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Total Chemical Synthesis of Palmitoyl-Conjugated Insulin

Mengjie Liu, Qingyang Li, Carlie Delaine, Hongkang Wu, Yanni Arsenakis, Barbara F. White, Briony E. Forbes, Chaitra Chandrashekar, and Mohammed Akhter Hossain*

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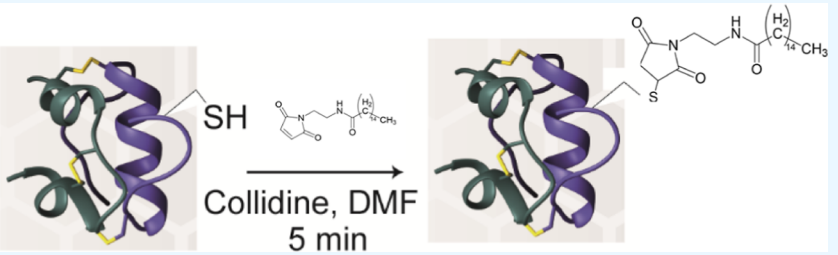

ABSTRACT: Commercially available insulins are manufactured by recombinant methods for the treatment of diabetes. Long-acting insulin drugs (e.g., detemir and degludec) are obtained by fatty acid conjugation at LysB29 ε-amine of insulin via acid−amide coupling. There are three amine groups in insulin, and they all react with fatty acids in alkaline conditions. Due to the lack of selectivity, such conjugation reactions produce non-desired byproducts. We designed and chemically synthesized a novel thiol-insulin scaffold (CysB^{29}-insulin **II**), by replacing the Lys^{B29} residue in insulin with the Cys^{B29} residue. Then, we conjugated a fatty acid moiety (palmitic acid, C16) to CysB^{29}-insulin **II** by a highly efficient and selective thiol−maleimide conjugation reaction. We obtained the target peptide (palmitoyl-insulin) rapidly within 5 min without significant byproducts. The palmitoyl-insulin is shown to be structurally similar to insulin and biologically active both in vitro and in vivo. Importantly, unlike native insulin, palmitoyl-insulin is slow and long-acting.

INTRODUCTION

The discovery of insulin 100 years ago remains a breakthrough in medical history, and it is still the only effective treatment option for type 1 diabetes and an important adjuvant therapy for treating type 2 diabetes at the advanced stage.[1] The advances in insulin therapy have led to the development of various structural analogues with different pharmacokinetic and pharmacodynamic profiles to better match the dynamic physiological demands of diabetic patients.[1,2] Diabetes is typically managed by a prandial (rapid- and short-acting) insulin analogue to provide an immediate glucose-lowering effect after a meal, in conjunction with a basal (intermediate- and long-acting) insulin analogue to maintain a sustained release over an extended period, especially during the sleep.

To date, multiple strategies have been successfully applied in developing various clinically available basal insulin analogues. The earliest strategy for obtaining a longer duration of insulin activity was to deploy a subcutaneous depot of insulin that enabled gradual dissolution after administration. The first commercial intermediate-acting analogue, neutral protamine Hagedorn (NPH) insulin, contains protamine and zinc additives to form slow-release insulin microcrystals, which enabled twice daily dosing.[3] The first marketed long-acting analogue insulin glargine incorporates an Asn^{A21} → Gly mutation and two Arg residues at B31 and B32.[4] The additional Arg shifted the isoelectric point to near neutral pH which causes precipitation in the subcutaneous space to provide a duration of action of up to 24 h.[5] The new generation of long-acting and ultralong-acting Lys^{B29}-lipidated insulin analogues such as detemir,[6] degludec,[7] and icodec[8] (duration of action up to 24, 42, and 196 h, respectively) has a fatty acid moiety at the Lys^{B29} ε-amine. Their prolonged action can be mainly attributed to the capability of the fatty acid side chain to form native insulin-like zinc hexamers and to bind to serum albumin.[9−11] In addition, the amino acid mutations in icodec also contribute to retarded insulin receptor clearance and enhanced resistance against proteolysis.[6−8,11] It is noteworthy to mention that these new analogues remain in the solution state and offer improved predictability of plasma glucose levels and less glycemic variability compared with NPH insulin and glargine.[12,13]

Commercially, the Lys^{B29}-lipidated insulin analogues are manufactured on a large scale by direct acylation of recombinant insulin with the desired fatty acid moiety in an alkaline environment.[14−17] Owing to the high pK_a of Lys ε-

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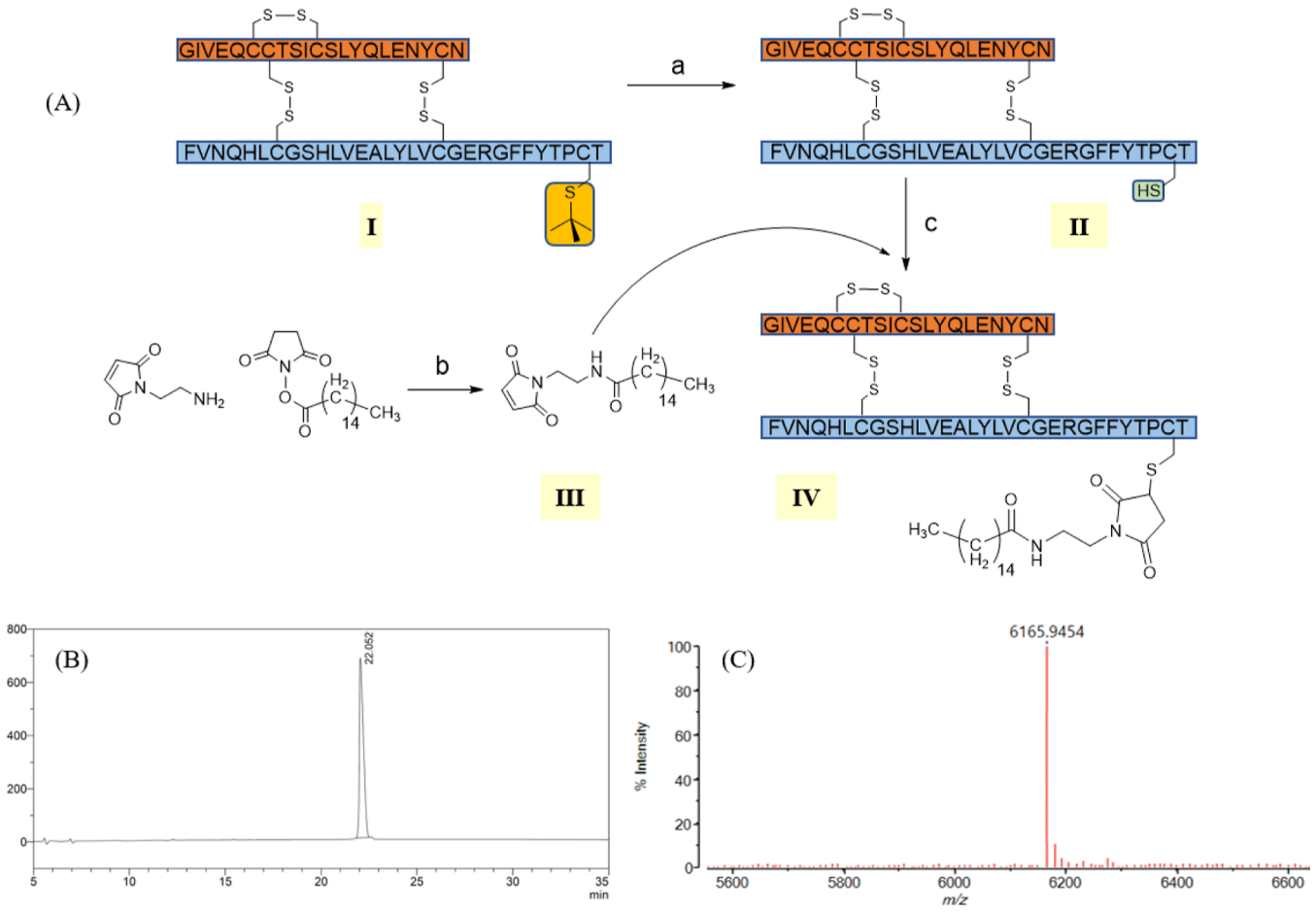

Figure 1. (A) Chemical synthesis of palmitoyl-insulin. (a) Cys(*t*Bu)^{B29}-insulin **I** was treated with TFMSA (10%) and anisole (5%) in TFA to obtain Cys^{B29}-insulin **II**. (b) 1-(2-Aminoethyl)maleimide was reacted with palmitic acid *N*-hydroxysuccinimide in DCM in the presence of DIEA to afford *N*-(2-(*N*-palmitoylaminoethyl)maleimide) **III**. (c) Lipidation of Cys^{B29}-insulin **II** was performed in DMF with collidine to generate palmitoyl-insulin **IV**. (B) HPLC profile of purified palmitoyl-insulin **IV**. (C) MALDI-TOF mass spectroscopy of purified palmitoyl-insulin **IV**.

amine, the required alkaline pH also promotes undesired acylation of both N$^{\alpha}$-amines at the termini of A- and B-chains. These non-selective reactions result in multiple byproducts and significant loss of the starting material.[18] A multi-step method of selective palmitoyl conjugation at the N-terminus of the B-chain was reported that resulted in an analogue with a low yield and poor activity.[19] To address this issue, we report here the production of a novel thiol insulin scaffold, Cys^{B29}-insulin **II** (Figure 1A), by substituting Lys^{B29} with a Cys^{B29} residue, where lipidation was achieved very rapidly and selectively by the highly efficient thiol−maleimide reaction. To our knowledge, such methods have never been reported.

MATERIALS AND METHODS

Reagents. N$^{\alpha}$-Fmoc-protected amino acids, rink amide resin (0.36 mmol/g), and *O*-(1*H*-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate were purchased from GL Biochem (China). Fmoc-Thr(*t*Bu)-preloaded TentaGel resin (0.18 mmol/g) was purchased from Rapp Polymere GmbH (Germany). *N*,*N*-Dimethylformamide (DMF), piperidine (PPD), trifluoroacetic acid (TFA), acetonitrile (MeCN), methanol, dichloromethane (DCM), and diethyl ether (Et_2O) were purchased from Merck (Australia). *N*,*N*-Diisopropylethylamine (DIEA), trifluoromethanesulfonic acid (TFMSA), 2,2′-(ethylenedioxy)-diethanethiol, triisopropylsilane, anisole, 2,2′-dipyridyl disulfide, 2,4,6-trimethylpyridine, 1-(2-aminoethyl)maleimide hydrochloride, and palmitic acid *N*-hydroxysuccinimide were purchased from Sigma-Aldrich (Australia). All solvents were of analytical grade.

Instruments. Syntheses of the insulin A-chain and B-chain were performed using an Initiator + Alstra automatic microwave-assisted peptide synthesizer (Biotage, Germany). Peptide purity was determined by a Shimadzu Nexera analytical high-performance liquid chromatography (HPLC) instrument incorporating a HALO C-18 column (4.6 × 250 mm, pore size 160 Å, particle size 5 μm). The aqueous buffer (buffer A) was 0.1% TFA in water, and the organic buffer (buffer B) was 0.1% TFA in acetonitrile. The detection wavelength was set at 214 nm. Peptide purification was performed using a Waters 600 semipreparative HPLC instrument coupled with a Waters 996 detector, on a Phenomenex Gemini C-18 column (150 × 21.2 mm, pore size 110 Å, particle size 5 μm). The molecular weight of the peptides was determined using a Shimadzu MALDI-8020 MALDI-TOF mass spectrometer using sinapinic acid as the matrix.

Synthesis. The synthesis of A- and B-chains and construction of three disulfide bonds in Cys(*t*Bu)^{B29}-insulin **I** were carried out using regioselective disulfide bond formation strategies.[20−23] The details of the synthesis protocols are described in the Supporting Information.

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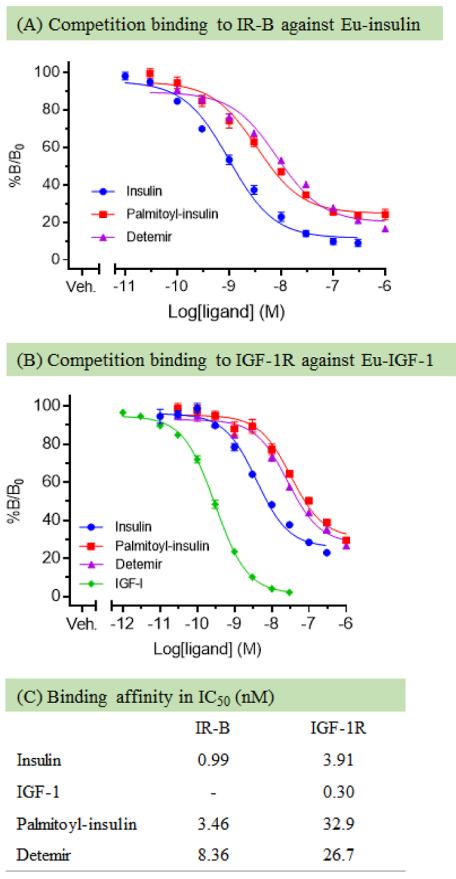
RESULTS AND DISCUSSION

The challenge of our proposed method is to produce the thiol-insulin precursor (Cys^{B29}-insulin **II**) with three existing disulfide bridges (Figure 1A-**II**). Inspired by our and others' recent success in producing stable thiol-insulin scaffolds (Cys^{B0}-insulin[20] and Cys^{B25}-insulin[24]), we hypothesized that our novel Cys^{B29}-insulin precursor **II** will also be stable in solution and will not be scrambled by disulfide shuffling (due to a free thiol). To synthesize the starting insulin scaffold, Cys^{B29}-insulin **I** (Figure 1A), we took the advantage of orthogonally protected Cys derivatives.[20,22,25] We used *tert*-butyl (*t*Bu)-protected Cys^{B29} to avoid undesired disulfide scrambling during the regioselective stepwise folding of Cys^{B29}-insulin **I** followed by facile acidolytic removal of *t*Bu.[20,26,27] Both the native A-chain and the modified B-chain were synthesized following the standard Fmoc-based solid-phase synthesis strategy. The A-chain incorporated trityl (Trt)-protected Cys residues at A6 and A11 to form the intramolecular disulfide bond, while a *t*Bu- and an acetamidomethyl (Acm)-protected Cys^{A7} and Cys^{A20}, respectively, to form the two interchain disulfide bonds with $Cys(Trt)^{B7}$ and $Cys(Acm)^{B19}$. After completing the insulin scaffold, $Cys(tBu)^{B29}$-insulin **I** (Figure 1A), the *t*Bu group was cleaved by treating with 10% TFMSA and 5% anisole in trifluoroacetic acid (TFA) to afford the desired thiol insulin precursor, Cys^{B29}-insulin **II**, in 3 min. The synthetic details are described in the Supporting Information. The maleimide-functionalized palmitic acid moiety, *N*-(2-(*N*-palmitoylaminoethyl)maleimide) **III**, was synthesized following the published method of Kuan et al.[28] by amide formation between 1-(2-aminoethyl)maleimide and the activated ester palmitic acid *N*-hydroxysuccinimide in the presence of DIEA. Subsequently, lipidation at Cys^{B29} was achieved in the presence of the weak base 2,4,6-trimethylpyridine (collidine) to obtain palmitoyl-insulin **IV**. The reaction completed in 5 min, and the purity of the crude peptide was 68% (Supporting Information) and that of the purified peptides was >99% (Figure 1B). The protocol and the crude HPLC profile of the reaction are shown in the Supporting Information. The characterization of purified palmitoyl-insulin **IV** by HPLC and MALDI-MS is illustrated in Figure 1B,C respectively. The free thiol group in Cys^{B29}-insulin **II** did not cause any noticeable disulfide bond shuffling or dimer formation,[25] which can be attributed to the stability of the folded insulin and the faster reaction kinetics of thiol–maleimide conjugation reaction.

Having successfully synthesized palmitoyl-insulin, we compared its binding affinities for the insulin receptor isoform B (IR-B) and insulin-like growth factor-1 receptor (IGF-1R) with that of the native insulin and commercially available long-acting insulin detemir. IR and IGF-1R are highly homologous receptors, yet they differ in the activity they elicit in vivo. As such, insulin can fully activate IR and can also bind to IGF-1R and activate it, however with much reduced affinity. For the receptor activation assay, we employed the strategy described by Denley et al.[29] As shown in Figure 2A, palmitoyl-insulin displaced europium-labeled insulin in a dose-dependent manner with an IC_{50} value of 3.46 nM. Importantly, the binding affinity of our palmitic acid (C16)-conjugated insulin is 2.4-fold higher than that of commercially available fatty acid (myristic acid, C14)-conjugated insulin, detemir (IC_{50} = 8.36 nM).



(C) Binding affinity in IC_{50} (nM)

	IR-B	IGF-1R
Insulin	0.99	3.91
IGF-1	-	0.30
Palmitoyl-insulin	3.46	32.9
Detemir	8.36	26.7

Figure 2. Competition binding of (A) palmitoyl-insulin and insulin detemir against Eu-labeled insulin to insulin receptor isoform-B (IR-B) and (B) insulin, palmitoyl-insulin, and insulin detemir against the Eu-labeled IGF-1 to IGF-1 receptor. (C) Binding affinity in IC_{50} values is shown in the table. Results are expressed as a ratio of binding percentage in the presence/absence of the competing ligand ($\%B/B_0$). Curves are plotted from at least three separate experiments, where each data point was performed in triplicate. Error bars are shown when greater than the size of the symbols.

Insulin has strong binding affinity for the IGF-1R. The activation of this receptor has been demonstrated to be involved in tumor growth and cancers.[30,31] Therefore, we performed competition binding assays to assess the IGF-1R-binding affinity of the palmitoyl-insulin (Figure 2B). Our data revealed that palmitoyl-insulin had a binding affinity of IC_{50} = 32.9 nM which is slightly lower (1.2-fold) than that of detemir, 8.4-fold lower than that of insulin, and 110-fold lower than that of IGF-1, suggesting that our palmitoyl-insulin is more selective for IR-B over IGF-1R, which might reduce the possibility of IGF-1R-related potential side-effects (e.g., tumor growth). The details of the experiment are described in the Supporting Information.

In vivo testing using the insulin tolerance tests (ITT, methods described in the Supporting Information) was performed to assess the glucose-lowering response of the palmitoyl-insulin compared to the commercially available insulin (Actrapid) and detemir, in normal 8 week-old C57Bl6/J mice, as previously described.[22]

Both intraperitoneal (IP, Figure 3) and subcutaneous (SC, Figure 4) routes were assessed, and results were plotted as absolute values and as a change from the baseline. Palmitoyl-insulin did not lower glucose to the same level as the Actrapid

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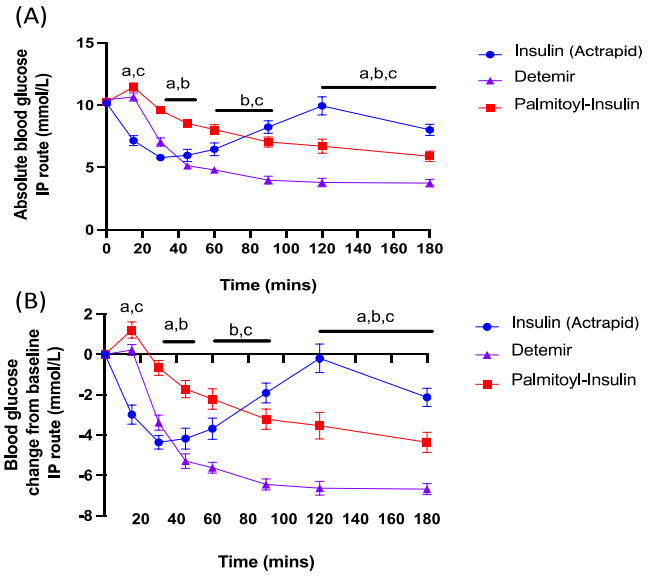

Figure 3. Blood glucose concentration in response to equimolar concentration of insulin (Actrapid), detemir, or palmitoyl-insulin administered IP (n = 10 per treatment). Data are presented as mean ± SEM. [a] $p < 0.05$ palmitoyl-insulin vs insulin (Actrapid); [b] $p < 0.05$ palmitoyl-insulin vs detemir; and [c] $p < 0.05$ insulin (Actrapid) vs detemir. (A) Absolute glucose levels over 180 min. Two-way ANOVA demonstrated significant effects of treatment, time, and an interaction between the two parameters on glucose response ($p < 0.05$). Tukey's post-hoc test showed that palmitoyl-insulin maintained reduced glucose levels compared to insulin (Actrapid) from 120 to 180 min. (B) Change from baseline glucose levels with the same statistically significant effects established.

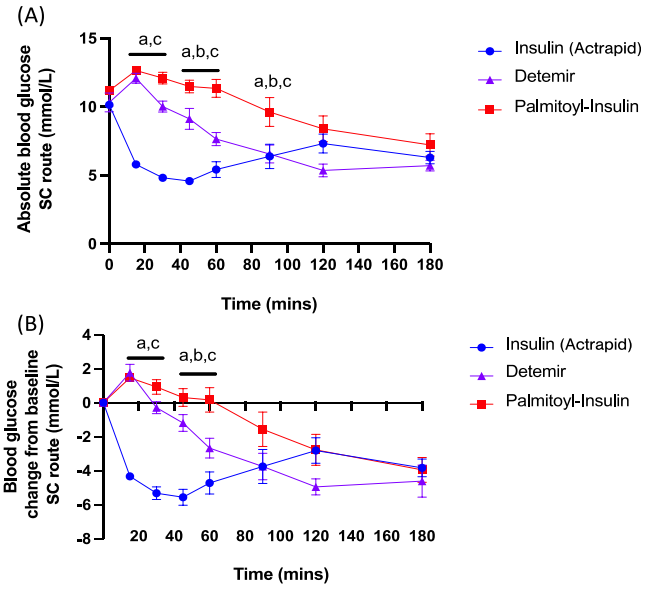

Figure 4. Blood glucose concentration in response to equimolar concentration of insulin (Actrapid), detemir, or palmitoyl-insulin administered SC (n = 5 per treatment). Data are presented as mean ± SEM. [a] $p < 0.05$ palmitoyl-insulin vs insulin (Actrapid); [b] $p < 0.05$ palmitoyl-insulin vs detemir; and [c] $p < 0.05$ insulin (Actrapid) vs detemir. (A) Absolute glucose levels over 180 min. Two-way ANOVA demonstrated significant effects of treatment, time, and an interaction between the two parameters on glucose response ($p < 0.05$). Tukey's post-hoc test showed that palmitoyl-insulin did not achieve reduced glucose levels compared to insulin (Actrapid) or detemir. (B) Change from baseline glucose levels.

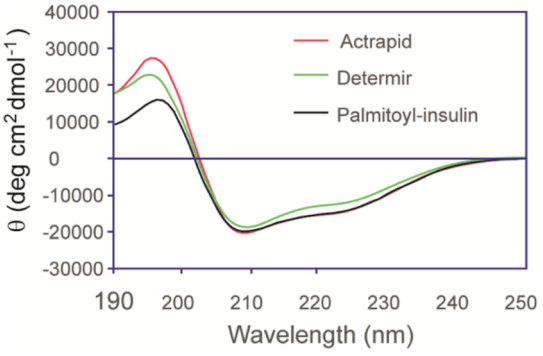

Figure 5. CD spectra of insulin and palmitoyl-insulin in 20% trifluoroethanol/water.

or detemir response within the first 90 min of the IP test (Figure 3A,B respectively); however, glucose was trending downward across these early time points. At 120 and 180 min, glucose levels were significantly lower in the palmitoyl-insulin group compared to the insulin (Actrapid) group ($p < 0.01$) but remained significantly higher compared to the detemir group. These IP results suggest that the glucose-lowering response to palmitoyl-insulin is more gradual (slower-acting) than both the rapid-acting insulin (Actrapid) and detemir but that it is longer-acting and sustains its lower glucose level to a better extent than insulin (Actrapid). Interestingly, the SC results demonstrated that palmitoyl-insulin has very little effect in lowering glucose in the first 90 min, remaining significantly higher than insulin (Actrapid) and detemir. By 120 min, blood glucose levels were lower than the baseline in the palmitoyl-insulin group but were not significantly better than the control insulins (Figure 4A,B). Our data suggest that the hypoglycemic effect of the palmitoyl-insulin is not as marked shortly after administration but has the ability to maintain low blood glucose levels for a longer period compared to insulin (Actrapid) when delivered IP, but not to the same extent as detemir. The details of the experiment are described in the Supporting Information.

To elucidate the effect of the fatty-acid side chain on the secondary structure of insulin, we performed circular dichroism (CD) spectrometry and compared it with native insulin and detemir (Figure 5). A typical α-helix pattern was observed for palmitoyl-insulin, similar to insulin and detemir with double minima around 208 and 222 nm (details of the experiment are described in the Supporting Information). The data suggest that fatty acid conjugation does not alter the secondary structure of insulin.

CONCLUSIONS

We have successfully synthesized palmitoyl-insulin using a thiol−maleimide reaction with no significant byproducts. The binding affinity of palmitoyl-insulin for IR-B is higher than that of the commercially available long-acting insulin detemir, and it was obtained through a quick (5 min), simple, and highly selective chemical method. The palmitoyl-insulin has a similar secondary structure as insulin and is slow- and long-acting in the animal model tested. Through our new chemical method, other maleimide-functionalized fatty acids, carbohydrates, polyethylene glycol, and biodegradable polymers varying in chain lengths and sizes can be attached to Cys^{B29}-insulin quickly and efficiently, and their improved pharmacokinetics

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and pharmacodynamics can be studied. A successful synthesis of the Cys^{B25}-insulin precursor by the recombinant method[24] and the high stability of Cys^{B0}-insulin[20] and Cys^{B29}-insulin precursors in aqueous solution suggest that our novel thiol-insulin precursor can also be produced by recombinant methods on an industrial scale.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c07918.

Description of peptide and palmitoyl-insulin synthesis, characterization by MALDI-TOF MS and analytical HPLC, methods for in vitro IR-B and IGF-1R assays, and CD spectroscopy and methods for ITT (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Mohammed Akhter Hossain − *The Florey Institute of Neuroscience and Mental Health, Parkville 3052, Australia; School of Chemistry, and Department of Biochemistry and Pharmacology, The University of Melbourne, Victoria 3010, Australia;* orcid.org/0000-0002-9961-0006; Email: akhter.hossain@unimelb.edu.au

Authors

Mengjie Liu − *Monash Institute of Pharmaceutical Sciences, Parkville, Victoria 3052, Australia; The Florey Institute of Neuroscience and Mental Health, Parkville 3052, Australia*

Qingyang Li − *The Florey Institute of Neuroscience and Mental Health, Parkville 3052, Australia; Sinochem Pharmaceutical Co., Ltd, Nanjing 210002, China*

Carlie Delaine − *Discipline of Medical Biochemistry, College of Medicine and Public Health, Flinders University, Adelaide, South Australia 5042, Australia*

Hongkang Wu − *The Florey Institute of Neuroscience and Mental Health, Parkville 3052, Australia*

Yanni Arsenakis − *Department of Medicine (Austin Health), The University of Melbourne, Heidelberg, Victoria 3084, Australia*

Barbara F. White − *Department of Medicine (Austin Health), The University of Melbourne, Heidelberg, Victoria 3084, Australia*

Briony E. Forbes − *Discipline of Medical Biochemistry, College of Medicine and Public Health, Flinders University, Adelaide, South Australia 5042, Australia;* orcid.org/0000-0003-4360-9927

Chaitra Chandrashekar − *The Florey Institute of Neuroscience and Mental Health, Parkville 3052, Australia;* orcid.org/0000-0002-0288-5952

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c07918

Author Contributions

All authors have given approval to the final version of the manuscript. M.A.H: conceived and designed the experiments. M.L., Q.L., C.D., H.W., Y.A., and B.F.W: performed the experiments. M.L., Q.L., C.D., B.F.W., and B.E.F: analyzed the data. M.A.H. and B.E.F: contributed reagents/materials. M.L., C.C., and M.A.H: wrote the paper.

Notes

The authors declare no competing financial interest.

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