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Site-specific fluorescein labeling of human insulin

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Three fluorescein derivatives of human insulin (HI, 1) labeled at positions $N^{\alpha A1}$, $N^{\alpha B1}$ and $N^{\varepsilon B29}$ respectively, were synthesized using an *N*-trifluoroacetyl-based protecting group scheme. The Tfa protecting group introduced by reaction with ethyl trifluoroacetate was found to be stable in aqueous and organic media and efficiently removed under mild basic conditions.

Supporting information can be found in the online version of this article.

Keywords: insulin; selective fluorescein labeling; *N*-trifluoroacetyl; selective protection

Introduction

Fluorescence-detection techniques have long been utilized for a wide range of basic life science applications and continue to play an indispensible role as chemical biology tools [1]. In addition to offering a sensitive, stable, low-cost alternative to radio labeling, fluorescent probes also have the advantage of being compatible with homogenous automated assay formats. As a result, fluorescence-detection techniques have seen a dramatic increase in their use for drug discovery, diagnostics and other biomedical applications [2,3]. Fluorescein (FSC) labeling is typically accomplished by treating the ligand with excess fluorescein isothiocyanate (FITC) [4] (Figure 1) and is generally straightforward providing the label is introduced regioselectively and does not sterically impair the molecular interaction being investigated.

Past experience with insulin illustrates the potential challenges involved in FSC labeling of complex peptides and proteins. Early attempts by several laboratories to label insulin (Figure 2) with FITC yielded complex mixtures with significantly reduced biological potencies [5–9]. Bromer and co-workers [10] prepared FSC derivatives of bovine insulin by treatment with FITC under basic aqueous conditions and chromatographically resolved the reaction mixture into monosubstituted, disubstituted and trisubstituted components, noting that the monosubstituted fraction retained approximately 40% of the native activity. A subsequent more extensive investigation by Hentz [11] isolated and characterized four distinct insulin species following FITC derivatization of human insulin (HI, **1**): monosubstituted derivatives at $N^{\alpha A1}$ and $N^{\alpha B1}$, disubstituted at $N^{\alpha A1}$ and $N^{\alpha B1}$ and trisubstituted at $N^{\alpha A1}$, $N^{\alpha B1}$ and $N^{\varepsilon B29}$. These four derivatives were evaluated in a fluorescence polarization-based assay and ranked with respect to their sensitivities in the following order: $N^{\alpha B1} > N^{\alpha A1} > N^{\alpha A1}$, $N^{\alpha B1} > N^{\alpha A1}$, $N^{\alpha B1}$, $N^{\varepsilon B29}$. Although the $N^{\alpha B1}$ derivative constituted a minor component of the product mixture, it retained nearly full activity in an autophosphorylation assay. We reasoned that selective labeling of $N^{\alpha B1}$ or one of the other two insulin amines would require reversible protection of two sites, leaving the third exposed for FITC derivatization. The use of the Boc group was originally adapted for insulin protection by Carpenter [12] and Geiger [13] and used in the course of semisynthetic studies of this hormone [14–16]. The use of TFA during the deprotection step however is incompatible with the thiourea-linked FSC group, which undergoes cyclization under strongly acidic conditions with concomitant loss of the N-terminal residue [17]. This limitation suggested instead the use of a base-labile protecting group, such as the *N*-trifluoroacetyl (Tfa), which was first successfully used for insulin protection by Borras and Offord [18]. The group is introduced by treatment with phenyl trifluoroacetate [18] or ethyl trifluoroacetate (Tfa-OEt) [19], is stable under strongly acidic conditions and efficiently cleaved in aqueous base. These characteristics, in addition to the group's demonstrated compatibility with phenylthiourea [18], encouraged us to utilize the Tfa protection strategy to selectively label the $N^{\alpha A1}$, $N^{\alpha B1}$ and $N^{\varepsilon B29}$ groups of HI **1** (Table 1) with FITC.

Results and Discussion

Initial attempts to introduce the Tfa group using trifluoroacetic anhydride were unsuccessful presumably because the anhydride is too reactive. The use of Tfa-OEt yielded promising results and in addition revealed an unexpected influence of solvent on the product composition. Conducting the trifluoroacetylation reaction in DMF or DMSO gave predominantly the $N^{\alpha A1}$, $N^{\alpha B1}$-di-Tfa-HI (**3a**), whereas aqueous condition yielded the $N^{\alpha A1}$, $N^{\varepsilon B29}$-di-Tfa-HI (**3b**) as the major product (Table 1). These observations prompted us to further optimize the yields as well as selectivity of the protocols (Experimental Section and Tables 2 and 3). Accordingly, treatment of 50 mg zinc-free HI (**1**) in 3.0 ml DMF with 40.0 eq of DIEA, 12.0 eq of Tfa-OEt produced the $N^{\alpha A1}$, $N^{\alpha B1}$-di-Tfa-HI (**3a)** in 69% yield along with 22% of $N^{\alpha A1}$, $N^{\alpha B1}$, $N^{\varepsilon B29}$-tri-Tfa-HI (**6**) (Table 2, entry 6). The use of 50 mg zinc-formulated HI (**1**) in a 1 : 1 mixture of acetonitrile (ACN)/H_2O (1.0 ml), 300.0 eq of DIEA and

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300.0 eq of Tfa-OEt afforded an 82% yield of $N^{\alpha A1}$, $N^{\varepsilon B29}$-di-Tfa-HI (**3b**) along with 7.6% of $N^{\varepsilon B29}$-Tfa-HI (**2b**) (Table 3, entry 20).

The optimized conditions were then applied to larger scale syntheses of FSC-labeled HIs (Scheme 1). Reaction of zinc-free HI in DMF with Tfa-OEt (6 eq) in the presence of DIEA (40 eq) for 4.0 h produced a 65% yield of $N^{\alpha A1}$, $N^{\alpha B1}$-di-Tfa-HI (**3a**) after chromatographic purification. Zinc-formulated insulin in ACN/H_2O treated with DIEA (300 eq) and Tfa-OEt (300 eq) for 1.0 h provided $N^{\alpha A1}$, $N^{\varepsilon B29}$-di-Tfa-HI (**3b**) in 96% yield after purification. A multistep procedure was required to prepare the $N^{\alpha B1}$, $N^{\varepsilon B29}$-di-Tfa-HI (**3c**). Zinc-formulated HI in DMSO in the presence of 40 eq TEA (Et_3N) was converted in a one-pot reaction to $N^{\alpha A1}$, $N^{\varepsilon B29}$-di-Boc-$N^{\alpha B1}$-Tfa-HI intermediate by sequential addition of di-*tert*-butyl dicarbonate (Boc_2O; 2 eq) and Tfa-OEt (30 eq). The intermediates were precipitated by cold acetone, dried and treated with TFA to furnish the $N^{\alpha B1}$-Tfa-HI (**2c**) in 50% yield. The $N^{\varepsilon B29}$ Lys group of **2c** was then protected by treatment with Tfa-OEt in ACN/H_2O providing the $N^{\alpha B1}$, $N^{\varepsilon B29}$-di-Tfa-HI (**3c**) in 50% yield, the overall yield of **3c** from HI was 25%. The Tfa-protected insulins (Table 1) were characterized by mass spectral analysis (Experimental Section and Figure S2, Supporting Information), and sites of Tfa modification were unequivocally confirmed through Glu-C (*Staphylococcus aureus* V8 protease) mapping (Table 4 and Figure S1).

di-Tfa-protected intermediates **3a**, **3b** and **3c** were converted to their corresponding FSC derivatives **4a**, **4b** and **4c** by treatment with FITC (1.5 eq) and DIEA (10 eq) in DMF. Final deprotection was accomplished using 15% NH_4OH at 4 °C or 25 mM NaOH at 4 °C, followed by HPLC purification to afford homogenous FSC-labeled HI derivatives **5a**, **5b** and **5c** (Figure 3).

Conclusion

This methodology, in addition to enabling straightforward, unambiguous synthesis of $N^{\alpha A1}$, $N^{\alpha B1}$ and $N^{\varepsilon B29}$ FSC-labeled HIs, will find utility in allowing for a wide range of selective chemical modifications at these sites.

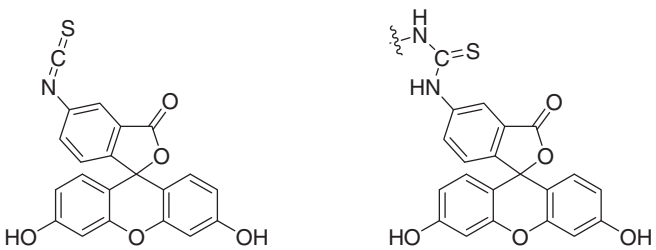

Figure 1. Chemical structures of FITC and FSC conjugate.

Table 1. Tfa-modified or FSC-modified insulin derivatives

Compound	$N^{\alpha A1}$	$N^{\alpha B1}$	$N^{\varepsilon B29}$	Name
1	H	H	H	HI
2a	Tfa	H	H	$N^{\alpha A1}$-Tfa-HI
2b	H	H	Tfa	$N^{\varepsilon B29}$-Tfa-HI
2c	H	Tfa	H	$N^{\alpha B1}$-Tfa-HI
3a	Tfa	Tfa	H	$N^{\alpha A1}$, $N^{\alpha B1}$-di-Tfa-HI
3b	Tfa	H	Tfa	$N^{\alpha A1}$, $N^{\varepsilon B29}$-di-Tfa-HI
3c	H	Tfa	Tfa	$N^{\alpha B1}$, $N^{\varepsilon B29}$-di-Tfa-HI
4a	Tfa	Tfa	FSC	$N^{\alpha A1}$, $N^{\alpha B1}$-di-Tfa-$N^{\varepsilon B29}$-FSC-HI
4b	Tfa	FSC	Tfa	$N^{\alpha A1}$, $N^{\varepsilon B29}$-di-Tfa-$N^{\alpha B1}$-FSC-HI
4c	FSC	Tfa	Tfa	$N^{\alpha B1}$, $N^{\varepsilon B29}$-di-Tfa-$N^{\alpha A1}$-FSC-HI
5a	H	H	FSC	$N^{\varepsilon B29}$-FSC-HI
5b	H	FSC	H	$N^{\alpha B1}$-FSC-HI
5c	FSC	H	H	$N^{\alpha A1}$-FSC-HI
6	Tfa	Tfa	Tfa	$N^{\alpha A1}$, $N^{\alpha B1}$, $N^{\varepsilon B29}$-tri-Tfa-HI

Experimental Section

General

HI of recombinant origin was obtained from Eli Lilly and Company (Indianapolis, IN, USA) in the zinc-formulated (zinc crystal) form. The zinc-free insulin was obtained by the following procedure: 500 mg of zinc crystal HI was dissolved in 10 mM HCl, 50 ml, loaded onto a preparative HPLC column, washed with 1% aqueous ACN (0.1% TFA) with a flow rate of 15 ml/min for 90 min, followed by 50% aqueous ACN (0.1% TFA) and lyophilization of the combined fractions to provide the zinc-free insulin as a TFA salt. Water (H_2O) was obtained from Milli-Q water purification system (Millipore, Billerica, MA, USA). LC-MS: 1100 series liquid chromatrograph mass spectrometer, model G1956A/B. General analytical HPLC condition: The reaction mixtures were analyzed by an RP-HPLC using a Waters SymmetryShield RP18 Column (Milford, MA, USA; cat. no. 186000179, 3.5 μm, 4.6 × 100 mm) with a linear gradient from 6% aqueous ACN (0.1% TFA) to 60% aqueous ACN (0.1% TFA) over 14 min at a flow rate of 1.0 ml/min. General preparative HPLC condition: The reaction mixtures were separated by RP preparative HPLC using a Waters SymmetryPrep C18 Column (cat. no. WAT066245, 7 μm, 19 × 300 mm) with a linear gradient from 2% aqueous ACN (0.1% TFA) to 60% aqueous ACN (0.1% TFA) over 80 min at a flow rate of 15 ml/min. After lyophilization, the peptides appeared as white or bright yellow (FSC labeled) amorphous solids. The yields are calculated on the basis of peptide weight.

$N^{\alpha A1}$-Tfa-HI (**2a**)

Zinc-free HI (100 mg) was dissolved in DMF (2 ml), DIEA (35 μl, 20 eq) and Tfa-OEt (4 μl, 2 eq) were added and the solution was gently shaken at r.t. (room temperature) for 4 h. The reaction

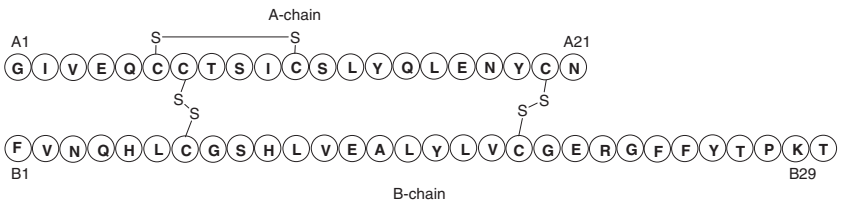

Figure 2. Structure of HI (**1**).

Table 2. Product distribution of Tfa protection reactions in a single solvent

	Solvent/HI	DIEA	Tfa-OET	HI	$N^{\alpha A1}$-Tfa-HI	$N^{\alpha B1}$-Tfa-HI	$N^{\varepsilon B29}$-Tfa-HI	$N^{\alpha A1}$, $N^{\varepsilon B29}$-di-Tfa-HI	$N^{\alpha A1}$, $N^{\alpha B1}$-di-Tfa-HI	$N^{\alpha B1}$, $N^{\varepsilon B29}$-di-Tfa-HI	$N^{\alpha A1}$, $N^{\alpha B1}$, $N^{\varepsilon B29}$-tri-Tfa-HI
Compound number				**1**	**2a**	**2c**	**2b**	**3b**	**3a**	**3c**	**6**
Retention time (min)				9.8	10.2	10.6	10.4	10.8	11.0	11.2	11.7
Entry											
1	DMSO, 3.0 ml zinc free	40 eq	20 eq	—[a]	21%	—	—	5.9%	51%	—	13%
2	DMF, 3.0 ml zinc free	40 eq	20 eq	—	—	—	—	—	62%	—	25%
3	ACN, 3.0 ml[b] zinc free	40 eq	20 eq	68%	21%	—	—	—	—	—	—
4	H2O, 3.0 ml zinc free	40 eq	20 eq	82%	—	—	—	—	—	—	—
5	DMF, 3.0 ml zinc free	40 eq	6 eq	—	12%	—	—	—	68%	—	12%
6	DMF, 3.0 ml zinc free	40 eq	12 eq	—	—	—	—	—	69%	—	22%
7	DMF, 3.0 ml zinc free	40 eq	20 eq	—	—	—	—	—	61%	—	28%
8	DMF, 3.0 ml[c] zinc crystal	40 eq	6 eq	—	20%	—	—	—	59%	—	12%
9	DMF, 3.0 ml[c] zinc crystal	40 eq	12 eq	—	—	—	—	—	61%	—	29%
10	DMF, 3.0 ml[c] zinc crystal	40 eq	20 eq	—	—	—	—	—	48%	—	41%

[a]Not detected or yield is less than 5%.
[b]Poor solubility was observed through 18 h.
[c]Not fully dissolved at the beginning, the solution becomes clear after 1–2 h.

mixture was diluted with H_2O (30 ml) and purified by an RP preparative HPLC. Lyophilization of the combined fractions gave the product $N^{\alpha A1}$-Tfa-HI **2a** (55 mg) in 50% yield, 99% purity (Figure S2) and ESI-MS: 5903.6 (calculated: 5903.7).

$N^{\varepsilon B29}$-Tfa-HI (**2b**)

Zinc crystal HI (100 mg) was dissolved in ACN (2 ml) and H_2O (2 ml), cooled to 0 °C. DIEA (0.5 ml) was added in one portion followed by dropwise addition of Tfa-OEt (0.3 ml). The mixture was diluted with H_2O (30 ml) after 1 h and purified by an RP preparative HPLC. Lyophilization of the combined fractions gave the product $N^{\varepsilon B29}$-Tfa-HI **2b** (55 mg) in 50% yield, 100% purity (Figure S2) and ESI-MS: 5903.0 (calculated: 5903.7).

$N^{\alpha B1}$-Tfa-HI (**2c**)

Zinc crystal HI (400 mg) was suspended in DMSO (8 ml) and Et_3N (0.40 ml); this suspension was gently shaken at r.t. until near dissolution (about 15 min), then Boc_2O (32 mg, 2.0 eq) was added. After 2 h at r.t., the LC-MS analysis indicated that the main product was the $N^{\alpha A1}$, $N^{\varepsilon B29}$-di-Boc-HI. Tfa-OEt (240 µl, 30 eq) was added, and the reaction mixture was gently shaken at r.t. for another 3 h. The reaction mixture is precipitated by addition of cold acetone (100 ml), centrifuged and dried under vacuum. The dried crude product was treated with a solution of TFA (9.5 ml), triisopropylsilane (TIS) (0.25 ml) and H_2O (0.25 ml) at r.t. for 1 h. The TFA was removed by rotary evaporation, the residue diluted with 20% ACN aq. (30 ml), purified by RP preparative HPLC. Lyophilization of selected fractions gave $N^{\alpha B1}$-Tfa-HI **2c** (200 mg) in 50% yield, 98% purity (Figure S2) and ESI-MS: 5903.2 (calculated: 5903.7).

$N^{\alpha A1}$, $N^{\alpha B1}$-di-Tfa-HI (**3a**)

Zinc-free HI (300 mg) was dissolved in DMF (9 ml), DIEA (200 µl, 40 eq) and Tfa-OEt (36 µl, 6 eq) were added, and the solution was gently shaken at r.t. for 4 h. The reaction mixture was diluted with H_2O (30 ml) and purified by an RP preparative HPLC. Lyophilization of the combined fractions gave the product $N^{\alpha A1}$, $N^{\alpha B1}$-di-Tfa-HI **3a** (200 mg) in 65% yield, 97% purity (Figure S2) and ESI-MS: 5998.0 (calculated: 5999.7) and $N^{\alpha A1}$, $N^{\alpha B1}$, $N^{\varepsilon B29}$-tri-Tfa-HI **6** (44 mg) in 14% yield, 99% purity (Figure S2) and ESI-MS: 6094.5, (calculated: 6095.7).

$N^{\alpha A1}$, $N^{\varepsilon B29}$-di-Tfa-HI (**3b**)

Zinc crystal HI (300 mg) was dissolved in ACN (3 ml) and H_2O (3 ml), the solution was cooled to 0 °C, and DIEA (2.88 ml) was added in one portion followed by dropwise addition of Tfa-OEt (1.88 ml). LC-MS indicated the reaction was complete after 1 h. The mixture was diluted with H_2O (30 ml) and purified by an RP preparative HPLC. Lyophilization of the combined fractions gave the product $N^{\alpha A1}$, $N^{\varepsilon B29}$-di-Tfa-HI **3b** (295 mg) in 96% yield, 99% purity (Figure S2) and ESI-MS: 5999.5 (calculated: 5999.7).

$N^{\alpha B1}$, $N^{\varepsilon B29}$-di-Tfa-HI (**3c**)

$N^{\alpha B1}$-Tfa-HI **2c** (60 mg) was dissolved in ACN (1.5 ml), H_2O (2.5 ml) and DIEA (1.0 ml). The solution was cooled to 0 °C, then Tfa-OEt (150 µl) was added. After 60 min, the reaction solution was diluted with H_2O (10 ml), adsorbed onto an RP preparative HPLC column. Purification and lyophilization of the combined fractions gave the final product $N^{\alpha B1}$, $N^{\varepsilon B29}$-di-Tfa-HI **3c** (31 mg) in 50% yield, 94% purity (Figure S2) and ESI-MS: 5999.0 (calculated: 5999.7). Overall yield of $N^{\alpha B1}$, $N^{\varepsilon B29}$-di-Tfa-HI **3c** from zinc crystal HI (**1**) was 25%.

$N^{\alpha A1}$, $N^{\alpha B1}$-di-Tfa-$N^{\varepsilon B29}$-FSC-HI (**4a**)

$N^{\alpha A1}$, $N^{\alpha B1}$-di-Tfa-HI **3a** (100 mg) was dissolved in anhydrous DMF (5.0 ml). DIEA (20 µl, 10 eq) and FITC (9.7 mg, 1.5 eq) were added to the solution, which was gently shaken at r.t. 2 h at which time LC-MS analysis indicated that the reaction was complete. The mixture was diluted with H_2O (30 ml) and purified by an RP preparative HPLC. Lyophilization of the combined fraction gave $N^{\alpha A1}$, $N^{\alpha B1}$-di-Tfa-$N^{\varepsilon B29}$-FSC-HI **4a** (85 mg) as a bright yellow powder with a yield of 80%, 84% purity (Figure S2) and ESI-MS: 6388.0 (calculated: 6388.7).

$N^{\alpha A1}$, $N^{\varepsilon B29}$-di-Tfa-$N^{\alpha B1}$-FSC-HI (**4b**)

$N^{\alpha A1}$, $N^{\varepsilon B29}$-di-Tfa-HI **3b** (100 mg, 0.017 mmol) was treated by the procedure previously described for **4a** providing bright

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Table 3. Product distribution of Tfa protection reactions in an aqueous mixed solvent

	Solvent/HI	DIEA	Tfa-OET	HI	$N^{\alpha A1}$-Tfa-HI	$N^{\alpha B1}$-Tfa-HI	$N^{\varepsilon B29}$-Tfa-HI	$N^{\alpha A1}$, $N^{\varepsilon B29}$-di-Tfa-HI	$N^{\alpha A1}$, $N^{\alpha B1}$-di-Tfa-HI	$N^{\alpha B1}$, $N^{\varepsilon B29}$-di-Tfa-HI	$N^{\alpha A1}$, $N^{\alpha B1}$, $N^{\varepsilon B29}$-tri-Tfa-HI
Compound number				**1**	**2a**	**2c**	**2b**	**3b**	**3a**	**3c**	**6**
Retention time (min)				9.8	10.2	10.6	10.4	10.8	11.0	11.2	11.7
Entry											
1	$ACN/H_2O = 1:1$ 3.0 ml, zinc free	100 eq	50 eq	52%	7.3%	—[a]	22%	—	—	—	—
2	$DMSO/H_2O = 1:1$ 3.0 ml, zinc free	100 eq	50 eq	22%	37%	—	21%	16%	—	—	—
3	$DMF/H_2O = 1:1$ 3.0 ml, zinc free	100 eq	50 eq	51%	17%	—	22%	6.9%	—	—	—
4	$DMSO/H_2O = 3:1$ 3.0 ml, zinc free	100 eq	50 eq	—	60%	—	—	25%	7.5%	—	—
5	$DMSO/H_2O = 1:3$ 3.0 ml, zinc free	100 eq	50 eq	62%	5.8%	—	24%	—	—	—	—
6	$DMSO/H_2O = 1:1$ 0.5 ml, zinc crystal	100 eq	50 eq	—	36%	—	5%	53%	—	—	—
7	$DMSO/H_2O = 1:1$ 1.0 ml, zinc crystal	100 eq	50 eq	8.3%	35%	—	17%	37%	—	—	—
8	$DMSO/H_2O = 1:1$ 2.0 ml, zinc crystal	100 eq	50 eq	26%	31%	—	25%	16%	—	—	—
9	$DMSO/H_2O = 1:1$ 3.0 ml, zinc crystal	100 eq	50 eq	39%	28%	—	20%	9.5%	—	—	—
10	$DMSO/H_2O = 1:1$ 1.0 ml, zinc crystal	100 eq	100 eq	—	40%	—	—	51%	—	—	—
11	$DMSO/H_2O = 1:1$ 1.0 ml, zinc crystal	150 eq	150 eq	—	27%	—	—	61%	—	—	6.8%
12	$DMSO/H_2O = 1:1$ 1.0 ml, zinc crystal	200 eq	200 eq	—	22%	—	—	60%	—	—	10%
13	$ACN/H_2O = 1:1$ 3.0 ml, zinc free	100 eq	50 eq	47%	12%	—	23%	—	—	—	—
14	$ACN/H_2O = 3:1$ 3.0 ml, zinc free	100 eq	50 eq	38%	17%	—	27%	10%	—	—	—
15	$ACN/H_2O = 1:3$ 3.0 ml, zinc free	100 eq	50 eq	55%	7%	—	26%	7.4%	—	—	—
16	$ACN/H_2O = 1:1$ 1.0 ml, zinc crystal	100 eq	100 eq	8.2%	13%	—	34%	43%	—	—	—
17	$ACN/H_2O = 1:1$ 1.0 ml, zinc crystal	150 eq	150 eq	—	8.8%	—	23%	62%	—	—	—
18	$ACN/H_2O = 1:1$ 1.0 ml, zinc crystal	200 eq	200 eq	—	6.4%	—	15%	73%	—	—	—
19	$ACN/H_2O = 1:1$ 1.0 ml, zinc crystal	250 eq	250 eq	—	5.3%	—	13%	77%	—	—	—
20	$ACN/H_2O = 1:1$ 1.0 ml, zinc crystal	300 eq	300 eq	—	—	—	7.6%	82%	—	—	—

[a]Not detected or yield is less than 5%.

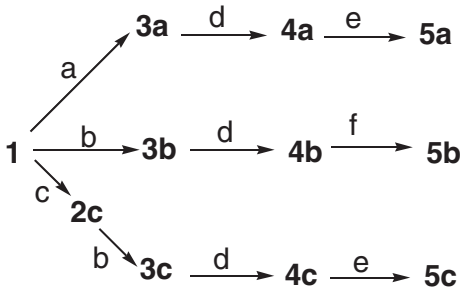
yellow lyophilized powder $N^{\alpha A1}$, $N^{\varepsilon B29}$-di-Tfa-$N^{\alpha B1}$-FSC-HI **4b** (80 mg) with 76% yield, 96% purity (Figure S2) and ESI-MS: 6387.0 (calculated: 6388.7).

$N^{\alpha B1}$, $N^{\varepsilon B29}$-di-Tfa-$N^{\alpha A1}$-FSC-HI (**4c**)

$N^{\alpha B1}$, $N^{\varepsilon B29}$-di-Tfa-HI **3c** (28 mg) was treated by the procedure previously described for **4a** providing $N^{\alpha B1}$, $N^{\varepsilon B29}$-di-Tfa-$N^{\alpha A1}$-FSC-HI **4c** (22 mg) as a bright yellow lyophilized powder with 74% yield and ESI-MS: 6387.0 (calculated: 6388.7).

$N^{\varepsilon B29}$-FSC-HI (**5a**)

$N^{\alpha A1}$, $N^{\alpha B1}$-di-Tfa-$N^{\varepsilon B29}$-FSC-HI **4a** (28 mg) was dissolved in a pre-cooled (0 °C) 15% NH_4OH solution (20 ml), and this mixture was stored at a 4 °C overnight. The next day LC-MS analysis indicated the reaction was complete. The mixture was then diluted with H_2O (100 ml) and purified by an RP preparative HPLC. Lyophilization of the combined fractions afforded the product $N^{\varepsilon B29}$-FSC-HI **5a** (24 mg) as bright yellow powder with 89% yield, 91% purity (Figure 3) and ESI-MS: 6196.5 (calculated: 6196.7)



Scheme 1. Synthetic routes towards Tfa-protected and/or FSC-labeled HIs. Reagents and Conditions: (a) Tfa-OEt, DIEA, DMF, 65%; (b) Tfa-OEt, DIPEA, ACN/H_2O, 96% for **3b**, 50% for **3c**; (c) i, $(Boc)_2O$, Et_3N, DMSO; ii, Tfa-OEt; iii, TFA, TIS, H_2O; 50% over three steps; (d) FITC, DIEA, DMF, 80% for **4a**, 76% for **4b** and 74% for **4c**; (e) 15% NH_4OH, 4°C, 89% for **5a**, 68% for **5c**; (f) 25 mM NaOH, ACN/H_2O, 4°C, 86% for **5b**.

$N^{\alpha B1}$-FSC-HI (**5b**)

$N^{\alpha A1}$, $N^{\varepsilon B29}$-di-Tfa-$N^{\alpha B1}$-FSC-HI **4b** (30 mg) was dissolved in a pre-cooled (0 °C) 25 mM NaOH solution (40 ml, ACN/H_2O = 1 : 3), maintained at 4 °C. LC-MS analysis indicated that the reaction was complete after 4 h. The reaction mixture was diluted by H_2O (100 ml) and purified by an RP preparative HPLC. Lyophilization of the combined fraction afforded the product $N^{\alpha B1}$-FSC-HI **5b** (24 mg) as bright yellow powder with 86% yield, 98% purity (Figure 3) and ESI-MS: 6196.0 (calculated: 6196.7).

$N^{\alpha A1}$-FSC-HI (**5c**)

$N^{\alpha B1}$, $N^{\varepsilon B29}$-di-Tfa-$N^{\alpha A1}$-FSC-HI **4c** (22 mg) was treated by the procedure previously described for **5a** providing $N^{\alpha A1}$-FSC-HI **5c** (14 mg) as a bright yellow lyophilized powder with 68% yield, 93% purity (Figure 3) and ESI-MS: 6196.2 (calculated: 6196.7).

Optimization of Tfa protection conditions

All screening reactions used 50 mg zinc-free or zinc-formulated HI (**1**) as the starting material. The reactions were monitored by LC-MS, and the yields were calculated on the basis of the integration of each peak area in the analytical HPLC traces. All Tfa-protected insulins (Table 1) were characterized by mass spectral analysis (see aforementioned preparation and characterizations of **2a–6**), and sites of Tfa modification were confirmed through Glu-C (*S. aureus* V8 protease) digestion and LC-MS analysis of the fragments (Table 4 and Figure S1).

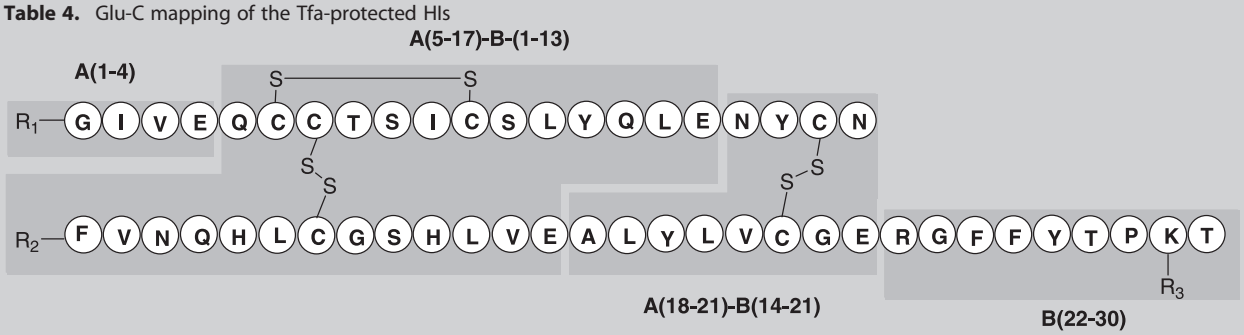
Single solvent

HI (zinc crystal or zinc free) 50 mg was dissolved or suspended in a total volume (3.0 ml or as indicated in Table 2) of a single solvent [DMF, DMSO, ACN or H_2O], with DIEA (40 eq, 60 µl or as indicated in Table 2) and Tfa-OEt (ethyl trifluoroacetate, 20 eq, 20 µl or as indicated in Table 2). The mixture was stirred at room temperature and monitored using LC-MS at 2 and 18 h. It appeared no further conversion after 18 h, so yields were calculated at 18 h. Among entries 1–10, entries 5 and 6 [50 mg of zinc-free insulin in DMF (3.0 ml) with 6–12 eq of Tfa-OEt and 40 eq DIEA] gave the highest yield of $N^{\alpha A1}$, $N^{\alpha B1}$-di-Tfa-HI (~70%) (Table 2).

Aqueous mixed solvent

HI (zinc crystal or zinc free) 50 mg was dissolved in a total volume (3.0 ml or as indicated in the below table) of mixture solvent (DMSO/H_2O, DMF/H_2O or ACN/H_2O). DIEA (100 eq,160 µl or as indicated in Table 3) and Tfa-OEt (50 eq, 52 µl or as indicated in Table 3) were added. The reaction mixture was stirred at room temperature and monitored by LC-MS at 2 and 18 h. It appeared no further conversion after 2 h, so the yields were calculated at 2 h. Among entries 1–20, entry 20 [50 mg of zinc HI in ACN/H_2O

Table 4. Glu-C mapping of the Tfa-protected HIs



	A(1–4)	Tfa- A(1–4)	A(5–17)-B(1–13)	(Tfa)-A(5–17)-B(1–13)	A(18–21)-B(14–21)	B(22–30)	Tfa-B(22–30)
	416.5[a]	513.5	2969.4	3066.4	1377.6	1116.3	1213.3
1	5.56/416.1[b]	/[c]	8.90/2969.6	/	8.12/1377.7	7.45/1116.7	/
2a	*[d]	9.88/513.2	8.89/2968.8	/	8.11/1376.8	7.45/1115.8	/
2b	5.52/416.1	/	8.90/2968.5	/	8.11/1376.8	/	9.24/1211/8
2c	5.57/416.1	/	/	10.49/3064.5	8.13/1376.8	7.47/1116.5	/
3a	*	9.86/513.2	/	10.47/3066.4	8.12/1376.4	7.45/1115.8	/
3b	/	9.82/513.2	8.91/2968.8	/	8.13/1376.8	/	9.25/1212.7
3c	5.46/416.1	/	/	10.50/3064.2	8.14/1377.7	*	9.27/1212.7
6	*	9.83/513.2	*	10.47/3064.5	8.11/1376.8	*	9.24/1211.8

[a]Calculated molecular weight.
[b]Retention time (min)/molecule weight of this fragment (Da).
[c]Not observed.
[d]Detectable by MS, presumably due to the slow hydrolysis of Tfa group at the Glu-C digestion condition (pH 8.4, 50 mM Tris buffer, r.t.).

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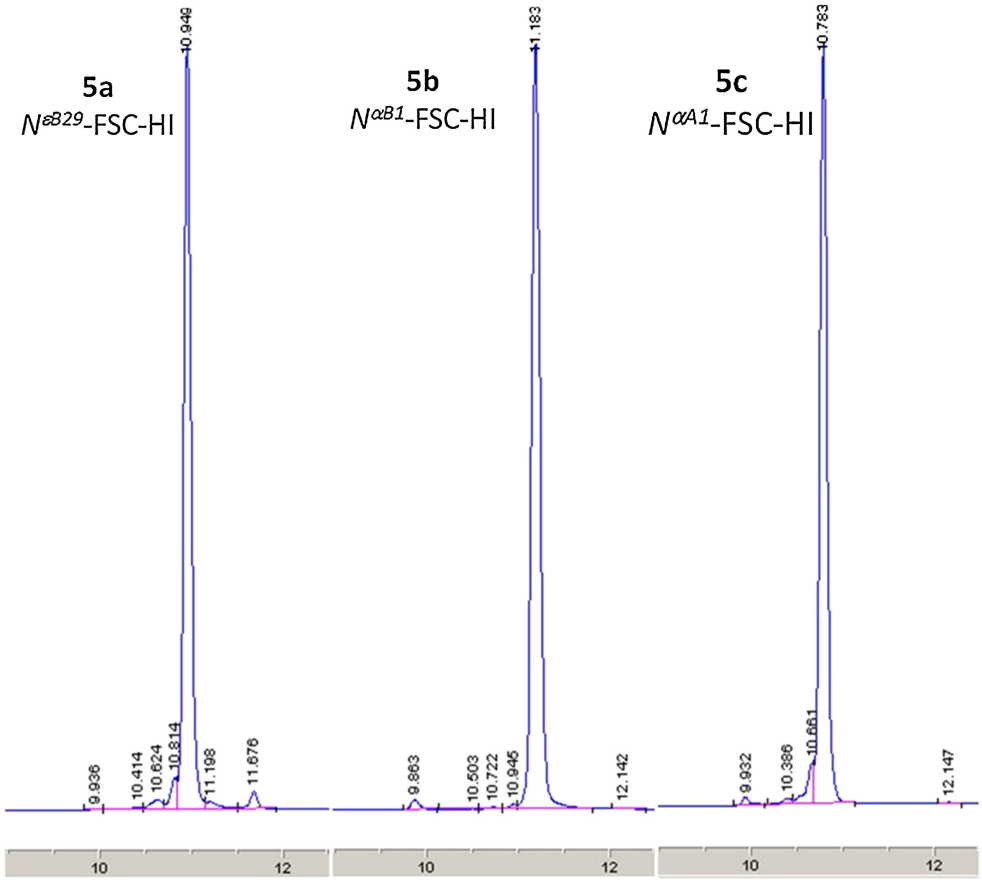

Figure 3. Analytical HPLC traces for **5a**, **5b** and **5c**.

(0.50/0.50 ml) with 300 eq of DIEA and 300 eq of Tfa-OEt] gave the highest yield of $N^{\alpha A1}$, $N^{\varepsilon B29}$-di-Tfa-HI (~80%) (Table 3).

S. aureus V8 protease digestion protocol

The Tfa-protected HIs (~100 μg) were mixed with 10 μg Glu-C in 100 μl of pH 8.4, 50 mM Tris buffer. The solution was incubated at r.t. for 4 h before analyzed by LC-MS (Table 4 and Figure S1).

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References

1 Jameson DM, Ross JA. Fluorescence polarization/anisotropy in diagnostics and imaging. *Chem. Rev.* 2010; **110**(5): 2685–2708.
2 Giepmans BNG, Adams SR, Ellisman MH, Tsien RY. The fluorescent toolbox for assessing protein location and function. *Science* 2006; **312**(5771): 217–224.
3 Lavis LD, Raines RT. Bright ideas for chemical biology. *ACS Chem. Bio.* 2008; **3**(3): 142–155.
4 Riggs JL, Seiwald RJ, Burckhalter JH, Downs CM, Metcalf TG. Isothiocyanate compounds as fluorescent labeling agents for immune serum. *Am. J. Pathol.* 1958; **34**(6):1081–1097.
5 Berns AW, Hirata Y, Blumenthal HT. Application of fluorescence microscopy to the study of possible insulin-binding reactions in formalin-fixed material. *J. Lab. Clin. Med.* 1962; **60**: 535–551.
6 Maggi V. The localization of fluorescent insulin in mouse tissues. *Exp. Cell Res.* 1966; **44**(2): 672–676.
7 Parker JW, Elevitch FR, Grodsky GM. Binding of fluorescent insulin to intracellular antibodies in guinea pigs immunized with insulin. *Proc. Soc. Exp. Biol. Med.* 1963; **113**(1): 48–53.
8 Tietze F, Mortimore GE, Lomax NR. Preparation and properties of fluorescent insulin derivatives. *Biochim. Biophys. Acta* 1962; **59**: 336–346.
9 Ciencialova A, Zakova L, Jiracek J, Barthova J, Barth T. Preparation and characterization of two LysB29 specifically labelled fluorescent derivatives of human insulin. *J. Pept. Sci.* 2004; **10**(7): 470–478.
10 Bromer WW, Sheehan SK, Berns AW, Arquilla ER. Preparation and properties of fluoresceinthiocarbamyl insulins. *Biochemistry* 1967; **6**(8): 2378–2388.
11 Hentz NG, Richardson JM, Sportsman JR, Daijo J, Sittampalam GS. Synthesis and characterization of insulin-fluorescein derivatives for bioanalytical applications. *Anal. Chem.* 1997; **69**(24): 4994–5000.
12 Levy D, Carpenter FH. Synthesis of triaminoacylinsulins and the use of the *tert*-butyloxycarbonyl group for the reversible blocking of the amino groups of insulin. *Biochemistry* 1967; **6**(11): 3559–3568.
13 Geiger R, Schone HH, Pfaff W. Bis (t-butyloxycarbonyl) insulin. *Hoppe-Seyler's Z. Physiol. Chem.* 1971; **352**(11): 1487–1490.
14 Geiger R, Geisen K, Regitz G, Summ HD. [A1-beta-alanine] insulin. *Hoppe-Seyler's Z. Physiol. Chem.* 1976; **357**(9): 1267–1270.
15 Canova-Davis E, Carpenter FH. Semisynthesis of insulin: specific activation of the arginine carboxyl group of the B chain of desoctapeptide-(B23-30)-insulin (bovine). *Biochemistry* 1981; **20**(24): 7053–7058.
16 Riemen MW, Pon LA, Carpenter FH. Preparation of semisynthetic insulin analogues from bis(*tert*-butyloxycarbonyl)-desoctapeptide-insulin phenylhydrazide: importance of the aromatic region B24-B26. *Biochemistry* 1983; **22**(6): 1507–1515.
17 Jullian M, Hernandez A, Maurras A, Puget K, Amblard M, Martinez J, Subra G. N-terminus FITC labeling of peptides on solid support: the truth behind the spacer. *Tetrahedron Lett.* 2009; **50**(3): 260–263.
18 Borras F, Offord RE. Protected intermediate for the preparation of semisynthetic insulins. *Nature* 1970; **227**(5259): 716–718.
19 Friesen HJ, Weimann J, Nowak J, Brandenburg D. Acylation and deacylation of insulins: analytical investigations and consequences for the preparation of derivatives. *Semisynth. Pept. Proteins, Pap. Int. Meet. Protein Semisynth*. 1978: 161–179.

