Journal of Peptide Science
*J. Pept. Sci.* 2007; **13**: 334–341
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.847

JOURNAL OF PEPTIDE SCIENCE

# The use of Fmoc-Lys(Pac)-OH and penicillin G acylase in the preparation of novel semisynthetic insulin analogs

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*Received 15 November 2006; Revised 30 January 2007; Accepted 31 January 2007*

**Abstract:** In this paper, we present the detailed synthetic protocol and characterization of Fmoc-Lys(Pac)-OH, its use for the preparation of octapeptides H-Gly-Phe-Tyr-*N*-MePhe-Thr-Lys(Pac)-Pro-Thr-OH and H-Gly-Phe-Phe-His-Thr-Pro-Lys(Pac)-Thr-OH by solid-phase synthesis, trypsin-catalyzed condensation of these octapeptides with desoctapeptide(B23-B30)-insulin, and penicillin G acylase catalyzed cleavage of phenylacetyl (Pac) group from $N^{\varepsilon}$-amino group of lysine to give novel insulin analogs [TyrB25, *N*-MePheB26,LysB28,ProB29]-insulin and [HisB26]-insulin. These new analogs display 4 and 78% binding affinity respectively to insulin receptor in rat adipose membranes. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

**Keywords:** insulin analogs; semisynthesis; phenylacetyl; penicillin G acylase

## INTRODUCTION

Despite that almost all medical applications of insulin are provided by its recombinant production, the synthetic or semisynthetic approach still remains important for the preparation of various insulin analogs. The advantage of chemical synthesis or semisynthesis of insulin analogs results from the possibility of a rapid incorporation of noncoded amino acids into insulin molecules [1–3]. Such nonstandard modifications may provide new insights into research on insulin function–structure relationships. Hence, insulin analogs may provide an excellent tool in the systematic study of insulin–insulin receptor interaction, and in the search for the 'active conformation' of insulin upon binding to the receptor [4,5]. Insulin analogs may also become new drugs for the treatment of diabetes or other diseases related to the impaired function of insulin or insulin receptor molecule [6,7].

The total chemical synthesis is the most flexible way for the preparation of insulin analogs [5,8,9], however, its low yield, extensive costs and laborious protocols prohibit its wide use. On the contrary, semisynthesis of this hormone is an elegant and relatively straightforward method. Semisynthesis of insulin analogs uses either the entire or some parts of the insulin molecule for chemical modifications, e.g. natural chain A is combined with a chemically prepared analog of chain B [10].

Enzymatic semisynthesis, one of the most interesting semisynthetic approaches, employs enzymes for cleavage and/or formation of covalent bonds in the insulin molecule and may be combined with the chemical synthesis of different insulin fragments. The disadvantage of the enzymatic semisynthesis is its limitation to changes only in the *C*-terminus of the B-chain due to restricted specificity of available enzymes, e.g. to ArgB22 and LysB29 sites for trypsin [11], LysB29 for *Achromobacter* protease I [12] and PheB25-TyrB26 site for pepsin [13]. Papain, chymotrypsin, and thermolysin are also used for formation of the peptide bond after cleavage by pepsin [14]. Fortunately, the *C*-terminus of the B-chain of insulin molecule is one of the most important segments, which is directly involved in the interaction of insulin with its receptor [15] and in the formation of insulin dimers [16]. The *C*-terminal octapeptide of the B-chain can be cleaved from insulin and attached again to desoctapeptide(B23-B30)insulin (DOI) with the use of trypsin [10,17,18]. This approach offers an extremely large variety of possible modifications that may produce interesting analogs.

Nevertheless, the method of enzymatic semisynthesis of insulin is fraught with several difficulties. One of the main problems is the necessity to protect the amino group of LysB29 upon the trypsin-catalyzed condensation of DOI and *C*-terminal synthetic peptide to avoid side reactions [19,20]. The major problem is the formation of undesirable condensation products via $N^{\varepsilon}$-amino group of lysine [21]. If $N^{\varepsilon}$-amino group of LysB29 is not protected during trypsin-catalyzed semisynthesis it can react with the carboxyl group of ArgB22. The protection of LysB29 is much more important than the protection of PheA1 and GlyB1

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because the synthetic peptide is in high molar excess compared to DOI [18]. In the 1970s, the first semisyntheses of insulin were carried out with lysine $N^{\varepsilon}$-protected by Boc [10,22–28] or Tfa [29,30] group. Moreover, PheA1 and GlyB1 of DOI were $N^{\alpha}$-protected by Boc groups or by methylsulfonylethyloxycarbonyl (Msc) groups [31]. Later, the PheA1 and GlyB1 protection turned out to be redundant, because only minor side reactions with the involvement of these residues were observed [18]. The protection of LysB29 by chemically cleavable groups as Boc, Tfa or Msc is not suitable because of the possible damaging effect of cleavage reagents on the insulin structure. Moreover, the instability of Boc in TFA or Tfa/Msc in piperidine complicates, or even disables, the solid-phase synthesis of peptides with such $N^{\varepsilon}$-Lys protection. This initiated a search for better protecting groups, which could be removed from the lysine side-chain under mild conditions. The enzymatically removable group seemed to be a method of choice for this purpose.

The enzymes usually operate at wide-ranging pH values and in many cases combine a high selectivity for the catalyzed reaction with a broad substrate tolerance [32,33]. Chymotrypsin and trypsin were first used for deprotection of *N*-protected peptides. For instance, the Bz group is removed from Bz-Phe by chymotrypsin [34] and the Z group is removed from Z-Arg by trypsin [35,36]. The disadvantage of using chymotrypsin and trypsin is that these endopeptidases catalyze the hydrolysis of specific peptide bonds, often present in synthesized peptide analogs.

Requirements for an enzymatically cleavable protecting group for primary amines and a 'deprotective' enzyme without peptidase activity are fulfilled by the penicillin G acylase (penicillin amidohydrolase or penicillin amidase, PA) from *Escherichia coli* (E.C.3.5.1.11). This enzyme, widely used in industry for large-scale synthesis of semisynthetic penicillin, attacks phenylacetyl amides and esters but does not hydrolyze peptide bonds [32,33,37]. Moreover, PA works under neutral conditions (pH 7–8), at room temperature (RT) and leaves other groups such as methyl, allyl, benzyl or *tert*-butyl esters unaffected [38–41]. An important condition for application of PA is that the enzyme must not be contaminated by endo- or exopeptidases [42]. Nowadays, PA is commercially available as an immobilized enzyme or as a soluble protein. The advantage of phenylacetyl protection of primary amines results from its relatively easy introduction and high chemical stability [38,39]. The disadvantage, however, is some level of racemization if phenylacetyl is introduced to $\alpha$-amino groups of amino acids (about 6%) [38,39], but this problem can be overcome if phenylacetyl moiety is introduced through an enzymatic route [43].

The concept of the use of $N^{\varepsilon}$-Pac-protected lysine in the synthesis of peptides was introduced in 1981 by Brtník *et al.* [37]. They prepared, and successfully used, Z-Lys(Pac)-OH for the solution synthesis of deaminolysine vasopressin. The Z group was cleaved by HBr in acetic acid and the final cleavage of the phenylacetyl group was achieved by PA isolated from *E. coli*. The disadvantage of Z-Lys(Pac)-OH is that it is hardly applicable in the solid-phase synthesis due to the necessity of cleavage of the Z group by hydrogenation or by HBr in acetic acid.

The phenylacetyl protection applied in insulin chemistry was first reported by the group of Wang *et al.* [44] in 1986. They examined the nonselective phenylacetyl protection of amines in the insulin molecule and subsequent cleavage by PA without apparent damage of the protein backbone. The authors proposed a phenylacetyl group and PA-deprotection for the semisynthetic preparation of protein and large peptide analogs.

For several years, our research team has been involved in the synthesis of insulin analogs with modification in the *C*-terminus of the B-chain. We prepared shortened or full-length insulin using the above described enzymatic semisynthesis [45–48]. The analogs of *C*-terminal octapeptide of the B-chain are synthesized by solid-phase synthesis chemistry using Fmoc-Lys(Pac)-OH if peptides contain lysine. However, the preparation of this amino acid derivative as well as the detailed description of PA-catalyzed cleavage of phenylacetyl from protected insulin analog has not been published as yet.

In this paper, we present the detailed synthetic protocol and characterization of ($N^{\alpha}$-fluorenylmethoxycarbonyl, $N^{\varepsilon}$-phenylacetyl)-L-lysine, its use for the preparation of synthetic octapeptides H-Gly-Phe-Tyr-*N*-MePhe-Thr-Lys(Pac)-Pro-Thr-OH and H-Gly-Phe-Phe-His-Thr-Pro-Lys(Pac)-Thr-OH by solid-phase synthesis, trypsin-catalyzed condensation of these octapeptides with DOI and penicillin G acylase catalyzed cleavage of phenylacetyl group from $N^{\varepsilon}$-amino group of lysine to give novel insulin analogs [TyrB25,*N*-MePheB26,LysB28,ProB29]-insulin and [HisB26]-insulin.

## EXPERIMENTAL

### Materials

For abbreviations see Ref [49]. All amino acids used were in their L form. Fmoc-Lys(Boc)-OH was purchased from Bachem. Other Fmoc-protected amino acids, HBTU and 2-chlorotrityl resin were purchased from Novabiochem. *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated bovine trypsin was purchased from Sigma-Aldrich (T-1426 or T-8802) and from Worthington (TRTVMF). PA immobilized on Eupergit C and soluble PA were from *E. coli* and purchased from Fluka (product code 76 429 and 76 427, respectively). All other chemicals and solvents were obtained from Sigma-Aldrich-Fluka. Human $^{125}$I-monoiodotyrosylA14-insulin was purchased from Amersham.

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## Fmoc-Lys(Pac)-OH

Reactions were monitored by thin-layer chromatography (TLC) on Merck silica gel 60 $F_{254}$ plates and visualized under UV light (254 nm) and/or by heating with phosphomolybdic-acid solution in ethanol. Preparative column chromatography was carried out on Merck silica gel 60. $^1$H-NMR spectra were measured on a Bruker Avance 400 spectrometer with TMS as an internal standard. Multiplicities are designated singlet (s), doublet (d), triplet (t), multiplet (m), or broad singlet (br s). Positive-ion FAB mass spectra were acquired on a ZAB-EQ (VG Analytical, UK) spectrometer. IR spectra (wavenumbers in cm$^{-1}$) were recorded on a Bruker IFS 88 spectrometer. Elemental analyses were determined on a PE 2400 Series II CHNS/O Analyzer (Perkin Elmer, USA). Optical rotations were run on an Autopol IV (Rudolph Research Analytical, USA) polarimeter. Melting points were obtained using a Kofler block and are uncorrected. Solvents were evaporated using vacuum rotary evaporator at 30 °C. Analytical samples were dried at 6.5 Pa and 40 °C for 8 h.

***Method A.*** H-Lys(Pac)-OH, was prepared in the yield of 64.9% according to the modified procedure of Brtník *et al.* [37], using aqueous EDTA disodium salt instead of hydrogen sulfide for decomposition of the bis[Lys(Pac)]copper(II) complex [50,51]. Fmoc-OSu (5060 mg, 15.0 mmol) and 1 M NaOH (to maintain pH 9.5) were added portion-wise within 30 min to a stirred slurry of H-Lys(Pac)-OH (3965 mg, 15.0 mmol) in a mixture of 1 M NaOH (15.0 ml), water (35.0 ml) and 1,4-dioxane (50.0 ml) at RT. The mixture was stirred at RT for 2 h and dioxane was removed *in vacuo*. The residue was partitioned between EtOAc and 1 M $KHSO_4$. Organic phase was washed by 10% NaCl, dried over anhydrous $MgSO_4$, filtered and evaporated *in vacuo*. The solid residue was purified by chromatography on a silica gel column in EtOAc-HCOOH 100 : 1 to afford 5678 mg (77.8%) of Fmoc-L-Lys(Pac)-OH as a white crystalline powder. $^1$H NMR (DMSO-$d_6$, 400 MHz): $\delta_{ppm}$ 12.58 (br s, 1H, COOH), 8.02 (t, 1H, $J$ 5.4 Hz, N$H$CH$_2$), 7.89 (d, 2H, $J$ 7.6 Hz, fluorene), 7.73 (d, 2H, $J$ 7.6 Hz, fluorene), 7.61 (d, 1H, $J$ 8.1 Hz, N$H$CH), 7.41 (t, 2H, $J$ 7.3 Hz, fluorene), 7.32 (t, 2H, $J$ 7.3 Hz, fluorene), 7.15–7.30 (m, 5H, Ph), 4.28 (d, 2H, $J$ 6.3 Hz, CHC$H_2$O), 4.23 (t, 1H, $J$ 6.5 Hz, C$H$CH$_2$O), 3.88–3.93 (m, 1H, NHC$H$), 3.38 (s, 2H, PhC$H_2$), 2.97–3.09 (m, 2H, NHC$H_2$), 1.55–1.74 (m, 2H, CHC$H_2$CH$_2$), 1.24–1.46 (m, 4H, CH$_2$C$H_2$C$H_2$CH$_2$). mp 145.5–146.5 °C (EtOAc). $[\alpha]_D^{25} - 11.99$ deg ml dm$^{-1}$ g$^{-1}$ ($c = 0.21$ in DMF). Anal. calcd. for $C_{29}H_{30}N_2O_5$ (486.57): C 71.59%, H 6.21%, N 5.76%, O 16.44%; found: C 71.26%, H 6.22%, N 5.59%. FAB-MS (thioglycerol-glycerol): $m/z$ 487.2 $(M + H)^+$. IR (KBr): 3341 (NH), 1729, 1699 (C = O), 1654 (amide I), 1615 (phenyl ring), 1602 (fluorene ring), 1535 (amide II).

***Method B.*** Fmoc-Lys(Boc)-OH (7028 mg, 15.0 mmol) was treated with TFA and lyophilized from 1,4-dioxane. The crude Fmoc-Lys-OH.TFA was dissolved in a mixture of dichloromethane-1,4-dioxane (1 : 1, 100 ml), cooled to 0 °C and triethylamine (8.4 ml, 60.4 mmol) followed by phenylacetyl chloride (2.4 ml, 18.1 mmol) was added drop-wise. The mixture was stirred at RT overnight. Solvents were evaporated *in vacuo*. The identical work up used as in Method A afforded 3650 mg (50%) of Fmoc-Lys(Pac)-OH as a white crystalline powder. An analytical sample was crystallized from EtOAc. The analytical data were in accordance with those of the product prepared by Method A.

## H-Gly-Phe-Tyr-(*N*-Me)Phe-Thr-Lys(Pac)-Pro-Thr-OH and H-Gly-Phe-Phe-His-Thr-Pro-Lys(Pac)-Thr-OH

The octapeptides were synthesized on a 2-chlorotrityl resin [52] using Fmoc-protected amino acids with *t*Bu protection of side-chains of Thr and Tyr and Trt protection of the side-chain of His [53]. Similar synthetic protocol was used for both peptides. Coupling was carried out using HBTU/DIPEA reagents [54] in 1-methyl-2-pyrrolidinone (NMP). Coupling after *N*-MePhe was performed with bromo-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBrop)/DIPEA reagents [55]. The completeness of the synthetic reaction was controlled by Kaiser test [56] and by measuring the absorbance of piperidine-dibenzofulvene complex after Fmoc group deprotection using extinction coefficient 7040 M$^{-1}$ cm$^{-1}$ at 301 nm [57]. The peptide was cleaved from the resin with a mixture of AcOH : TFE : DCM (2 : 2 : 6) (v/v/v) for 2 h [52]. The residue was evaporated to dryness and treated with a mixture of TFA : triisopropylsilane (TIS) : DCM : $H_2O$ (50 : 2 : 48 : 2) (v/v/v/v) for 30 min. The mixture was evaporated *in vacuo* and treated with diethylether. The solid residue after diethylether extraction was dissolved in 10% AcOH and peptide was purified using RP-HPLC (Phenomenex column Luna C-18, 250 × 21.2 mm, 10 μm). The purity of the peptides was controlled using analytical Nucleosil 120-5 C-18 column (250 × 4 mm, Watrex Praha, Czech Republic). The identity of the peptides was confirmed with MALDI-TOF mass spectrometry.

## Enzymatic Semisynthesis of (TyrB25,N-MePheB26,Lys(Pac)B28,ProB29)-insulin and (HisB26,Lys(Pac)B29)-insulin

The semisyntheses of these full-length insulin analogs were performed according to the method of Svoboda *et al.* [45] with a few modifications. The same protocol was used for both peptides. DOI was prepared from porcine insulin as described previously [47]. Typically, H-Gly-Phe-Tyr-(*N*-Me)Phe-Thr-Lys(Pac)-Pro-Thr-OH or H-Gly-Phe-Phe-His-Thr-Pro-Lys(Pac)-Thr-OH peptide (150 mM) and DOI (30 mM) were dissolved in a solution (the total volume of 200 μl) containing 55% aq. dimethylformamide, 20 mM calcium acetate and 4.7 mg of TPCK-trypsin (enzyme/substrate molar ratio of 1 : 30). The pH value was adjusted by *N*-methylmorpholine (usually 0.5–6 μl) to 6.9–7.0 and checked by ISFET pH Meter IQ125 (IQ Scientific Instruments, Carlsbad, CA, USA) or by pH testing paper. The resulting reaction mixture was incubated at RT (20–25 °C). After 7–24 h, the reaction, monitored by analytical RP-HPLC, was stopped by the addition of acetone (4 °C). The sediment was dissolved in 10% acetic acid and the product was separated from trypsin, DOI and side products by preparative RP-HPLC using gradient of acetonitrile. The peptide was purified by RP-HPLC (Nucleosil 120-5 C-18 column, 250 × 8 mm, Watrex Praha, Czech Republic). The purity of peptide was verified using analytical RP-HPLC (Nucleosil 120-5 C-18 column, 250 × 4 mm, Watrex Praha, Czech Republic). The fractions containing the product were pooled and lyophilized. The identity of insulin analog was confirmed with MALDI-TOF mass spectrometry (Reflex IV, Bruker).

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DOI: 10.1002/psc

### Enzymatic Deprotection of (TyrB25,N-MePheB26,Lys(Pac)B28,ProB29)-insulin and (HisB26,Lys(Pac)B29)-insulin

[TyrB25,*N*-MePheB26, Lys(Pac)B28,ProB29]-insulin or [HisB26,Lys(Pac)B29]-insulin (5 mg) was dissolved in 1 ml of 50 mM K-phosphate buffer pH 7.5 and soluble PA (10 IU in 6.6 μl of the buffer) or PA immobilized on Eupergit C (30 mg of the resin prewashed with the buffer) was added. The reaction proceeded at RT (22 °C) and the progress of the reaction was followed with RP-HPLC. If needed, another portion of PA was to be added. After completion of the deprotection (12–24 h), the insulin analog was purified and characterized as described above.

### Isolation of Rat Adipose Tissue Plasma Membranes

Plasma membranes [58] were prepared from epididymal adipose tissue of adult male Wistar rats weighing 210–250 g. The adipose tissue (5–6 g) was homogenized using ultra-turrax with 20 ml of buffer consisting of 10 mM tris(hydroxymethyl)aminomethane (Tris)/HCl, 250 mM sucrose, 1 mM phenylmethylsulfonylfluoride (PMSF), and 1 mM benzamidine, pH 7.4. The mixture was centrifuged at 3000 × g for 15 min at 4 °C and then the supernatant was centrifuged again at 17 000 × g for 15 min at 4 °C. The resulting pellet was resuspended in 1 ml of 0.05 M Tris/HCl buffer, pH 7.6, and the protein concentration was determined according to Bradford [59].

### Receptor Binding Studies

Plasma membranes (50 μg of proteins) were incubated in 5 ml tubes with $^{125}$I-insulin at a concentration of about $5 \times 10^{-10}$ M (∼ 57 000 dpm) and various concentrations (range, $10^{-13} - 10^{-5}$ M) of insulin or insulin analogs in the buffer composed of 100 mM Tris/HCl, 2 mM *N*-ethylmaleimide, 13.2 mM $CaCl_2$, and 0.1% (w/v) BSA, pH 7.6, in a total volume of 250 μl. The solution was incubated at 4 °C for 21 h and then the reaction was terminated by the addition of ice-cold 120 mM NaCl followed by a quick filtration on a Brandel cell harvester (Biochemical Research and Development Laboratories, Gaithersburg, MD, USA). Bound radioactivity was determined by $\gamma$-counting (Wizard 1470 Automatic Gamma Counter, Perkin Elmer, Wellesley, MA, USA). The total binding (the binding in the absence of the competitor) was 8–10% of the total radioactivity. The nonspecific binding (the binding in the presence of $10^{-5}$ M insulin) was determined to be about 30% of the total binding.

### Analysis of Binding Data

Competitive binding curves were plotted using Graph-Pad Prizm 3 Software (San Diego, CA, USA), comparing best fits for single-binding site models. Half-maximal inhibition values of binding of $^{125}$I-insulin to receptor ($IC_{50}$) were obtained from nonlinear regression analysis.

## RESULTS AND DISCUSSION

We used two different synthetic protocols for the preparation of Fmoc-Lys(Pac)-OH. Method A employed modified the procedure of Brtník *et al.* [37] for the preparation of H-Lys(Pac)-OH starting from H-Lys-OH. We used aqueous EDTA disodium salt solution instead of toxic and malodorous hydrogen sulfide for decomposition of the bis[Lys(Pac)]copper(II) complex [50,51]. Our procedure gave a somewhat better yield (65%) than the original protocol (50%). Subsequent introduction of $N^{\alpha}$-Fmoc group proceeded in a satisfactory yield of 78%.

Method B started from commercially available Fmoc-Lys(Boc)-OH and involved the cleavage of $N^{\varepsilon}$-Boc group and introduction of $N^{\varepsilon}$-phenylacetyl protecting group. This route is simpler and much less laborious than Method A. The relatively moderate yield of 50% is probably caused by a partial cleavage of base-labile $N^{\alpha}$-Fmoc group in the course of the introduction of phenylacetyl group in the presence of triethylamine. The fact that Method B is more straightforward than Method A compensates the higher cost of Fmoc-Lys(Boc)-OH compared to free lysine.

Recently, Žáková *et al.* [48] published the synthesis, binding affinity and the crystal structure of [TyrB25, *N*-MePheB26]-insulin analog. The analog was designed to address the problem of structural and biological switch from mostly metabolic activity of insulin to predominant growth factor activities of insulin-like growth factor I IGF-I. The modifications were expected to introduce a kink in the main chain, as observed at residue PheB25 in the IGF-I structure. Surprisingly, the changes are well accommodated within insulin R6 hexamer. Only one molecule of each dimer in the hexamer responds to the structural TyrB25 and *N*-MePheB26 alterations, the other remains very similar to the wildtype insulin. In this study, we decided to synthesize and characterize the binding potency of insulin analog in which the swap TyrB26 ↔ PheB25 and the *N*-methylation of resulting PheB26 is accompanied with the swap LysB29 ↔ ProB28. LysB29 ↔ ProB28 swap was shown to cause the monomerization of insulin [60]. X-ray crystallography study should confirm if the more IGF-I-like sequence of [TyrB25, *N*-MePheB26,LysB28,ProB29]-insulin is followed by IGF-like conformational distortion of the B-chain.

Recently, we published a series of novel des-tetrapeptide(B27-B30)-shortened insulin analogs (B26-amides) [47]. We demonstrated that the substitution of TyrB26 with His or *N*-MeHis resulted in potent shortened insulin analogs. In this study, we decided to synthesize full-length insulin analog with His in the position B26 and to investigate its binding affinity for insulin receptor in rat adipocyte membranes.

The solid-phase synthesis of H-Gly-Phe-Tyr-(*N*-Me)Phe-Thr-Lys(Pac)-Pro-Thr-OH or H-Gly-Phe-Phe-His-Thr-Pro-Lys(Pac)-Thr-OH requires coupling with

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DOI: 10.1002/psc

Fmoc-Lys(Pac)-OH as a third or second synthetic step, respectively. We observed fully satisfactory couplings using this lysine derivative as proved by monitoring the absorbance of piperidine–dibenzofulvene complex after Fmoc group deprotection (see Experimental). We found similar satisfactory results for the coupling of protected threonine or proline in the course of the subsequent synthetic step. The octapeptides were obtained in the yield of 40–45% after HPLC purification (150 μmole synthetic scale).

The crucial step in our semisynthetic approach is the trypsin-catalyzed condensation of DOI with synthetic peptide. We performed the semisynthesis of [TyrB25, *N*-MePheB26,Lys(Pac)B28,ProB29]-insulin ten times and the synthesis of [HisB26,Lys(Pac)B29]-insulin six times in a 200 or 100 μl synthetic scale (see Experimental). The time-courses of typical semisynthetic reactions of both insulin analogs are shown in Figure 1(A). The concentration of DOI in a reaction mixture progressively decreases and the concentration of a product increases. Figure 1(B) shows HPLC chromatogram of purification of [TyrB25,*N*-MePheB26,Lys(Pac)B28,ProB29]-insulin from a semisynthetic reaction mixture. During semisynthesis not only the desired product (peak 3 in Figure 1(B)) was formed, but also various side products appeared as well (peaks 4 and 5 in Figure 1(B)), with the products of selfcondensation of DOI as the most frequent species. The coupling of the carboxyl group of ArgB22 either with the *N*-terminus of the same molecule results in SC-DOI (single-chain DOI) or with the *N*-terminus of another DOI molecule gives $(DOI)_2$ (covalent DOI dimer) [17,18,45]. The average yield of our semisyntheses of [TyrB25,*N*-MePheB26,Lys(Pac)B28,ProB29]-insulin was about 11% calculated from the starting amount of DOI, which is a limiting reactant. However, in some cases the yield was as high as 32% or as low as 2%. The average yield of semisyntheses of [HisB26,Lys(Pac)B29]-insulin was about 15% (3–26%). In general, the yield of semisynthesis of any insulin analog strongly depends on the quality of trypsin and on the structure and length of the peptide. We have been using several different TPCK-treated trypsins (see Materials). Interestingly, we observed that after some time, trypsin may lose its 'synthetic' activity but still keeps its hydrolytic capacity; in such a case it is necessary to purchase a fresh batch of this enzyme.

The enzymatic cleavage of phenylacetyl group from [Lys(Pac)]-insulin is the final step of the preparation of a new analog. Our laboratory has the experience with the use of several different forms of PA. At the beginning of 1990s, we used PA isolated from *E. coli* by affinity chromatography [42] and immobilized on porous glass [61]. These enzyme preparations were of a relatively low specific activity and it was necessary to circulate the solution of substrate through a column of Dowex $1 \times 8$ ($OH^-$ form), devised to trap

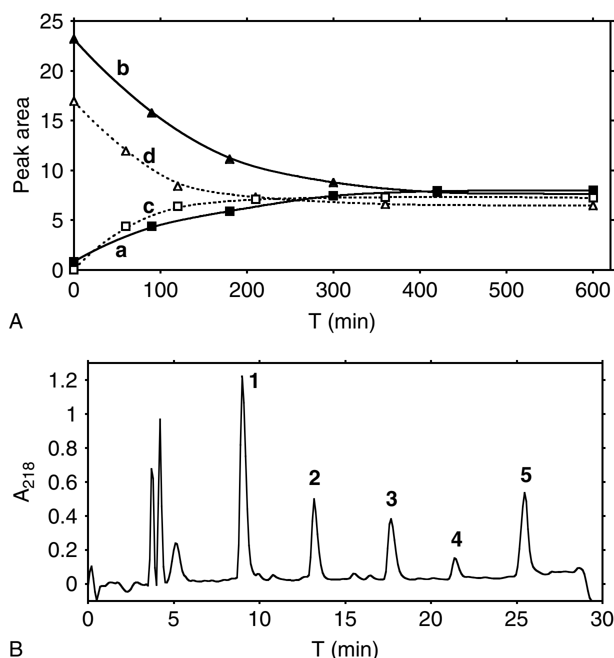


**Figure 1** (A) The time-course of the trypsin-catalyzed enzymatic semisynthesis of [TyrB25,*N*-MePheB26,Lys(Pac)B28, ProB29]-insulin (■ and **a**) from DOI (▲ and **b**) and H-Gly-Phe-Tyr-(*N*-Me)Phe-Thr-Lys(Pac)-Pro-Thr-OH peptide (not shown) calculated from integration of respective peaks at 218 nm. The time-course enzymatic semisynthesis of [HisB26,Lys(Pac)B29] -insulin (□ and **c**) from DOI (Δ and **d**) and H-Gly-Phe-Phe-His-Thr-Lys(Pac)-Pro-Thr-OH peptide is also shown. (B) RP-HPLC chromatogram of the purification of [TyrB25,*N*-MePheB26,LysB28(Pac),ProB29]-insulin from the semisynthetic reaction mixture. Peak 1 is DOI, peak 2 is H-Gly-Phe-Tyr-(*N*-Me)Phe-Thr-Lys(Pac)-Pro-Thr-OH peptide, peak 3 is the product and peaks 4 and 5 are side-products. For details see Experimental and Results.

the released phenylacetyl acid, which can inhibit the immobilized PA [45]. However, the addition of an anion exchange support had a negative effect on the isolation yields of the deprotected insulin analog. We have also been using commercial preparations of PA, *E. coli* enzyme immobilized on Eupergit C and soluble PA (see Materials). The use of these preparations is simple and due to the high specific activity does not require additional application of the Dowex resin for removal of the inhibiting phenylacetyl acid. Interestingly, both PAs, soluble and insoluble, give similar results and yields but soluble PA is overall simpler to handle. The use of soluble PAs is not accompanied by the generation of byproducts and we have not observed any insulin degradation. The time courses for deprotections of [TyrB25,*N*-MePheB26,Lys(Pac)B28,ProB29]-insulin and of [HisB26,Lys(Pac)B29]-insulin are shown in Figure 2(A). There is no significant difference between the courses of both reactions. The yield of phenylacetyl deprotection was usually between 60 and 80%, and any losses here are very likely due to the HPLC purification.

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DOI: 10.1002/psc

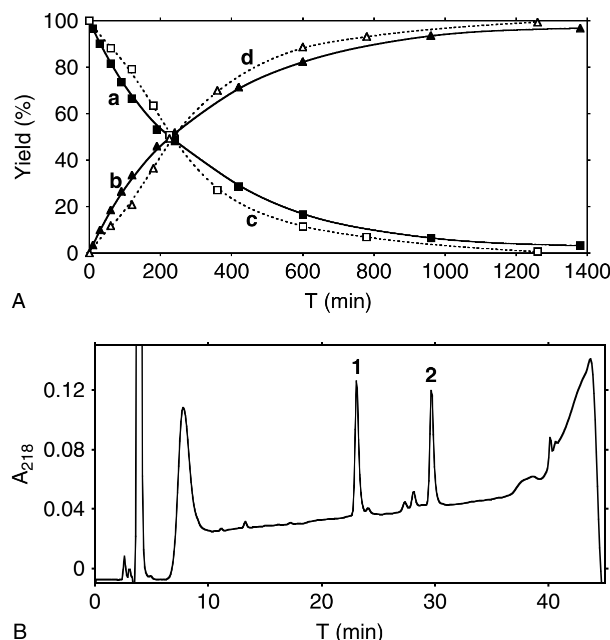


**Figure 2** (A) The time-course of the soluble PA-catalyzed enzymatic deprotection of [TyrB25,*N*-MePheB26,Lys(Pac)B28, ProB29]-insulin (■ and **a**) to yield [TyrB25,*N*-MePheB26, LysB28,ProB29]-insulin (▲ and **b**) expressed as a relative yield in % and calculated from integration of respective peaks at 218 nm. The time course of enzymatic deprotection of [HisB26,Lys(Pac)B29]-insulin (□ and **c**) to give [HisB26]-insulin (Δ and **d**) is also shown. (B) RP-HPLC analytical chromatogram of the deprotection mixture after 240 min. Peak 1 is [TyrB25,*N*-MePheB26,LysB28,ProB29]-insulin and peak 2 is [TyrB25,*N*-MePheB26,Lys(Pac)B28,ProB29]-insulin. For details see Experimental.

Figure 2(B) shows HPLC analysis of the deprotection of [TyrB25,*N*-MePheB26,Lys(Pac)B28,ProB29]-insulin after 240 min.

The receptor-binding affinity of [TyrB25, *N*-MePheB26,LysB28,ProB29]-insulin is only 4% of that of human insulin (Table 1 and Figure 3). This affinity is as low as the affinity of previously published [TyrB25, *N*-MePheB26]-insulin analog [48]. Hence it will be very interesting to investigate the structural and conformational changes occurring in this new mutant insulin. These experiments are in progress.

The receptor-binding affinity of [HisB26]-insulin is about 78% of that of human insulin (Table 1 and Figure 3). During the preparation of this manuscript, we found out that DiMarchi *et al.* had already mentioned the same analog in the 19th American Peptide Symposium Proceedings [62]. They reported a 75% binding affinity on membranes from the engineered cells, which is in close agreement with our results. Our results show that the substitution which leads to a very potent shortened insulin analog (des-tetrapeptide(B27-B30)-B26-amide) [47] has not the same positive effect in the case of a full-length analog.

**Table 1** Values of $IC_{50}$[a] and Relative Receptor Binding Affinities[b] of Human Insulin, [TyrB25,*N*-MePheB26,LysB28, ProB29]-insulin and [HisB26]-insulin

| Ligand | $IC_{50} \pm$ SEM (nM) | *n* | Potency (%) |
|---|---|---|---|
| Human insulin | $0.70 \pm 0.19$ | 5 | 100 |
| [TyrB25,*N*-MePheB26,LysB28, ProB29]-insulin | $17.36 \pm 3.12$ | 3 | 4.03 |
| [HisB26]-insulin | $0.90 \pm 0.21$ | 4 | 77.7 |

[a] $IC_{50}$ values represent concentrations of insulin or the analog causing half-maximal inhibition of binding of human $^{125}$I-monoiodotyrosylA14-insulin to the insulin receptor. Each value represents the mean ± SEM of multiple determinations (*n*).
[b] Relative receptor binding affinity defined as ($IC_{50}$ of human insulin/$IC_{50}$ of analog) ×100. For details see Experimental.

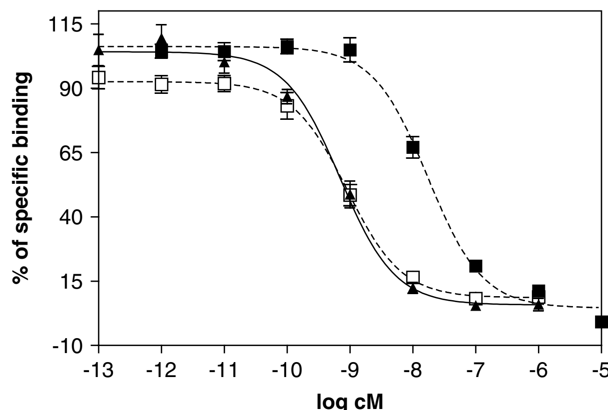


**Figure 3** Inhibition of binding of $^{125}$I-insulin to adipose tissue plasma membranes by human insulin (▲), [TyrB25,*N*-MePheB26,LysB28,ProB29]-insulin analog (■) and [HisB26]-insulin analog (□). Quantitative information is provided in Table 1. For details, see Experimental.

## CONCLUSIONS

In this paper, we present the detailed synthetic protocol and characterization of Fmoc-Lys(Pac)-OH, its use for the preparation of two different synthetic octapeptides by solid-phase synthesis, trypsin-catalyzed condensation of respective octapeptides with desoctapeptide(B23-B30)-insulin and penicillin G acylase catalyzed cleavage of phenylacetyl group from $N^{\varepsilon}$-amino group of lysine to give novel insulin analogs. The first prepared analog is [HisB26]-insulin, which has about 78% binding potency compared to human insulin. We applied the above described strategy also for the preparation and characterization of a novel insulin analog [TyrB25,*N*-MePheB26,LysB28,ProB29]-insulin in which the swap TyrB26 ↔ PheB25 and

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DOI: 10.1002/psc

the *N*-methylation of resulting PheB26 is accompanied with the swap LysB29 ↔ ProB28. The analog was designed to address the problem of a structural and biological switch from the mostly metabolic activity of insulin to the predominant growth factor activities of IGF-I. The modifications of [TyrB25, *N*-MePheB26,LysB28,ProB29]-insulin are expected to introduce IGF-like conformational distortion of the *C*-terminus of the B-chain. Experiments to determine the crystal structure of this novel insulin analog are in progress.

## Acknowledgements

This work was supported by a grant from the Ministry of Education, Youth and Sports of the Czech Republic (Chemical Genetics Consortium LC06077) and Research Project of the Academy of Sciences of the Czech Republic (Z40550506).

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DOI: 10.1002/psc