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A novel method for repetitive peptide synthesis in solution without isolation of intermediates

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Abstract: A novel method was developed for the large-scale manufacture of peptides in solution, called DioRaSSP – Diosynth Rapid Solution Synthesis of Peptides. This method combines the advantages of the homogeneous character of classical solution-phase synthesis with the universal character and the amenability to automation inherent to the solid-phase approach. The process consists of repetitive cycles of coupling and deprotection in a permanent organic phase and is further characterized by the fact that intermediates are not isolated. Couplings are mediated by water-soluble carbodiimide. Several types of function may be applied for temporary amino protection depending on the sequence of the actual peptide, including Z, Fmoc, Msc and Nsc. Formate is the preferred hydrogen donor during hydrogenolysis of the Z function, while 1,8-diazabicyclo[5.4.0]undec-7-ene is used to deprotect Fmoc, Msc and Nsc. Morpholine is added during the deprotection of Msc and Nsc to scavenge the arising alkenes. Processes according to this highly efficient synthesis method are easy to scale up and yield products of reproducible high purity, which is guaranteed by a new quenching method for residual activated compounds, applying an anion-forming amine such as a β-alanine ester. This ester should display a lability similar to that of the temporary amino-protecting function, allowing simultaneous deprotection of the growing peptide and the quenched compound. The DioRaSSP approach assures the completely quantitative removal of deprotected quenched compounds before the coupling step of the next cycle of the synthesis by basic aqueous (that is active) extraction, while the growing peptide remains anchored in the organic phase due to the presence of hydrophobic protecting functions.

Keywords: DioRaSSP; solution-phase synthesis; large-scale manufacture; quenching; hydrogenolysis; automation

INTRODUCTION

Ever-increasing pressure is being imposed upon the pharmaceutical industry to reduce the time-to-market for new drugs. Whereas on a laboratory scale, the toolbox provided by the two classical approaches to peptide synthesis, that is, classical solution-phase peptide synthesis (CSPS) and solid-phase peptide synthesis (SPPS), is sufficient to arrive at almost any type of peptide, commercial peptide manufacture is subject to additional incentives [1–3]. From the API-manufacturer's point of view, time-to-market comprises the development of synthesis routes for new compounds, the scale-up of the ensuing processes, and their subsequent validation and registration. During the development of a synthesis route, speed is primarily achieved through the application of a general protocol, while integral homogeneity during a synthesis expedites its development as well as its scale-up and validation. Manufacturing efficiency is largely increased by omitting the isolation of intermediates.

Based on these premises and combining the advantages of CSPS and SPPS, a novel method was developed for the synthesis of peptides known as DioRaSSP – Diosynth Rapid Solution Synthesis of Peptides [4–12].

Product Quality Assurance

Besides commercial competitiveness, the robustness of a manufacturing process, and hence quality assurance, is a highly important requirement in API manufacturing [13,14]. Quality guidelines are becoming ever more stringent. Typical specifications for a modern peptide-based drug include a purity by HPLC of 98% with the largest individual impurity of 0.5%; all impurities of 0.1% should be characterized.

Impurities in the final product of a peptide synthesis may be roughly divided into five different categories: epimers, impurities arising from modifications of functional side-chains (and/or terminal functions) on the actual peptide, insertion sequences, deletion sequences and truncated sequences. Epimers originate from racemization of amino acid derivatives during their coupling and are essentially independent of the synthesis approach, being determined rather by the conditions and reagents applied during coupling. In general, the extent of racemization is negligible when the peptide is assembled in the *N*-terminal direction

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through consecutive couplings of amino acids whose α-amino functions carry a urethane-type protecting group [15].

Modifications of functional side-chains (and/or terminal functions) on a 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 also occur during the assembly of the actual sequence [16].

Insertion, deletion and truncated sequences, on the other hand, always originate from assembly of the (protected or semi-protected) sequence. Insertion sequences containing one or more additional amino acid residues are mainly encountered in products originating from CSPS. In order 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 believed 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 CSPS shows this assumption to be incorrect.

In classical synthesis approaches, quenching of residual activated carboxylic compound sometimes occurs with a polyamine [17]. 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, since reactions of the growing peptide on the resin are diffusion controlled and sometimes very slow depending on the actual sequence. Moreover, a thoroughly quantitative in-process analysis of single synthesis steps is not practicable due to the heterogeneous character of the synthesis [18].

Reproducibility of the impurity profile of a peptide product is dependent on the robustness of the production process and its parameters. In CSPS, reproducibility is compromised during the isolations, whereas in SPPS, reproducibility is difficult to achieve in terms of the 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 these diverse impurities and to assure peptide products of reproducible high quality.

DioRaSSP Characteristics

With 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 performed according to the DioRaSSP protocol is completely homogeneous and its intermediates are not isolated. Excess reagents and by-products are intermittently removed by aqueous extractions, and no organic waste streams are generated during performance of the synthesis, which is a big advantage for a manufacturing scale.

As shown in Figure 1, one cycle of the DioRaSSP protocol consists of a coupling step, quenching of residual activated carboxylic compound, aqueous extractive work-up, deprotection of the *N*-terminal amino function, and finally another aqueous extractive work-up. Couplings are mediated by water-soluble carbodiimide to allow aqueous extractive work-up and to avoid an additional filtration step.

After completion of a coupling, residual activated carboxylic compound, if hydrophobic, is quenched with a compound containing a nucleophilic moiety, which is able to convert an activated carboxylic moiety (e.g. an amine or thiol), as well as an anion-forming moiety, which can be deprotonated under mildly basic conditions compatible with peptide synthesis (e.g. a protected or free carboxylate, sulfonate or

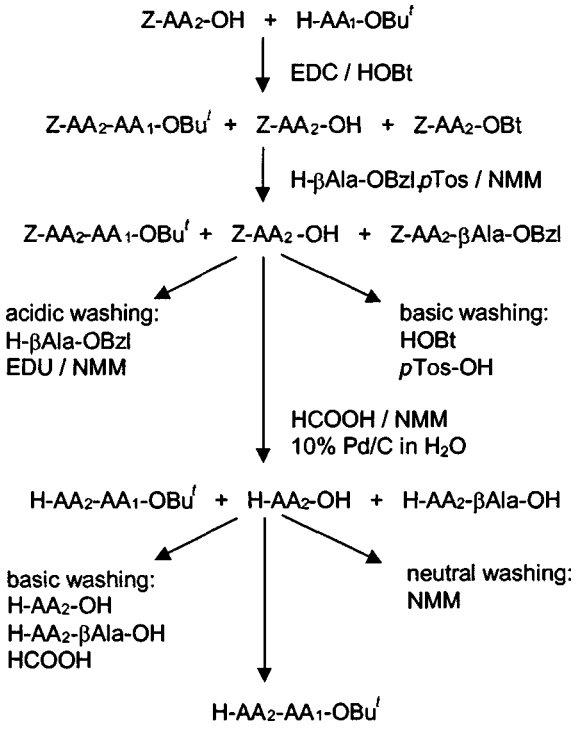

Figure 1 A synthesis cycle according to Z-DioRaSSP.

phosphonate). The preferred quenching compound is a β-alanine ester, whose lability is similar to that of the temporary amino-protecting function, allowing simultaneous deprotection of the growing peptide and the quenched compound. The DioRaSSP approach assures 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, while the growing peptide remains anchored in the organic phase due to the presence of hydrophobic protecting functions. β-Alaninate was selected for its inability to form diketopiperazines as well as its higher nucleophilicity with respect to α-amino compounds.

With the DioRaSSP method, functional 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 application of appropriate protecting functions accounts for the fact that the actual length of a peptide (fragment) is not a limiting factor in terms of its solubility. *Tert*-butyl-type functions, or functions of similar lability, are generally applied for the semi-permanent protection of functional side-chains.

The DioRaSSP concept may be applied in combination with several types of temporary amino-protecting functions. The ideal function for application in DioRaSSP on a manufacturing scale would typically meet the following specifications:

1. Its application should be economically feasible.
2. It should be easy to introduce (without racemization or cleavage of *tert*-butyl-type functions).
3. It should be stable during all steps of the synthesis (that is, stable under mildly acidic and basic conditions) with the exception of the deprotection step.
4. Deprotection reagents and byproducts should be easily removable by filtration and/or (basic) aqueous extraction, or chemically inert without accumulating.
5. It should be cleavable under mild conditions to ensure the integrity of the formed product.
6. It should be selectively cleavable in the presence of *tert*-butyl-type protecting functions.
7. It should be cleavable in the presence of sulfur.
8. Cleavage (as well as other synthesis steps) should preferably occur in ethyl acetate. The application of ethyl acetate as the general solvent in a continuous synthesis is favourable from both an environmental and a practical point-of-view. Since ethyl acetate is the lighter phase during aqueous extractions, only one reaction vessel is required as opposed to two for dichloromethane, which is a big advantage on a manufacturing scale.
9. Coupling of protected amino acid derivatives should occur fast and without racemization.
10. An ester function of similar lability should be available (for the quenching compound).
11. The protected amino acid derivatives should be available in a stable crystalline form.

In Z-DioRaSSP, the benzyloxycarbonyl (Z) function is applied for temporary amino protection. This function is removed by hydrogenolysis in each cycle of the process, using formate as the preferred hydrogen donor. Several alternative amino-protecting functions have been applied in the DioRaSSP protocol, including Fmoc (9-fluorenylmethyloxy-carbonyl), Msc (methylsulfonylethyloxy-carbonyl) and Nsc (2-(4-nitrophenyl)sulfonyl-ethyloxy-carbonyl), thus enabling the incorporation of sulfur-containing residues [19–21]. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) is used to effect fast cleavage of these protecting functions in ethyl acetate (Fmoc-DioRaSSP, Msc-DioRaSSP and Nsc-DioRaSSP, respectively). Morpholine is added during the deprotection of Msc and Nsc to scavenge the arising alkenes. Due to its lower lability towards base, the former is cleaved at 40 °C. Coupling and deprotection may be monitored by RP-HPLC and/or TLC and complete conversion is generally achieved within 30–60 min for all of these approaches.

Combined approaches (combi-DioRaSSP) are also encompassed by the scope of the DioRaSSP methodology and maximize its flexibility: the approach may be adapted to the actual sequence of the growing peptide at any stage of a synthesis. The applied protecting schemes in the various DioRaSSP protocols justify the commercial viability of application on a manufacturing scale. Moreover, on account of the homogeneous character, reagents and amino acid derivatives may be applied in low molar excess. Deletion sequences are avoided in the DioRaSSP protocol, since all reactions can be closely monitored.

Due to its homogeneous character, the DioRaSSP method is perfectly suitable for combinatorial synthesis approaches. Reaction mixtures may be split during the coupling and quenching step and subsequently combined for the remaining steps of a cycle, to arrive at an equimolar mixture of peptides.

Comparison of DioRaSSP with Other Repetitive Methods in Solution

Several repetitive methods for peptide synthesis in solution have been reported in the literature, none of which, however, have found widespread application in contemporary peptide manufacturing [22–33]. One of the most recent examples is the method developed by Carpino and co-workers, which employs the 1,1-dioxobenzo[*b*]thiophene-2-ylmethyloxy-carbonyl (Bsmoc) as the amino-protecting function [34]. In a single step of this protocol, the Bsmoc function is cleaved and the residual activated carboxylic compound is quenched by the use of a primary amine.

A comparison of DioRaSSP with the reported methods for repetitive peptide synthesis in solution clearly manifests the following combined benefits of DioRaSSP with respect to the benefits of the individual alternative methods:

1. DioRaSSP is essentially independent of the applied protecting scheme, allowing the application of relatively cheap, commercially available amino acid derivatives, and offering maximum flexibility with the applied (mild) chemistry, depending on the sequence of the actual peptide.
2. Neither pre-activated amino acid derivatives nor exotic handle molecules are applied in the DioRaSSP protocol. Moreover, whereas in some of the reported methods, solid supports are applied containing a quenching function, no solid supports whatsoever are used in the DioRaSSP protocol.
3. Ethyl acetate is generally the solvent in syntheses performed according to DioRaSSP. Concentration, drying and isolation steps, which are time-consuming and laborious on a manufacturing scale, are not part of the DioRaSSP protocol.
4. DioRaSSP assures complete inertia of residual (activated) carboxylic compound, independent of the hydrophobicity of the actual compound. The application of an anion-forming amine in the quenching step, accompanied by the appropriate work-up procedures, thus accounts for absolute prevention of the formation of insertion and/or truncated sequences.
5. The quenching step and the deprotection step are separated in the DioRaSSP protocol. Approaches based on simultaneous quenching and deprotection inadvertently presuppose that quenching is a faster process than deprotection, thus running the risk of formation of insertion sequences.
6. With DioRaSSP, the avoidance of acidic aqueous work-up following the deprotection step implicates negligible dissipation of peptide material during syntheses, resulting in high overall yields.

MATERIALS AND METHODS

General

Most amino acid derivatives were purchased from Bachem, Novabiochem or Senn Chemicals. Nsc-Phe-OH was purchased from A&PEP Inc. Msc-Phe-OH and quenching compounds H-βAla-ONse.HCl (2-(4-nitrophenyl)sulfonylethyl β-alaninate hydrochloride salt) and H-βAla-OFm.HCl (9-fluorenylmethyl β-alaninate hydrochloride salt) were prepared according to standard procedures [20,31,35].

Analytical RP-HPLCs were performed on a Waters HPLC System using a Vydac C18 column (250 × 4.6 mm ID; 5 μm particle size). The peptides were eluted applying a linear gradient of 2% to 75% acetonitrile in 0.1% trifluoroacetic acid in 48 min. The flow rate was 2.0 ml/min and peptide peaks were detected photometrically at 220 nm.

Molecular masses were determined by electrospray MS on a Perkin-Elmer Sciex API 165. The ^{1}H NMR spectrum was recorded on a Bruker DPX400 using deuterochloroform as the solvent and tetramethylsilane as the internal standard.

The general protocol of a synthesis is described for the first cycle. For subsequent cycles of the synthesis, merely adaptations with respect to the protocol of the first cycle are mentioned, unless stated otherwise. The detection of quenched compounds during in-process analyses following quenching demonstrates the validity of the quenching procedure of the DioRaSSP process.

Boc-Gly-Phe-Phe-Tyr(Bu^t)-Thr(Bu^t)-Pro-Lys(Boc)-Thr(Bu^t)-OBu^t According to Z-DioRaSSP

1st Cycle. To a stirred solution of H-Thr(Bu^t)-OBu^t (5.00 g, 21.6 mmol) in 128 ml ethyl acetate at 20 °C, were added 1-hydroxybenzotriazole (HOBt, 3.50 g, 25.9 mmol), Z-Lys(Boc)-OH (9.87 g, 25.9 mmol) and 1-(3′-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 4.56 g, 23.8 mmol). After stirring the resulting solution until completion of the reaction, 4-methylmorpholine (NMM, 1.31 ml, 11.9 mmol) and H-βAla-OBzl *p*-tosylate salt (3.80 g, 10.8 mmol) were added. The mixture was stirred for another 30 min and then extracted with 10% Na_2CO_3, 10% $KHSO_4$ and 10% Na_2CO_3.

The organic layer containing the protected dipeptide Z-Lys(Boc)-Thr(Bu^t)-OBu^t was subjected to catalytic hydrogenolysis using HCO_2H (2.77 ml, 73.5 mmol) and NMM (8.89 ml, 80.8 mmol) in the presence of 10% Pd/C (1.28 g) and water (6.75 ml). Upon completion of the reaction, 10% Na_2CO_3 was added and the resulting suspension was filtered. The residue was washed with ethyl acetate, and the combined organic filtrates were extracted with 10% Na_2CO_3 and 30% NaCl.

2nd Cycle. Z-Pro-OH (6.46 g, 25.9 mmol) was used during coupling, 3-dimethylamino-1-propylamine (DMAPA, 1.36 ml, 10.8 mmol) for quenching, and HCO_2H (1.95 ml, 51.9 mmol), NMM (6.28 ml, 57.1 m mol), 10% Pd/C (1.49 g) and water (8.3 ml) during hydrogenolysis. The basic washings following hydrogenolysis were performed at 35 °C.

3rd Cycle. Z-Thr(Bu^t)-OH (previously liberated from the dicyclohexylamine salt by standard procedures, 12.72 g, 25.9 mmol) was used during coupling, and 10% Pd/C (1.83 g) and water (9.8 ml) during hydrogenolysis.

4th Cycle. Z-Tyr(Bu^t)-OH (previously liberated from the dicyclohexylamine salt by standard procedures, 14.33 g, 25.9 mmol) was used during coupling, and 10% Pd/C (2.31 g) and water (11.3 ml) during hydrogenolysis.

5th Cycle. Z-Phe-OH (7.76 g, 25.9 mmol) was used during coupling, and 10% Pd/C (2.62 g) and water (12.8 ml) during hydrogenolysis.

6th Cycle. Z-Phe-OH (7.76 g, 25.9 mmol) was used during coupling, and 10% Pd/C (2.94 g) and water (14.3 ml) during hydrogenolysis.

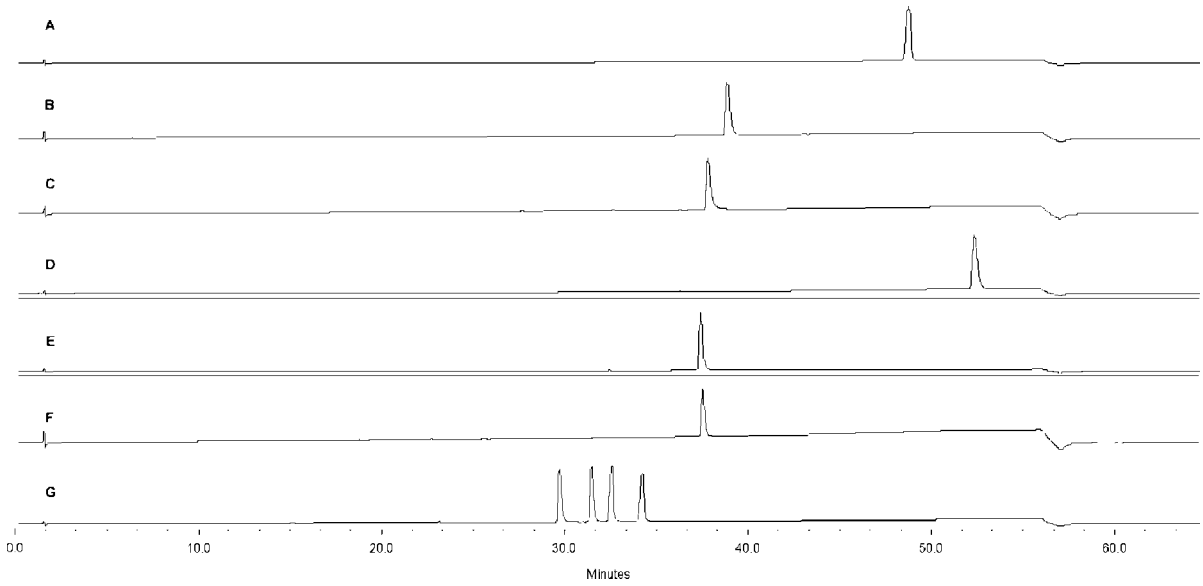

Figure 2 Analytical RP-HPLC chromatograms of (A) Boc-Gly-Phe-Phe-Tyr(Bu^t)-Thr(Bu^t)-Pro-Lys(Boc)-Thr(Bu^t)-OBu^t according to Z-DioRaSSP; (B) Boc-Gly-Phe-Asp(OBu^t)-Ser(Bu^t)-OBu^t according to Z-DioRaSSP using Z-Lys-OBzl as quenching compound; (C) Boc-Gly-Phe-Leu-Ser(Bu^t)-OBu^t according to Fmoc-DioRaSSP; (D) Boc-Ile-Phe-Cys(Trt)-Pro-Phe-Leu-OBu^t according to combi-DioRaSSP (Z/Fmoc); (E) Boc-Gly-Phe-Phe-Leu-OBu^t according to Msc-DioRaSSP; (F) Boc-Gly-Phe-Phe-Leu-OBu^t according to Nsc-DioRaSSP; (G) an equimolar mixture of Boc-Gly-Ala-Ile-Phe-OBu^t, Boc-Gly-Val-Ile-Phe-OBu^t, Boc-Gly-Ala-Val-Phe-OBu^t and Boc-Gly-Val-Val-Phe-OBu^t according to combinatorial Z-DioRaSSP.

7th Cycle. To the organic layer containing the heptapeptide H-Phe-Phe-Tyr(Bu^t)-Thr(Bu^t)-Pro-Lys(Boc)-Thr(Bu^t)-OBu^t at 20 °C, were added HOBt (3.50 g, 25.9 mmol), Boc-Gly-OH (4.54 g, 25.9 mmol) and EDC (4.56 g, 23.8 mmol). After stirring the resulting solution until completion of the reaction, DMAPA (1.36 ml, 10.8 mmol) was added. The mixture was stirred for another 30 min and was extracted with 10% Na_2CO_3, 10% $KHSO_4$, 10% Na_2CO_3, 30% NaCl and water. The organic layer was evaporated to dryness and dried to give the desired protected octapeptide in 83% yield based on the starting material H-Thr(Bu^t)-OBu^t, corresponding to an average yield of 98.6% per chemical conversion.

Purity: 97.3% by RP-HPLC (Figure 2A). Identity: m/z 1385.2 $[M + H]^+$ by electrospray MS.

Boc-Gly-Phe-Asp(OBu^t)-Ser(Bu^t)-OBu^t According to Z-DioRaSSP using Z-Lys-OBzl as Quenching Compound

1st Cycle. To a stirred solution of H-Ser(Bu^t)-OBu^t (3.26 g, 15.0 mmol) in 79 ml ethyl acetate at 20 °C, were added HOBt (2.43 g, 18.0 mmol), Z-Asp(OBu^t)-OH (5.82 g, 18.0 mmol) and EDC (3.16 g, 16.5 mmol). After stirring the resulting solution until completion of the reaction, NMM (0.91 ml, 8.3 mmol) and Z-Lys-OBzl benzenesulfonate salt (3.96 g, 7.5 mmol) were added. The mixture was stirred for another 30 min and then extracted with 5% Na_2CO_3/10% NaCl, 5% $KHSO_4$/10% NaCl and 5% Na_2CO_3/10% NaCl.

The organic layer containing the protected dipeptide Z-Asp(OBu^t)-Ser(Bu^t)-OBu^t was subjected to catalytic hydrogenolysis using HCO_2H (2.49 ml, 66.0 mmol) and NMM (7.98 ml, 72.6 mmol) in the presence of 10% Pd/C (0.79 g) and water (4.2 ml). Upon completion of the reaction, 5% Na_2CO_3/10% NaCl was added and the resulting suspension was filtered. The residue was washed with ethyl acetate, and the combined organic filtrates were extracted with 5% Na_2CO_3/10% NaCl and 30% NaCl.

2nd Cycle. Z-Phe-OH (5.39 g, 18.0 mmol) was used during coupling, and 10% Pd/C (1.01 g) and water (5.3 ml) during hydrogenolysis.

3rd Cycle. To the organic layer containing the tripeptide H-Phe-Asp(OBu^t)-Ser(Bu^t)-OBu^t at 20 °C, were added HOBt (2.43 g, 18.0 mmol), Boc-Gly-OH (3.15 g, 18.0 mmol) and EDC (3.16 g, 16.5 mmol). After stirring the resulting solution until completion of the reaction, DMAPA (0.95 ml, 7.5 mmol) was added. The mixture was stirred for another 30 min and then extracted with 5% Na_2CO_3/10% NaCl, 5% $KHSO_4$/10% NaCl, 5% Na_2CO_3/10% NaCl, 30% NaCl and water. The organic layer was evaporated to dryness and dried to give the desired protected tetrapeptide in 93% yield based on the starting material H-Ser(Bu^t)-OBu^t, corresponding to an average yield of 98.6% per chemical conversion.

Purity: 97.3% by RP-HPLC (Figure 2B). Identity: m/z 693.4 $[M + H]^+$, 715.4 $[M + Na]^+$, 691.6 $[M - H]^-$, 727.6 $[M + Cl]^-$ by electrospray MS.

The synthesis was repeated using H-βAla-OBzl p-tosylate salt as the quencher in the first two cycles, yielding similar results.

Boc-Gly-Phe-Leu-Ser(Bu^t)-OBu^t according to Fmoc-DioRaSSP

1st Cycle. To a stirred solution of H-Ser(Bu^t)-OBu^t (5.43 g, 25.0 mmol) in 140 ml ethyl acetate at 20 °C, were added HOBt (4.50 g, 30.0 mmol), Fmoc-Leu-OH (10.60 g,

30.0 mmol) and EDC (5.27 g, 27.5 mmol). After stirring the resulting solution until completion of the reaction, NMM (1.51 ml, 13.8 mmol) and H-βAla-OFm.HCl (3.80 g, 12.5 mmol) were added. The mixture was stirred for another 30 min. Upon extraction with 10% Na_2CO_3, 10% $KHSO_4$ was added and the resulting suspension was filtered. The residue was washed with ethyl acetate, and the combined organic filtrates were extracted with 10% $KHSO_4$ and 10% Na_2CO_3.

The organic layer containing the protected dipeptide Fmoc-Leu-Ser(Bu^t)-OBu^t was subjected to basic deprotection using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 7.61 ml, 50.0 mmol). Upon completion of the reaction, the mixture was extracted with 5% Na_2CO_3/10% NaCl and 30% NaCl.

2nd Cycle. Fmoc-Phe-OH (11.61 g, 30.0 mmol) was used during coupling.

3rd Cycle. To the organic layer containing the tripeptide H-Phe-Leu-Ser(Bu^t)-OBu^t at 20 °C, were added HOBt (4.50 g, 30.0 mmol), Boc-Gly-OH (5.26 g, 30.0 mmol) and EDC (5.27 g, 27.5 mmol). After stirring the resulting solution until completion of the reaction, DMAPA (1.56 ml, 12.5 mmol) was added. The mixture was stirred for another 30 min and then extracted with 10% Na_2CO_3, 10% $KHSO_4$, 10% Na_2CO_3, 30% NaCl and water. The organic layer was concentrated *in vacuo*. The product crystallized at 0 °C, and was subsequently filtered, washed with cold ethyl acetate and dried. The protected tetrapeptide was obtained in 86% yield based on the starting material H-Ser(Bu^t)-OBu^t, corresponding to an average yield of 97.0% per chemical conversion.

Purity: 95.3% by RP-HPLC (Figure 2C). Identity: *m/z* 635.4 $[M+H]^+$, 633.4 $[M-H]^-$, 679.4 $[M+HCOO]^-$ by electrospray MS.

Boc-Ile-Phe-Cys(Trt)-Pro-Phe-Leu-OBu^t According to combi-DioRaSSP (Z/Fmoc)

1st Cycle. To a stirred suspension of H-Leu-OBu^t.HCl (3.36 g, 15.0 mmol) in 70 ml ethyl acetate at 20 °C, were added NMM (1.65 ml, 15.0 mmol), HOBt (2.43 g, 18.0 mmol), Z-Phe-OH (5.39 g, 18.0 mmol) and EDC (3.16 g, 16.5 mmol). After stirring the resulting solution until completion of the reaction, NMM (0.91 ml, 8.3 mmol) and H-βAla-OBzl *p*-tosylate salt (2.64 g, 7.5 mmol) were added. The mixture was stirred for another 30 min and then extracted with 10% Na_2CO_3, 10% $KHSO_4$ and 10% Na_2CO_3.

The organic layer containing the protected dipeptide Z-Phe-Leu-OBu^t was subjected to catalytic hydrogenolysis using HCO_2H (1.92 ml, 51.0 mmol) and NMM (6.17 ml, 56.1 mmol) in the presence of 10% Pd/C (0.95 g) and water (4.0 ml). Upon completion of the reaction, 5% Na_2CO_3/10% NaCl was added and the resulting suspension was filtered. The residue was washed with ethyl acetate, and the combined organic filtrates were extracted with 5% Na_2CO_3/10% NaCl and 30% NaCl.

2nd Cycle. Z-Pro-OH (4.49 g, 18.0 mmol) was used during coupling, no NMM was added during coupling, and 10% Pd/C (1.09 g) and water (5.0 ml) were used during hydrogenolysis. The pH of the organic layer at the end of this cycle was adjusted to 6.5 through addition of 10% $KHSO_4$ to the washings with 30% NaCl.

3rd Cycle. To the organic layer containing the tripeptide H-Pro-Phe-Leu-OBu^t at 20 °C, were added HOBt (2.43 g, 18.0 mmol), Fmoc-Cys(Trt)-OH (10.05 g, 18.0 mmol) and EDC (3.16 g, 16.5 mmol). After stirring the resulting solution until completion of the reaction, NMM (0.91 ml, 8.3 mmol) and H-βAla-OFm.HCl (2.28 g, 7.5 mmol) were added. The mixture was stirred for another 30 min. Upon extraction with 10% Na_2CO_3, 10% $KHSO_4$ was added and the resulting suspension was filtered. The residue was washed with ethyl acetate, and the combined organic filtrates were extracted with 10% $KHSO_4$ and 10% Na_2CO_3.

The organic layer containing the protected dipeptide Fmoc-Cys(Trt)-Pro-Phe-Leu-OBu^t was subjected to basic deprotection using DBU (4.60 ml, 30.0 mmol). Upon completion of the reaction, the mixture was extracted with 5% Na_2CO_3/10% NaCl and 30% NaCl.

4th Cycle. Analogous to the third cycle, using Fmoc-Phe-OH (6.97 g, 18.0 mmol) during coupling.

5th Cycle. To the organic layer containing the pentapeptide H-Phe-Cys(Trt)-Pro-Phe-Leu-OBu^t at 20 °C, were added HOBt (2.43 g, 18.0 mmol), Boc-Ile-OH (4.16 g, 18.0 mmol) and EDC (3.16 g, 16.5 mmol). After stirring the resulting solution until completion of the reaction, DMAPA (0.94 ml, 7.5 mmol) was added. The mixture was stirred for another 30 min and then extracted with 10% Na_2CO_3, 10% $KHSO_4$, 10% Na_2CO_3, 30% NaCl and water. The organic layer was concentrated *in vacuo*. The product crystallized upon addition of diethyl ether at 0 °C, and was subsequently filtered, washed with cold diethyl ether and dried. The protected hexapeptide was obtained in 69% yield based on the starting material H-Leu-OBu^t.HCl, corresponding to an average yield of 96.0% per chemical conversion.

Purity: 97.6% by RP-HPLC (Figure 2D). Identity: *m/z* 1137.6 $[M+H]^+$, 1136.0 $[M-H]^-$, 1181.8 $[M+HCOO]^-$ by electrospray MS.

Boc-Gly-Phe-Phe-Leu-OBu^t According to Msc-DioRaSSP

1st Cycle. To a stirred solution of H-Leu-OBu^t.HCl (1.79 g, 8.0 mmol) in 38 ml ethyl acetate at 20 °C, were added NMM (0.88 ml, 8.0 mmol), HOBt (1.30 g, 9.6 mmol), Msc-Phe-OH (3.02 g, 9.6 mmol) and EDC (1.69 g, 8.8 mmol). After stirring the resulting solution until completion of the reaction, NMM (0.44 ml, 4.0 mmol) and H-βAla-ONse.HCl (1.36 g, 4.0 mmol) were added. The mixture was stirred for another 30 min and then extracted with 10% Na_2CO_3, 10% $KHSO_4$ and 10% Na_2CO_3.

The organic layer containing the protected dipeptide Msc-Phe-Leu-OBu^t was subjected to basic deprotection at 40 °C using DBU (2.41 ml, 16.0 mmol) and morpholine (2.08 ml, 24.0 mmol). Upon completion of the reaction, the mixture was extracted with 5% Na_2CO_3/15% KNO_3 and 30% NaCl, and ethyl acetate was added.

2nd Cycle. Analogous to the first cycle.

3rd Cycle. To the organic layer containing the tripeptide H-Phe-Phe-Leu-OBu^t at 20 °C, were added HOBt (1.30 g,

9.6 mmol), Boc-Gly-OH (1.68 g, 9.6 mmol) and EDC (1.69 g, 8.8 mmol). After stirring the resulting solution until completion of the reaction, DMAPA (0.51 ml, 4.0 mmol) was added. The mixture was stirred for another 30 min and was extracted with 10% Na_2CO_3, 10% $KHSO_4$, 10% Na_2CO_3, 30% NaCl and water. The organic layer was concentrated *in vacuo* and the residue dissolved in methanol. The product crystallized upon addition to water, and was subsequently filtered, washed with water and dried. The protected tetrapeptide was obtained in 84% yield based on the starting material H-Leu-OBu^t.HCl, corresponding to an average yield of 96.6% per chemical conversion.

Purity: 96.1% by RP-HPLC (Figure 2E). Identity: *m/z* 639.4 $[M+H]^+$, 661.4 $[M+Na]^+$, 637.4 $[M-H]^-$, 683.4 $[M+HCOO]^-$ by electrospray MS.

Boc-Gly-Phe-Phe-Leu-OBu^t According to Nsc-DioRaSSP

1st Cycle. To a stirred solution of H-Leu-OBu^t.HCl (1.79 g, 8.0 mmol) in 47 ml ethyl acetate at 20 °C, were added NMM (0.88 ml, 8.0 mmol), HOBt (1.30 g, 9.6 mmol), Nsc-Phe-OH (4.06 g, 9.6 mmol) and EDC (1.69 g, 8.8 mmol). After stirring the resulting solution until completion of the reaction, NMM (0.44 ml, 4.0 mmol) and H-βAla-ONse.HCl (1.36 g, 4.0 mmol) were added. The mixture was stirred for another 30 min. Then 10% $NaHCO_3$ was added and the resulting suspension was filtered. The residue was washed with ethyl acetate, and the combined organic filtrates were extracted with 10% $NaHCO_3$, 10% $KHSO_4$ and 10% $NaHCO_3$.

The organic layer containing the protected dipeptide Nsc-Phe-Leu-OBu^t was subjected to basic deprotection using DBU (2.41 ml, 16.0 mmol) and morpholine (2.08 ml, 24.0 mmol). Upon completion of the reaction, 10% $NaHCO_3$ was added and the resulting suspension was filtered. The residue was washed with ethyl acetate, and the combined organic filtrates were extracted with 10% $NaHCO_3$ and 30% NaCl.

2nd Cycle. Analogous to the first cycle. During the coupling 20 v/v% NMP was added. Following quenching, 30% NaCl was added and the resulting suspension was filtered. The residue was washed with ethyl acetate, and the combined organic filtrates were extracted with 10% $NaHCO_3$, 10% $KHSO_4$ and 10% $NaHCO_3$ in the presence of each 5 v/v% NMP.

3rd Cycle. To the organic layer containing the tripeptide H-Phe-Phe-Leu-OBu^t at 20 °C, were added HOBt (1.30 g, 9.6 mmol), Boc-Gly-OH (1.68 g, 9.6 mmol) and EDC (1.69 g, 8.8 mmol). After stirring the resulting solution until completion of the reaction, DMAPA (0.51 ml, 4.0 mmol) was added. The mixture was stirred for another 30 min. Then 10% $NaHCO_3$ was added and the resulting suspension was filtered. The residue was washed with ethyl acetate, and the combined organic filtrates were extracted with 10% $NaHCO_3$, 10% $KHSO_4$, 10% $NaHCO_3$, 30% NaCl and water. The organic layer was concentrated *in vacuo* and dried. The protected tetrapeptide was obtained in 78% yield based on the starting material H-Leu-OBu^t.HCl, corresponding to an average yield of 95.2% per chemical conversion.

Purity: 97.2% by RP-HPLC (Figure 2F). Identity: *m/z* 639.4 $[M+H]^+$, 661.4 $[M+Na]^+$, 637.4 $[M-H]^-$, 683.4 $[M+HCOO]^-$ by electrospray MS.

Equimolar Mixture of Boc-Gly-Ala-Ile-Phe-OBu^t, Boc-Gly-Val-Ile-Phe-OBu^t, Boc-Gly-Ala-Val-Phe-OBu^t and Boc-Gly-Val-Val-Phe-OBu^t According to Combinatorial Z-DioRaSSP

1st Cycle. To a stirred suspension of H-Phe-OBu^t.HCl (2.58 g, 10.0 mmol) in 50 ml ethyl acetate at 20 °C, were added NMM (1.10 ml, 10.0 mmol), HOBt (1.62 g, 12.0 mmol), Z-Ile-OH (3.18 g, 12.0 mmol) and EDC (2.10 g, 11.0 mmol). After stirring the resulting solution until completion of the reaction, NMM (0.61 ml, 5.5 mmol) and H-βAla-OBzl *p*-tosylate salt (1.76 g, 5.0 mmol) were added. The mixture was stirred for another 30 min. The same reaction was performed in parallel using Z-Val-OH (3.02 g, 12.0 mmol) instead of Z-Ile-OH. The two reaction mixtures were combined upon addition of 5% Na_2CO_3/10% NaCl, followed by extraction with 5% Na_2CO_3/10% NaCl, 5% $KHSO_4$/10% NaCl and 5% Na_2CO_3/10% NaCl.

The organic layer containing the protected dipeptides Z-Ile-Phe-OBu^t and Z-Val-Phe-OBu^t was subjected to catalytic hydrogenolysis using HCO_2H (2.57 ml, 68.0 mmol) and NMM (8.22 ml, 74.8 mmol) in the presence of 10% Pd/C (0.92 g) and water (5.3 ml). Upon completion of the reaction, 5% Na_2CO_3/10% NaCl was added and the resulting suspension was filtered. The residue was washed with ethyl acetate, and the combined organic filtrates were extracted with 5% Na_2CO_3/10% NaCl and 30% NaCl. The organic layer was then split into two equal portions.

2nd Cycle. Z-Ala-OH (2.68 g, 12.0 mmol) and Z-Val-OH (3.02 g, 12.0 mmol) were used during coupling, no NMM was added during coupling, and 10% Pd/C (1.09 g) and water (6.3 ml) were used during hydrogenolysis. The organic layer was not split at the end of the cycle.

3rd Cycle. To the organic layer containing the four tripeptides H-Ala-Ile-Phe-OBu^t, H-Val-Ile-Phe-OBu^t, H-Ala-Val-Phe-OBu^t and H-Val-Val-Phe-OBu^t at 20 °C, were added HOBt (3.24 g, 24.0 mmol), Boc-Gly-OH (4.20 g, 24.0 mmol) and EDC (4.22 g, 22.0 mmol). After stirring the resulting solution until completion of the reaction, DMAPA (1.25 ml, 10.0 mmol) was added. The mixture was stirred for another 30 min and then extracted with 5% Na_2CO_3/10% NaCl, 5% $KHSO_4$/10% NaCl, 5% Na_2CO_3/10% NaCl, 30% NaCl and water. The organic layer was evaporated to dryness and dried to give the desired equimolar mixture of protected tetrapeptides in 89% yield based on the starting material H-Phe-OBu^t.HCl, corresponding to an average yield of 98.3% per chemical conversion.

Purity: 22.3%, 23.3%, 26.0% and 26.8% by RP-HPLC (Figure 2H). Identity: *m/z* 593.6, 607.4, 621.4, 635.4 $[M+HCOO]^-$ by electrospray MS; ^{1}H NMR ($CDCl_3$) d 0.80–0.96 (m, 36H), 1.02–1.16 (m, 2H), 1.33 (dd, 6H), 1.38 (s, 36H), 1.42–1.53 (m, 2H), 1.46 (s, 36H), 1.74–1.87 (m, 2H), 1.99–2.32 (m, 4H), 2.94–3.14 (dm, 8H), 3.71–3.94 (m, 8H), 4.36–4.46 (m, 6H), 4.56–4.67 (m, 2H), 4.74–4.83 (m, 4H), 5.40–5.52 (m, 4H), 6.80 (dd, 4H), 7.02–7.48 (m, 28H). The equimolarity of the four peptides in the mixture is corroborated by the proton ratios as detected by ^{1}H NMR, e.g. $H^{\alpha}{}_{Gly}$ (d 3.71–3.94) : $H^{\beta}{}_{Ala}$ (d 1.33) : $H^{\beta}{}_{Ile}$ (d 1.02–1.16) : $H^{\beta}{}_{Val}$ (d 1.99–2.32) : $H^{\beta}{}_{Phe}$ (d 2.94–3.14) = 8 : 6 : 2 : 4 : 8.

RESULTS AND DISCUSSION

Syntheses performed according to DioRaSSP proceed by a general and fast protocol. In the past four years, a considerable number of protected peptides have thus been synthesized, varying from tripeptides to a dodecapeptide. Purities and yields are generally high, as exemplified by the described syntheses. Average yields of more than 96% per chemical conversion are typically obtained in fast first-trial syntheses. Several DioRaSSP processes have been directly scaled up after a preliminary feasibility study at the laboratory scale, achieving reproducible results in terms of both yield and purity. Peptides manufactured according to the DioRaSSP method include Buserelin, Deslorelin, Goserelin, Histrelin, Leuprolide, Octreotide and Triptorelin. The application of DioRaSSP implies the same process and impurity profile throughout all stages of development, that is, from the first laboratory sample to production batches, combined with intrinsically short process times. The size of the available reaction vessels should prove the only limiting factor in the scale-up towards multi-100-kg batches.

Issues addressed to optimize and extend the scope of the DioRaSSP concept comprise the development of alternative protecting schemes, the preparation of fragments for convergent synthesis schemes, prevention of diketopiperazine formation, assessment of the extent of racemization of carboxylic compounds during coupling and of *C*-terminal esters during basic deprotections, assessment of the extractability of amino acid derivatives, the applicability of residues with unprotected functional side-chains, and impediment of aspartimide formation during basic deprotections. The results of these investigations have been published or will appear in forthcoming publications and/or patents [36].

Moreover, we have recently implemented the first fully automated solution-phase peptide synthesizer, which increases the potential of the DioRaSSP method even further. The DioRaSSP Synthesizer is equipped to perform reagent additions, phase separations, hydrogenolysis and sampling (for HPLC-analysis at every stage of a synthesis) in an automated mode and according to general protocols, 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, investigation of critical process parameters and for kinetic studies.

CONCLUSION

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

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