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Communications to the Editor

DioRaSSP: Diosynth Rapid Solution Synthesis of Peptides

Ivo F. Eggen,* Frits T. Bakelaar, Annet Petersen, and Paul B. W. Ten Kortenaar
Diosynth bv, P.O. Box 20, 5340 BH Oss, The Netherlands

Abstract:
We have developed a novel method for the large-scale manufacturing of peptides in solution called DioRaSSP. It combines the advantages of the homogeneous character of classical solution-phase synthesis with the generic character and the amenability to automation inherent to the solid-phase approach. DioRaSSP 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, we have recently implemented the first fully automated solution-phase peptide synthesizer for application in processes according to DioRaSSP.

Introduction

Ever-increasing pressure is imposed upon the pharmaceutical industry to reduce time-to-market for new drugs. 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, and their validation and registration. An additional challenge lies in the eventual manufacturing of API's of increasingly higher and reproducible purity in a commercially competitive way, which is environment-friendly and compatible with ever more stringent guidelines regarding cGMP. These incentives prompted us to perform a thorough revaluation of the two classical approaches of peptide synthesis, that is classical solution-phase peptide synthesis (CSPS) and solid-phase peptide synthesis (SPPS), taking into account the extensive knowledge of impurity profiles built up by the company in the course of 50 years of peptide manufacturing.

Time-to-Market. During the development of a synthesis route, speed is first achieved through the application of a generic protocol, which is moreover a prerequisite for the eventual automation of said synthesis. A generic protocol has never been achieved for CSPS, 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 CSPS and the SPPS approach. Finally, the heterogenic nature of SPPS inhibits the proper characterization of intermediates and therefore complicates 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 past decade, there has been a shift from CSPS towards SPPS for the manufacturing of peptides on a large scale.[1] Two intrinsic properties of the SPPS approach contribute to its commercial competitiveness. First of all, no intermittent isolations occur during the synthesis on a solid support. Furthermore, SPPS follows a generic protocol and is therefore automatable. Both of these aspects increase the manufacturing efficiency and are not applicable to CSPS.

Despite said benefits of the SPPS approach, manufacturing efficiency in this approach is somewhat compromised by the cost of starting materials. These are relatively high due to application of expensive nonreusable resins and amino acid derivatives. The intrinsic heterogeneity of SPPS is often reflected in retarded coupling rates, thus necessitating the application of a large molar excess of reagents and amino acid derivatives during SPPS couplings. Moreover, all functional side chains must be protected in SPPS to make the hydrophobic resin accessible to the otherwise polar amino acids and to avoid the accumulation of sequences containing modified side chains.

Product Quality Assurance. As well as its commercial competitiveness, the robustness of a manufacturing process, and hence quality assurance, is a highly important requirement in API manufacturing.[2] Impurities in the final product of a peptide synthesis may be roughly divided into five different categories: epimers, insertion sequences, deletion sequences, truncated sequences, and impurities arising from modifications of functional side chains (and/or terminal functions) on the actual peptide. Epimers originate from racemization of amino acid derivatives during their coupling and are essentially independent of the synthesis approach,

* To whom correspondence should be addressed. Phone: +31 (0)412 663220. Fax: +31 (0)412 662563. E-mail: ivo.eggen@diosynth.com.

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Table 1. **Comparison of methods for peptide synthesis**

aspect	determined by	CSPS	SPPS	DioRaSSP
	Time-to-Market			
route development	generic protocol	−	+	+
scale-up & validation	homogeneous synthesis	+/−	−	+
	Manufacturing Efficiency			
cycle times	no isolations	−	+	+
	automation	−	+	+
materials	small excess reagents	+	−	+
	no solid support	+	−	+
	minimal side-chain protection	+	−	+/−
	Product Quality Assurance			
high purity	no insertion sequences	−	+	+
	no deletion sequences	+	−	+
	no side-chain reactions	−	+	+
reproducibility	reproducible isolations	−	n.a.	n.a.
	reproducible supports	n.a.	−	n.a.
	Environmental Demands			
organic waste streams	no solvent changes	−	+	+
	no organic washings	+	−	+

being rather determined by the conditions and reagents applied during coupling. In general, the extent of racemization 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.[3] Modifications of functional side chains (and/or terminal functions) on the peptide are often introduced 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. However, in CSPS these side chains are often not protected and modifications may thus in addition occur during the assembly of the actual sequence.[4]

Insertion, deletion, and truncated sequences, on the contrary, always originate from the assembly of the (protected or semiprotected) sequence. Insertion sequences containing one or more additional amino acid residues are mainly encountered in products originating from CSPS. To impede their formation, all residual unactivated carboxylic compounds should be removed before the coupling step of the next cycle of the synthesis, while all residual activated carboxylic compounds should be removed even before the following deprotection step. It is generally assumed that residual activated carboxylic compound is destroyed during the aqueous workup after coupling and as such removed by aqueous workup 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 CSPS 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, and which 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.

Deletion sequences lacking one or more amino acid residues 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.[5]

Reproducibility of the impurity profile of a peptide product is dependent on the reproducibility of the production process and its parameters. Reproducibility in CSPS 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. Consequently, an adaptation of the classical methods for peptide synthesis is required to prevent the formation of said impurities and to vouch for peptide products of reproducible high quality.

A final aspect of CSPS 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.

DioRaSSP characteristics. Based on the above considerations, we have developed a new and patented method for the preparation of peptides in solution, called DioRaSSP, Diosynth Rapid Solution Synthesis of Peptides.[6,7] The characteristics of this and the two classical methods for peptide synthesis are summarized in Table 1. In the DioRaSSP approach, the growing peptide is essentially anchored in a permanent organic phase (generally ethyl acetate) by means of its hydrophobic C-terminal and side-chain protecting groups. A synthesis according to the DioRaSSP protocol is completely homogeneous, and its intermediates are not isolated. Excess reagents and byproducts are intermittently

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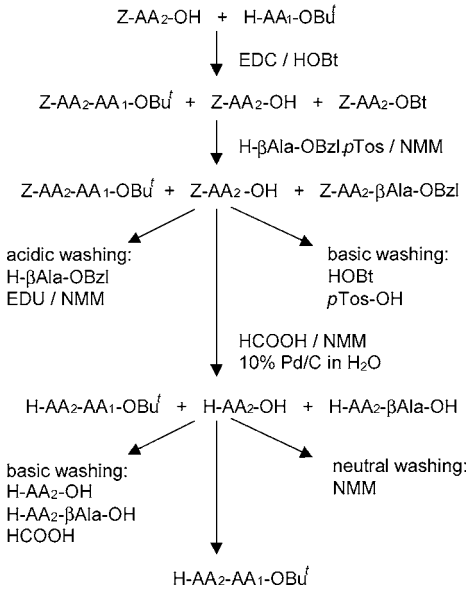
Table 2. **Results of model syntheses according to various DioRaSSP approaches**

peptide product	DioRaSSP approach	product purity (%)	average yield per chemical conversion (%)
Boc-Gly-Phe-Phe-Tyr(Bu^t)-Thr(Bu^t)-Pro-Lys(Boc)-Thr(Bu^t)-OBu^t	Z-DioRaSSP	97.3	98.6
Boc-Gly-Phe-Leu-Ser(Bu^t)-OBu^t	Fmoc-DioRaSSP	95.3	97.0
Boc-Ile-Phe-Cys(Trt)-Pro-Phe-Leu-OBu^t	combined Z/Fmoc-DioRaSSP	97.6	96.0
Boc-Gly-Phe-Phe-Leu-OBu^t	Msc-DioRaSSP	96.1	96.6
Boc-Gly-Phe-Phe-Leu-OBu^t	Nsc-DioRaSSP	97.2	95.2

Scheme 1. **A synthesis cycle according to Z-DioRaSSP**



removed by aqueous extractions. No organic waste streams are generated during the performance of the synthesis.

As represented in Scheme 1, one cycle of the DioRaSSP protocol consists of a coupling step, a quenching of the residual activated carboxylic compound, an aqueous extractive workup, a deprotection of the N-terminal amino function, and finally another aqueous extractive workup. In the Z-DioRaSSP 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 semipermanent protection of certain functional side chains. The former is removed by hydrogenolysis in each cycle of a DioRaSSP process. Complete conversion during coupling and deprotection is generally achieved within 30−60 min. Alternative DioRaSSP approaches apply Fmoc (9-fluorenylmethyloxycarbonyl), Msc (methylsulfonylethyloxycarbonyl), and Nsc (2-(4-nitrophenyl)sulfonylethyloxycarbonyl) for temporary amino protection, thus enabling the incorporation of sulfur-containing residues.[8−10] Combined approaches have also proven successful. Side chains are only protected if this is required from a chemical point of view or to ensure the anchorage of the growing peptide in the organic phase. The applied protection schemes justify the commercial viability of its application on a manufacturing scale. Moreover, on account of the homogeneous character, reagents and amino acid derivatives may be applied in low molar excess.

After completion of a coupling, the 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 DioRaSSP 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 DioRaSSP protocol accompanied by the appropriate workup procedures accounts for absolute impediment of formation of insertion and/or truncated sequences.

Deletion sequences are avoided in the DioRaSSP protocol, since all reactions can be minutely monitored. Functional side chains on the growing peptide, being shielded by protecting groups, are not modified during the assembly of the sequence. The selection of appropriate protecting groups, moreover, accounts for the fact that the actual length of a peptide (fragment) is not a limiting factor in terms of its solubility.

DioRaSSP State-of-the-Art. Syntheses according to DioRaSSP proceed by a generic and fast protocol. In the past four years, we have thus synthesized a considerable number of protected peptides, varying from tripeptides to a dodecapeptide.[11] High purities and yields are generally obtained, which is corroborated by the results of model syntheses according to various DioRaSSP approaches as summarized in Table 2. Several DioRaSSP 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. Figure 1 shows an overlay of the HPLC chromatograms of a Leuprolide heptapeptide precursor obtained in syntheses according to Z-DioRaSSP on a 100-g and a 5-kg scale. Other peptides manufactured at Diosynth

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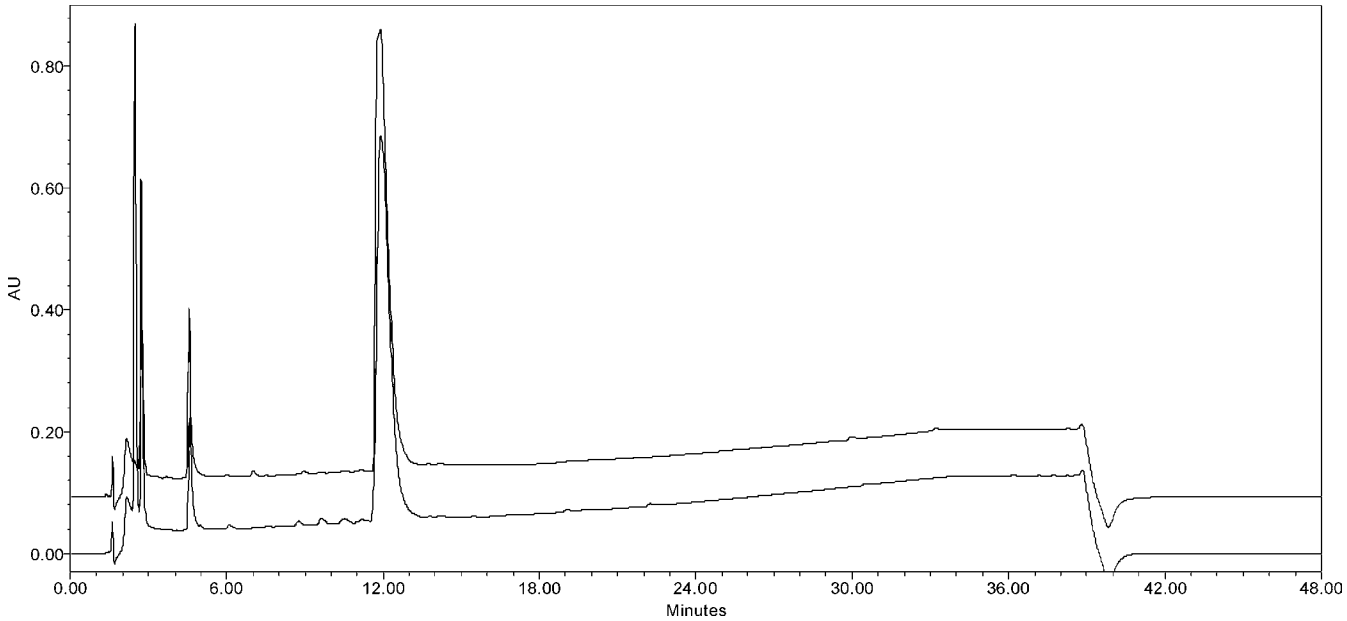

Figure 1. **Overlay of HPLC chromatograms of a Leuprolide heptapeptide precursor obtained in syntheses according to Z-DioRaSSP on a 100-g (upper graph) and a 5-kg scale (lower graph).**

Figure 2. **DioRaSSP Synthesizer: the first fully automated solution-phase peptide synthesizer.**

according to the DioRaSSP method include Buserelin, Deslorelin, Goserelin, Histrelin, and Triptorelin. The application of DioRaSSP 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. The size of the available reaction vessels should prove to be the only limiting factor during scale-up towards multi-100-kg batches.

We have recently implemented the first fully automated solution-phase peptide synthesizer, which applies generic DioRaSSP protocols. The DioRaSSP synthesizer, as depicted in Figure 2, is equipped to perform reagent additions, phase separations, hydrogenations, and sampling (for HPLC-analysis at every stage of a synthesis) in a fully automated mode, yielding first samples of up to 20 g of peptide material within a very short time frame. It may furthermore be applied for route scouting and optimization, the investigation of critical process parameters, and kinetic studies.

It may be concluded that DioRaSSP offers substantial benefits concerning time-to-market, manufacturing efficiency, quality assurance, and environment and thus meets all specifications for peptide manufacturing of the 21st century.

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