment with only 3–12 % ThT fluorescence loss, and retained their assembly status afterwards (Figure 5 B and C, Figure S32). Transmission electron microscopy (TEM) analysis of ASN samples with or without DNIm-mediated nitration was in agreement with results of ThT assays. ASN samples that were nitrated at day-0 displayed small spherical-shaped oligomeric intermediates without any fibril structures after agitation (Figure S33). In contrast, ASN samples that were nitrated at day-8 retained

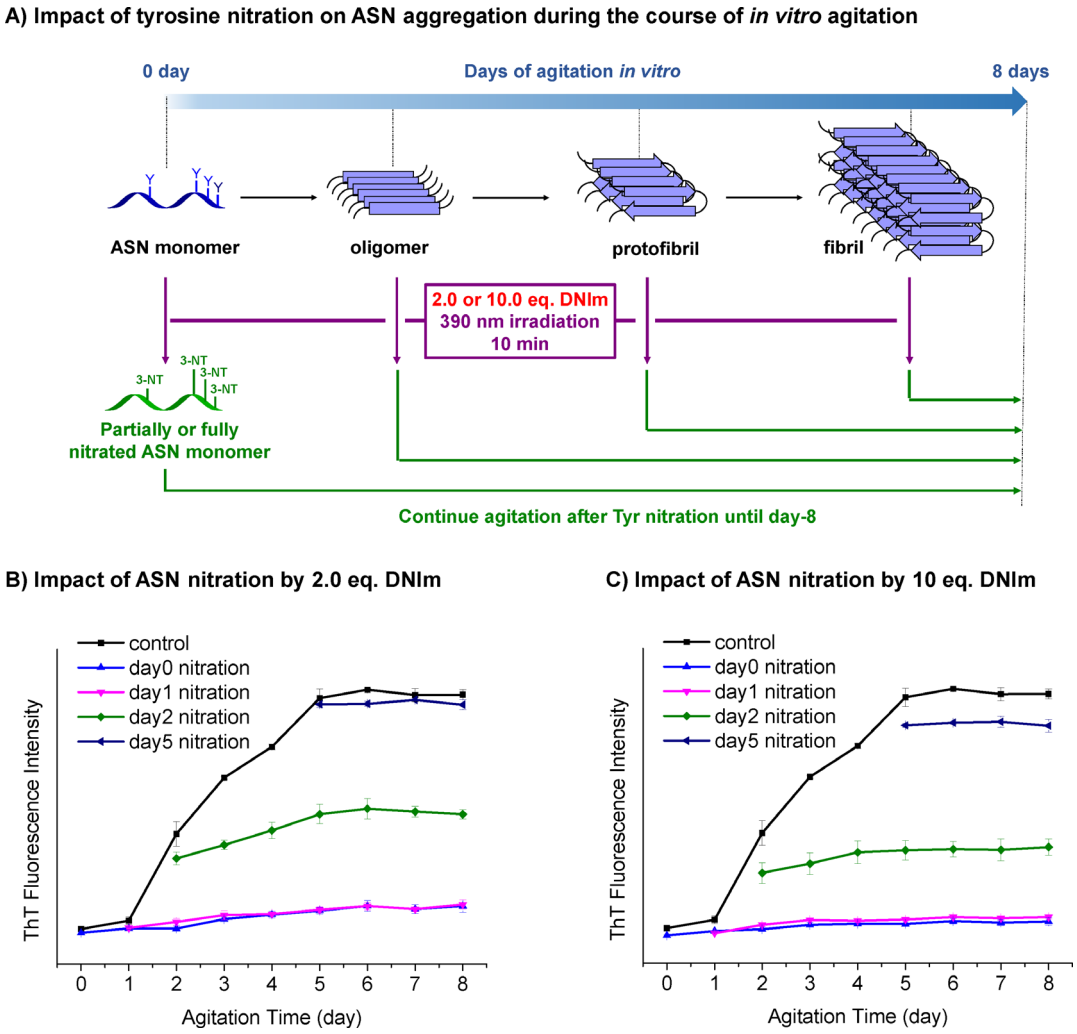

Figure 5. The impact of tyrosine nitration on ASN aggregation during the course of in vitro agitation. A) Illustration of the experimental design. ASN sample was first agitated for a period of time (0, 1, 2 and 5 days) to allow aggregation to occur before DNIm-mediated tyrosine nitration. After nitration, ASN samples were agitated in vitro until the 8th day. The formation of β-sheet-rich oligomers was monitored by ThT fluorescence assay at intervals of every 24 hours. B–C) Impact of tyrosine nitration on the progress of ASN aggregation by 2.0 equiv. and 10 equiv. DNIm.

characteristic fibril structures under TEM (Figure S33). Together, our data indicates that tyrosine nitration suppresses the ability of ASN monomers to form β-sheet-rich aggregates or be recruited into existing aggregates. Nitration of ASN had limited impact on the β-sheet-rich aggregates that are already stably formed. Thus, our tyrosine nitration method provides a significantly improved platform to mimic and investigate how this oxidative PTM affects the biophysical behavior of proteins with time-resolution compared with traditional protein nitration methods.

Finally, we applied this method to prepare (3-NT)-containing proteins with therapeutic potential. Incorporation of nitroaryl groups, such as *p*-nitrophenylalanine and 3-NT residues, often enhances the immunogenicity of peptides and proteins (Figure 6A). DNIm-mediated Tyr nitration may provide a facile method to prepare (3-NT)-containing proteins as epitopes. As an alternative method to the genetic code expansion strategy, introduction of multiple 3-NT residues in one protein by DNIm may also allow us to investigate the correlation between the average number of 3-NT residues in a protein and the resulting immunogenicity. We selected murine tumor necrosis factor-α (mTNF-α) as an example. Site-specific incorporation of a single 3-NT residue in mTNF-α has been reported to break its self-tolerance in mice, and such effect is not dependent on the site of 3-NT incorporation.[11,23] We prepared nitrated mTNF-α proteins with 2.0 to 25 equiv. DNIm under standard nitration conditions. As a result, nitrated mTNF-α proteins carrying 0.5 to 3.5 3-NT residues on average were acquired. The nitration sites of these mTNF-α proteins were analyzed by LC-MS after trypsin digestion. For mTNF-α proteins carrying one nitration on average, Tyr86, Tyr114/118 and Tyr140/150 were determined as the major nitration sites (Figure 6B, Table S3). The immunogenicity of the resulting nitrated mTNF-α proteins was evaluated in vivo. 36 C57BL/6 mice were randomized into six groups and injected with PBS buffer, wild-type (WT) mTNF-α and nitrated mTNF-α samples by using RIMMS (repetitive immunization at multiples sites) on day-0, day-7 and day-14. Antibody titers against WT mTNF-α were determined by ELISA using a horseradish peroxidase

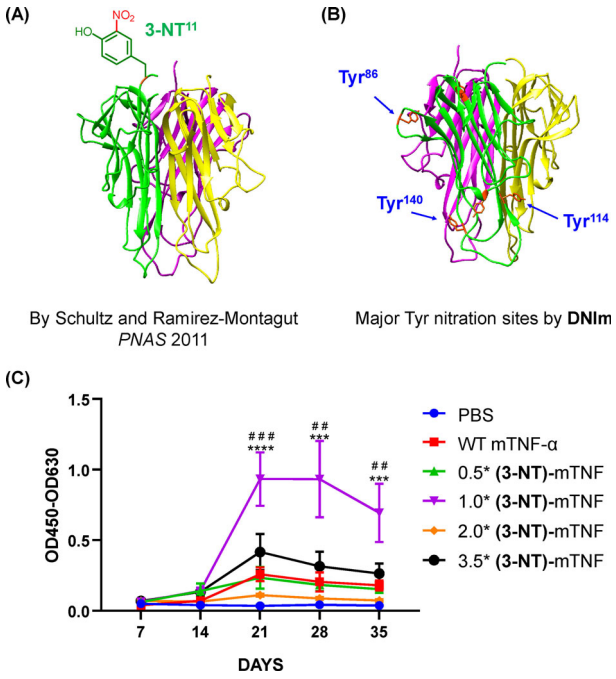

Figure 6. Preparation of tyrosine-nitrated mTNF-α as a protein epitope to break immunological self-tolerance. A) (3-NT)[11]-mTNF-α prepared by genetic incorporation of an unnatural amino acid. B) Major tyrosine nitration sites for DNIm-mediated nitration in mTNF-α. C) Serum titer for C57BL/6 mice immunized with PBS, WT mTNF-α and mTNF-α carrying various numbers of 3-NT residues. The data indicate autoantibody responses of mTNF-α at a 25 600 titer measured by ELISA. One-way ANOVA followed by Tukey's multiple comparison test, ***$p<0.01$ compared with PBS group, ###$p<0.01$, ##$p<0.05$ compared with WT mTNF-α group. ($n=6$ mice per group).

conjugate of goat anti-mouse IgG secondary antibody on day 7, 14, 21, 28 and 35. As expected, PBS buffer and WT mTNF-α induced no significant serum IgG titers against WT mTNF-α as a self-protein (Figure 6C). Although incorporation of 0.5 equiv. 3-NT in mTNF-α had no observable impact on its immunogenicity, the (1.0*3-NT)-mTNF-α sample induced significantly high serum IgG titers against WT mTNF-α, indicating the generation of antibodies that are highly cross-reactive with WT mTNF-α. Interestingly, increasing the average number of 3-NT incorporation in mTNF-α proteins strongly inhibited or completely abolished their ability to stimulate the generation of antibodies against WT mTNF-α, as indicated by the cases of (2.0*3-NT) and (3.5*3-NT)-mTNF-α (Figure 6). Together, these results demonstrate that DNIm-mediated Tyr nitration is a robust method in the preparation of (3-NT)-containing proteins, although as a mixture, with increased immunogenicity. Importantly, our study provides the first evidence that the immunogenicity of mTNF-α is highly dependent on the number of 3-NT residues incorporated and demonstrated that an increased number of 3-NT residues does not necessarily lead to stronger immunogenicity.

Conclusion

We have developed a simple yet effective dinitroimidazole reagent for light-controlled tyrosine nitration in peptides and proteins. Our nitration method is featured by its high chemoselectivity towards tyrosine residues and fast reaction kinetics, which is superior to conventional protein nitration reagents. The applicability of the reagent was demonstrated by tyrosine nitration of various phenol derivatives, complex peptides and proteins. In addition to providing a new tool for tyrosine nitration, our light-controlled approach may offer a general strategy for investigating the impact of tyrosine nitration on biophysical and immunogenic properties of proteins. The light-controlled chemical strategy described in the present study paves the way for developing a new class of chemical tools to investigate the role of protein tyrosine nitration and may offer opportunities to photo-manipulate protein nitration.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

Keywords: dinitroimidazole · light-induced reaction · nitration of phenol · protein nitration · tyrosine nitration

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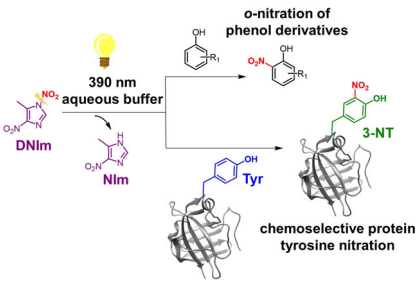
Research Articles

Protein Nitration

T. Long, L. Liu, Y. Tao, W. Zhang, J. Quan, J. Zheng, J. D. Hegemann, M. Uesugi, W. Yao, H. Tian,* H. Wang* _____ ▮▮▮▮–▮▮▮▮

Light-Controlled Tyrosine Nitration of Proteins



A simple yet effective dinitroimidazole reagent for light-controlled nitration of tyrosine residues in peptides and proteins was developed.