Labeling and Protecting *N*-Terminal Protein Positions by β-Peptidyl Aminopeptidase-Catalyzed Attachment of β-Amino-Acid Residues – insulin as a first example

Preliminary communication

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We have shown for the first time that a natural protein (human insulin) can be acylated at the *N*-terminus with a β-amino acid (H-β^{3}hAla-), in a process catalyzed by the β-peptidyl aminopeptidase 3-2W4-BapA. This

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[2]) Parts of the projected PhD thesis of J. W. are described herein.

selective modification, which could also be applied for protein labeling and tagging, should be generally useful, also to protect peptides and proteins from attack by common aminopeptidases.

Keywords: post-translational protein modification, reversible enzymatic *N*-terminal H-(β^3hAla) attachment to a protein, β-peptidyl aminopeptidase 3-2W4-BapA, insulin, peptide synthesis, SSPS, coupling reagent DMT/NMM/TsO$^-$

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1. Introduction. - a) ***Post-Translational Protein Modifications***.- Enzymatic post-translational modifications of proteins, such as phosphorylation, sulfonylation, glycosylation, prenylation, methylation, epimerization *etc*. are most important processes in the chemistry of life. The targets for modifications are generally the OH-groups of Ser, Thr, Tyr, the SH group of Cys, the NH_2-groups of Lys and Arg, and the aromatic moieties of Tyr and Trp (Fig. 1) [1][3]). Enzymes catalyzing these reactions can be employed for modifications of proteins other than their natural substrates [3]. In addition, many *purely* chemical, site-selective modifications of native proteins have been discovered[4]).

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[3]) For a spectacular array of post-translational modifications of a protein (49 amino acids), discovered by genome mining of an uncultivated bacterium, see [2].

[4]) For an excellent review article entitled „Unglaublich chemoselektiv: Modifikation nativer Proteine“, with references to seminal contributions by *Waldmann, Davis, Francis, and Barbas,* see [4].

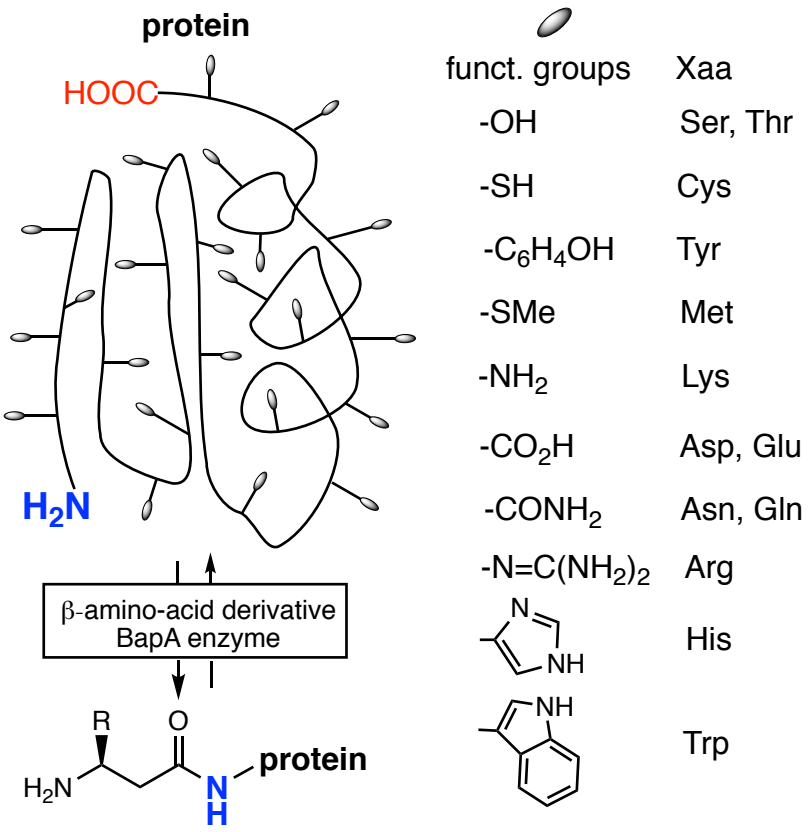
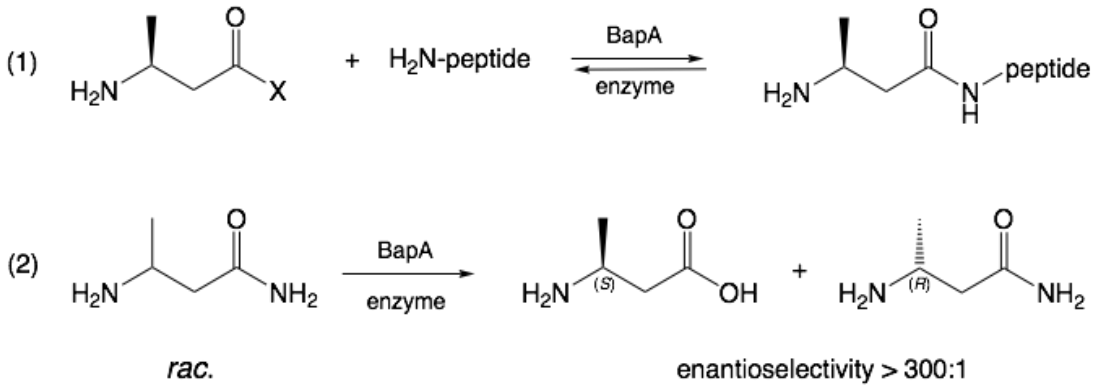


Fig. 1. Site-selective reactions of proteins containing many side-chain functional groups, and possible enzymatic *N*-terminal modification by attachment of a β-Amino acid (*vide infra*, experiments with insulin, Figs. 2-4, Tab.). Except for Met and Asp all the side-chain functional groups shown here are present in insulin.

Also, for labeling and tagging of certain amino-acid positions in proteins unnatural amino acids (UAAs), carrying azido- or alkynyl-groups [5] are incorporated (by common chemical peptide-synthesis methodology, or by UAA addition to the genetic code [6] of, for instance, *E. coli*), thus allowing for so-called bio-orthogonal couplings [7].

b) ***The β-Peptidyl Aminopeptidase 3-2W4-BapA***.- In the course of studies testing the enzymatic stability of β-peptides we discovered a microorganism (*Sphingosinicella xenopeptidilytica*, strain 3-2W4) that is able to survive with a simple β-tripeptide as sole carbon and nitrogen source; the enzyme BapA, cleaving the β-peptide was identified, isolated, expressed and processed in *E. coli* [8-11][5]. The enzyme BapA cleaves *N*-terminal β- but not α-amino-acid residues [12] from peptides, it was also shown to be able to attach β-amino-acid moieties to an α-aminoacid and to the *N*-termini of α-di- and α-tripeptides [13] (Eqn. (1)), and it generally prefers β^3-amino acid derivatives of (*S*)-configuration (Eqn. (2)) [14].



Since *N*-terminal β-amino-acid residues protect peptides from degradation by common amino-peptidases [15], it occurred to us that we could possibly perform a BapA-catalyzed acylation of a protein, for instance with β^3hAla (cf. Fig. 1), and thus make the protein more stable under physiological conditions.

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[5]) For an extensive review article, with emphasis on microbiological aspects, see especially reference [11].

c) ***Survey of Insulin Modifications***.- As an example, we chose human insulin, a protein consisting of 51 amino-acid residues, 24 of which have functionalized side chains (not counting 6 cysteins, see Fig. 1 and 2)). Furthermore, due to its role in the treatment of diabetes and concomitant diseases insulin is perhaps the protein, the chemistry of which has been investigated most intensively. Accordingly, the corresponding literature is vast, and it is impossible to give due attention to seminal contributions about insulin modifications, herein; for some more or less randomly chosen, more general review articles published in the last two decades see references [16-19]. The goal of investigations of insulin is, of course, to improve its bioavailability and activity profile, and to possibly make it orally available. Non-covalent modifications [17] of the insulin molecule as such have been performed, and the resulting formulations tested: the interactions exploited for improved or alternative administration modes and delivery systems include a kind of salt formation (for instance with the green-tea tannin component gallate EGCS [20], with anionic surfactants and macromolecular hydrophobic ions [21], with the hydrophilic glucosamino-glycan hyaluronic acid [22]), or association with the amphipathic cyclodextrins [21], and, of course, deliveries with nanoparticles [22-25].

Structural modifications of the insulin molecule by replacement, addition or omission of certain amino-acid residues have been carried out by application of enzymes used for transformation of porcine insulin into human insulin [26], classical peptide synthesis (Fmoc methodology) of the A- and/or B-chain units [27-29], or by the rDNA technology [30-32]. In the case of insulin (51 amino acids) selective chemical modifications of the type alluded to above can be carried out with the entire molecule or on the A- and B-chain separately: the two disulfide-bridged peptides (21 and 30 amino acids, respectively, see the primary structure in Fig. 2) can be reductively separated, and, after modification, oxidatively reassembled.

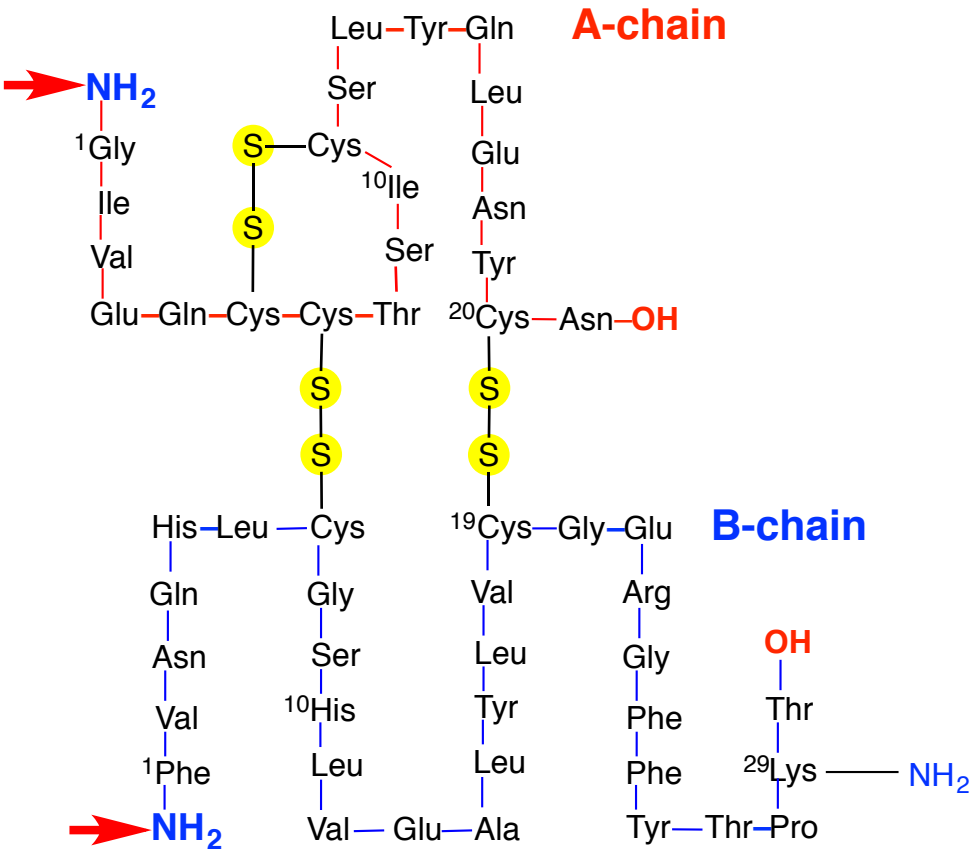

Fig. 2. Molecular *formula* ("primary" structure) of human insulin with an intramolecular disulfide bond in the A-chain and two intermolecular disulfide bridges connecting A- and B-chains. The *N*-terminal amino groups of GlyA1 and PheB1 and the primary amino group in the ε-position of LysB29 stand out.

Two types of side-chain modifications can be distinguished: the first one occurs without attachment of groups to the side chains, for instance deamidation of Asn to Asp (a hydrolysis that has been observed on AsnA21 or AsnB3 of insulin under certain conditions of formulation, pH, temperature, or amyloid aggregation [33,34]), or the formation of a DOPA residue from Tyr by oxidation with Fe^{III} or Cu^{II} [35]. In the much more frequently employed derivatizations substituents are attached to the functional groups of the side chains. These include *Michael* additions of the benzylic amine ABS to oxidized Tyr groups [35], phosphorylations with $(^{i}PrO)_2POH_2$ [36] (on Lys and

N-terminal H_2N-groups, as well as on His and Arg residues), glycosylations with the succinic mixed-anhydride method (on the terminal and Lys H_2N-groups) [37]. The most readily accessible, *i.e.* least hindered and most nucleophilic primary amino group of the one and only Lys moiety in insulin is the prime target for derivatizations by acylation (*e.g.* palmitoylation [38], lipoylation [39], myristoylation [40], PEG-acylation [41], acylation with undecanoic acids bearing a phenyl-boronic acid group in the ω-position [42], or Boc-protection for directing nucleophilic reactivity to other centers [41]); another example is the reductive amination of aldehydes with the LysB29 H_2N-group [43], and thus N-alkylation on the Lys side chain.

2. Experiments with Insulin and BapA. - To test for possible BapA-catalyzed reactions of insulin, solutions of human insulin and racemic H-β^3hAla-NH_2 were treated with a BapA enzyme [11] solution, at certain time intervals samples were withdrawn from the reaction mixture, the enzyme was removed by precipitation, and the supernatant analyzed by HPLC; for details of the procedure see caption of Figure 3. As can be seen from the trace obtained after a reaction time of 8 h, essentially only three new compounds are formed under the chosen conditions; they have longer retention times (18.04, 19.32, 20.44 min) than insulin (16.83 min), and their amount increases within the first eight hours (Table). After 24 hours, however, they have more

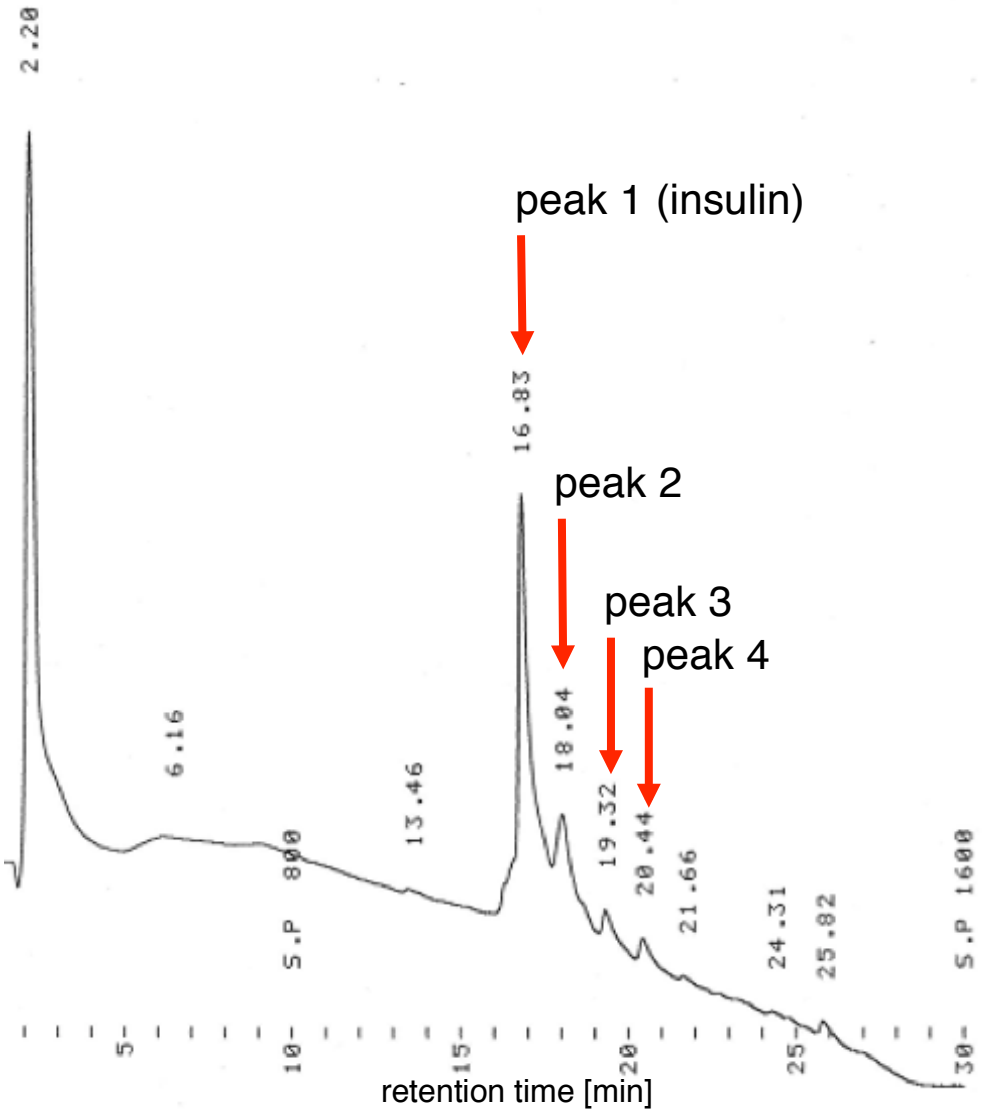

Fig. 3. *HPLC Trace of the reaction mixture obtained when a solution of human insulin (purchased as dry powder from Sigma-Aldrich) was treated with rac. 3-amino-butanoic acid amide (rac. H-β³hAla-NH₂) in the presence of the enzyme BapA for 8 h at 36°C*. Concentrations: insulin 0.95 mM, amide 1.5 mM, BapA 20 ug/ml (activity units), buffer 100 mM (carbonate buffer, pH 9.6). Analytical HPLC: Waters 600S HPLC system using a Supelco Discovery BIO Wide Pore® C18 column (25 cm x 4.6 mm, 5 mm, Sigma). HPLC was performed with a gradient (3-97) of 0.1% TFA in H_2O (A) and 0.08% TFA in MeCN (B), at a flow rate of 1 ml/min with UV detection at 220 nm; the ramping time was 30 min.

Table. *HPLC Analysis of the reaction mixtures obtained upon treatment (for up to 24 h) of human insulin with rac.-3-amino-butanoic acid amide in the presence of the enzyme BapA.* The peak integrations were calculated for the peaks 1 – 4, with exclusion of the input peak (retention time 2.20 min) and of the small peak at 25.82 min, see Fig. 3. For the reaction conditions and analytical details see the specifications in the caption of Fig. 3.

Reaction time [h]	Peak integration [%]			
	Insulin (peak 1)	Compound I (peak 2)	Compound II (peak 3)	Compound III (peak 4)
0	100	-	-	-
2	90.3	5.0	2.5	2.3
4	71.5	14.5	7.2	6.9
8	51.7	26.2	11.6	10.5
24	94.1	2.1	0.8	3.0

or less disappeared from the reaction mixture and insulin is back, indicating that after consumption of H-(β^3hAla)-NH_2 amide, the acyl-donor substrate of the enzyme, BapA starts hydrolytically cleaving the products, the formation of which it had been catalyzing at the beginning of the process[6]). There is, of course, no competing non-enzymatic background acylation by the amide.

[6]) The rate of product (H-βhAla-insulin) cleavage exceeds the rate of coupling when the concentration of the acyl donor (H-βhAla-NH_2) decreases, an effect we had observed before in oligomerizations of β-amino acids and in attachments of β-aminoacids to α-aminoacids and to α-di- and tripeptides, catalyzed by BapA and other β-amino-peptidases [11, 13].

What are the products (compounds **I** - **III**, corresponding to peaks 2 - 4 in the Table and in Figure 3, respectively)? By electron-spray (ESI) mass-spectrometric analysis we detected only insulin molecules, to which one or two H-β^3hAla-moieties have been attached (Figure 4), and we expect that these β-amino-acid residues have been placed only to the *N*-termini of the protein – after all, BapA is a β-amino-acid specific member of the Ntn family of peptidases (*N*-terminal nucleophile hydrolases) [11, 44], which should not "recognize" the LysB29 $(CH_2)_4$-NH_2 side chain as a substrate (*cf.* Fig.2). We would further assume that compound **I**, formed most rapidly, may carry the β^3h-Ala group on the sterically less hindered GlyA1 of insulin, and that one of the compounds **II** and **III** is modified on PheB1 and the other one on both termini. Knowing of the high enantio-preference of BapA for the (*S*)-enantiomer of 3-amino-butanoic-acid amide (Eqn. 2), we take it for granted without proof that in the observed enzymatic reaction only the (*S*)-form, *i.e.* H-β^3hAla- is attached to insulin: the process is expected to be accompanied by kinetic resolution of the acyl donor used.

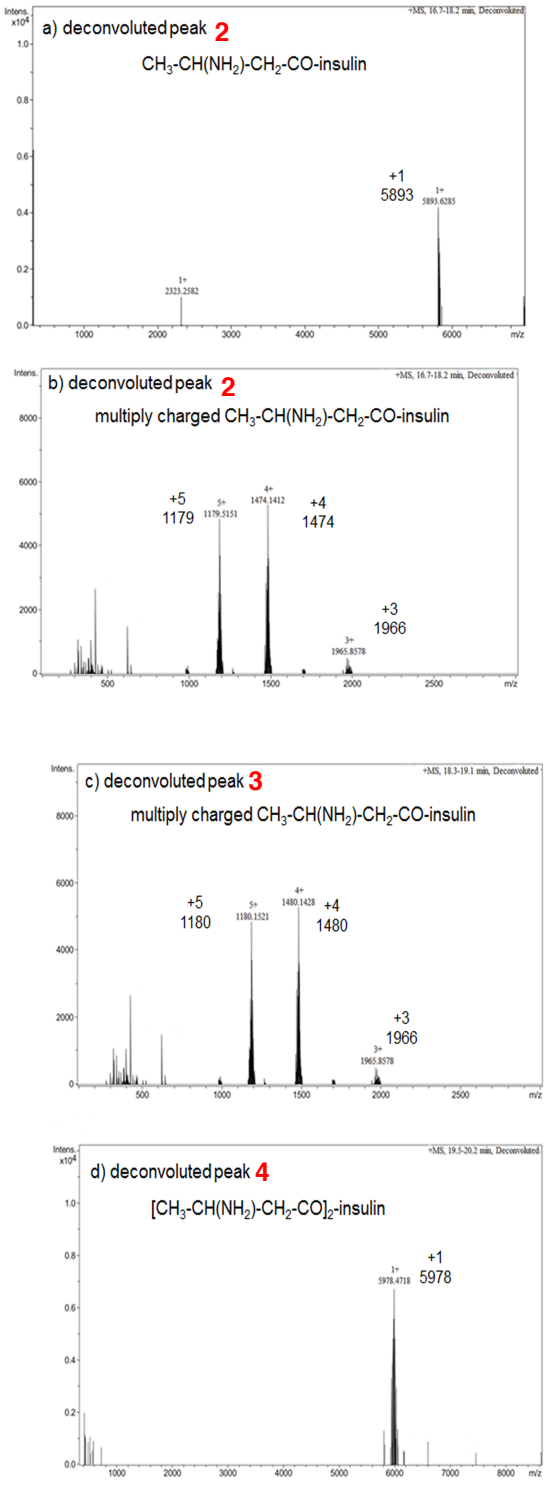

Fig. 4. *LC-ESI-MS Analysis (Bruker Daltonics maXis ESI-QTOF) of the new compounds **I – III** formed after 8 h from human insulin (mol. weight 5808 Da) and 3-amino-butanoic acid amide (H-(β^3hAla)-NH_2) under BapA catalysis*. a), b), c) Mono-acylated (5808 + 85 Da), and d) di-acylated (5808 + 170 Da) insulin derivative. The MS spectra a) and b) are deconvoluted from peak 2, c) from peak 3, and d) from peak 4, see Fig. 3.

3. - Proof of Structure of the Three Products, formed by Enzymatic β-Amino-butanoylation of Insulin, by Independent Total Synthesis. - To prove the structures of compounds **I** - **III** (Table) and to assign the corresponding HPLC peaks 2 - 4 (Fig.3) we have synthesized the three insulin derivatives **1** – **3** (Fig.5), with an (*S*)-3-amino-butanoyl (H-β^3hAla) group on GlyA1 (**1**), on PheB1 (**2**), and on both *N*-termini of insulin (**3**), suspected to be the products of the enzymatic reaction.

1 H-β^3hAla—GlyA1-**INSULIN**

2 H-β^3hAla—PheB1-**INSULIN**

3 (H-β^3hAla—GlyA1)(H-β^3hAla—PheB1)-**INSULIN**

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4 H-β^3hAla—GlyA1-**A-CHAIN**-AsnA21-OH

5 H-β^3hAla—PheB1-**B-CHAIN**-ThrB30-OH

6 H-GlyA1-**A-CHAIN**-AsnA21-OH

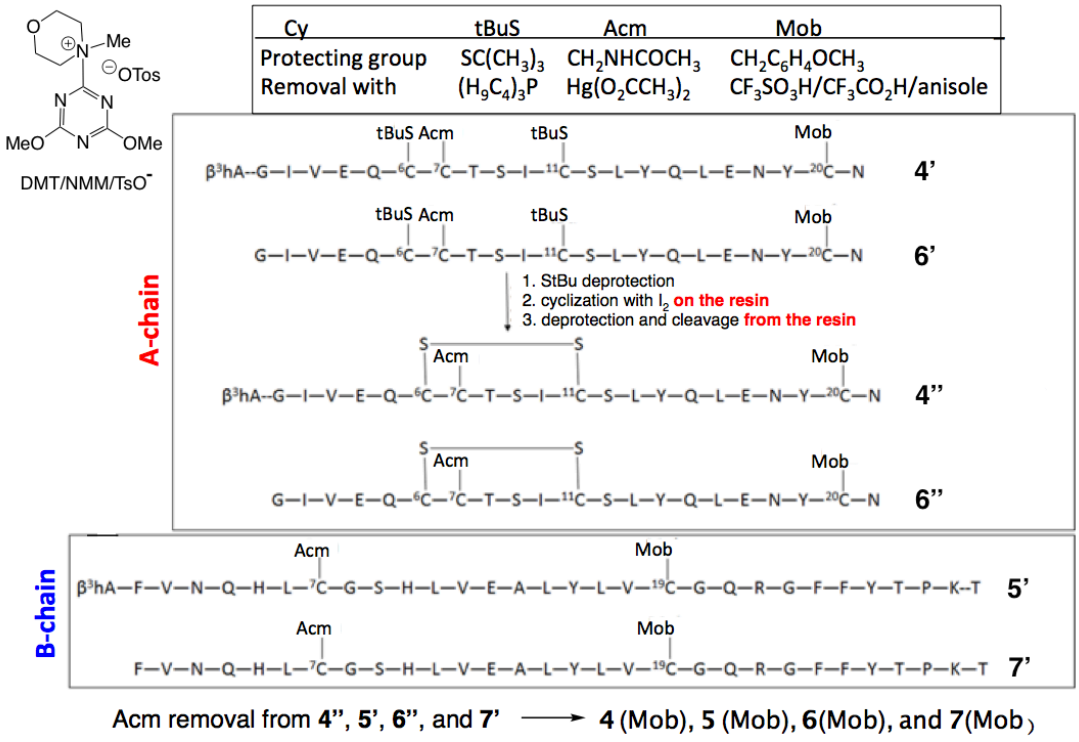
7 H-PheB1-**B-CHAIN**-ThrB30-OH

4 + **7** —> **1** ; **5** + **6** —> **2** ; **4** + **5** —> **3**

Fig. 5. *Schematic presentation of the peptide syntheses performed, in order to assign structures to compounds **I** – **III** (Fig. 3, 4, and Table).*- Peptides **1** – **3** are the expected products of insulin 3-amino-butanoylation by BapA; they

are synthetically accessible by coupling of Hβ^3Ala-chain-A (**4**) and Hβ^3Ala-chain-B (**5**) derivatives with the insulin chain-B (**7**) (–> **1**) and chain-A (**6**) derivatives (–> **2**), respectively, and by coupling of derivatives of **4** and **5** with each other (–> **3**). The selective coupling of H-β^3hAla-substituted insulin chain-A and chain-B parts requires tedious syntheses of suitably protected derivatives as shown in Scheme 1 and discussed in the accompanying text.

In order to obtain the final products **1** - **3**, it was necessary to synthesize derivatives of the A- and B-chains of insulin containing *N*-terminal β^3hAla residues (**4'**, **4''**, **5'**) and derivatives of A- and B-chains of insulin with "free" *N*-termini (**6'**, **6''**, **7'**). Standard solid-phase Fmoc-peptide synthesis on 2-chlorotrityl resin provided the B-chain components **5'** and **7'**, and synthesis on *Wang* resin gave the A-chain components **4'** and **6'** (Scheme 1). As coupling reagent in the SPP syntheses of all four peptide chains we used DMT/NMM/TsO^-, the reagent introduced by one of our groups [45] (Scheme 1). In order to secure selective formation of the intramolecular disulfide bond in the A-chains, the S*t*Bu/Mob/Acm-strategy was employed for protection of the cysteine residues (Scheme 1). This strategy circumvents the necessity of using high dilution techniques [46] and allows for selective removal of the tBuS protecting groups on CysA6 and CysA13 in the peptide chains **4'** and **6'**, and for cyclizing oxidative disulfide formation to give the resin-bound peptides **4''** and **6''** (Scheme 1).



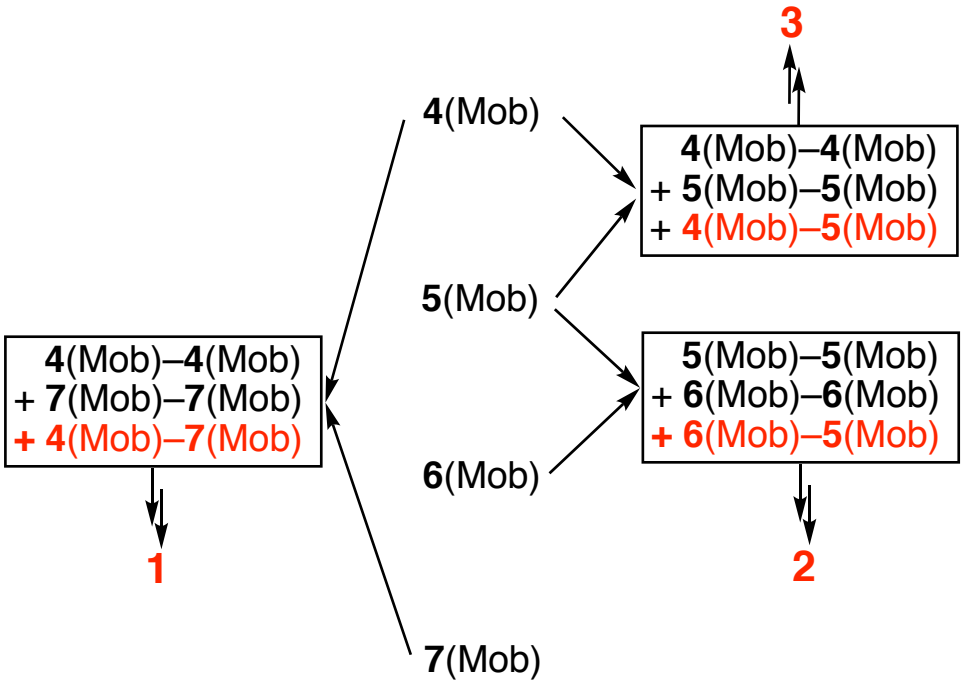
Scheme 1. *Synthesis of the building blocks for couplings of A- with B-chain derivatives, using the tBu/Acm/Mob protection scheme for the cysteins and the DMT/NMM/ TsO^- coupling reagent. Preparation of the Mob-protected A- and B-chains **4**(Mob), **5**(Mob), **6**(Mob), and **7**(Mob).*- For the SSPS the Fmoc technique was used with *Wang* resin (–> **4'**, **6'**) and chloro-trityl resin (–> **5'**, **7'**), and all coupling steps were achieved with [4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium-toluene-4-sulfonate] (DMT/NMM/TsO^-) [45]. All Fmoc-building blocks, including Fmoc-β^3hAla-OH, Fmoc-Cys(StBu)-OH, Fmoc-Cys(Acm)-OH, Fmoc-Cys(Mob)-OH are commercially available. The StBu groups are removed from the peptide chains **4'** and **6'** with tri-butyl-phosphine [47] and the disulfide bonds are formed by subsequent oxidation with iodine (8 eq. I_2 and 8 eq. di-isopropyl-ethyl-amine (DIEA) in CH_2Cl_2 [48]) on the resin (–> **4''**, **6''**). After removal of the peptides from the resin the Acm protecting groups are cleaved from the four Cys(7) residues with Hg(II) in HOAc (aq.) to give the four free peptides with Mob-protection.

After cleavage of the peptides from the resins, preserving the protecting groups Acm and Mob, it was possible to remove these two types of orthogonal protecting groups selectively and connect the A-chains and B-chains by inter-chain S-S bonds in solution. Thus, the Acm groups were removed first (with Hg(II))[7]. In this way, four substrates (containing the peptide chains of **4**, **5**, **6**, **7** protected with the Mob groups only) were obtained for the subsequent intermolecular disulfide formation and cyclization steps. The oxidation leading to the first - intermolecular - disulfide bond formation was performed following a standard procedure (with I_2 in MeOH)[8]. In this way, S-S bonds between the Cys thiol group at position 7 of **4**(Mob) (chain A) and the Cys thiol group at position 7 of 7(Mob) (chain B) are formed, to give **4**(Mob)–**7**(Mob); analogously, **6**(Mob)–**5**(Mob) and **4**(Mob)–**5**(Mob)

[7]) Mercury(II) acetate in 10% (v/v) aqueous acetic acid (100 mL/mg peptide) and carefully adjusted pH 4.0 with glacial acetic acid or ammonia solution; 1.0 eq. Hg(II) acetate per Acm group; stirring for 1 hour under an argon atmosphere; then β-mercaptoethanol (0.5 mL per 100 mmol of peptide) was added and the mixture was left to stand at room temperature for 5 hours; finally, the mixture was centrifuged (to remove the precipitate) and the supernatant lyophilized. For literature procedures see [49, 50].

[8]) The A and B chain precursors were dissolved in methanol (1.25 mL/mmol), then a 0.4 M methanolic iodine solution (2.5 equivalents per thiol group) was added and the mixture was stirred for 30 minutes at room temperature. After completion of the oxidation 1 M aqueous ascorbic acid (100 mL/mmol peptide) was added. The final stage involved evaporation of solvent under reduced pressure to approximately one third of the original volume, removal of salts and lyophilization to give the crude peptides. For a representative literature procedure for producing asymmetrical peptidic disulfides by iodine oxidation see [50]. For mechanistic interpretations of asymmetrical coupling reactions of this type see [50,51].

were produced by the corresponding combinations. As expected, the symmetrical dimers containing two A-chains and two B-chains are also found in the reaction mixtures (Scheme 2), but the desired asymmetrical products were prevailing in all three cases (*cf.* [50, 51]). Separation and purification by preparative HPLC (C18 column) provided the three compounds marked red in the boxes of Scheme 2.



Scheme 2. Iodine *Oxidation of equimolar mixtures of insulin chain-A and chain-B derivatives, Mob-protected on CysA20 and CysB19, respectively, with and without terminal βhAla group, to access the products* **4**(Mob)–**7**(Mob), **6**(Mob)–**5**(Mob) and **4**(Mob)–**5**(Mob) *of mixed coupling, which were formed in excess and separated by HPLC.*

The last stage involved deprotection of the Mob groups (with CF_3SO_3H/CF_3CO_2H/anisole) and oxidation with I_2 in MeOH[9]), forming the second, intramolecular S-S-bonds between the A- and B-chains of the insulin derivatives to yield the target molecules **1**, **2**, and **3** (see Scheme 2 and Fig. 5). LC-MS Analyses confirmed the identity of the species with m/z = 5893.63, calculated for the insulin derivatives **1** and **2** with one β^3hAla residue attached to the *N*-terminus of chain A or chain B. Peptide **3** gave rise to a peak m/z = 5978.48, corresponding to the insulin derivative containing two β^3hAla residues.

With the unambiguous syntheses of compounds **1**, **2**, and **3** we can now assign the structures of the three compounds formed by the BapA-catalyzed 3-amino-butanoylation (Fig. 3): On the basis of comparing HPLC analyses (Fig.6) and co-injections, compound **I** (Table) with shortest retention time (peak 2 in Fig. 3) belongs to insulin with an H-β^3hAla residue attached to GlyA1, *i.e.* **1**; peak 3 in Fig. 3 (compound **II** in the Table) can be assigned to the protein derivative **2**, containing β^3hAla attached to PheB1 of the insulin B-chain; and, finally, the compound with largest retention time (compound **III** in the Table and peak 4 in Fig. 3) has structure **3**, i.e. insulin with H-β^3hAla residues attached to both, GlyA1 and PheB1. Thus, the assignment

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[9]) The cleavage cocktail (TFMSA/TFA/anisole 1:8:1) for removing the Mob groups was cooled in an ice bath and added to the peptide solutions (3 mg/ml); the resulting mixture was stirred at 0°C for 30 min, diluted 50-fold with cold water and extracted 3 times with ether. The cyclization was performed by iodine titration (addition of an I_2 solution in MeOH up to persistent yellow color) and quenching of the I_2 excess with ascorbic acid. For model procedures see [49,50].

corresponds to what we had expected on the basis of steric hindrance of the two *N*-termini and on the basis of the rates of formation of the three enzymatic products (Table) (see last paragraph of section 2, above).

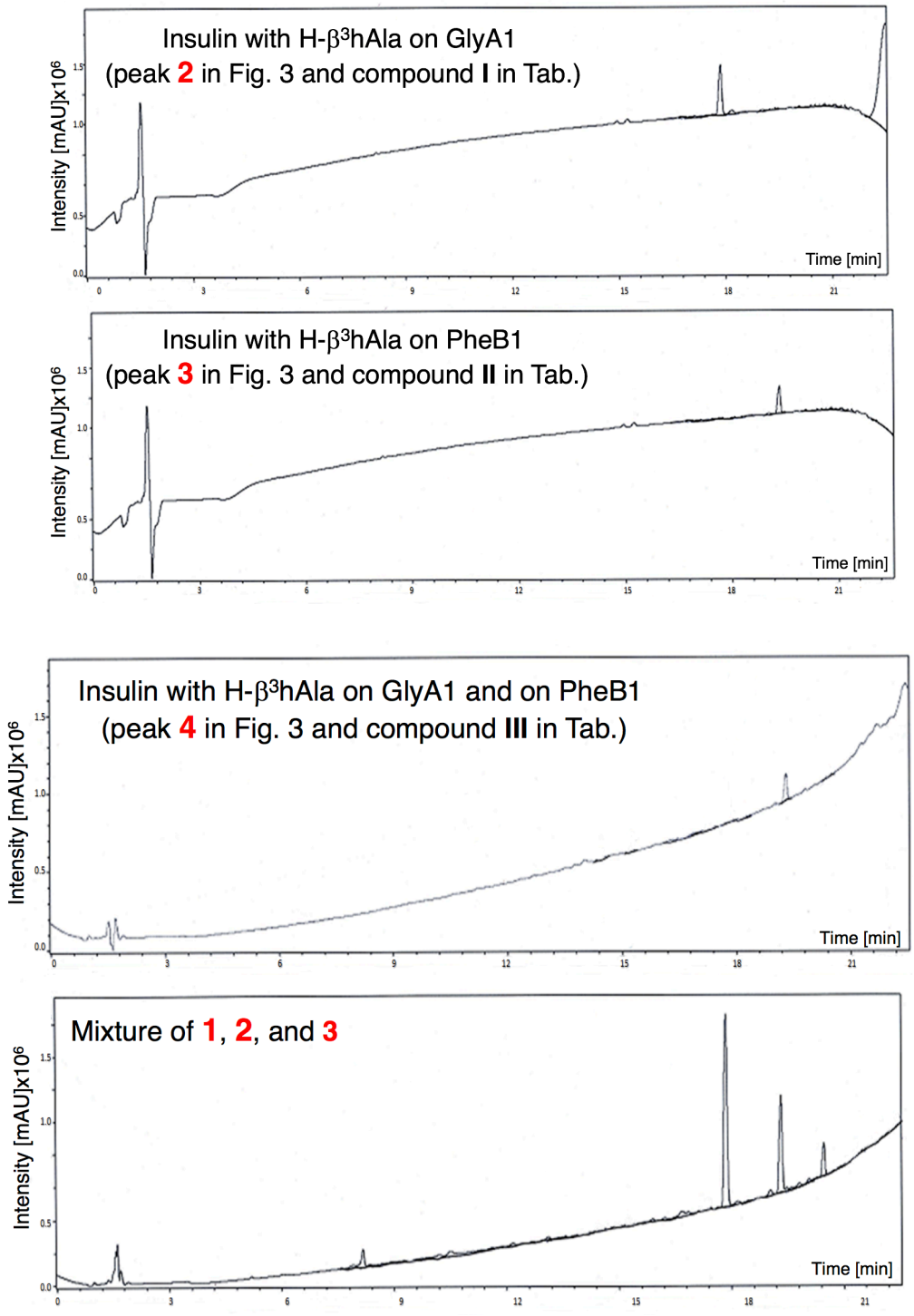

Fig. 6. *HPLC Traces of the Peptides **1**, **2**, **3** and of their mixture*. Dionex UltiMate 3000, Thermo Scientific HPLC system, using a Kintex 2.6u, C18, 100A column (100 x 4.6 mm). HPLC was performed with a gradient (3-97) of 0.1% TFA in H_2O (A) and 0.08% TFA in MeCN (B), at a flow rate of 1 ml/min with the UV detection at 220 nm.

4. ***Conclusion***.- We have been able to selectively attach a β-amino-acid residue to the *N*-terminus of a protein in a - reversible - BapA-enzyme-catalyzed process. This post-translational modification should be applicable to proteins other than insulin, as long as their *N*-termini are located on the surface of their tertiary structures, so that they can enter the active site [44] of the enzyme. Besides β^3hAla other β^3- and also β^2-amino-acid moieties with proteinogenic and non-proteinogenic side chains [14] can be processed by BapA, so that the R-group in the acyl donors R-CH(NH_2)-CH_2-$CONH_2$ could be sterically more demanding than the methyl group of β^3hAla, and we envision that R might also contain a functional group (such as azido or alkynyl) for further tagging procedures [5-7]. An advantage of this protein modification is that the acylating β-amino-acid amides can be applied as racemic mixtures (see Fig.1, Eqn. (2), and caption of the Table). The 3-2W4 BapA enzyme is readily available: it can be prepared by expression and processing in *E. coli*, and consensus-sequence searches have led to a number of microorganisms producing β-peptidyl aminopeptidases of variable

activity profiles [11], providing a tool kit for carrying out the general transformation outlined in Fig. 1[10]).

The most important next step is the preparation of larger amounts of the three modified insulin derivatives **1** – **3**, to allow for tests of their activities in comparison with natural insulin. Due to the fact that N-terminal β-amino-acid residues in peptides are not cleaved by common aminopeptidases [8, 10, 12 15] it is expected that the *in*-vivo half life of the H-β^{3}hAla-modified insulin derivatives described herein will be larger than that of insulin, and that the physiological profile of the three compounds **1** - **3** will differ substantially.

Author Contribution Statement

The experiments were carried out by B. K., J. W., and B. G.. The research was conceived by D. S. and B. K.. The manuscript was written by D.S..

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[10]) For *N*-terminal attachment to proteins of a series of α-amino-acid residues with the naturally split GOS-TerL intein (protein trans-splicing) see [52].

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