GREEN CONTINUOUS LPPS

The future in peptide manufacturing

Ivo F. Eggen
Senior Project Manager CDMO

November 2024 - version 2

Summary

Some 25 years ago, the Aspen API labs in Oss, the Netherlands, developed a method for the large-scale manufacturing of peptides in solution, called GREEN CONTINUOUS LPPS, which has been continuously improved ever since. This method combines the advantages of the homogeneous character of classical liquid-phase synthesis with the generic character inherent to the solid-phase approach.

GREEN CONTINUOUS LPPS is characterized by the fact that intermediates are not isolated. Processes according to this highly efficient synthesis method are easy to scale up and yield products of reproducible high purity. Moreover, due to the use of limited amounts of ethyl acetate as the solvent, it offers significant advantages in terms of sustainability with respect to the more generally applied solid-phase protocols, which use vast amounts of reprotoxic solvents DMF or NMP. New additions to our peptide product portfolio according to the GREEN CONTINUOUS LPPS method include Goserelin and Lanreotide.

Introduction

Ever-increasing pressure is imposed upon the pharmaceutical industry to reduce time-to-market for new drugs and at the same time meet sustainability goals. Time-to-market, from the API-manufacturer's point of view, comprises the development of synthesis routes for new compounds, the scale-up of the ensuing processes, their validation and registration.

An additional challenge lies in the eventual manufacturing of APIs with increasingly higher and reproducible purity in a commercially competitive way, which is compatible with ever more stringent regulatory guidelines regarding control strategies.

These incentives prompted us in the year 2000 to perform a thorough revaluation of the two classical approaches of peptide synthesis, that is classical liquid-phase peptide synthesis (LPPS) and solid-phase peptide synthesis (SPPS).

Combining "the best of both worlds" and taking into account the extensive knowledge of impurity profiles built up by our company in the course of 60 years of peptide manufacturing, we have developed our Green Continuous Liquid-Phase Synthesis of Peptides (GREEN CONTINUOUS LPPS) method, which we have been using and optimizing since then (fig. 1).

Comparison of peptide synthesis methods

Process	Green	Lean	Clean
ASPEN GREEN CONTINUOUS LPPS	★ ★ ★	★ ★ ★	★ ★ ★
LPPS	★	★	★ ★
SPPS	-	★ ★ ★	★ ★

ASPEN GREEN CONTINUOUS LPPS

Green
- *Continuous EtOAc phase, almost 400 times less solvent consumption w.r.t. SPPS*
- *Low excess reagents*

Lean
- *Continuous synthesis, no isolations*
- *18 Days for 9-mer*
- *Easy scalability*

Clean
- *U(H)PLC in-process controls in solution*
- *Patented quenching method*
- *Reproducibility in solution*

LPPS

Less Green
- *Less green solvents*
- *Intermediate isolations*

Less Lean
- *Intermediate isolations*
- *21 days for 5-mer*

Less Clean
- *U(H)PLC in-process controls in solution*
- *Reproducibility in solution*

SPPS

Not Green
- *High volumes DMF*
- *High excess reagents*

Lean
- *Automated continuous synthesis*
- *Scalability challenging*

Less Clean
- *No quantitative in-process controls*
- *Low reproducibility resins*

Figure 1: Comparison of peptide synthesis methods.

GREEN CONTINUOUS LPPS characteristics

In our GREEN CONTINUOUS LPPS method, the growing peptide is essentially anchored in a permanent organic phase, generally ethyl acetate, by means of its hydrophobic C-terminal and sidechain protecting groups. A synthesis according to the GREEN CONTINUOUS LPPS protocol is completely homogeneous and its intermediates are not isolated. Excess reagents and byproducts are intermittently removed by aqueous extractions. No organic waste streams are generated during the performance of the synthesis, thus resulting in a reduction factor of approximately 400 for organic waste streams with respect to SPPS protocols. Moreover, reprotoxic solvents N,N-dimethylformamide (DMF) and N-methylpyrrolidin-2-one (NMP) are substituted by one of the greenest solvents available, ethyl acetate (fig. 2).

One cycle of the GREEN CONTINUOUS LPPS protocol consists of a coupling step, quenching of residual activated carboxylic compound and deprotection of the N-terminal amino function with intermittent aqueous extractive work-up steps. In one GREEN CONTINUOUS LPPS approach, the benzyloxycarbonyl (Z) function is applied for temporary amino protection, whereas tert-butyl-type functions or functions of similar lability are applied for the semi-permanent protection of certain functional side chains. The former is removed by hydrogenolysis in each cycle of a GREEN CONTINUOUS LPPS process (fig. 3).

Alternative GREEN CONTINUOUS LPPS approaches apply Fmoc (9-fluorenylmethyloxycarbonyl) and Nsc (2-(4-nitrophenyl) sulfonylethyloxycarbonyl) for temporary amino protection, thus enabling the incorporation of sulphur-containing residues. Combined approaches have also proven successful. Side chains are protected to ensure the solubility of the growing peptide in the organic phase. On account of the homogeneous character, coupling reagents and amino acid derivatives may be applied in low molar excess, resulting in a reduction of approximately 60% with respect to SPPS protocols.

Starting materials and reagents

Typical production of 1 kg of protected peptide

	AA	CR	SS	Ratio materials
GREEN CONTINUOUS LPPS	2.4 kg	2.4 kg		1
LPPS	1.5 kg	2.6 kg		0.9
SPPS	4.3 kg	5.7 kg	1.3 kg	2.4

AA – amino acid derivatives
CR – coupling reagents
SS – solid support

Solvents

Typical production of 1 kg of protected peptide

Ratio organic solvents	EtOAc	Water	DMF	*n*BuOH	DEE	NMP	EtOH
1	15 L	165 L	-	-	-	-	-
15	28 L	55 L	55 L	28 L	110 L	-	-
377						3967 L	1690 L

Figure 2: Comparison of material usage in various peptide synthesis methods.

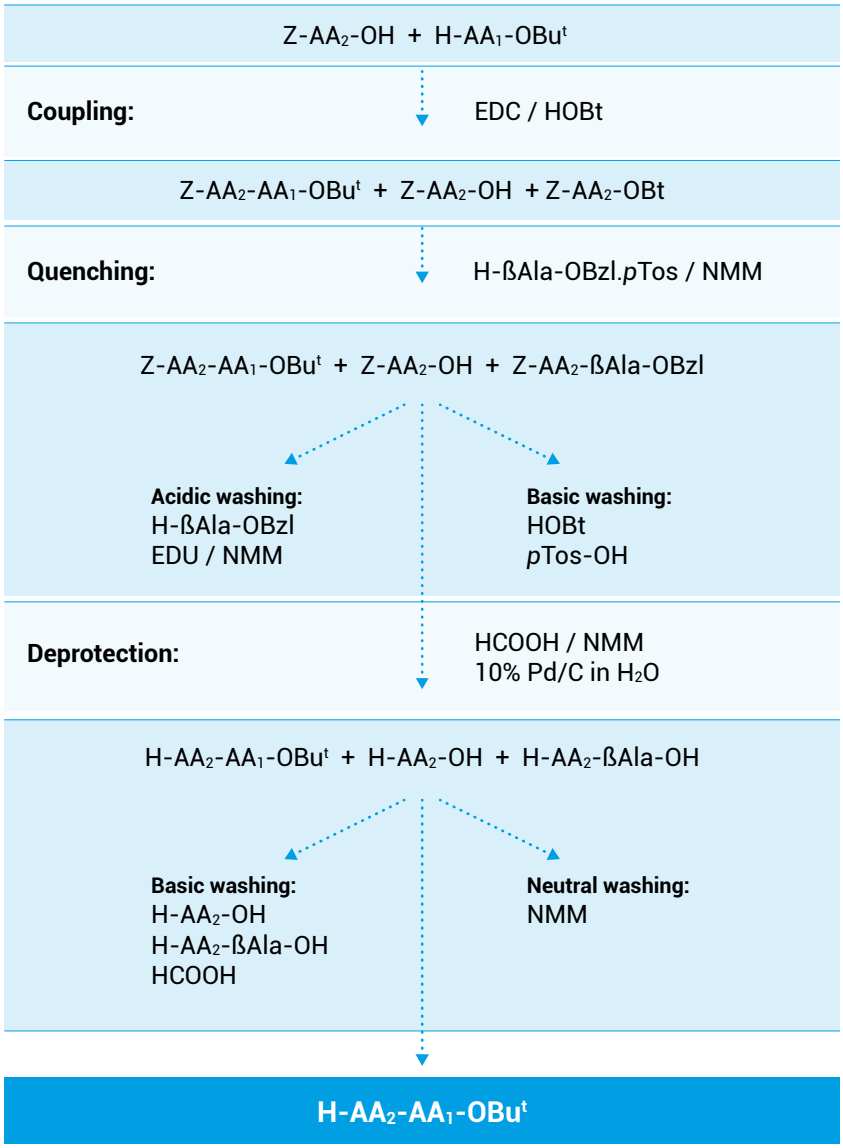

Figure 3:
Typical GREEN CONTINUOUS LPPS cycle using Z chemistry.

After completion of a coupling, residual activated carboxylic compound, if hydrophobic, is quenched using an anion-forming amine, preferably a β-alaninate ester. This ester should display a lability similar to that of the temporary amino protecting function to allow simultaneous deprotection of the growing peptide and the quenched compound. β-Alaninate was selected for its inability to form diketopiperazines as well as its higher nucleophilicity with respect to α-amino compounds. The GREEN CONTINUOUS LPPS approach allows the completely quantitative removal of quenched compounds before the coupling step of the next cycle of the synthesis by basic aqueous (that is active) extraction. Accordingly, the application of an anion-forming amine in the quenching step of the GREEN CONTINUOUS LPPS protocol accompanied by the appropriate work-up procedures accounts for absolute impediment of formation of insertion and/or truncated sequences.

Deletion sequences are avoided in the GREEN CONTINUOUS LPPS protocol, since all reactions can be minutely monitored. Functional side chains on the growing peptide, being shielded by protecting groups, are less likely to be modified during the assembly of the sequence.

In general, the strict control of the quality of starting materials is one important aspect of the regulatory control strategy to secure the reproducible high quality of the peptide API. However, GREEN CONTINUOUS LPPS offers additional advantages in view of the control strategy: the feasibility to monitor and optimize process conditions, define the design space and study critical process parameters (CPPs) at the intermediate stages (which is more complicated in SPPS protocols) and the application of tailored quenching and aqueous extractive washing protocols.

Revaluation of the classical approaches

Time-to-market

During the development of a synthesis route, speed is in the first place achieved through the application of a generic protocol. A generic protocol has never been achieved for classical LPPS, since functional side chains of amino acid residues are often not protected in this approach, thus accounting for intermediates (that is the growing peptide) with strongly varying chemical and, more important, physical properties. Intermediates of such syntheses are usually isolated by precipitation and filtration. Especially during these isolations, when a transition from a homogeneous to a heterogeneous system occurs, a great variation in the applied protocols is inevitable. Heterogeneity is also the major cause of complications during the scale-up of a synthesis in both the classical LPPS and the SPPS approaches. Also, the heterogenic nature of SPPS inhibits the proper characterization of intermediates and may therefore complicate the validation of the process. It is obvious that integral homogeneity during a synthesis expedites its development as well as its scale-up and validation.

Manufacturing efficiency

In the last decades, there has been a shift from classical LPPS towards SPPS for the large-scale manufacturing of peptides. Two intrinsic properties of the SPPS approach contribute to its commercial competitiveness. First, no intermittent isolations occur during the synthesis on a solid support. Furthermore, SPPS follows a generic protocol and is therefore automatable. Both aspects increase the manufacturing efficiency and do not apply to classical LPPS.

Despite the mentioned benefits of the SPPS approach, the manufacturing efficiency is somewhat compromised by the cost of starting materials. These are relatively high due to application of expensive non-reusable resins and amino acid derivatives. The intrinsic heterogeneity of SPPS is often reflected in retarded coupling rates, thus necessitating the application of large molar excess of reagents and amino acid derivatives during SPPS couplings.

Quality control strategy

Not only its commercial competitiveness, but also the robustness of a manufacturing process, and hence the quality control strategy, is a highly important requirement in API manufacturing. Impurities in the final product of a peptide synthesis may be roughly divided into five different categories:

- diastereomers,
- insertion sequences,
- deletion sequences,
- truncated sequences,
- impurities arising from modifications of functional side chains on the actual peptide.

Impurities either originate from the synthesis protocol towards the API, or from the starting amino acid derivatives. In the latter case, the impurities are controlled by setting appropriate specifications on these derivatives.

Diastereomers either originate from the presence of the optical antipode in the starting amino acid derivatives or from epimerization of amino acid derivatives during the peptide assembly. They are essentially independent of the synthesis approach, being rather determined by the conditions and reagents applied during coupling. In general, the extent of epimerization is negligible when the peptide is assembled in N-terminal direction through consecutive couplings of amino acids whose α-amino functions carry a urethane-type protecting group.

Depending on the actual peptide sequence, modifications of functional side chains on the peptide

may be introduced during the peptide assembly or in the later stages of a synthesis, hence after the assembly of the actual sequence when protecting groups on the constituting functional side chains are being or have been removed. In classical LPPS the functional side chains are often not protected and modifications are thus more likely to occur during the assembly of the actual sequence.

Insertion sequences containing one or more additional amino acid residues either originate from the presence of the free amino acid or dipeptide in the starting amino acid derivatives or from the assembly of the peptide. The latter are mainly encountered in products originating from classical LPPS. To impede their formation, all residual unactivated carboxylic compound must be removed before the coupling

step of the next cycle of the synthesis. All residual activated carboxylic compound must be removed even before the following deprotection step. It is generally assumed that residual activated carboxylic compound is destroyed during the aqueous work-up after coupling and as such removed by aqueous work-up or precipitation before the coupling step of the next cycle of the synthesis. However, the detection of substantial quantities of insertion sequences in peptide products originating from classical LPPS proves this assumption to be incorrect. In classical synthesis approaches, quenching of residual activated carboxylic compound sometimes occurs with a polyamine. This type of quenching generates basic quenched compounds which are, depending on their hydrophobicity, only partly removed prior to the following deprotection step. They cannot be actively removed (that is by means of acidic aqueous extraction) before the coupling step of the next cycle of the synthesis due to the risk of loss of peptide material. This approach therefore necessarily results in the formation of C-terminally truncated sequences. In SPPS protocols, N-terminally truncated sequences may be formed due to the application of capping procedures.

Like truncated sequences, deletion sequences lacking one or more amino acid residues always originate from the assembly of the (protected or semi-protected) sequence. Deletion sequences are primarily encountered in peptide products originating from SPPS, in which thoroughly quantitative in-process analysis of single synthesis steps is not practicable due to the heterogeneous character of the synthesis.

Reproducibility of the impurity profile of a peptide product is dependent on the reproducibility of the production process and its parameters. Reproducibility in classical LPPS is compromised during the isolations, whereas reproducibility is difficult to achieve in SPPS in terms of swelling properties and loadings of the applied solid supports. Moreover, monitoring and optimization of process conditions and critical process parameters studies at the intermediate stages as part of the (development of the) quality control strategy are complicated in SPPS protocols.

Sustainability

A final pivotal aspect of classical LPPS and especially SPPS that demands revaluation pertains to the reduction of organic waste streams originating from the application of these syntheses on a manufacturing scale. In the last decade, there has been a strong focus from the peptide manufacturing industry on development of protocols, which better comply with sustainability goals laid down in the 12 principles of green chemistry and reduce Process Mass Intensity (PMI). Increasing atom efficiency using smaller equivalents of starting materials and reagents is one focus point, but by far most advantages can be gained if reprotoxic solvents such as DMF and NMP, which are used in vast amounts in SPPS protocols, can be substituted by greener alternatives. However, the search for generally applicable greener solvents has been hampered by various aspects, like solubility, reactivity, resin properties in these solvents as well as commercial availability of these solvents.

GREEN CONTINUOUS LPPS state-of-the-art

Syntheses according to GREEN CONTINUOUS LPPS proceed by a generic and fast protocol. In the last twenty years, a considerable number of protected peptides has thus been synthesized in our labs, varying from tripeptides to dodecapeptides. Purities and yields are generally high, as exemplified by the synthesis of the protected human Insulin fragment Boc-Gly-Phe-Phe-Tyr(Bu^t)-Thr(Bu^t)-Pro-Lys(Boc)-Thr(Bu^t)-OBu^t, which was obtained in 98% purity and 85% yield (corresponding to an average yield of 99% per chemical conversion) in a first 7 days' trial. Several GREEN CONTINUOUS LPPS processes have been directly scaled-up after a preliminary feasibility study on a laboratory scale, achieving reproducible results in terms of both yield and purity. Peptides manufactured at Aspen API according to the GREEN CONTINUOUS LPPS method include Leuprorelin. Other peptides, including Goserelin and Lanreotide, are under development. We are able to produce a nonamer peptide on a commercial scale within a time period of 18 days. Recent research is focused on the introduction of automated phase separation techniques, so that manufacturing efficiency can be further increased.

The application of GREEN CONTINUOUS LPPS implies the same process and impurity profile throughout all stages of development, hence from the first laboratory sample to production batches, combined with intrinsically short process times. It may be concluded that GREEN CONTINUOUS LPPS offers substantial benefits concerning sustainability, time-to-market, manufacturing efficiency and quality control strategy and thence meets all requirements for peptide manufacturing of the future.

GREEN CONTINUOUS LPPS: The principle (simplified)

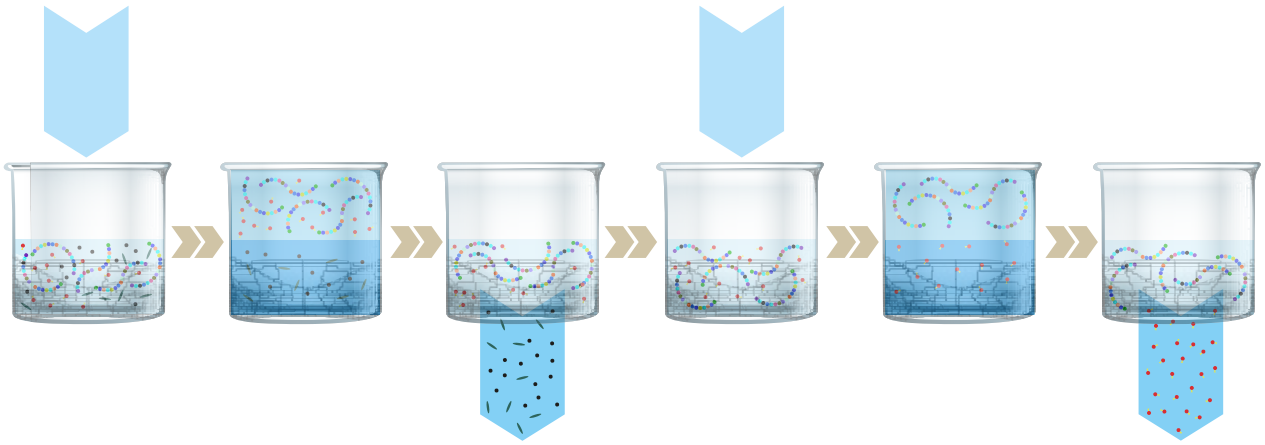

Figure 4: Simplified process GREEN CONTINUOUS LPPS.

November 2024 - version 2

Contact us

Global sales office

PO Box 98
5340 AB Oss
The Netherlands

Tel. +31 (0) 88 2779 779
api@nl.aspenpharma.com

We also have local distributors in several countries. Please contact our commercial department for more information.

Local sales offices

Sales Office USA / Canada

2136 South Wolf Road
Des Plaines
IL 60018
United States of America

Tel. +1 847 635 09 85
api-usa@us.aspenpharma.com

Liaison Office

Level 11, Aoyama Palacio Tower
3-6-7 Kita-Aoyama, Minato-ku
Tokyo 107-0061
Japan

Tel. +81 3-5464-8634
aspenapi-japan@nl.aspenpharma.com

Fine Chemicals Corporation

15 Hawkins Avenue
Epping Industria
Epping
Cape Town
South Africa

Tel. +27 21 530 8100
fccinfo@aspenpharma.com