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Research Article

Total Synthesis of Semaglutide Based on a Soluble Hydrophobic-Support-Assisted Liquid-Phase Synthetic Method

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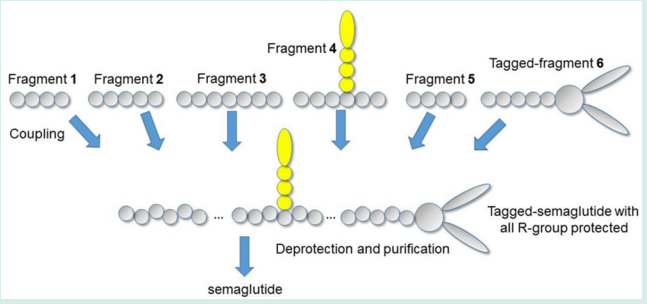
ABSTRACT: Considering the high cost of the production of semaglutide, which is currently the most promising antidiabetic drug especially for the treatment of type 2 diabetes mellitus, a new synthetic route of semaglutide production that possesses excellent yield and high purity is of vital importance. Herein, we reported a newly developed synthetic route of semaglutide that is simple and efficient, based on a soluble hydrophobic-support-assisted liquid-phase synthetic method by applying Alloc-chemistry to the synthesis of the main chain peptide and side chain peptide of semaglutide. With careful optimization of the reaction conditions and innovative strategy of post-synthetic treatments, the total yield and purity of the crude semaglutide was improved satisfactorily.



KEYWORDS: *peptide synthesis, semaglutide, Alloc-chemistry, diatomite, post-synthetic treatment*

INTRODUCTION

Glucagon-like peptide-1 receptor agonists (GLP-1 RAs) that exhibit efficacy for glycemic control, decrease of body weight and blood pressure, and low risk of hypoglycemia, are widely used for the treatment of type 2 diabetes mellitus (T2DM).[1−3] Among them, semaglutide is a newly discovered, long acting GLP-1 RA with 94% homology to natural GLP-1 in structure.[4−6] However, semaglutide was obtained through modifying three amino acid residues of natural GLP-1 (Ala8 altered to Aib, Lys34 altered to Arg, and a side chain introduced to Lys26, respectively) (Figure 1).[4] These modifications confer to semaglutide comprehensive promotion of efficacy and stability (half-life) compared to the most effective GLP-1 RA on the market, liraglutide.[7] Distinguished from normal peptides, semaglutide has a side chain on its Lys26 that contains four residues to guarantee its high performance and efficacy toward blood glycemic control.[7] On the other hand, this side chain on lysine also makes the synthesis of semaglutide more difficult. Since Novo Nordisk invented semaglutide, different routes have been explored to complete the synthesis of semaglutide. Till now, however, the synthetic routes of semaglutide are all based on solid-phase peptide synthesis (SPPS), which leads to high production costs. Thus, researchers have paid more attention to the optimization of the reaction conditions of existing synthetic routes to improve the yield or purity of semaglutide, thereby reducing the product cost. Unfortunately, most of the attempts have achieved little improvement to the synthesis of semaglutide.

Recently, a soluble, hydrophobic-support-assisted, liquid-phase synthetic method was reported.[8,9] The principle of this peptide synthesis method is similar to that of SPPS, but the support in this method has good solubility in nonpolar solvents. This property converts all the coupling and deprotecting reactions to homogeneous reactions, which can be monitored through common analytical techniques, such as thin layer chromatography (TLC) method.[8,10] Because of the poor solubility of the hydrophobic support in polar solvents, the desired product will be precipitated by diluting the reaction mixtures with such poor solvents. After filtration, the precipitated product can be easily collected, and the undesirable impurities such as excess amino acids, catalysts, and coupling agents can be rinsed away. This soluble hydrophobic-support-assisted liquid-phase synthetic method is one of the most promising high-efficiency strategies for peptide synthesis that can be utilized to synthesize almost every kind of peptide, such as cyclic peptides and disulfide bond-containing peptides.[11−13]

In this context, we applied this soluble hydrophobic-support-assisted liquid-phase synthetic method to successfully accomplish the total synthesis of semaglutide. Strategies for total synthesis of semaglutide are outlined in Figure 2. Specifically,

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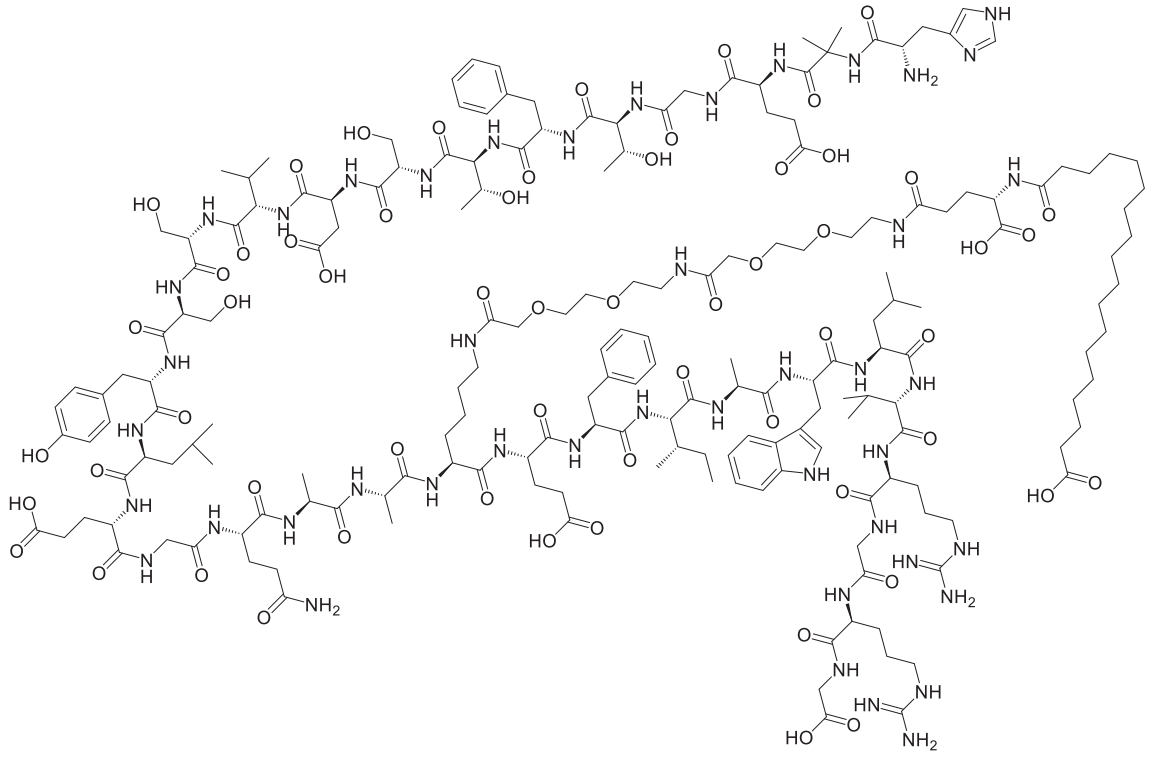

Figure 1. Structure of semaglutide.

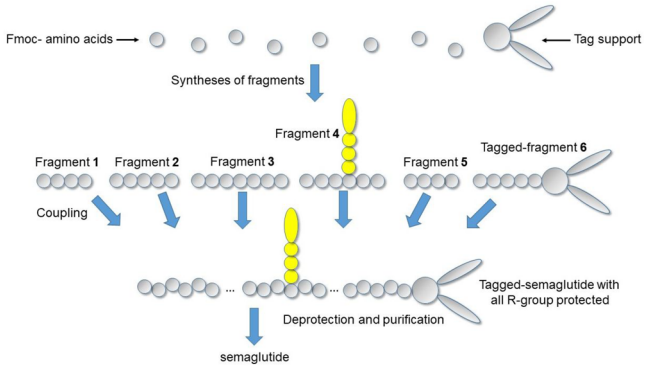

Figure 2. Strategies for the total synthesis of semaglutide.

we divided the structure of semaglutide into six fragments based on the above strategies (Table 1). We first completed synthesis of these six fragments and then coupled these fragments together in sequence, and finally obtained semaglutide through easy deprotection of the protecting groups and the tag support. It is worth mentioning that we coupled one-by-one a side chain consisting of four residues on the main chain of fragment 4 by using Alloc-chemistry, which has not been used in this method for the synthesis of peptides. Although a little support was detached from fragment 4 caused by the existence of tetrakis(triphenylphosphine)palladium, the yield of the Alloc-deprotecting reaction was still excellent compared with that of the well studied Fmoc-deprotecting reactions.[14]

Table 1. Division of Six Fragments of Semaglutide

fragment	amino acid sequence
fragment 1	Boc-His(Trt)-Aib-Glu(OtBu)-Gly-OH
fragment 2	Fmoc-Thr(tBu)-Phe-Thr(tBu)-Ser(tBu)-Asp(OtBu)-OH
fragment 3	Fmoc-Val-Ser(tBu)-Ser(tBu)-Tyr(tBu)-Leu-Glu(OtBu)-Gly-OH
fragment 4	Fmoc-Gln(Trt)-Ala-Ala-Lys[-AEEA-AEEA-γ-Glu(α-OtBu)-Oct(OtBu)]-Glu(OtBu)-Phe-OH
fragment 5	Fmoc-Ile-Ala-Trp(Boc)-Leu-OH
tagged fragment 6	Fmoc-Val-Arg(Pbf)-Gly-Arg(Pbf)-Gly-OH

RESULTS AND DISCUSSION

This work began with the feasibility of integrating Alloc-chemistry into the soluble hydrophobic-support-assisted liquid-phase synthetic method mentioned above. We chose Tag-OH (**1**) as the support for the peptide synthesis because this support has excellent stability under the conditions of Fmoc-chemistry. The trityl group at the histidine residue of fragment 1 and other protective groups (R groups) like Boc, Pbf, and OtBu groups were easily cleaved through acidic cleavage. Thus, Boc-chemistry was not used to complete the coupling of the fragments. Here, we put emphasis on the synthesis of fragment 4 with a four-residue side chain on its lysine residue. First, Fmoc-Phe-OH, Fmoc-Glu(OtBu)-OH, and Alloc-Lys(Fmoc)-OH were coupled to give the tagged tripeptide (**2**). After the deprotection of Fmoc group on lysine residue of the tagged tripeptide with piperidine, two Fmoc-AEEA-OH, Fmoc-Glu-OtBu, and octadecanedioic acid mono (1,1-dimethylethyl) ester were coupled to the free amino group of the lysine residue in proper sequence to get the tagged tripeptide with a four-residue side chain (**3**) (Scheme 1).

The tagged tripeptide with a four-residue side chain (**3**) was treated with $Pd(PPh_3)_4$ to give the tagged peptide (**4**). However, we found that the tag support of **4** was partly detached to produce the detached peptide (**5**), a byproduct. The free carboxyl group of **5** could react with the α-amino group on the lysine residue during treatment with HBTU and

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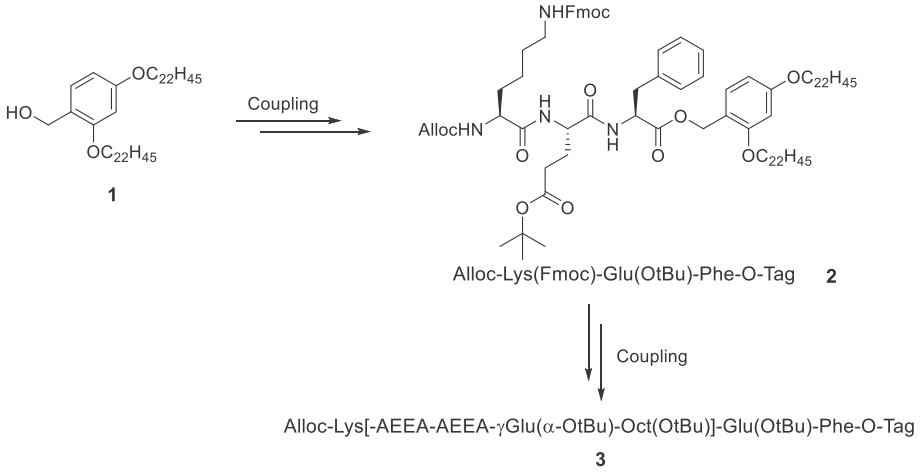
Scheme 1. Synthesis of the Tagged Tripeptide with a Side Chain Consisting of Four Residues (3)



Scheme 2. Synthesis of Fragment 4

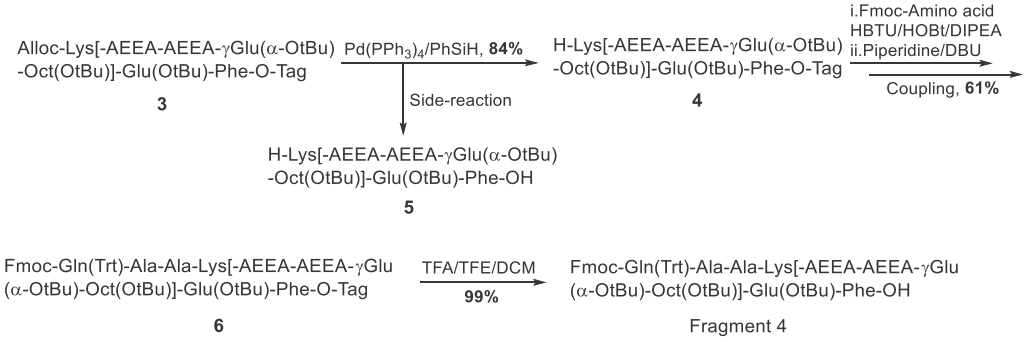
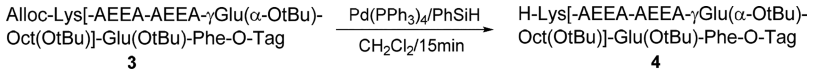


Table 2. Optimization of the Deprotection Reaction Conditions



entry	$Pd(PPh_3)_4$ [equiv]	PhSiH [equiv]	yield [%]
1	0.2	8	76
2	0.1	8	49
3	0.15	8	71
4	0.3	8	53
5	0.2	12	84
6	0.2	4	55

HOBt in the next step. Fortunately, **5** exhibited good solubility in polar solvent and was rinsed away with polar solvent. On the other hand, Tag-OH (**1**) from the above detachment reaction could not react with the carboxyl group of amino acid residues to form an amide bond, which would lead to generation of extra impurities. Finally we coupled the last three residues to **4** to get tagged fragment 4 (**6**), which was followed by treatment with trifluoroacetic acid (TFA) to detach the tag support to provide fragment 4 (Scheme 2).

We found that a moderate feeding rate of $Pd(PPh_3)_4$ and a high concentration of phenylsilane (PhSiH) gave a good yield of **4** in the deprotecting reaction of **3**. This is because a large amount of $Pd(PPh_3)_4$ caused severe tag-support detachment reaction of **4**, while low feeding rate could slow this detachment reaction but increase the exposure time of **3** and **4** to $Pd(PPh_3)_4$, finally leading to more **5**. On the other hand, increasing the feeding rate of PhSiH could speed up the deprotecting reaction and minimize the exposure time of **3** to the palladium catalyst, resulting in a low rate of tag-support detachment reaction. However, considering that too much PhSiH introduced unnecessary impurities related to PhSiH, we did not further carry out the experiment in which the feeding rate of phenylsilane was more than 12 equiv (Table 2).

Other fragments including tagged fragment 6 are all straight-chain peptides, which were synthesized through these steps of coupling, deprotection, and tag-support detachment reactions in the soluble hydrophobic-support-assisted liquid-phase method. In the presence of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride n-hydrate (DMT-MM) and diisopropylethylamine (DIPEA), tagged fragment 6 was effectively coupled with fragment 5, 4, 3, 2, and 1 in turn to provide tagged R-group-protected semaglutide in satisfactory yields. The tagged R-group-protected semaglutide was then treated with 2.5% TIS and 2.5% H_2O in TFA to give crude semaglutide quantitatively as a precipitate.[8] This crude product was further purified by reversed-phase high performance liquid

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chromatography (RP-HPLC) using H_2O−MeCN as mobile phase to produce pure semaglutide.

Additionally, we also found that the physical properties of the precipitates obtained after almost every coupling or deprotecting reaction were too sticky to be separated from reaction mixtures by vacuum filtration, which caused a decrease in the efficiency of post-synthesis treatment.[15,16] In order to solve this technical problem, a magnetic nanoparticle assisted post-synthetic treatment method was put forward by Okada et al.[16] In sharp contrast to this, in our study, we found a simple and effective postprocessing method to solve the above problems. Specifically, moderate quantities of diatomite were added to the precipitates in the reaction mixture and well mixed. Then, particle powders of tagged peptides with diatomite were obtained through filtering in vacuum and rinsing away impurities (Figure 3). Less-polar solvent was added to this particle powder to dissolve the tagged peptides, and after sonication treatment and filtration, we could get a solution of the tagged peptides that could be directly used in the next step of coupling or deprotecting reaction. This "catch and release" strategy significantly increased the efficiency of post-synthesis treatment of the soluble hydrophobic-support-assisted liquid-phase method. Therefore, our goal was achieved by using a cheap material, diatomite, in the post-synthesis treatment of coupling or deprotecting reactions, which guarantees the cost control of the target peptide.

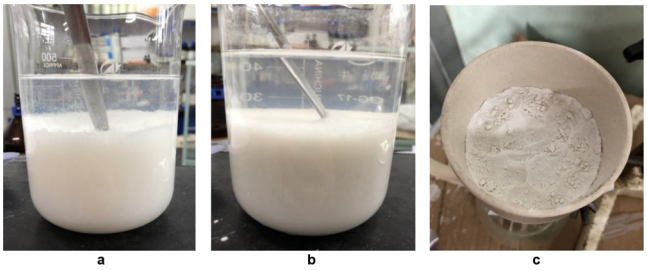

Figure 3. (a) Precipitates in the mixture before adding diatomite, (b) precipitates in the mixture after mixing well with diatomite; (c) powder of peptides tagged with diatomite after vacuum filtration.

CONCLUSIONS

In summary, we applied a newly developed soluble hydrophobic-support-assisted liquid-phase synthetic method to the total synthesis of semaglutide and established a simple and efficient route for the total synthesis of semaglutide through elaborate optimizations of the reaction conditions and innovation of the post-synthesis treatment method. This provides a new direction for the industrial production of semaglutide that was once thought to be produced only by SPPS. More importantly, Alloc-chemistry was introduced to a simple and efficient synthetic route of semaglutide, which further broadens the application of the soluble hydrophobic-support-assisted liquid-phase method. In addition, this method could also be applied for the synthesis of the side-chain peptide of semaglutide without introducing unnecessary impurities.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscombsci.0c00134.

Detailed methods and mass spectra, NMR spectra, and HPLC chromatograms of the fragments (PDF)

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Author Contributions

The manuscript was written through contributions of the authors Xingbang Liu and Shutao Ma. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

T2DM, type 2 diabetes mellitus; GLP-1 RAs, glucagon-like peptide-1 receptor agonists; SPPS, solid-phase peptide synthesis; TLC, thin layer chromatography; Alloc, allyloxycarbonyl; Boc, *tert*-butoxycarbonyl; Fmoc, 9-fluorenylmethoxycarbonyl; Pbf, 2,2,4,6,7-pentamethyldihydrobenzo-furan-5-sulfonyl; OtBu, *tert*-butoxy; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate; HOBt, 1-hydroxyben-

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zotriazole; $Pd(PPh_3)_4$, tetrakis(triphenylphosphine) palladium; PhSiH, phenylsilane; equiv, equivalent; AEEA, 8-amino-3,6-dioxaoctanoic acid; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; DIPEA, diisopropylethylamine; DMT-MM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride n-hydrate; TIS, tris-isopropylsilane; RP-HPLC, reversed-phase high performance liquid chromatography; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene

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