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Protein Modifications

Bioinspired Synthesis of Allysine for Late-Stage Functionalization of Peptides

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Abstract: Inspired by the enzyme lysyl oxidase, which selectively converts the side chain of lysine into allysine, an aldehyde-containing post-translational modification, we report herein the first chemical method for the synthesis of allysine by selective oxidation of dimethyl lysine. This approach is highly chemoselective for dimethyl lysine on proteins. We highlight the utility of this biomimetic approach for generating aldehydes in a variety of pharmaceutically active linear and cyclic peptides at a late stage for their diversification with various affinity and fluorescent tags. Notably, we utilized this approach for generating small-molecule aldehydes from the corresponding tertiary amines. We further demonstrated the potential of this approach in generating cellular models for studying allysine-associated diseases.

Introduction

Aldehydes are reactive carbonyl groups that are widely utilized for the site-specific functionalization of biomolecules with varying tags such as dyes, drugs and affinity tags using diverse chemistries thus act as a valuable handle for varying biological applications including reversible covalent inhibitors, synthesis of antibody-drug conjugates (ADCs), selective drug delivery, cellular imaging of biomolecules and many more (Figure 1a).[1–4] Although there is a wide applicability of aldehydes, the current chemical methods are limited to introducing aldehydes at the N and/or C-terminus of proteins.[5–6] This is mainly due to the unique reactivity of the N and C-terminus thus aldehydes can be introduced in the last step without modifying any reactive amino acids on proteins. In nature, several enzymes selectively convert side chains of a few amino acids to aldehydes as posttranslational modifications (PTMs) for multiple biological functions,[7–9] for example arginase and ornithine aminotransferase together convert the Arg side chain to L-glutamate semialdehyde,[8] formyl glycine enzyme (FGE) converts the side chain of Cys to formyl glycine,[9] and lysyl oxidase converts the lysine side chain to allysine,[7] and many others (Figure 1b).[9] In contrast, chemical methods to selectively convert amino acid side chains to aldehydes are completely lacking. Inspired by the activity of lysyl oxidase to convert lysine side-chain to allysine where amine from lysine is replaced with formyl group, we herein introduce the first chemical method for the selective conversion of the side chain of dimethyl lysine Kme_2 to allysine by the oxidation (Figure 1c). To the best of our knowledge, there are no other chemical methods for the generation of allysine. Our study describes the late-stage conversion of the side-chain of dimethyl lysine Kme_2 to allysine in peptides without epimerization in a selective manner for further diversification of pharmaceutically active linear and cyclic peptide aldehydes with varying tags such as dye and affinity tags using hydroxylamine,[10] hydrazine,[11] thiolamine[12] and reductive amination approaches.[13] We expanded the application of this biomimetic approach for synthesizing small molecule aldehydes starting from complex tertiary amines thus further displaying the structural and synthetic significance of these methods in drug design and discovery. Notably, overexpression of allysine is associated with age-related diseases thus we demonstrated the potential of this approach in generating allysine PTM inside cells as a model system for determining the role of allysine in diseases.[7b]

Results and Discussion

A Chemical Approach for Modification of Dimethyl Lysine to Allysine

Inspired by the activity of lysyl oxidase to carry out selective oxidation of lysine to allysine,[7] we explored varying oxidizing reagents on a lysine-containing model peptide FKV but were unable to modify it to the corresponding aldehyde (Figure 2a, Figure S1). The observed lack of reactivity prompted us to calculate intrinsic reactivity of lysine and its different methylation states, monomethyl lysine Kme_1 and dimethyl lysine Kme_2 towards oxidation using DFT. To achieve this, we focused on chemical properties such as bond dissociation energy (BDE), free energy for iminium ion formation, and the change in electrostatic potential (ESP) of the C=N bond (Figure 2b, Figure S2). Our extensive DFT calculations showed significantly higher bond dissociation energy 96.7 kcal/mol for lysine as compared to 96.4 kcal/mol and 94.9 kcal/mol for monomethyl and dimethyl lysine, respectively.

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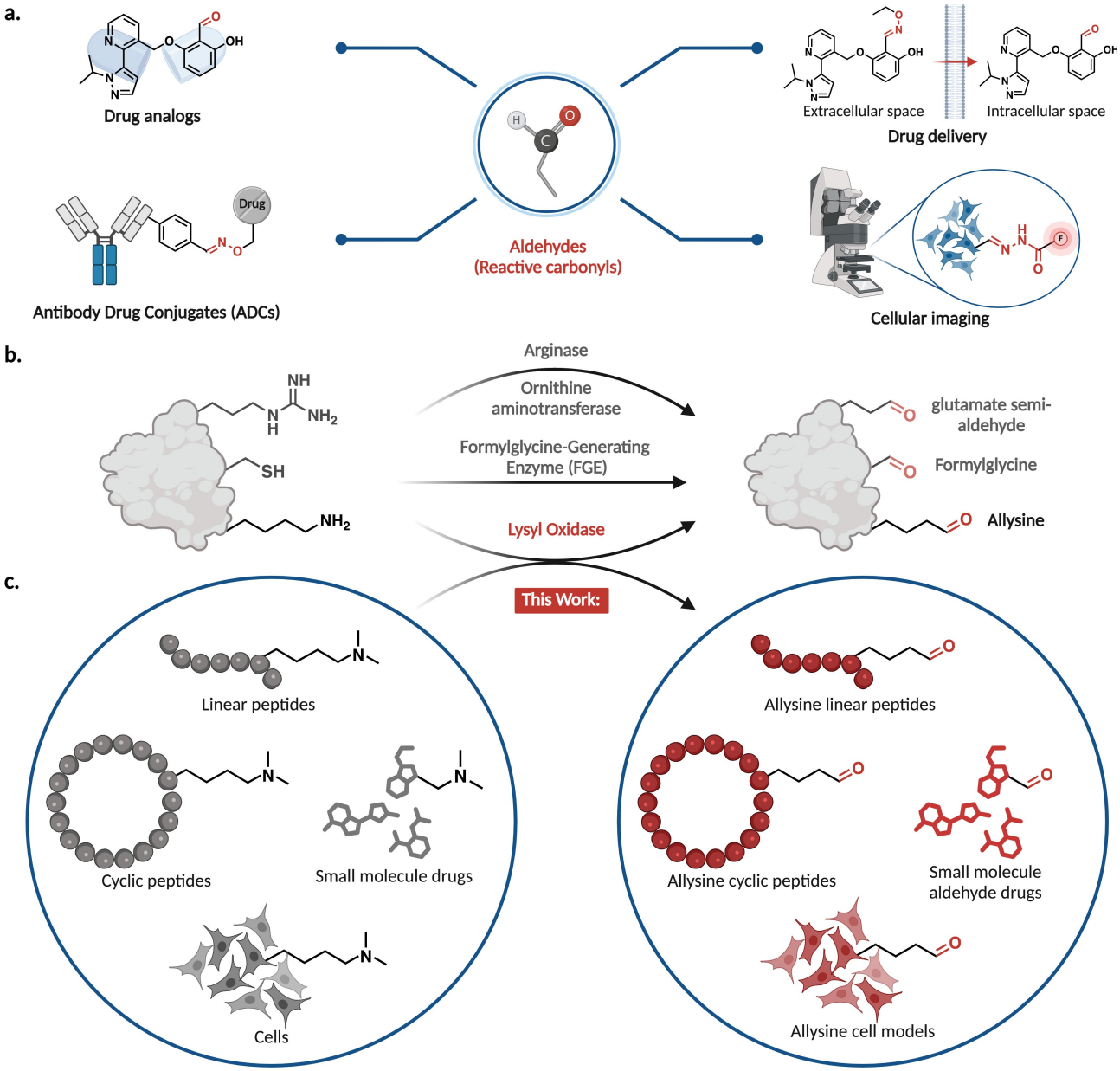

Figure 1. Chemical approach for the synthesis of allysine. **a**. Aldehydes as reactive handles in reversible covalent drugs, drug delivery systems, antibody-drug conjugates, and cellular imaging and tracking of biochemical processes. **b**. Enzyme-mediated generation of aldehydes at selective amino acids. **c**. This work: A chemical approach for the synthesis of aldehydes by selective modification of the side chain of dimethyl lysine and tertiary amines.

Furthermore, the free energy for the formation of the iminium ion intermediate from lysine was significantly higher (165 kcal/mol), than monomethyl lysine Kme_1 (160 kcal/mol) and dimethyl lysine Kme_2 (158 kcal/mol). Analysis of the change in the electrostatic potential (ESP) of C=N bonds clearly showed a net negative charge on lysine (−0.998) and monomethyl lysine Kme_1 (−0.355), while a significant positive charge was observed for dimethyl lysine Kme_2 (+0.209), suggesting the potential of Kme_2 to generate reactive electrophilic iminium ions. Taken together, these computational observations encouraged us to use dimethyl lysine Kme_2 for chemical oxidation to allysine. Therefore, we evaluated the oxidation of dimethyl lysine in a model peptide LLDVLLKme_2 **1a** by screening varying oxidizing reagents such as NADP+, Selectfluor, hydrogen peroxide (H_2O_2) and photoreactive Rose Bengal under physiological conditions (Figure S3). Interestingly, we observed the formation of a small amount of allysine-peptide **2a** (~5 % conversion) with Selectfluor (Figure 2c). We hypothesized that the formation of allysine-peptide **2a** was observed with Selectfluor only because of its ability to generate a DABCO-like base in situ, that enables the hydrolysis of more substituted iminium ion generated by the oxidation of Kme_2.[14]

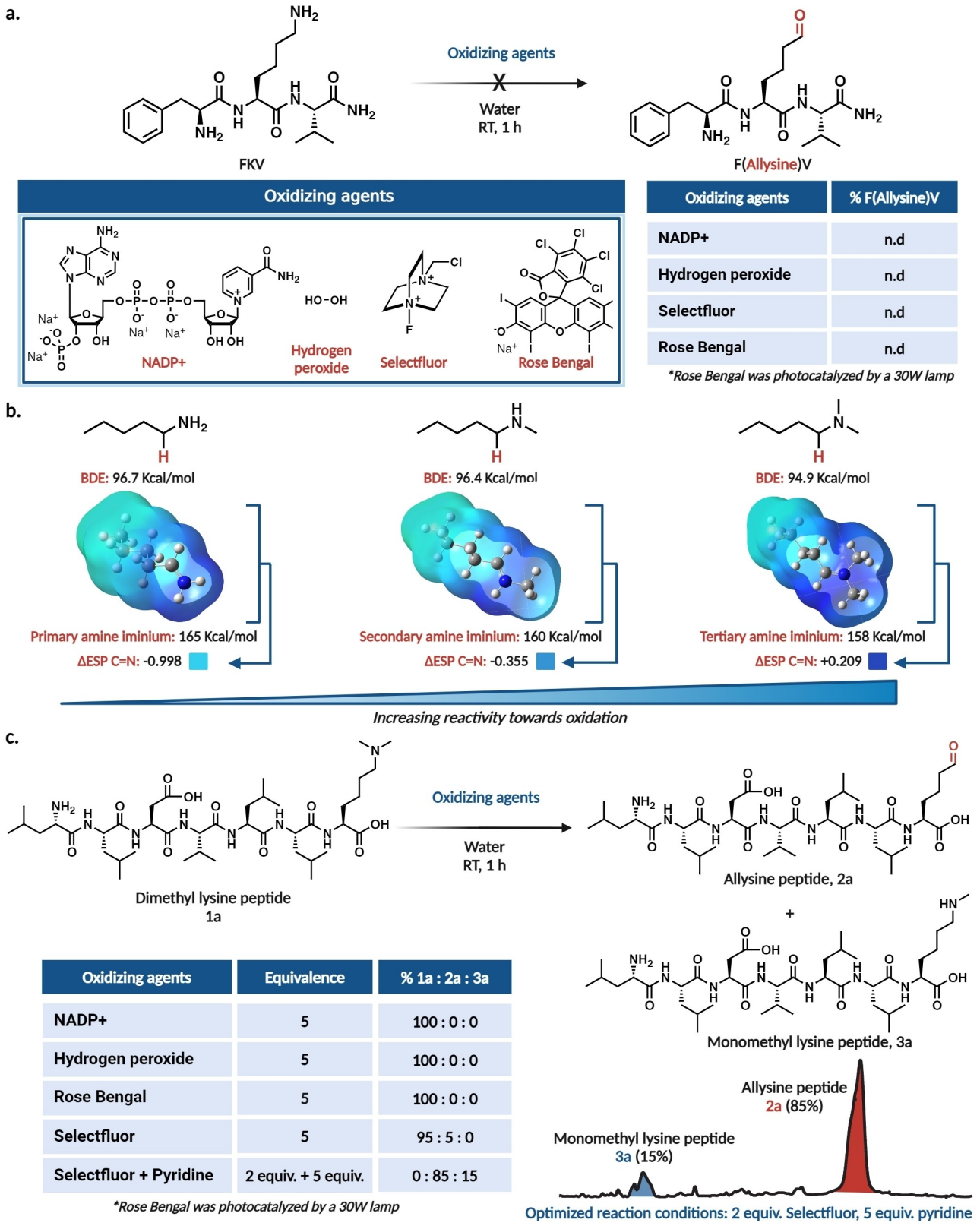

Figure 2. Development of a chemical method for generating allysine by selective oxidation of dimethyl lysine. **a**. Oxidation of lysine to allysine in peptide FKV. No reaction was observed with various oxidizing agents. **b**. Density functional theory (DFT) calculations of the intrinsic reactivity of lysine, and the methylated analogs such as monomethyl lysine and dimethyl lysine, highlight the suitability of dimethyl lysine to generate allysine by oxidation. **c**. Optimization of dimethyl lysine oxidation to allysine through screening of different oxidizing agents. Selectfluor was observed to be the best oxidant and the addition of pyridine significantly enhanced the formation of allysine.

To increase the hydrolysis of the iminium ion, we added a stronger base such as pyridine and observed a significant increase in the conversion to allysine-peptide **2a** (85 %) as analyzed by LCMS. We also observed the formation of small amounts of monomethyl lysine (15 %) due to the hydrolysis of the less-substituted iminium ion (Figure 2c, Figure S3).

Chemoselectivity and Substrate Scope for Modification of Linear and Cyclic Peptides

With the optimized conditions in hand, we next explored the synthesis of allysine on several linear peptides of different sizes and amino acid compositions with Kme_2 at varying positions. Using Fmoc-solid-phase peptide synthesis,[15] we synthesized several bioactive linear peptides such as LILKme_2PF **1b**, RLPYMPYGGKme_2G **1c**, LDKme_2VNR **1d**, FSDKKIKme_2K **1e**, AKme_2GSKAF(Pra)A **1f**, VFKme_2RN **1g**, and LKme_2VPFLKme_2VPF **1h** some of which exhibit anticancer **1c**,[16] anti-inflammatory **1d**,[17] antimicrobial **1e**[18] and antihypertensive **1g**[19] properties and subjected them to the optimized conditions. The dimethyl lysine Kme_2 in all the peptides converted to allysine (CHO) **2b**–**2g** with high conversions (58–89 %) along with the formation of small amounts of monomethyl lysine peptides **3b**–**3g** (11–42 %) without the formation of any byproducts with reactive amino acids including Lys, Ser, Asp, Asn and Pra (propargyl glycine) (Figure 3a, Figure S4). However, the oxidation of methionine to sulfoxide was observed with peptide **1c**. Interestingly, peptide LKme_2VPFLKme_2VPF **1h** containing two Kme_2 generated the double modified allysine product **2h** (37 %) and a single modified allysine product **2h'** (43 %) by the selective oxidation of both Kme_2, along with small amount of their monomethyl analog **3h** (~20 %). Together, these results confirmed the high chemoselectivity and pan-specificity of this chemistry in generating allysine from dimethyl lysine, making this approach ideal for the late stage functionalization of cyclic peptides.[20] To achieve this goal, we proceeded with Kme_2-analogs of varying bioactive cyclic peptides containing different amino acid compositions and ring sizes (18–30 atoms) such as Phepropeptin cyc(LILKme_2PF) **1i**,[21] a proteasome inhibitor, Vasopressin cyc-(CYFNQCPRKme_2)-disulfide **1j**,[22] an antidiuretic hormone (ADH), Baceridin cyc(IKme_2WLLV) **1k** a proteasome inhibitor inducing apoptosis in tumor cells,[23] cyc(FSKRKme_2PFES) **1l** and Gramicidin S cyc(LKme_2VpfLKme_2VPF) **1m** an antibiotic,[24] and incubated them under optimized conditions (Figure 3b, Figure S5). All the cyclic peptide drugs (**1i**–**1m**) regardless of the ring size and composition generated allysine-cyclic peptides (**2i**–**2m**) from Kme_2 in high conversions (60–99 %) along with the small amounts of monomethyl lysine-cyclic counterparts (**3i**–**3m**) (1–40 %, Figure 3b, Figure S5). Interestingly, Vasopressin cyc(CYFNQCPRKme_2) **1j** containing a disulfide bridge at the site of cyclization successfully formed allysine-containing cyclic peptide **2j** with very high conversion (99 %) without any ring opening due to the cleavage of the disulfide bond, however free thiol containing FCV was readily oxidized to disulfide and sulfenic acid that regenerated the free thiol peptide upon addition of tris(2-carboxyethyl)phosphine (TCEP) (Figure S5). With Baceridin cyc(IKme_2WLLV) **1k** containing Trp, we also observed the fluorination of the Trp side chain in the allysine-cyclic peptide **2k** (60 %) and monomethyl lysine-cyclic peptide **3k** (40 %, Figure 3b, Figure S5). Similar to the linear peptide, Gramicidin S containing two Kme_2 cyc(LKme_2VDFLKme_2VPF) **1m**, generated a mixture of di-allysine-cyclic peptide **2m** (52 %), and mono-allysine cyclic peptide **2m'** (38 %) along with small amounts of corresponding monomethyl lysine cyclic counterparts **3m** (~10 %) (Figure 3b, Figure S5).

Substrate Scope for Modification of Small Molecules

Since this approach is highly selective for Kme_2, which is a tertiary amine, we extended its application to install aldehyde handles in complex small molecules containing tertiary amines at the last step for converting non-covalent inhibitors to covalent inhibitors because of the ability of aldehyde handle to form reversible bonds with biomolecules.[25] We preceded our studies with a simple small molecule 4-phenyl *N,N*-dimethyl butylamine **1n** and obtained the high yield of the corresponding 4-phenyl butanal **2n** (80 %) (Figure 3c, Figure S6). The reaction with *N,N*-dimethyl benzylamine **1o** generated the corresponding benzaldehyde **2o** in a very high yield (88 %) (Figure 3c, Figure S6). Next, we applied this reaction to generate bioactive aromatic aldehyde, vanillin **2p** a key flavoring agent in sweets,[25] in high yields (84 %) by the selective oxidation of a corresponding tertiary amine-containing small molecule **1p**. Finally, we utilized this approach for the selective modification of a complex small molecule citalopram hydrogen bromide **1q** containing tertiary amine, a serotonin reuptake inhibitor used as an antidepressant for the treatment of anxiety,[26] to the corresponding aldehyde **2q** in high yields (93 %, Figure 3c, Figure S6). Notably, the presence of reactive functional groups in citalopram such as an aromatic nitrile and a complex three-dimensional structure comprising a fused tetrahydrofuran ring remains unaffected under the reaction conditions. All the small molecule aldehyde products were characterized by NMR and HRMS (Figure S6).

Late-Stage Diversification of Peptides

With the allysine installed on peptides, we further diversified them with varying chemoselective reactions using oxime chemistry, hydrazone chemistry, reductive amination and thiazolidine chemistry.[10–11] We diversified a linear peptide **2h** containing two allysine groups by reactions with aminoxy-functionalized molecules, *o*-2-propynylhydroxylamine and *o*-(2,3,4,5,6-pentafluorobenzyle)hydroxylamine in 2 % acetic acid in water and observed the full conversion (> 93 %) to double oxime-conjugation products **4a** and **4b** at

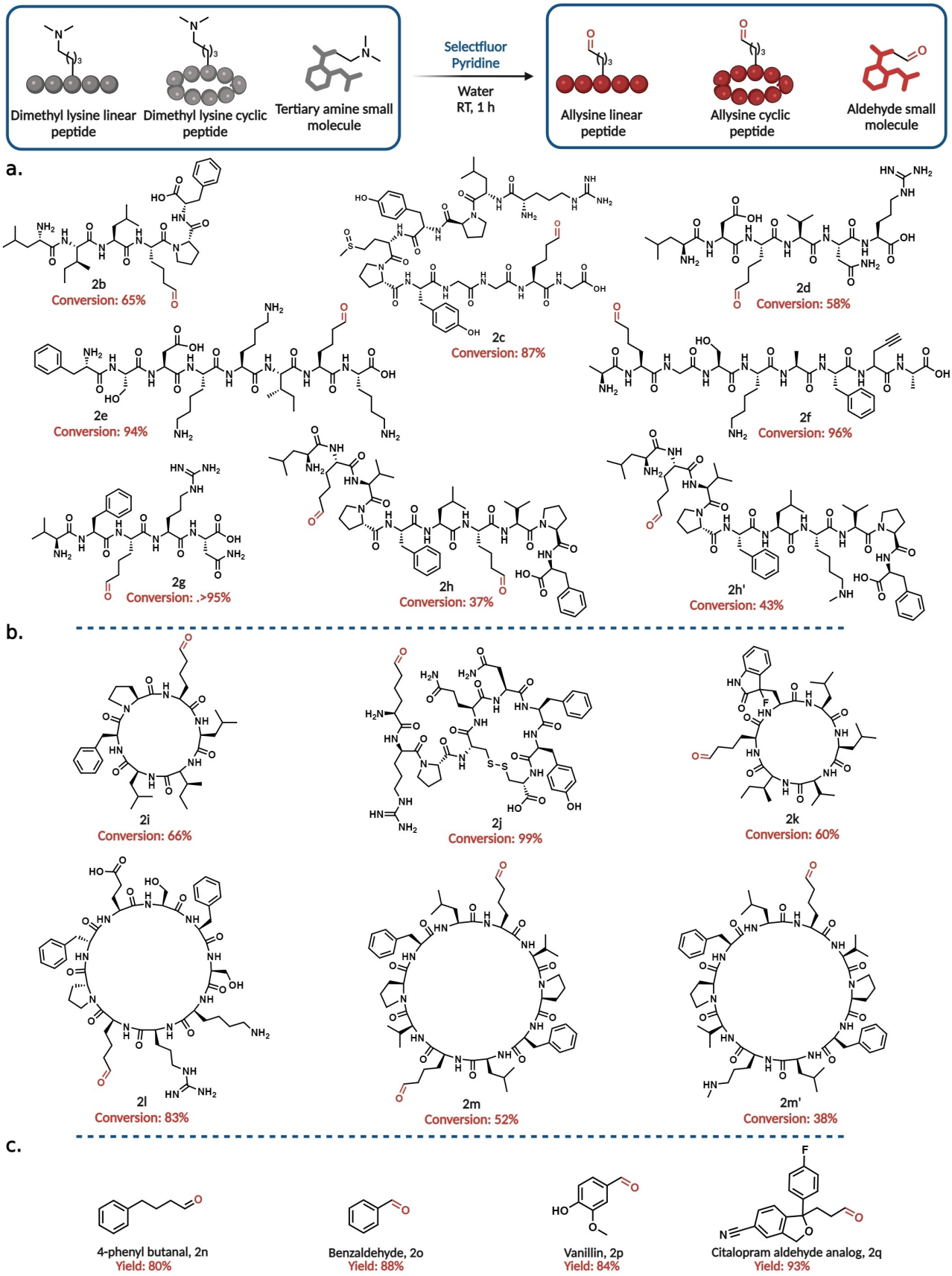

Figure 3. Substrate scope of a biomimetic approach for generating aldehydes by selective oxidation. **a**. Selective modification of bioactive linear Kme_2-containing peptides to allysine. **b**. Selective modification of bioactive cyclic Kme_2-containing peptides to allysine analogs. **c**. Selective modification of small molecules containing tertiary amines to aldehydes.

both the allysine as analyzed by LCMS (Figure 4a, Figure S7) under the reaction conditions.

Next, we modified allysine-containing linear peptide **2f** with o-2-propynylhydroxylamine and observed a single oxime-conjugation product **4c** in high conversion (98 %). The reaction of a linear peptide **2f** with hydrazide-functionalized molecules, such as biotin hydrazide and dansyl hydrazide in 2 % acetic acid in water generated corresponding mono-modified hydrazone-products **4d** (57 %) and **4e** (73 %) (Figure 4b, Figure S7).[11] We next modified allysine peptide **2f** by carrying out reductive amination with propargyl amine[13] and observed the formation of alkyne ligated-peptide product **4f** (67 %) (Figure 4b, Figure S7). The functionalization of allysine peptides **2f** and **2d** with cysteine methyl ester in 2 % acetic acid in water generated thiazolidine-modified peptides **4g** and **4h** in full conversion (~94 %) (Figure 4c, Figure S7).[12b]

Solid Support Synthesis of Allysine

To further demonstrate the robustness of the bioinspired chemical method for the selective formation of allysine on a Kme_2-side chain, we attempted the reaction on a solid support. Initial attempts focused on the modification of resin-bound ($WKme_2A$) peptide on polystyrene-based rink amide resin using Selectfluor and pyridine did not lead to a decent conversion to the allysine peptide (< 10 %).

We reasoned that the utilization of water-soluble resins would significantly improve the conversion to aldehyde. To evaluate this, we synthesized a peptide $FKme_2HV$ **1r** on a water-soluble rink amide PEGA resin and subjected it to Selectfluor and pyridine and observed the modification of dimethyl lysine peptide **1r** to allysine peptide **2r** in high conversion 87 % in 6 h (Figure 5a, Figure S8). With the optimized reaction conditions on a solid support, we further modified PEGA resin-bound Kme_2-tetrapeptides containing

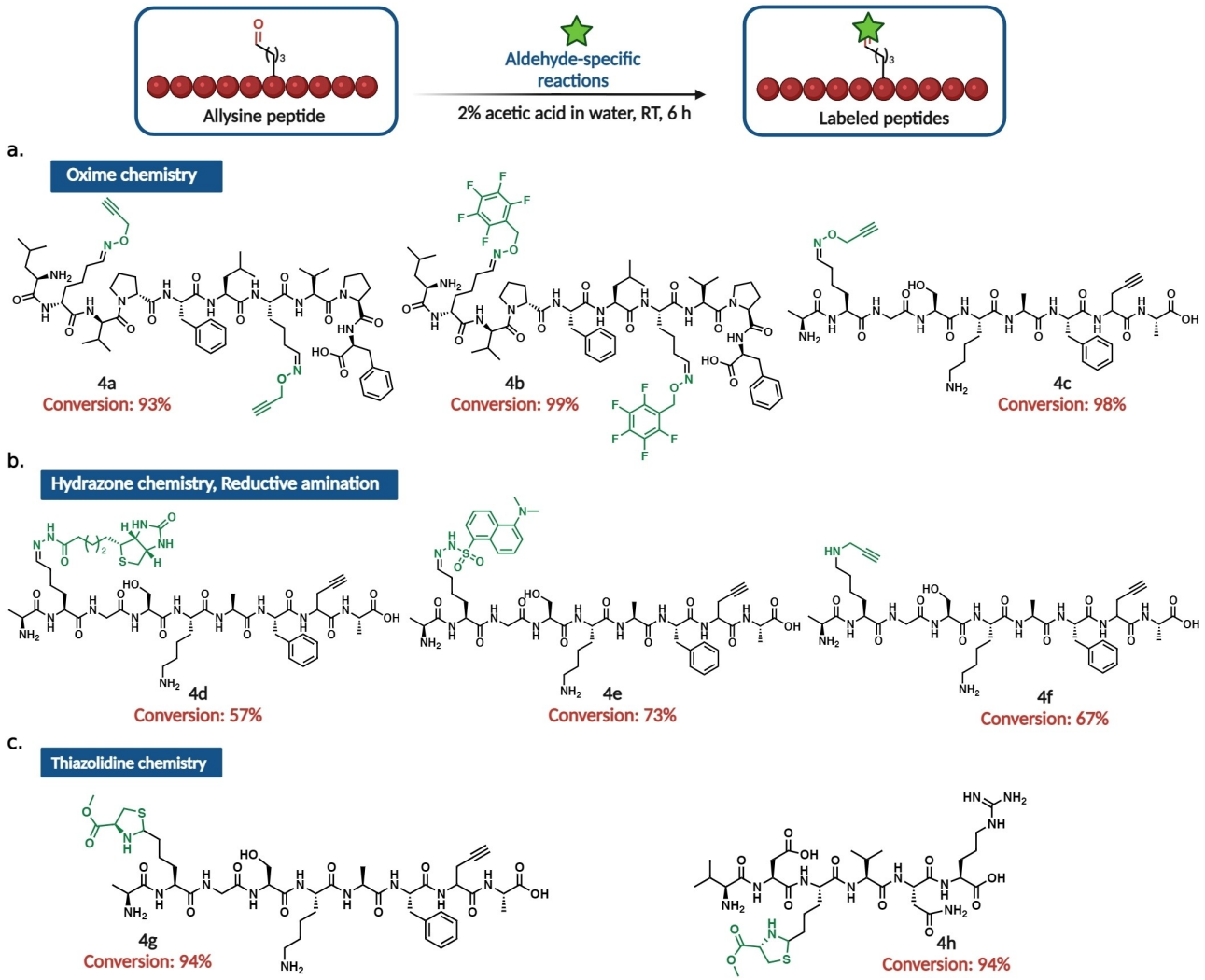

Figure 4. Late-stage derivatization of allysine peptides with aldehyde-specific reactions. **a**. Derivatization of allysine peptides with *o*-2-propynylhydroxylamine hydrochloride and *o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride to generate oxime modified peptides. **b**. Derivatization of allysine peptides via hydrazone and reductive amination chemistries using biotin hydrazide, dansylhydrazine, and propargylamine. **c**. Thiazolidine functionalization of allysine peptides using cysteine methyl ester.

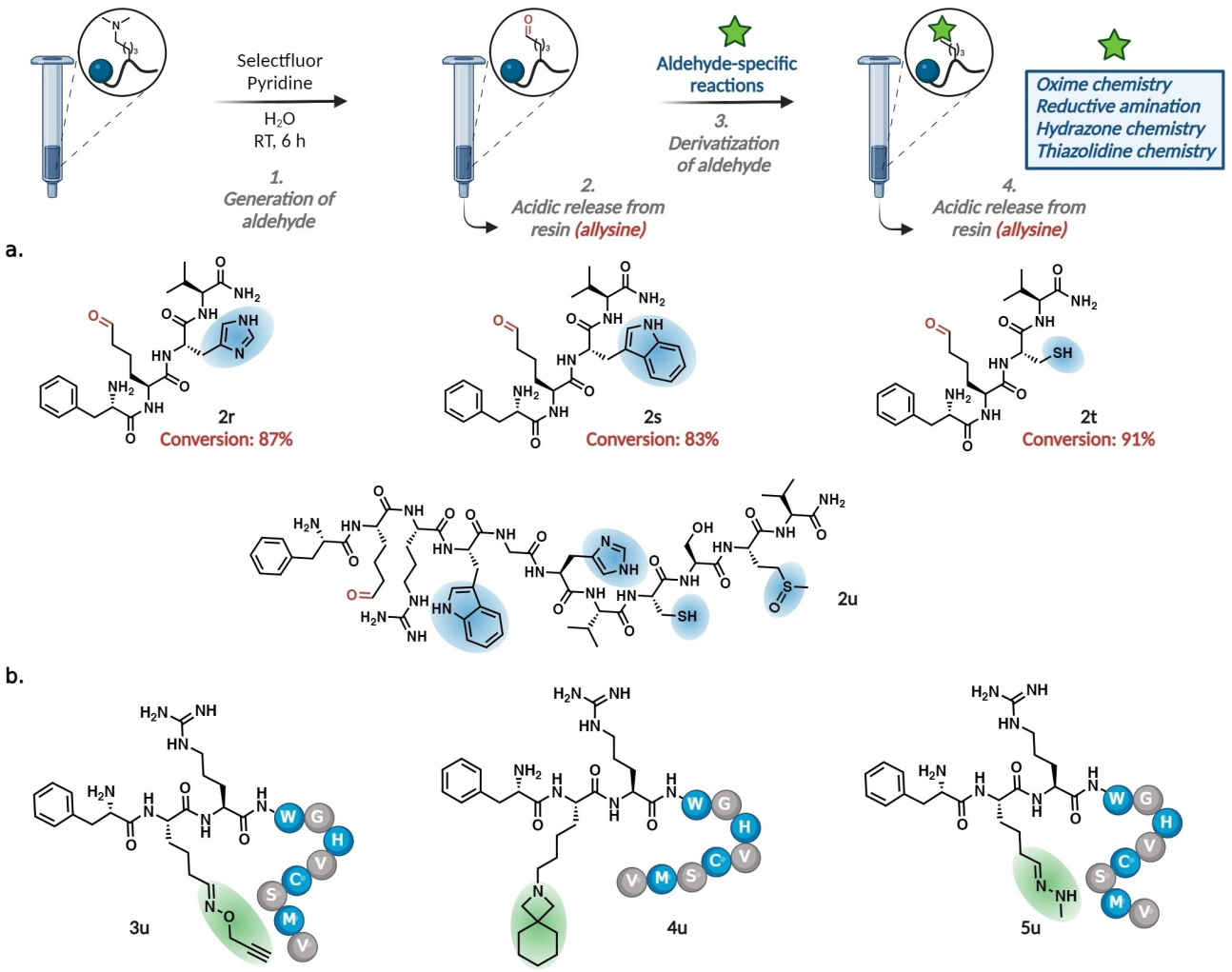

Figure 5. On resin synthesis of allysine by selective oxidation of dimethyl lysine. **a**. Selective conversion of Kme_2 to aldehyde without fluorination and oxidation of sensitive residues, such as histidine, tryptophan and cysteine. **b**. On-resin derivatization of allysine peptides using oxime chemistry, reductive amination, and hydrazone chemistry.

oxidizable groups, tryptophan ($FKme_2WV$) **1s**, and cysteine ($FKme_2CV$) **1t** to allysine peptides ($FKme_2(CHO)WV$, 83 %) **2s** and ($FKme_2(CHO)CV$, 91 %) **2t** in high conversions without any oxidation and fluorination of Trp and Cys, (Figure 5a, Figure S8). Notably, peptide $FKme_2RWGHVCSMV$ **1u** containing all the oxidizable residues, generated allysine peptide **2u** exclusively as analyzed by LCMS without the formation of any fluorinated and oxidized byproducts which contrasts with the solution phase reaction generating fluorinated product with Trp and oxidation of cysteine. This is mainly due to the protecting groups on Trp and Cys on a solid support. To demonstrate the utility of the solid-support pipeline in late-stage diversification of peptides, we further functionalized resin-bound allysine peptide FK(CHO)RWGHVCSMV **2u**, with *o*-2-propynylhydroxylamine hydrochloride, 2-azaspiro-[3.5]nonane hydrochloride, and methylhydrazine (Figure 5b, Figure S9). The cleavage from the resin led to the direct synthesis of derivatized peptides **3u**, **4u** and **5u** exclusively without modification of any other side chains as analyzed by LCMS (Figure 5b, Figure S9).[27]

Cellular Imaging of a Peptide-Fluorophore Conjugate

To demonstrate the utility of allysine handles on peptides for selective labeling with a fluorophore for tracking inside cells, we conjugated Alexafluor-647 hydroxylamine dye to allysine linear peptide **2b** followed by imaging the fluorophore labeled peptides inside cells (Figure 6a, Figure S10).

Incubation of peptide-fluorophore conjugate with T47D cells for 12 h followed by confocal imaging clearly showed the delivery of peptide-fluorophore conjugate into the cells (Figure 6a, Figure S10). No fluorescence was observed with negative control samples without the peptide-fluorophore conjugate.

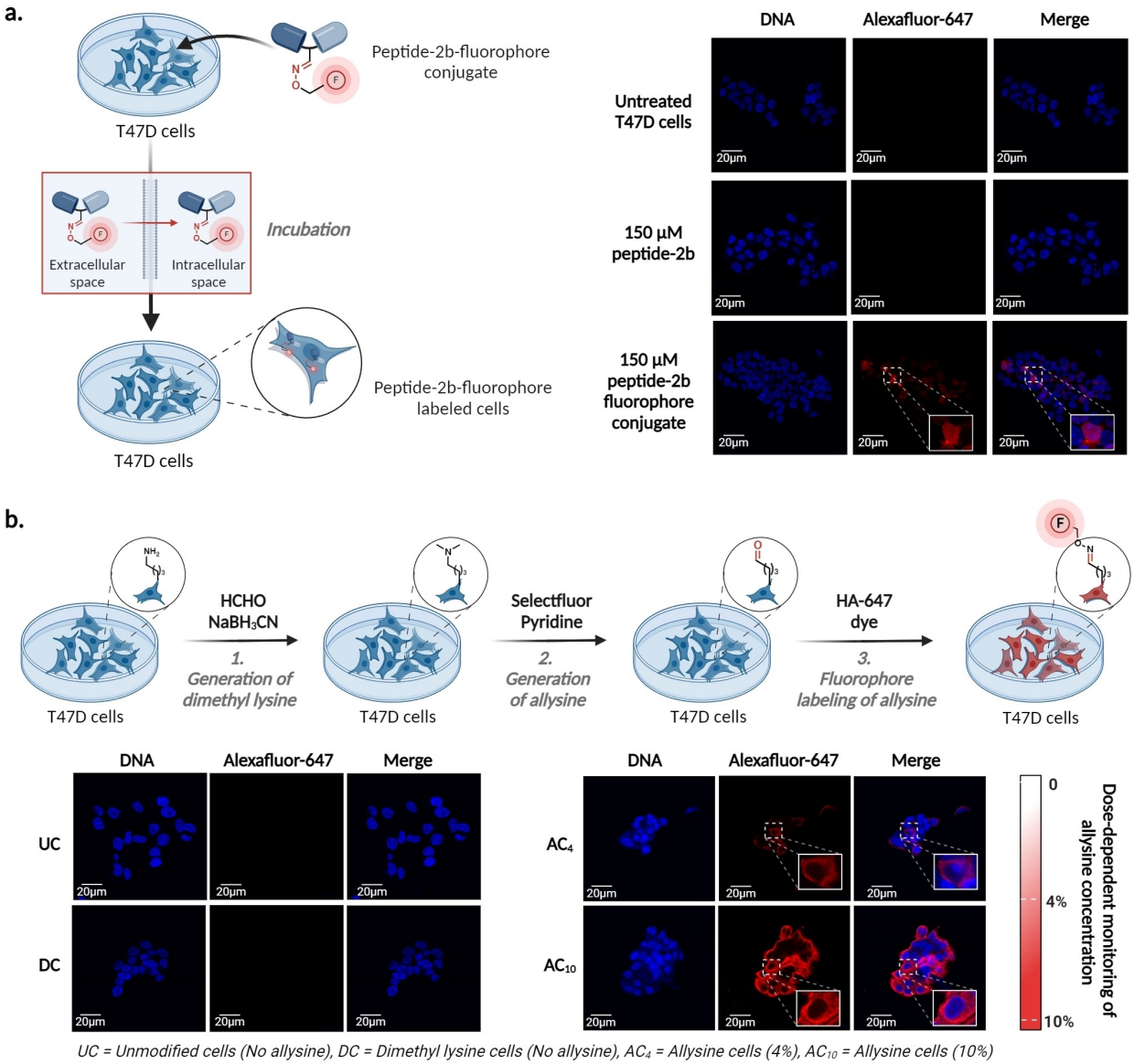

Figure 6. Cellular imaging of allysine using aldehyde-reactive fluorophore. **a**. Cellular tracking of the peptide-fluorophore conjugate in T47D cells. **b**. Concentration dependent cellular imaging of allysine proteins using hydroxylamine-647 dye. No fluorescence signal was observed in cells with no allysine protein. A dose-dependent increase in fluorescence signal was observed for cells exhibiting allysine proteins (4% to 10%).

Cellular Imaging of Allysine Proteins

Understanding the molecular basis of allysine impact on the production and extracellular maturation of connective tissues, and its pivotal role in pathological conditions such as cancer, fibrosis, and diabetes is of utmost interest and represents a relatively understudied research area.[28–30] This is largely due to the lack of appropriate model systems for overexpressing allysine. To generate cellular models for studying allysine-associated diseases, we chemically generated dimethyl lysine in breast cancer cells (T47D) in a dose-dependent manner under reductive amination conditions followed by the modification of dimethyl lysine proteins in cells using Selectfluor and pyridine to allysine proteins. The treatment of allysine proteins in cells with hydroxylamine-647 dye led to a dose-dependent fluorescent signal response thus indicating the presence of different concentrations of allysine proteins in the different cell populations (Figure 6b, and Figure S11). Interestingly, no fluorescent signal was observed in unmodified cells and cells with dimethyl lysine thus indicating that the observed signal is due to the presence of allysine. Further, spatiotemporal visualization of allysine adducts will lead to an increase in our understanding of allysine-mediated cellular events.

Conclusion

Inspired by the enzyme lysyl oxidase, we have developed a biomimetic chemical approach for the generation of allysine,

an aldehyde in which an amine of lysine is replaced with a formyl group, by selective oxidation of dimethyl lysine Kme_2. The reaction is highly chemoselective and modifies only Kme_2 to allysine in the presence of all the reactive amino acid residues. This is the first approach that selectively converts the side chain of an amino acid into an aldehyde, which is in contrast to other chemical methods limited to the N- or C-terminus. We have demonstrated the application of this chemistry for the late-stage generation of allysine on therapeutically relevant linear and cyclic peptides. We showed the successful site-selective modifications of allysine-peptides with a variety of different tags, such as fluorescent dyes and affinity tags, using hydroxylamine, hydrazine, thiolamine and reductive amination. Notably, we extended the application of this approach for the synthesis of small-molecule aldehydes by the late-stage selective modification of corresponding tertiary amine small-molecule drugs. We also demonstrated a robust solid-support pipeline for the synthesis of allysine on peptides without the modification of oxidation sensitive residues, such as histidine, tryptophan, and cysteine. Lastly, we showed the application of this biomimetic approach for the generation of cellular models with overexpressed allysine. Owing to the high chemoselectivity and mild reaction conditions required for transforming tertiary amines into orthogonal aldehyde handles, this reaction will find multiple applications in various fields, such as medicinal chemistry, proteomics, biochemistry, material chemistry and the synthesis of antibody-drug conjugates ADCs.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Aldehydes · Dimethyl lysine · protein modification · Chemoselectivity · Late-stage modification

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Research Articles

Protein Modifications

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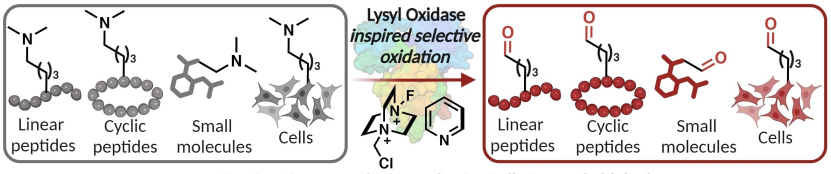
Bioinspired Synthesis of Allysine for Late-Stage Functionalization of Peptides



Biomimetic approach for synthesis of allysine and aldehydes

A biomimetic approach was developed for the synthesis of allysine by selective oxidation of the side chain of dimethyl lysine. The reaction exhibits broad substrate scope and modifies dimethyl lysine and tertiary amines to form aldehydes in small molecules, peptides, proteins and cells.