Synthesis of the nonapeptide (B_{22}–B_{30}) of insulin B-chain using liquid and modified solid-phase methods

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Background and objective
This work embraced a systematic search for potentiated methodologies for peptide synthesis through a di-dimensional approach for convenient synthesis of the nonapeptide (B_{22}–B_{30}) of porcine insulin B-chain. Liquid-phase peptide synthesis (LPPS) and liquid–solid-phase peptide synthesis (modified SPPS) were used for the synthesis of this active part.

Materials and methods
A nonapeptide Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala-OH corresponding to B_{22}–B_{30} of porcine insulin B-chain was synthesized using different methods: LPPS and modified SPPS. Time consumption, yield and purity of all products using the different methods were compared with each other.

Results and conclusion
The results indicated that modified SPPS is advantageous, as it consumes less solvent per coupling and deprotection reaction, thereby reducing operating costs and solvent waste. Reducing side reactions result in racemization, cyclization or premature peptide formation. Modified SPPS produces peptides with yields and purity better than LPPS. Also, it can reduce the time of coupling and deprotection reactions.

Keywords:
amino acids, insulin, liquid phase, modified solid phase, peptide synthesis

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Introduction

Insulin monomer is a peptide hormone produced by the pancreatic β-cells of the islets of Langerhans. It regulates the uptake of glucose into the cells from the blood for use as fuel, the formation of glycogen for storage in cells, and many anabolic processes such as cell growth and cellular protein synthesis [1]. Insulin is produced as a response to a high concentration of glucose that occurs after eating a meal or injection of dextrose fluid. However, when the level of glucose comes down to the usual physiological value, Insulin release from β-cells slows or stops, and glucose returns to the normal 'fasting' level.

Insulin hormone consists of two main chains called the A-chain and the B-chain. The A-chain has 21 amino acids, whereas the B-chain has 30 amino acids. These two chains are linked together by disulfide bonds formed between A7–B7 and A20–B19. In addition, the A-chain contains an intrachain disulfide bridge between A7 and A11 (Fig. 1). These disulfide bonds play an important role in the construction of its three-dimensional structure.

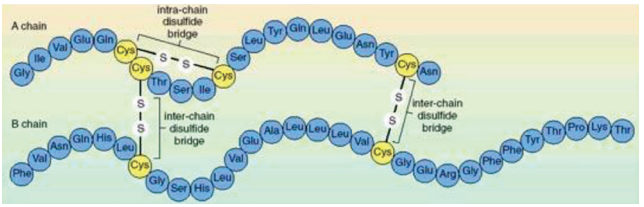
The biological activity of insulin is known to be closely related to the C-terminal nonapeptide (B_{22}–B_{30}) fragment of its B-chain [2].

In the last few years, many attempts have been made to prepare synthetic insulin [3].

Merrifield's ingenious idea was to use an insoluble and filterable polymeric support such as cross-linked polystyrene, which functions at the same time as the carboxyl protecting group for the C-terminal amino acid. Thus, the N^{α}-protected C-terminal amino acid is attached to the chloromethylated polystyrene-divinylbenzene [4]. After removal of the N^{α} protection, the next N^{α}-protected amino acid is coupled and the process was repeated until the entire desired peptide was assembled on the polymeric support [5].

9-Fluorenylmethoxycarbonyl (Fmoc) amino acids were first used for solid-phase peptide synthesis (SPPS) a little more than a decade ago. Since that time, the Fmoc-SPPS

Figure 1



Simplified Chemical Structure of Insulin Hormone.

methodology has been enhanced considerably by the introduction of a variety of solid supports, linkages and side chain-protecting groups, as well as by increased understanding of solvation conditions. These advances have led to much impressive synthesis, such as those of biologically active and isotopically labeled peptides and small proteins. The significant variety of conditions under which Fmoc-SPPS and liquid-phase peptide synthesis (LPPS) may be carried out represents a truly 'orthogonal' scheme, and thus offers many unique opportunities for bioorganic chemistry.

The matrix effect of the cross-linked polymer and the heterogeneous reaction can be avoided if a linear and soluble polymer is used. This goal was achieved by Bayer, who used linear polyethylene glycol (PEG) [6]. Linear polystyrene is very easily cross-linked and difficult to handle. PEG is more suitable, which, owing to its good solubility in many organic solvents, can solubilize even otherwise insoluble peptides (LPPS). The graft copolymers of cross-linked polystyrene and PEG show pressure stability, similar swelling behavior in different solvents, chemical stability, a spherical form, and equal bead size. All of these properties are prerequisites for a rapid peptide synthesis, thus combining the advantages of liquid-phase and solid-phase methods (liquid–solid-phase peptide synthesis).

Materials and methods

Chemicals

The Organic solvents and the chemicals used in this part were obtained from Aldrich Chemical Company (Taufikirchem, Germany), E. Merck (Schwalbach/Ts, Germany), Sigma (LOUIS, Mo, USA), and Fluka (LOUIS, Mo, USA). Otherwise stated, the amino acids used are of l-configuration. TentaGel-resin (PS-PEG_{3000}-NH_2) was supplied by Rapp polymer (Tübingen, Germany) [7].

Synthesis

Liquid-phase method of the nonapeptide (B_{22}–B_{30}) of insulin B-chain

Coupling of monomethyl polyethylene glycol-6000 ($MPEG_{6000}$) with the first amino acid: $MPEG_{6000}$ (7.92 g, 1.58 mmol) was dissolved in 10 ml dichloromethane; symmetrical anhydride of the N^a-Boc-Ala was prepared in a separate vessel by adding 5 mmol of dicyclohexylcarbodiimide (DCCI) to 10 mmol of Boc-Ala in dichloromethane.

Symmetrical anhydride was filtered upon the $MPEG_{6000}$ solution, 5 ml pyridine was then added to the reaction mixture, the volume was reduced to 3 ml under vacuum and the reaction mixture was stirred for 12 h. The product was precipitated by dropwise addition of dry ether to the concentrated solution under vigorous stirring at 0°C; the precipitate was filtered off and washed with dry ether. The powder produced was dissolved in the least amount of proper solvent (dimethylformamide, dichloromethane or methanol) and precipitated by dropwise addition of dry diethyl ether while cooling several times till the chromatographically pure product was obtained.

Removal of the (Boc group) N^a-protected group: for cleavage of the tert-butyloxycarbonyl group (Boc group), 2 mmol of MEG_{6000}-Ala-Boc was dissolved in 20 ml of trifluoroacetic acid/dichloromethane (1 : 1), and the solution was stirred for 30 min at room temperature; the reaction mixture was then reduced under vacuum, the oil produced was dissolved in dichloromethane and the product was precipitated by dropwise addition of dry ether while cooling with vigorous stirring. The pure crystals were filtered off and dried under vacuum. Thin layer chromatography, R_f = 0. Solvent system (butanol : acetic acid : water, 30 : 10 : 10), Kaiser test: positive.

The coupling reaction of $MPEG_{6000}$ – Ala-H with Boc-Lys: (2 mmol) $MPEG_{6000}$ – Ala-H was dissolved in 20 ml dichloromethane; in another flask, 10 mmol Boc-Lys was dissolved in 10 ml dichloromethane and cooled to 0°C; 5 mmol of DCCI dissolved in dichloromethane was added and the reaction mixture was allowed to stand with stirring for 30 min at 0°C. The dicyclohexylurea precipitate was removed by filtering off the anhydride solution directly into the flask containing the deprotected amino component in dichloromethane, and then the solution was neutralized with *N*-methyl morpholine and concentrated to about 10 ml and stirred overnight at room temperature. The product was precipitated by dropwise addition of dry ether while vigorously stirring under cooling; the product was then recrystallized twice till pure crystals were obtained. The coupling was controlled by the quantitative ninhydrin test, the Kaiser test, and thin layer chromatography.

Coupling and deprotection reactions were continued as described before to obtain the full sequence.

Synthesis of the nonapeptide by the liquid–solid-phase method

Coupling of the first amino acid to the polymer anchor: To a solution of polymer anchor (0.15 g, 0.22 mmol) in 2 ml dimethyl formamide (DMF)/CH_2Cl_2(1 : 1), a solution of Fmoc-Ala-OH (0.082 g, 1.76 mmol), *N*-hydroxybenzotriazole (HOBt) (0.036 g, 1.76 mmol), *N,N*-diisopropylcarbodiimide (DIC) (0.033 g, 1.76 mmol), and dimethylaminopyridine (20 mg, 0.3 mmol) in 5 ml DMF/CH_2Cl_2, (1 : 1) was added. The

mixture was shaken at room temperature until the Kaiser test became negative. The solution was then filtered off and washed three times with DMF, CH_2Cl_2, DMF, CH_2Cl_2, MeOH, and ether [8].

Deprotection of Fmoc-N^{α}-protecting group: Fmoc-Ala-anchor polymer (0.22 mmol) was suspended in the least amount of DMF. Then, a solution of 25% piperidine/DMF was added and the mixture was shaken at room temperature for 45 min, until the Kaiser test became positive. The reaction mixture was then filtered off and washed three times with DMF, CH_2Cl_2, MeOH, DMF, CH_2Cl_2, and ether.

Coupling of Fmoc-Lys to the peptide chain: To a solution of NH_2-Ala-anchor polymer (0.15 g, 0.22 mmol) in 2 ml DMF/CH_2Cl_2, (1 : 1), a solution of Fmoc-Lys (0.17 g, 1.76 mmol), HOBt (0.036 g, 1.76 mmol), and DIC (0.033 g, 8 mmol) in 4 ml DMF/CH_2Cl_2, (1 : 1) was added. The mixture was shaken at room temperature, until the Kaiser Test became negative. The solution was then filtered and washed three times with DMF, CH_2Cl_2, DMF, CH_2Cl_2, MeOH, and ether.

The nonapeptide Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala-Anchor-Resin was synthesized according to the above steps.

Results and discussion

The liquid-phase method of synthesizing noanapeptide (B_{22}–B_{30}) of porcine insulin B-chain

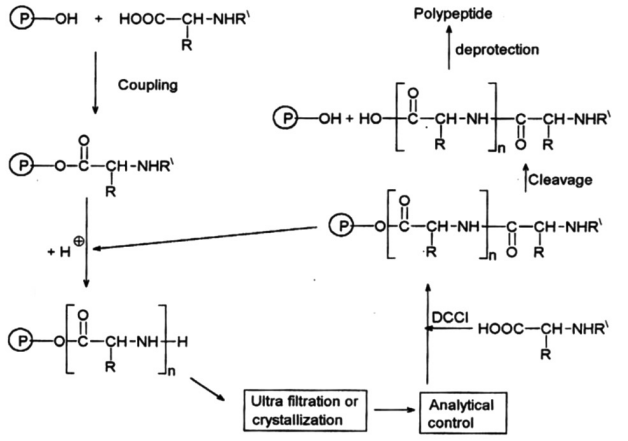
The present work deals with the synthesis of the model peptide chain bounded to PEG according to LPPS, which is the nonapeptide (B_{22}–B_{30}) of porcine insulin B-chain: H-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala-$MPEG_{6000}$.

The model peptides are covalently bounded to the monofunctional PEG of molecular weight 6000, which have a strong solublizing effect on the attached peptide chain in most solvents (Scheme 1).

The first amino acid (Boc-Ala) was covalently bounded to the MPEG chain with molecular weight 6000. Coupling reactions were carried out in a symmetrical anhydride separately prepared by the reaction of 2 mol of Boc-amino acid with 1 mol of DCCI dissolved in dichloromethane [9]. The mixture was allowed to stand for 30 min at 0°C. The precipitated dicyclohexylurea was removed by filtering off the anhydride solution directly into a flask containing the polymer dissolved in dichloromethane. The extent of the coupling was monitored by the Kaiser test.

The Boc group was removed by treatment of the polymer-bounded peptide by trifluoroacetic acid/dichloromethane

Scheme 1



Synthesis of the nonapeptide using the liquid-phase method.

(1 : 1) using 10 ml of the deprotecting agent per 1 g of PEG peptide. The volume of the solution was then reduced by flash evaporation to oil; then the PEG peptide was precipitated by the addition of dry ether under vigorous stirring. The mixture was stirred over 15–30 min at 30°C, the precipitate was filtered off, washed with ether and dried under vacuum. Quantitative cleavage of the Boc group was tested by the ninhydrin reaction.

The coupling of the second Boc-amino acid was carried out by the symmetrical anhydride method using excess anhydride component. To this end, the Boc-protected amino acid derivative was dissolved in a minimum amount of dichloromethane and the solution was cooled to 0°C. About 0.48 equivalent of DCCI in a 2 mol/l stock solution of dichloromethane was added, and the mixture was allowed to stand for 30 min at 0°C. The precipitated dicyclohexylurea was removed by filtering the anhydride solution directly into a flask containing the deprotected amino component in dichloromethane.

Test for coupling was monitored by the Kaiser test after isolation of the protected polymer-bounded peptide by precipitation.

The purity of the synthesized peptide was tested using thin layer chromatography after each step. Crude polymer-bounded peptide chains were dissolved in distilled water and subjected to HPLC.

Thin layer chromatography

R_f=0, the solvent system was butanol: acetic acid: water (3 : 1 : 1).

The time required for the complete coupling and deprotection reaction in the synthesized nonapeptide (B_{22}–B_{30}) of insulin B-chain is represented in Table 1.

The liquid–solid-phase method of synthesizing noanapeptide (B_{22}–B_{30}) of porcine insulin B-chain

It is evident that the correct choice of the synthetic strategy, the protecting groups and the polymeric support could contribute most fundamentally to the improvement of the method used. The following nonapeptide (B_{22}–B_{30}) of porcine insulin B-chain is the synthetic model.

H-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala-anchor resin

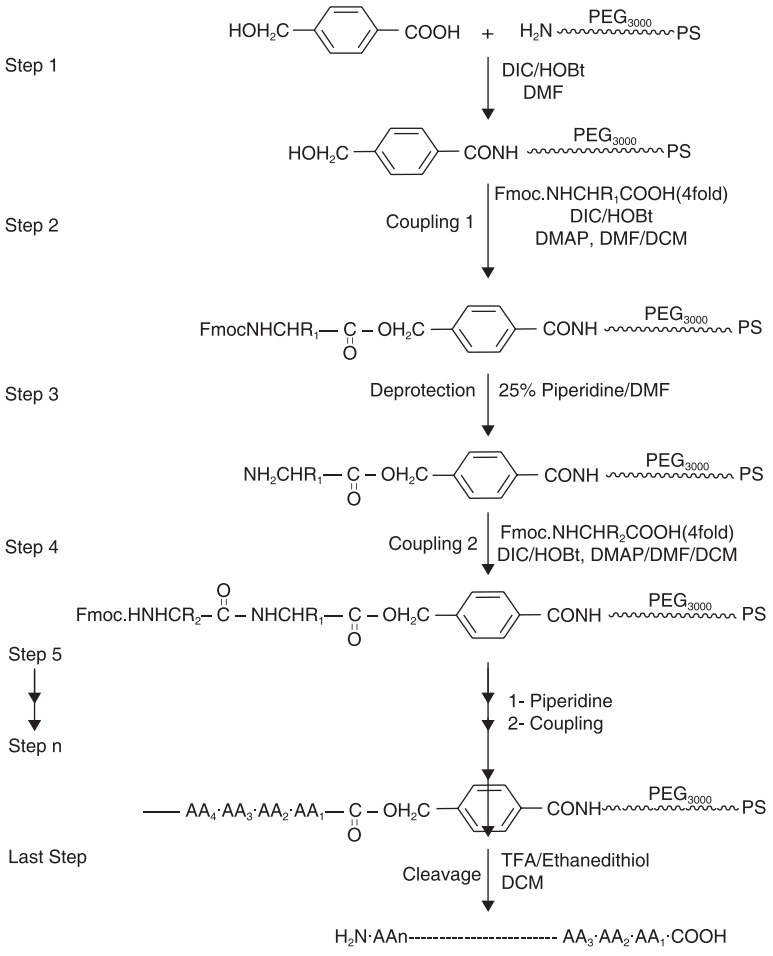
In the present work, the protocol used for the synthesis of the model peptide chain by the slow batch LSPPS (Scheme 2) includes the use of the Fmoc group for protection of the amino group. The Fmoc group can be cleaved with alkalis such as piperidine and can therefore be used in conjunction with acid-labile side

Table 1 The time required for maximum coupling and deprotection reactions of nonapeptide (B_{22}–B_{30}) using liquid-phase peptide synthesis and modified solid-phase peptide synthesis

Activated amino acid	LPPS (slow batch) method I		Modified SPPS (slow batch) method II	
	Coupling/min	Deprotection/min	Coupling/min	Deprotection/min
Ala	1440	30	1200	45
Lys	1200	30	960	45
Pro	1320	30	1200	45
Thr	1320	30	1080	45
Tyr	1440	30	960	45
Phe	1200	30	960	45
Phe	1200	30	960	45
Gly	1080	30	600	45
Arg	1440	30	1320	45

LPSS, liquid-phase peptide synthesis; SPSS, solid-phase peptide synthesis.

Scheme 2



Synthesis of the nonapeptide (B_{22}–B_{30}) of porcine insulin B-chain using modified solid-phase peptide synthesis.

chain-protecting groups such as t-Bu, compared with the solely acid-labile Boc group; the Fmoc group thus furnishes decisive improvements. Its UV absorption also opens up the possibility of spectroscopically monitoring coupling and deprotection reactions during the peptide synthesis process.

The Fmoc group was deprotected using 25% piperidine/DMF [10]. The electron-withdrawing fluorene ring system of the Fmoc group renders the lone hydrogen on the β-carbon very acidic, and therefore susceptible to removal by weak bases. After β-hydrogen extraction, the Fmoc group proceeds through a carbanion intermediate to form dibenzofulvene.

Coupling reactions were carried out [11] using the 1-hydroxybenzotriazole (HOBt) ester of Fmoc amino acids introduced by DIC, which forms soluble urea.

The interest in HOBt esters of Fmoc amino acids is based on well-established properties of these esters, such as suppression of racemization during coupling and rapid coupling of either *in situ* or the preformed esters. HOBt esters of Fmoc amino acids are easily formed with carbodiimides (e.g. DIC).

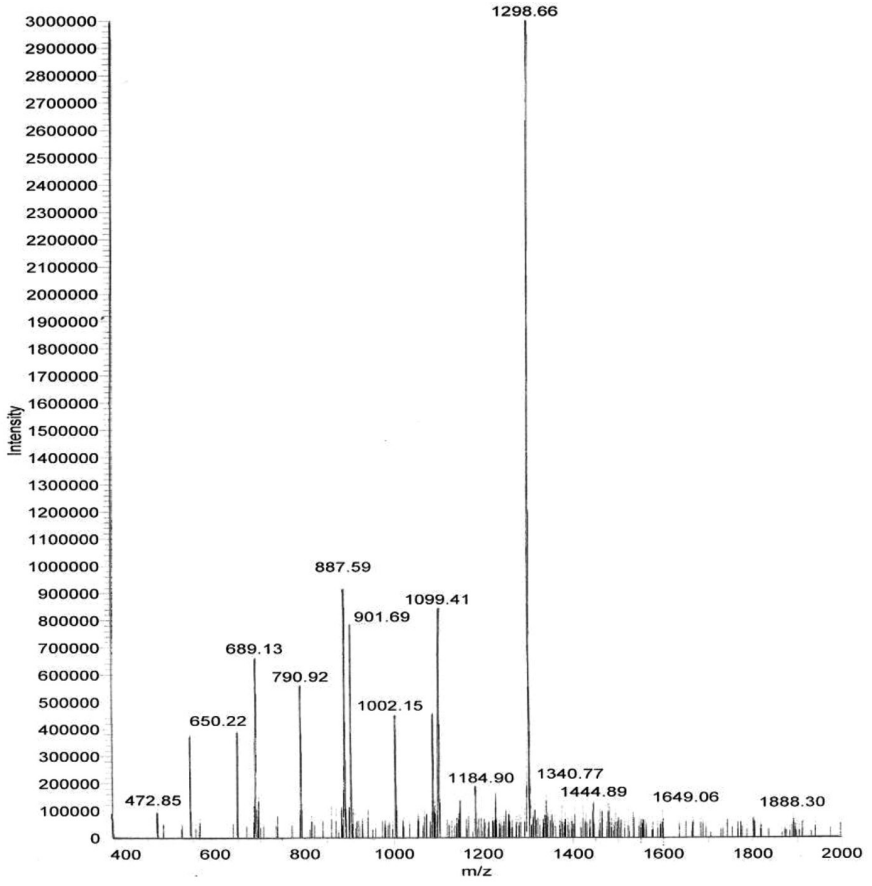
Cleavage of the peptide linker was carried out using a 10 ml mixture of TFA/ethanedithiol (4/1) for each gram of resin. 1,2-Ethanedithiol has been shown to be the most efficient scavenger for t-butyl trifluoroacetate compared with anisole/phenol and thiophenol, which are normally used for this purpose. All side-chain-protecting groups were cleaved simultaneously. The time required for maximum coupling and deprotection reactions is represented in Table 1.

On comparing LPPS (I) and LSPPS (II), the above results indicate clearly that the rate of coupling reactions is reduced by modified SPPS (600–1200 min) as compared with 1080–1440 min LPPS for all used amino acids.

The crude peptide synthesized by the slow batch-modified SPPS was analyzed by HPLC and positive-mode electrospray ionization mass spectrometry.

Figure 2 shows the electrospray ionization mass spectra of the crude peptide chain. The target peptide is

Figure 2



Electrospray ionization mass spectrometry data for the crude nonapeptide (B_{22}–B_{30}) of insulin B-chain synthesized using modified solid-phase peptide synthesis (slow batch).

Table 2 Amino aid analysis of the target peptide using two different methods

Methods	Ala	Lys	Pro	Thr	Tyr	Phe	Gly	Arg
LPPS (slow batch)								
Calculated	1	1	1	1	1	2	1	1
Found	0.9	0.83	0.92	0.90	0.80	1.9	1	0.89
Modified SPPS (slow batch)								
Calculated	1	1	1	1	1	2	1	1
Found	1	0.96	0.94	0.81	0.84	1.8	1	0.86

LPSS, liquid-phase peptide synthesis; SPSS, solid-phase peptide synthesis.

represented by the peak at 1298.66 *m*/*z*. The spectrum indicates the presence of impurities, immature, branched peptides and the remaining particles of the polymeric support.

The two chains were identified by amino acid analysis (Table 2).

From Table 2, we can observe the results of amino acid analysis equal of the theoretical and calculated amino acids except tyrosine due to the destruction of tyrosin during splitting of the peptide by conc acid.

Conclusion

Modified SPPS is the modification of the solid phase, which depends on cross-linking the polystyrene with diphenyl benzene, and the liquid phase, which is PEG; hence, this modification resulted in less consumption of solvent and no racemization and reducing side reactions, preventing cyclization during reactions.

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Conflicts of interest

There are no conflicts of interest.

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