SCS Swiss Chemical Society

HELVETICA
chimica acta

Accepted Article

Title: Chemoselective derivitization of folded synthetic insulin variants with potassium acyltrifluoroborates (KATs)

Authors: Gabor N. Boross, Dominik Schauenburg, and Jeffrey W. Bode

To be cited as: *Helv. Chim. Acta* 10.1002/hlca.201800214

Link to VoR: http://dx.doi.org/10.1002/hlca.201800214

10.1002/hlca.201800214

HELVETICA

Chemoselective derivitization of folded synthetic insulin variants with potassium acyltrifluoroborates (KATs)

Gábor N. Boross[a], Dominik Schauenburg [a] and Jeffrey W. Bode*[, a, b]

[a] Laboratorium für Organische Chemie, Department of Chemistry and Applied Biosciences, ETH–Zürich, 8093 Zürich, Switzerland (bode@org.chem.ethz.ch)

[b] Institute of Transformative bio-Molecules (WPI–ITbM), Nagoya University, Chikusa, Nagoya 464-8602, Japan

Dedicated to Professor François Diederich

Synthetic folded insulin variants containing an ornithine-hydroxylamine residue are readily modified in aqueous buffers by amide-forming ligations with potassium acyltrifluoroborates (KATs). The synthetic insulin analogs were prepared by Fmoc-SPPS, α-ketoacid–hydroxylamine (KAHA) ligation, and a prosthetic C-peptide that delivers the correct disulfide pattern and allows facile incorporation at the Bo position of Glargine M2 of a new ornithine hydroxylamine protected with a photolabile group. The folded insulin is readily modified by photo-deprotection followed by amide-forming KAT ligation to give insulin variants labeled with dyes, lipids, and PEGs as well as the formation of a covalent dimer.

Keywords: chemoselective ligation, insulin, protein modification, amide-formation, acylboronates

Introduction

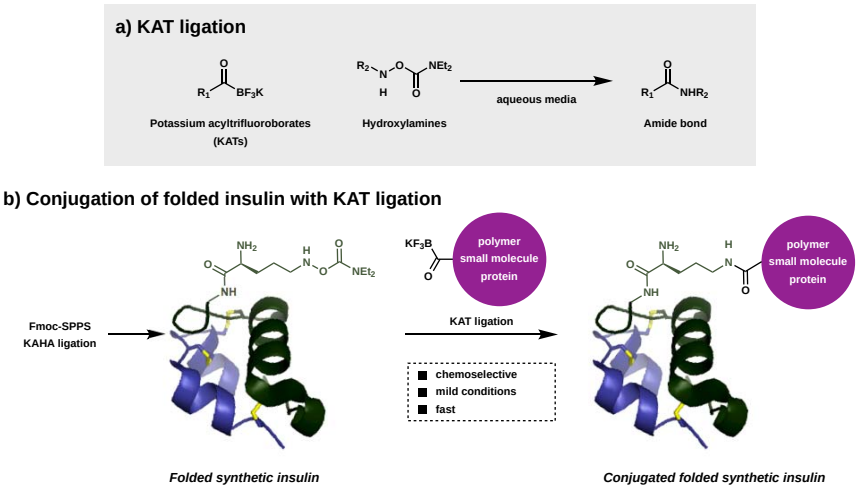
Peptides and proteins are key medicines for the treatment of human diseases. While early protein therapeutics were largely replacement therapies based on isolated or recombinant variants of the natural proteins, it is now widely recognized that modifications including PEGylation, lipidation, glycosylation, or fusion to other proteins is advantageous and often required for safety and dosing. For example, most peptides and small proteins currently used in medicine have modifications to improve the half-life and stability.[1]

Chemistry and chemical biology offer several approaches to improve the unsatisfactory physiochemical properties of peptides and proteins. Incorporation of non-canonical amino acids or modifications at the C- or N-termini of the polypeptide chain can lead to increased stability against peptidases.[2] Attachment of polyethylene glycol (PEG) units to the peptide can also contribute to the enzymatic stability, tune the solubility and extend the circulating time.[3] Introduction of *N*-methylated amino acids can also improve the pharmacokinetic properties.[4] Lipidation of peptides and proteins can increase the blood plasma half-life leading to prolonged therapeutic effect and also increase membrane permeability or retention.[5]

A handful of conjugation techniques have been developed for introduction of pendant groups to peptide and protein therapeutics. Despite the enormous synthetic effort invested in this field, most of the reported methods rely on the inherent reactivity of lysine and cysteine residues (e.g. thiol conjugation by maleimides). These approaches often limit the ability to effect the conjugation in a controlled and selective fashion, as most proteins contain multiple lysine and cysteine residues. Site-specific modifications of other residues including tyrosines,[6] arginines,[7] and methionines[8] have been reported, but these are better suited for labeling or introducing a handle for a subsequent conjugation reaction such as Cu-promoted Click reactions. [9] The recombinant incorporation of unnatural amino acids by a variety of methods provides access to small amounts of proteins with suitable conjugation handles,[10] but is still limited by the paucity of chemoselective ligation handles that operate on folded proteins at micromolar concentrations.[11] The Sortase A enzyme – from *Staphylococcus aureus* – becomes an important tool for enzymatically ligating synthetic or expressed proteins and biomolecules together.[12]

These methodologies provide an indispensable toolkit for chemical–biologists for gaining understanding of basic biological processes.[13] However, there is still an unmet synthetic need for a reliable, amide-forming method that can be broadly applied for the preparation of conjugates.

10.1002/hlca.201800214



Scheme 1. a) General scheme of the KAT ligation. **b)** Schematic concept of protein modification via KAT ligation.

As part of a larger program aimed at rapid, chemoselective amide forming reactions that operate at low concentrations in aqueous buffers with near equimolar amounts of the ligation partners, our group has developed the coupling of potassium acyltrifluoroborates (KATs[14]) and *O*-carbamoylhydroxylamines – known as KAT ligation (Scheme 1. a).[15,16] We demonstrated that this ligation proceeds in a chemoselective fashion in the presence of all common, unprotected amino acid side chains and is fast enough to operate at low micromolar concentrations. The KAT ligation was successfully applied in PEGylation, lipidation, biotinylation and dye labeling of peptides bearing a synthetically incorporated hydroxylamine.[17] More recently, we reported the PEGylation and dimerization of expressed proteins using KAT ligation; in this case the hydroxylamines were attached to a surface exposed cysteine using a labeling reagent.[18]

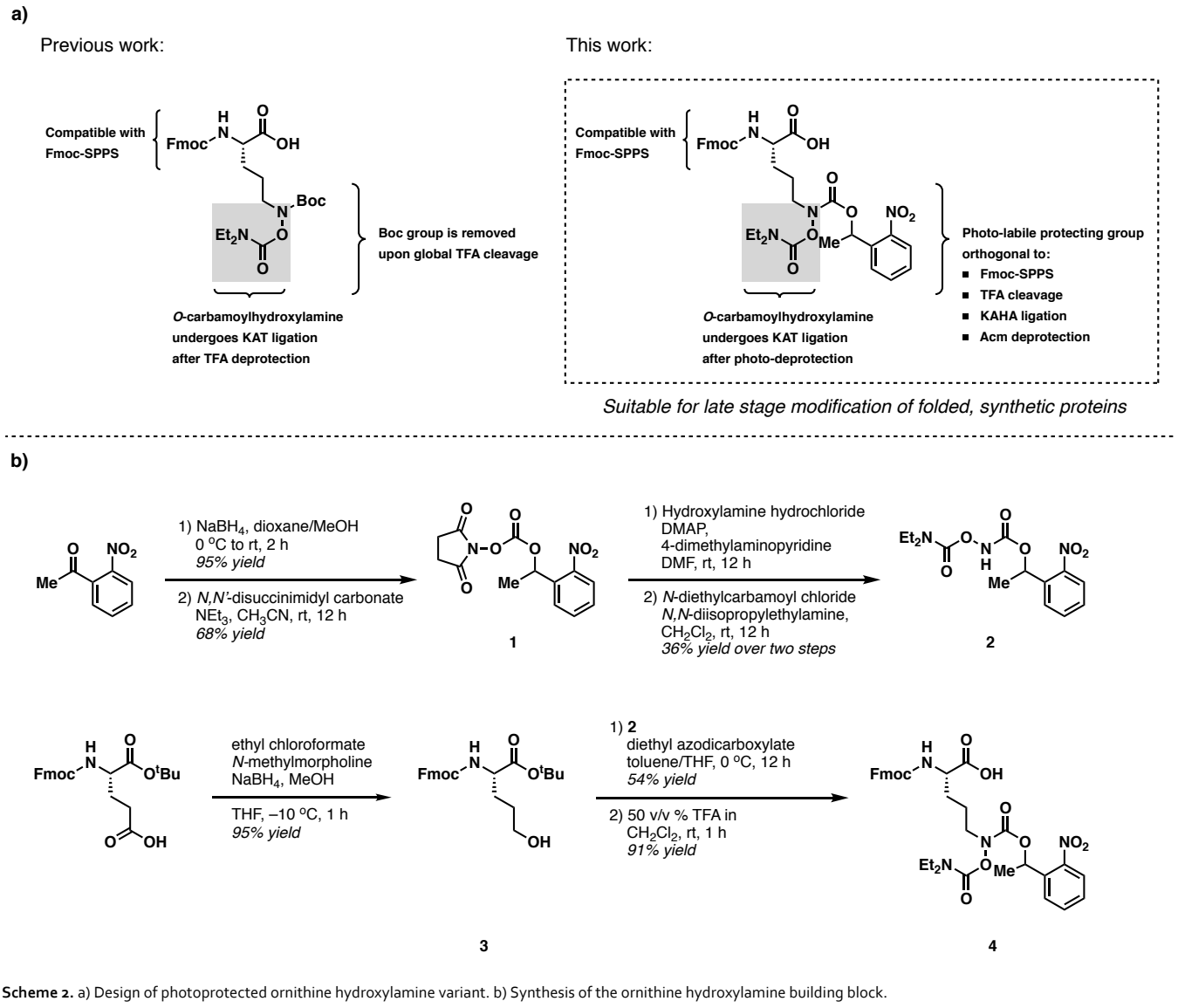
In this report, we document the preparation of a synthetic, folded protein bearing a protected ornithine hydroxylamine, which following facile deprotection with UV light undergoes conjugation with KAT reagents (Scheme 1. b). Advances in chemical protein synthesis make it competitive to expression for the production of small proteins (less than 200 residues) and allows the facile incorporation of non-canonical residues. Key to the success of this approach was the identification of a suitable, protected hydroxylamine side chain, as unprotected *O*-carbamoylhydroxylamines are unsuitable for protein synthesis by α-ketoacid–hydroxylamine (KAHA) ligations and are somewhat unstable under basic conditions. In contrast, the photocaged variant proved to be completely stable to all standard manipulations for resin cleavage, peptide purification, assembly with KAHA ligation, Acm deprotection with silver, and protein folding. This finding allowed facile construction of synthetic folded Glargine M2 insulin variant by KAHA ligation[19] and its conjugation to a variety of KATs suitable for protein labeling, PEGylation, lipidation, and dimerization.

Results and Discussion

Synthesis of ornithine with photoprotected side chain hydroxylamine

We previously reported the synthesis of a Fmoc-protected ornithine derivative with a side chain Boc-hydroxylamine (Scheme 2. a) which readily undergoes amide-forming KAT ligation after peptide synthesis and trifluoroacetic acid (TFA) cleavage.[17] We choose to prepare ornithine hydroxylamine rather than lysine hydroxylamine, simple due to its shorter synthesis route. This building block could be rapidly incorporated into peptides by Fmoc-SPPS, however the unprotected hydroxylamine formed after resin cleavage precluded its use in protein synthesis by segment coupling using KAHA ligation. We also found the unprotected form to be somewhat unstable to basic buffers and lyophilization. Encouraged by success with other photoprotected hydroxylamines and α-ketoacids for peptide assembly by KAHA ligation,[20] we designed a photoprotected variant of the Fmoc-ornithine hydroxylamine (**4**) (Scheme 2 a). This building block would stay protected during SPPS and TFA cleavage, allowing the assembly of longer peptides by KAHA ligation and further synthetic steps, such as Acm-deprotection and protein folding. By UV irradiation at 365 nm the photoprotecting group can be removed and the resulting free *O*-carbamoylhydroxylamine can undergo KAT ligation.

10.1002/hlca.201800214



Scheme 2. a) Design of photoprotected ornithine hydroxylamine variant. b) Synthesis of the ornithine hydroxylamine building block.

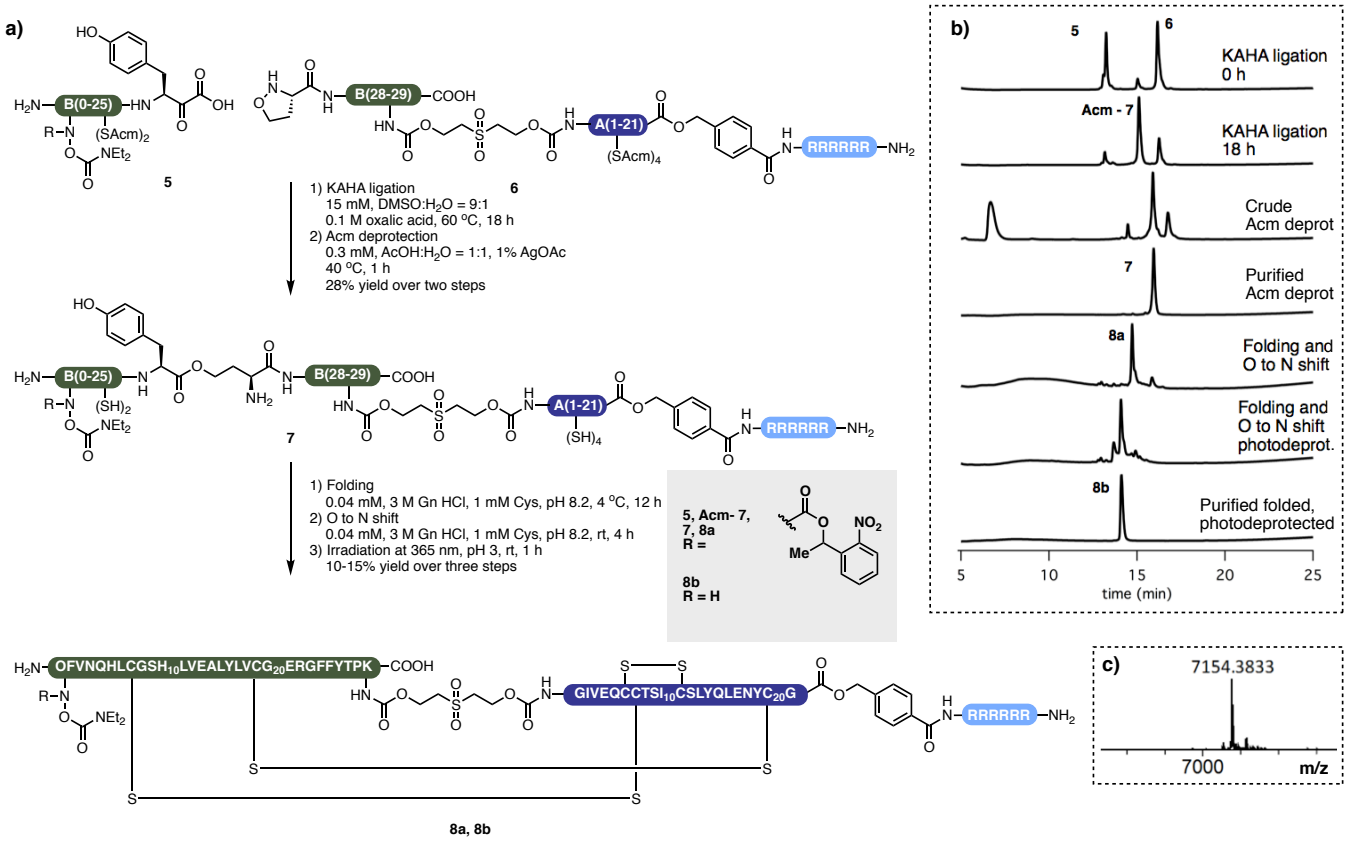
We initiated the synthesis of the ornithine hydroxylamine building block (**4**) with the reduction of the commercially available 2-nitroacetophenone (Scheme 2 b). The resulting racemic alcohol (**S1**) was isolated on a gram scale and activated with *N,N'*-disuccinimidyl carbonate followed the procedure of Thuaud *et. al.*[20] Hydroxylamine hydrochloride was *N*-acylated utilizing the activated carbonate (**1**), prepared in the previous step, and *O*-acylated using *N*-diethylcarbamoyl chloride to give hydroxylamine **2** with the appropriate functionalities. Fmoc-Glu(OH)O^{t}Bu was transformed to the corresponding side-chain alcohol (**3**) in excellent yield.[21] In the key step of the synthesis the *N*- and *O*-acylated hydroxylamine (**2**) was attached to the side-chain of **3** via a Mitsunobu reaction in satisfactory yield.[22] Finally, the ^{t}Bu ester was removed by TFA treatment to give the desired hydroxylamine building block (**4**).

Synthesis of Glargine M2 insulin with a photoprotected ornithine hydroxylamine

Although there are a myriad of different insulins on the market, there is constant interest in novel insulins with altered therapeutic properties including PEGylated variants or lipidated insulins.[23] The Friedman group developed photoactive depots for recombinant insulin storage and controlled, light mediated release.[24] Most recently Seebach *et. al.* described an elegant enzymatic modification for the N-terminal amino acids of insulin chains.[25] These works contribute to the methods for preparing modified insulins, but there is still an unmet need for a reliable method that could be introduced to fine-tune insulin in a selective and efficient fashion.

Our group recently reported a convergent, modular synthesis for the homoserine (Hse) variants of insulins (ThrB27Hse), which allows the on-demand incorporation of any additional functionality into the amino acid sequence.[26] Importantly, ThrB27Hse replacement showed no detriment to the biological activity across a number of different insulin variants.

10.1002/hlca.201800214



Scheme 3. Synthesis of M2 Glargine insulin variant equipped with ornithine side chain hydroxylamine on the Bo position. Mutation: ThrB27Hse.

We utilized our established synthesis for the preparation of the pepide segments **5** and **6**. The ornithine hydroxylamine building block was coupled onto the insulin B-chain at the Bo position (Scheme 3). The peptide segment could be cleavage from resin and purified without decomposition or premature deprotection of the hydroxylamine moiety. We decided to place the conjugation site at the Bo position because it is far from the biologically active region of insulin and offers an orthogonal alternative to the commonly modified LysB29 position.

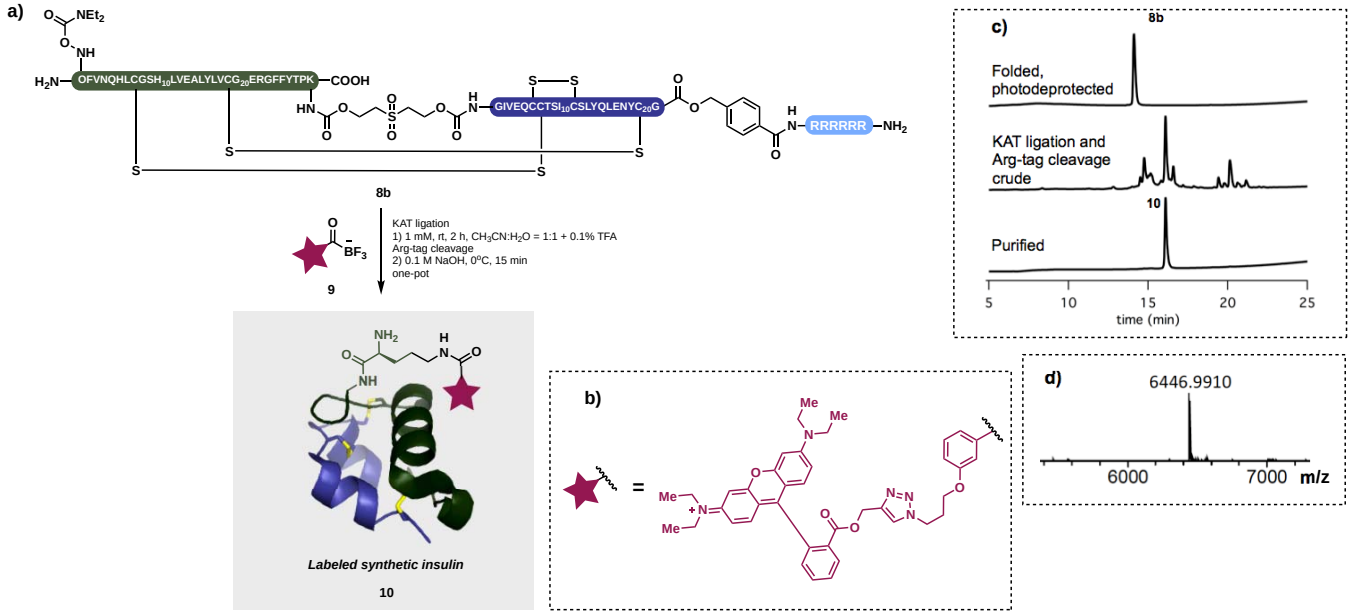
With the required peptide segments in hand (73 mg of α-ketoacid segment **5** and 85 mg (*S*)-5-oxaproline segment **6**) we began protein assembly with KAHA ligation. We were pleased to see that the established conditions (60 °C in a mixture of DMSO/H_2O containing 0.1 M oxalic acid over 18 h) worked well and gave the ligated peptide (Acm-**7**), which was submitted to Acm-deprotection without purification. The folding precursor (**7**, 42 mg) was obtained in 28% yield over two steps after RP-HPLC purification. The reduced linear depsi insulin, containing the photoprotected hydroxylamine functionality was folded and rearranged to the amide in one-pot fashion. Compared to the already published synthetic Glargine M2 insulin, we observed precipitation during the folding and O to N shift. We attribute this to the increased hydrophobicity of the peptide due to the aromatic protecting group. In order to improve handling and solubility of the insulin in both its protected and unprotected form, we elected to retain the C-terminal arginine tag, which can be readily cleaved at a later stage. UV irradiation of **8a** for 60 minutes at 40 μM converted the ornithine side chain to the free *O*-carbamoylhydroxylamine functional group. **8b** was isolated on semi-preparative RP-HPLC, typically in 10–15% yields over three steps.

Conjugation of folded Glargine M2 insulin hydroxylamine by KAT ligation

Hydroxylamine **8b** serves as the key structure for rapid functionalization of the folded insulin under dilute, aqueous, acidic buffers that are ideal for handling this highly hydrophobic protein. The unique features of the KAT ligation – extremely fast reaction rates, the formation of amide bonds, and equimolar ligation stoichiometry – makes it perfectly suited for conjugation of diverse groups including dyes, lipids, and PEGs.

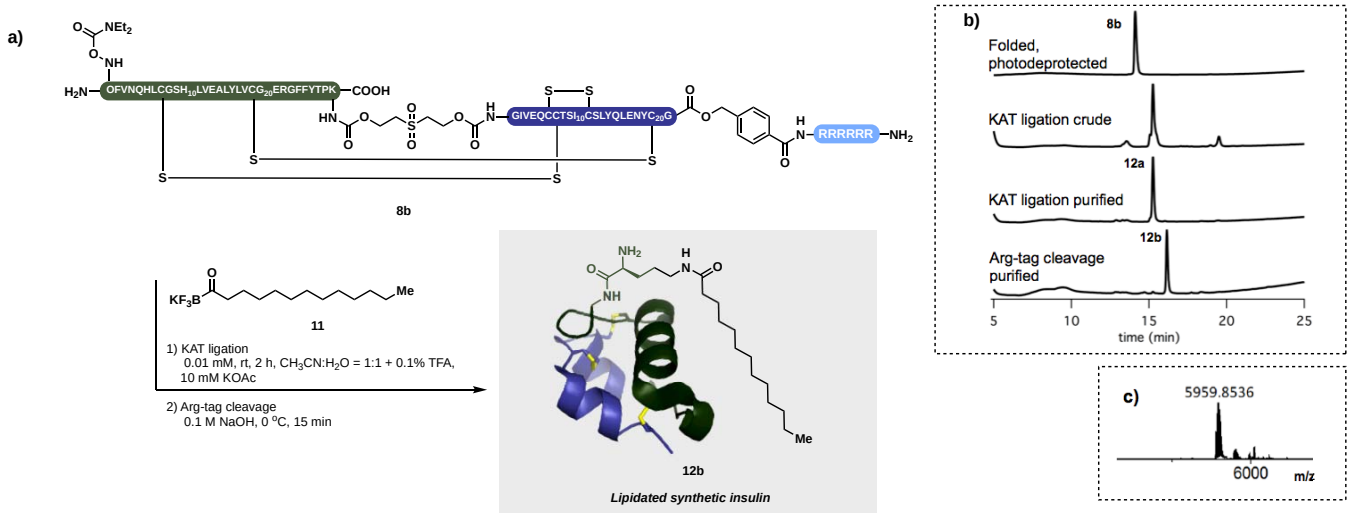
Conjugation with KAT Dye. The labeling of peptides and proteins with fluorescent dyes is an important tool for many biotechnological applications including fluorescence microscopy and fluorescence correlation spectroscopy. One of the most commonly used dyes is rhodamine, due to its red shifted fluorescence (λ_{ex} 553 nm; λ_{em} 627 nm in methanol) and high fluorescence quantum yield (0.98)[27,28].

10.1002/hlca.201800214



Scheme 4. a) Preparation of rhodamine labeled insulin variant. **b)** Structure of the rhodamine dye. **c)** HPLC trace of the KAT ligation. **d)** HR-MS trace of the final product **10**.

We performed KAT ligation with 1.5 equivalents of rhodamine KAT reagent **9** in an aqueous CH_3CN mixture with 0.1 v/v% TFA at room temperature. After 2 hours the reaction was cooled to 0 °C and treated with aqueous NaOH. The basic treatment led to the cleavage of the Arg-tag and the linkage between the insulin A- and B-chain (prosthetic C-peptide). The crude KAT ligated and base cleavage product was directly purified by RP-HPLC to give rhodamine labeled Glargine M2 insulin (ThrB27Hse) (**10**) (Scheme 4).

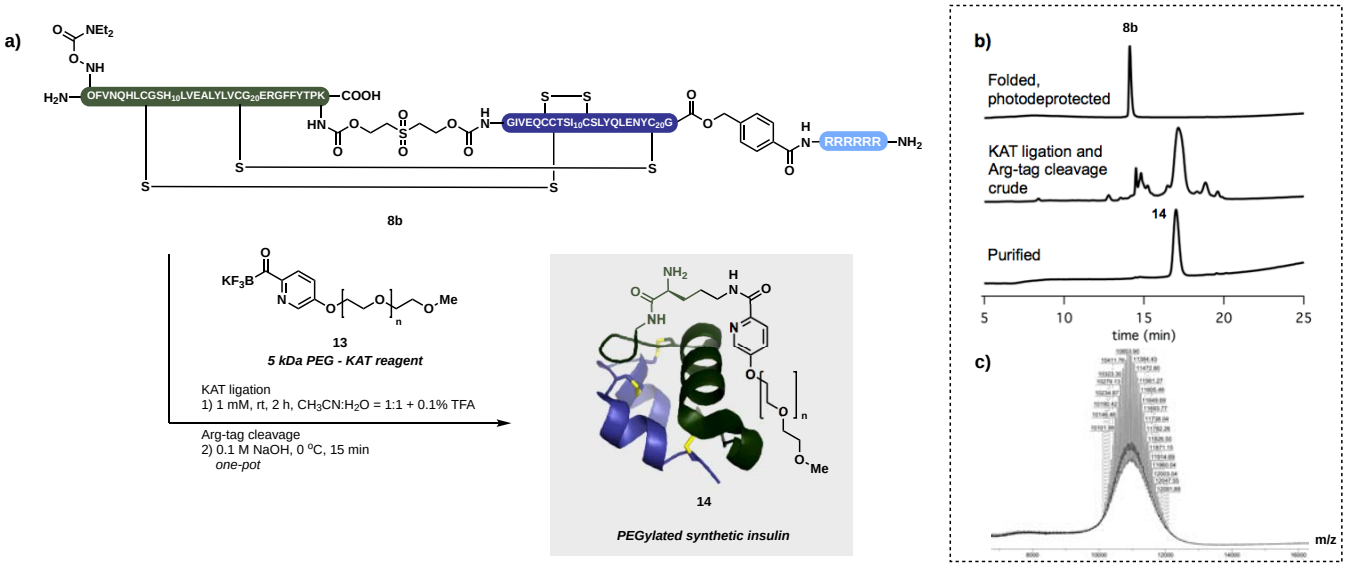


Scheme 5. a) Preparation of lipidated insulin variant **b)** HPLC trace of the KAT ligation. **c)** HR-MS trace of the final product **12b**.

Lipidation of insulin with a KAT lipid. Lipidation of insulin is one of the most efficient ways to achieve a prolonged time-action profile, due to the slow absorption from the site of injection into the circulation and half-life non-covalent albumin binding.[29]

We anticipated that a lipidated insulin variant would be highly hydrophobic and difficult to keep in aqueous buffers even at 1 mM concentration, the conditions at which the rhodamine labeling was established. Therefore, we decided to perform the KAT ligation at approximately 10 μM concentration directly in the aqueous acetonitrile obtained from HPLC purification of the previous synthetic step. The reaction mixture was buffered by the addition of potassium acetate and acetic acid (pH 2–3). Myristic acid KAT reagent (11) (2.0 equiv) was added from a DMF stock solution. After 2 hours of gentle agitation at room temperature the KAT ligation was complete. The ligated, single chain insulin (**12a**) was isolated and converted to the final, lipidated two chain insulin (**12b**) by treatment with base (Scheme 5).

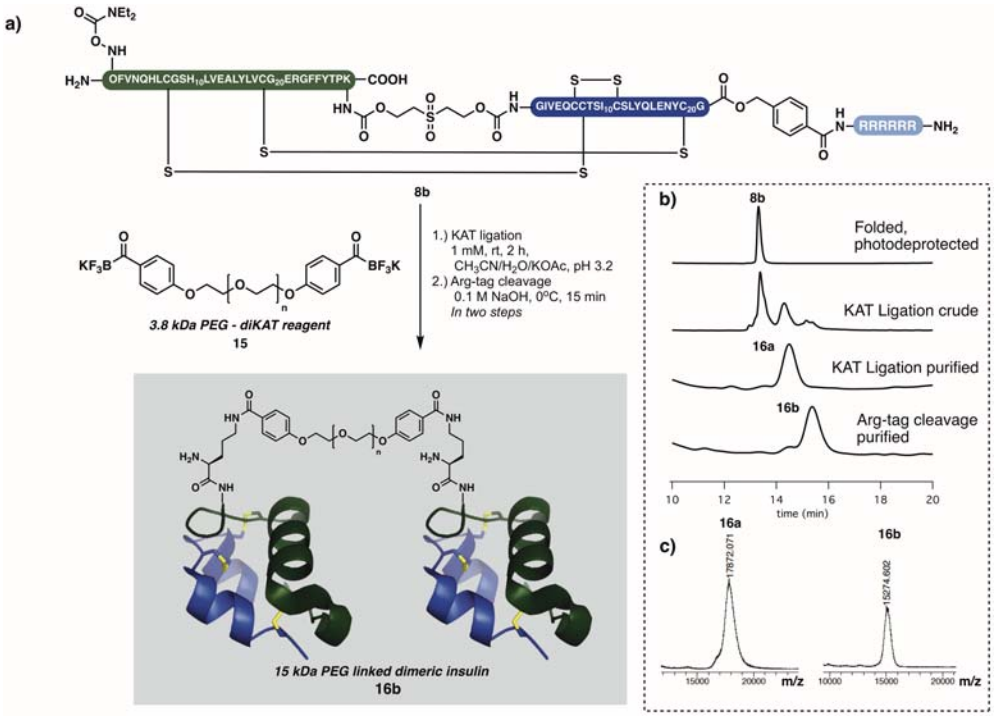
10.1002/hlca.201800214



Scheme 6. a) Preparation of PEGylated insulin variant **b)** HPLC trace of the KAT ligation. **c)** HR-MALDI trace of the final product **14**.

PEGylation of insulin hydroxylamine. PEGylation of insulin has been shown to lower the immunogenicities accompanying insulin treatment and slow the clearance of the peptide, resulting in a prolonged therapuetic effect. Furthermore, PEGylation on the N-teminus of the B chain has been shown to increase the physical stability to a higher degree than PEGylation on the Lys B29 side chain.[30] We wanted to demonstrate that our approach can offer alternative sites for selective PEGylation of insulin, compared to the Lys side chain amino group, as was employed on the recently discontinued peglispro.[31]

In contrast to lipidation, PEGylation typically improves the solubility of proteins; accordingly, the PEG modification of insulin could be conducted at 1 mM concentration. The protein was dissolved in aqueous acetonitrile containing 0.1 v/v% TFA. Two hours after the addition of the PEG_{5000} KAT (**13**) reagent[18] the mixture was treated with aqueous NaOH. The analytical HPLC showed the formation of the PEGylated insulin and the removal of the Arg-tag in a one-pot fashion (**14**). The broad peak on the analytical HPLC and on the mass spectrum is attributed to the heterodispersity nature of the PEG reagent (Scheme 6).



Scheme 7. a) Preparation of dimerized insulin variant. **b)** HPLC trace of the KAT ligation. **c)** MALDI trace of the intermediate **16a** and of the final product **16b**.

Synthesis of PEG-linked insulin dimer. It is well known that the most stable form of insulin is hexameric. The nascent protein associates into dimers and trimers of these dimers constitute the stable hexamers.[32] It is believed that the major consequence of these non-covalent insulin dimers is the stabilization of the peptide during storage. In 2012, a covalently linked dimeric insulin – via an additional disulfide bond (B25) close to the binding region – was reported, which showed increased physical stability but lacked biological activity.[33] We were interested in the preparation of covalent insulin dimers without interfering with the binding site (N-terminal region of the B chain).

For synthesis of a covalent insulin dimer linked at the Bo position, PEG bearing two KAT groups (diKAT) was required. We attempted the dimerization using a tetraethylene glycol diKAT (**S3**) derivative. Unfortunately, we were not able to observe the formation of insulin dimer. Reasoning that the short linker length may negativly affect the dimerization, we choose a longer PEG_{3500} diKAT for another attempt at covalent dimer formation. With this heterodisperse diKAT (**15**) dimerization of folded, synthetic insulin was achieved with nearly equimolar ratio of reaction partners (1 equivalent of diKAT **15** and 2.6 equivalents of insulin **8b**). Due to difficulties during the HPLC purification of the dimerized intermediate (**16a**), the ligation and removal of the Arg-tag was performed in two separate steps. After HPLC purification of the final product, dimeric two-chain insulin (**16b**) was isolated (Scheme 7).

Conclusions

In summary, we reported the synthesis of a new photoprotected hydroxylamine amino acid and its incorporation into synthetic insulin. It is stable to Fmoc-SPPS, final cleavage, KAHA ligation, Acm-deprotection and protein folding. It can be cleanly deprotected under mild conditions by irradiation with 365 nm UV-light. This amino acid was incorporated at the Bo position of a Glargine M2 insulin variant and the folded, synthetic protein was modified by amide-forming KAT ligation, with dyes, PEGs and lipids. Due to the fast kinetics of KAT ligation, the modification could be carried out in the isolated fractions from preparative HPLC under dilute conditions. We anticipate that the incorporation of this photoprotected hydroxylamine and subsequent late stage KAT ligation will be useful for the synthesis of other protein derivatives.

Experimental Section

The experimental details are available in the Supporting information.

Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/MS-number

Acknowledgements

We thank Simon Baldauf, Raphael Hofmann and Jakob Farnung for the helpful discussions Haewon Song for proofreading the manuscript, the MS and the NMR service of the ETH LOC for analyses and Sanofi for the financial support.

Author Contribution Statement

G.N.B., D.S., and J.W.B. designed the studies. G.N.B. and D.S. performed the experiments. G.N.B., D.S., and J.W.B. wrote the manuscript. G.N.B. and D.S. contributed equally to this work.

References

[1] D. Goodwin, P. Simerska, I. Toth 'Peptides as therapeutics with enhanced bioactivity', *Curr. Med. Chem.* **2012**, *19*, 4451–4461.
[2] L. T. Nguyen, J. K. Chau, N. A. Perry, L. de Boer, S. A. J. Zaat, H. J. Vogel, '*Serum stabilities of short tryptophan-and arginine-rich antimicrobial peptide analogs*', *PLOS ONE*, **2010**, *5*, 12684.
[3] G. H. Zhang, B. Z. Han, X. Y. Lin, X. Wu, H. S. Yan, 'Modification of antimicrobial peptide with low molar mass poly(ethylene glycol)', *J. Biochem.* **2008**, *144*, 781–788.
[4] J. Chatterjee, C. Gilon, A. Hoffmann, H. Kessler, 'N-methylation of peptides: A new perspective in medicinal chemistry', *Acc Chem. Res.* **2008**, *41*, 1331–1342.
[5] M. D. Resh, 'Covalent lipid modifications of proteins', *Curr. Biol.* **2013,** 23, R431–5.
[6] S. D. Tilley, M. B. Francis, 'Tyrosine-selective protein alkylation using π-allylpalladium complexes', *J. Am. Chem. Soc.* **2006**, *128*, 1080–1081.
[7] I. Dovgan, S. Erb, S. Hessmann, S. Uruegui, C. Michel, C. Muller, G. Chaubet, S. Cianférani, A. Wagner, 'Argininge-selective bioconjugation with 4-azidophenyl glyoxal: application to the single and dual functionalisation of native antibodies', *Org. Biomol. Chem.* **2018**, *16*, 1305–1311.

[8] S. Lin, X. Yang, S. Jia, A. M. Weeks, M. Hornsby, P. S. Lee, R. V. Nichiporuk, A. T. Iavarone, J. A. Wells, F. D. Toste, C. J. Chang, 'Redox-based reagents for chemoselective methionine bioconjugation', *Science*, **2017**, *335*, 597–602.
[9] C. P. R. Hackenberger, D. Schwarzer, 'Chemoselective Ligation and Modification Strategies for Peptides and Proteins', *Angew. Chem. Int. Ed.* **2008**, *47*, 10030–10074.
[10] K. Wals, H. Ovaa, 'Unnatural amino acid incorporation in E. coli: current and future applications in the designe of therapeutic proteins', *Front. Chem.* **2014**, *2*, 15.
[11] F. Saito, H. Noda, J. W. Bode, 'Critical evaluation and rate constants of chemoselective ligation reactions for stoichiometric conjugations in water' *ACS Chem. Biol.* **2015**, *10*, 1026–1033.

[12] A. W. Jacobitz, M .D. Kattke, J. Wereszczynski, R. T. Clubb, 'Sortase transpeptidase: Structural biology and catalytic mechanism', *Adv. Protein Chem. Struct. Biol.* **2017**, *109*, 223-264.
[13] E. M. Sletten, C. R. Bertozzi, 'Bioorthogonal chemistry: Fishing for selectivity in a sea of functionality', *Angew. Chem. Int. Ed.* **2009**, *48*, 6974–6998.
[14] G. A. Molander, J. Raushel, N. M. Ellis, 'Synthesis of an acyltrifluoroborate and its fusion with azides to form amides', *J. Org. Chem.* **2010,** *75*, 4304–4306.
[15] A. M. Dumas, J. W. Bode, 'Synthesis of acyltrifluoroborates', *Org. Lett.* **2012**,*14*, 2138–2141.
[16] A. M. Dumas, G. A. Molander, G. A., J. W. Bode, 'Amide-forming ligation of acyltrifluoroborates and hydroxylamines in water', *Angew. Chem. Int. Ed.* **2012**, *51*, 5683–5686.
[17] H. Noda, G. Erős, J. W. Bode, 'Rapid ligations with equimolar reactants in water with the potassium acyltrifluoroborate (KAT) amide-formation', *J. Am. Chem. Soc.* **2014**, *136*, 5611–561.
[18] C. J. White, J. W. Bode, 'Pegylation and dimerization of expressed proteins under near equimolar conditions with potassium 2-pyridyl acyltrifluoroborates', *ACS Cent. Sci.* **2018**, *4*, 197–206.
[19] J. W. Bode 'Chemical protein synthesis with the α-ketoacid–hydroxylamine ligation', *Acc. Chem. Res.* **2017**, *50*, 2104–2115.
[20] F. Thuaud, F. Rohrbacher, A. Zwicky, J. B. Bode, 'Photoprotected peptide α-ketoacids and hydroxylamines for iterative and one-pot KAHA Ligations: Synthesis of NEDD8', *Helv. Chim. Acta* **2016**, *99*, 868–894.
[21] S. Jiang, P. Li, C. C. Lai, J. A. Kelley, P. P. Roller, 'Design and concise synthesis of fully protected analogues of l-γ-carboxyglutamic acid', *J. Org. Chem.* **2006***, 71*, 7307–7314.
[22] K. Kitahara, T. Toma, J. Shimokawa, T. Fukuyama, '*O*-TBS-*N*-tosylhydroxylamine: A reagent for facile conversion of alcholols to oximes', *Org. Lett.* **2008**, *10*, 2259-2261.
[23] V. P. Sinha, S. Choi, K. Mace, K. P. Yeo, D. C. Howey, 'Single-dose pharmacokinetics and glucodynamics of the novel, long-acting basal insulin LY2605541 in healthy subjects', *J.Clin. Pharmacol.,* **2014**, *54*, 792–799.
[24] P. K. Jain, D. Karunakaran, S. H. Friedman, 'Construction of a photoactivated insulin depot', *Angew. Chem. Int. Ed.* **2013**, *52*, 1404 – 1409.
[25] B. Kolesinska, J. Wasko, Z. Kaminski, B. Geueke, H.-P. E. Kohler, D. Seebach, 'Labeling and protecting *N*-terminal protein positions by β-peptidyl aminopeptidase-catalyzed attachment of β-amino-acid residues – Insulin as a first example', *Helv. Chim. Acta*, **2018**, *101*, e1700259.
[26] G. N. Boross, S. Shimura, M. Besenius, N. Tennagels, K. Rossen, M. Wagner, J. W. Bode, 'Facile folding of insulin variants bearing a prosthetic C-peptide prepared by α-ketoacid-hydroxylamine (KAHA) ligation', *Chem. Sci.* **2018**, DOI:10.1039/C8SC03738H
[27] D. Setiawan, A. Kazaryan, M. A. Martoprawiro, M. Filatov, 'A first principles study of fluorescence quenching in rhodamine B dimers: how can quenching occur in dimeric species?', *Chem. Chem. Phys.*, **2010**, *12*, 11238.
[28] T. Karstens, K. Kobs, 'Rhodamine B and rhodamine 101 as reference substances for fluorescence quantum yield measurements', *J. Phys. Chem.* **1980,** *84*, 1871–1872.
[29] S. Havelund, A. Plum, U. Ribel, I. Jonassen, A. Vølund, J. Markussen, P. Kurzhals, 'The mechanism of protraction of insulin detemir, a long-acting, acylated analog of human insulin', *Pharm. Res.* **2004**, 21, 1498–1504.
[30] D. Kenneth, H. S. W. Kim, 'Effects of PEG conjugation on insulin properties', *Advanced Drug Delivery Reviews*, **2002**, 54 505–530.
[31] T. Hirose, 'Development of new basal insulin peglispro (LY2605541) ends in a disappointing result', *Diabetology International*, **2016**, *7*, 16–17.
[32] D. T. Birnbaum, M. A. Kilcomons, M. R. DeFelippis, J. M. Beals, 'Assembly and dissociation of human insulin and LysB28ProB29-insulin hexameres: a comparison study', *Pharm. Res.* **1997**, *14*, 25–36.
[33] N. T.Vinther, M. Norrmam, H. M. Strauss, K. Huus, M. Schlein, T. M. Pedersen, T. Kjeldsen, K. J. Jensen, F. Hubálek, 'Novel Covalently Linked Insulin Dimer Engineered to Investigate the Function of Insulin Dimerization', *PLOS One,.* **2012**, *7*, (2), e30882.

10.1002/hlca.201800214