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An Optimized Scalable Fully Automated Solid-Phase Microwave-Assisted cGMP-Ready Process for the Preparation of Eptifibatide

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ABSTRACT: We investigated several strategies, based on the use of microwave-assisted solid-phase peptide synthesis (MW-SPPS) and scalable to kilogram-scale manufacturing, for the preparation of Eptifibatide, a disulfide-bridged cyclo-heptapeptide drug approved as an antithrombotic agent. Following the very fast microwave-assisted Fmoc/tBu synthesis of the linear precursor, we explored both the solution (off-resin) and the solid-phase (on-resin) disulfide formation. In order to optimize the oxidation in solution, we focused our attention on the mild disulfide formation procedure based on the use of air, observing some drawbacks, such as the formation of unwanted oxidation byproducts, such as dimers, or the use of large volumes of an environmentally unfriendly solvent (CH_3CN). In order to overcome these difficulties, we studied four different on-resin strategies, with the final aim to develop a fully automated, single reactor procedure, exploring different strategies to protect the thiol side-chain functional group on the C-terminal Cys residue and to form the Eptifibatide ring. The main difference among these strategies is represented by the final cyclization mode that was obtained either by direct formation of an S−S disulfide bridge or by head to MPA on cysteine side-chain amide bond formation. In conclusion, the optimization of the latter strategy enabled us to devise an optimized scalable fully automated solid-phase microwave-assisted cGMP-ready process to prepare Eptifibatide.

KEYWORDS: *active pharmaceutical ingredient, peptide, scale-up, oxidation, automated on-resin disulfide bond formation, off-resin disulfide bond formation, single reactor solid-phase synthesis*

INTRODUCTION

The need of new, specific, safe and well-tolerated medicines led several pharmaceutical companies to move toward peptide-based drugs.[1,2] In the past few years the U.S. Food and Drug Administration (FDA) approved 15 new peptides or peptide-containing drugs (7% of the total number of drugs).[3] Moreover, since many patents of peptide drugs are expiring, a large number of generic peptide drugs are also entering the market or are expected to do so in the near future. Accordingly, the few Contract Manufacturing Organizations (CMOs) specialized in production of peptides as Active Pharmaceutical Ingredients (APIs) constantly strive to meet an increasing need for large quantities of different new peptide APIs in compliance with current Good Manufacturing Practice (cGMP).[4]

Of the approximately 60 approved peptide APIs available on the market, roughly half are manufactured by 9-fluorenylmethyloxycarbonyl/*tert*-butyl (Fmoc/tBu) solid-phase peptide synthesis (SPPS),[5] although, generally speaking, solid-phase strategies are commonly used to prepare small volumes of short peptides, whereas hybrid technologies (combining solid-phase and solution methods) are applied to produce longer peptide sequences and/or higher volumes.[6]

The large-scale manufacturing of peptides as APIs, requiring full compliance with cGMP regulations,[7] leads pharmaceutical companies to face many challenges related to the complexity of peptide synthesis in terms of size, modifications, conjugation methods, stability, and purity. This requires on one hand the development of robust manufacturing processes and on the other hand keeping production costs at a reasonable level.

An additional critical factor is the use of large quantities of nonaqueous hazardous organic solvents, such as dimethylformamide (DMF) and *N*-methyl-2-pyrrolidone (NMP), restricted under REACH (European Regulation on Registration, Evaluation, Authorization, and Restriction of Chemicals). For this reason, there is a growing research interest in Green Solid-Phase Peptide Synthesis (GSPPS), looking for an alternative to DMF, commonly employed for washings, couplings, and Fmoc-removal steps;[8−10] still, SPPS in water is a very demanding procedure.[11]

If the synthetic target is a cyclopeptide, further difficulties arise. Cyclization is typically used as a method to constrain structures and stabilize the putative bioactive peptide folding, thus enhancing selectivity toward the target receptor. Last but not least, cyclic peptides are less degraded by proteases, and therefore, they display an in vivo higher stability. Indeed on average, one new cyclopeptide drug is approved each year.[12]

Among the different and sometimes very unusual cyclization strategies available in the tool-box of the peptide medicinal

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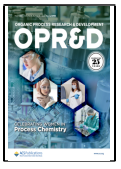

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chemist, the first to be mentioned is the formation of disulfide bridge(s) by oxidation of suitably located cysteines and/or thiol-containing unnatural amino acid residues. Recently, an extensive review reported the most commonly used oxidation methods to form disulfide bridges by oxidation mediated by I_2, Tl(III) trifluoroacetate, potassium ferricyanide, dimethyl sulfoxide (DMSO), both in acidic and basic conditions and/or simply in air in basic conditions or in the presence of activated charcoal.[13−19] However, not all of these methods can be applied to the kilogram-scale production. In developing a robust, large-scale cGMPs-compliant chemical process, it is crucial to take into account and due consideration critical factors such as reactants toxicity, raw materials costs, and optimization of the oxidative conditions (concentration, pH, etc.).[20]

This paper reports a scalable cGMP-ready multigram synthesis of the disulfide-bridge containing cyclopeptide Eptifibatide as API, marketed in the United States under the trademark INTEGRILIN (Figure 1). This drug is used as an antithrombotic agent to treat patients with acute coronary syndrome such as unstable angina and/or acute myocardial infarction.

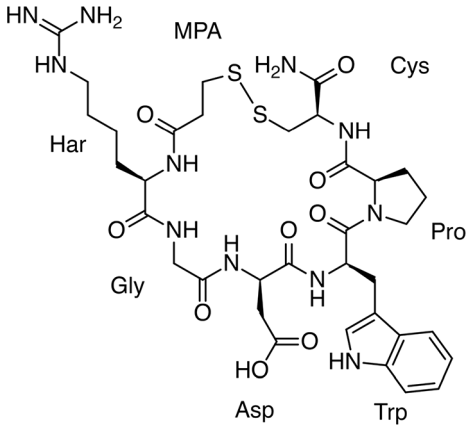

Figure 1. Eptifibatide: N^6-(aminoiminomethyl)-N^2-(3-mercapto-1-oxopropyl)-L-lysylglycyl-L-α-aspartyl-L-tryptophyl-L-prolyl-L-cysteinamide, cyclic (1 → 6)-disulfide.

This synthetic heterodetic cyclo-heptapeptide is characterized by the presence of the unnatural amino acid homoarginine (Har) and a single disulfide bridge between the C-terminal cysteinamide and the N-terminal deamino-cysteine residue, 3-mercaptopropionic acid (MPA). Eptifibatide is currently prepared on a large scale applying a hybrid strategy, based on SPPS combined with in-solution strategies.[21−24] All these procedures are generally designed to prevent the occurrence during the process of major side-reactions, such as racemization, deletion(s), Har deguanidylation, and dimer sequences formation. In particular, the latter undermines formation of the disulfide bridge, which is often the crucial and final step of the synthesis.

The aim of the present paper is to present an optimized and fast protocol for the synthesis of Eptifibatide at a satisfactory purity degree, suitable for an effective process transfer to kilogram-scale manufacturing in the pharmaceutical industry. In particular, we focused on production needs, investigating different Fmoc/tBu automated microwave-assisted solid-phase peptide synthesis (MW-SPPS) protocols, using the high-speed and high-efficiency CEM technology (Charlotte, NC, U.S.A.), widely used for R&D, available for both gram-scale (Liberty Prime and Liberty Blue systems) and more recently for kilogram-scale synthesis thanks to the newly developed Liberty PRO, especially designed for cGMP compliance. Moreover, we compared the thiol-free off-resin disulfide bond formation in Eptifibatide production process with four novel and different on-resin approaches, in which ring closure was obtained both by disulfide bond and head to MPA on cysteine side-chain by a simple amide bond formation.

RESULTS AND DISCUSSION

The possibility of developing efficient and reliable scale-up processes is becoming more and more crucial for all pharmaceutical companies. Peptide manufacturers need to improve their processes both chemically and economically, that is, in terms of profitability: such remarkable advancement can be achieved being able to optimize the solid-phase synthesis of an active peptide as pharmaceutical ingredient on a laboratory scale and then to perform the same synthesis on the larger scale, up to hundreds of times, with the same efficiency in terms of peptide quality and impurities profile. This unique goal can be reached by automated microwave-assisted solid-phase peptide synthesis (MW-SPPS), as proven by several experimental evidence (Jon Collins' personal communications from CEM, U.S.A.). Therefore, once a synthetic protocol has been optimized at the laboratory scale (up to 5 mmol), the same quality (indeed higher, usually) can be achieved directly on a much bigger scale. Therefore, intermediate-scale peptide synthesis is no longer required and the optimized synthetic strategy can be directly scaled up from grams to multigrams/kilograms. With these considerations in mind, in the present paper we focused our interest to the scale-up from R&D multigram-scale of the active peptide ingredient Eptifibatide by the microwave-assisted solid-phase synthesizer (Liberty Blue CEM, Charlotte, NC, U.S.A.), affording and solving both technical and economic issues, to transfer the technology to an industrial kilogram-scale production plant based on the recently engineered Liberty PRO (CEM, Charlotte, NC, U.S.A.), allowing fully automated, production scale, microwave synthesis with full cGMP compatibility. Our strategy differs considerably from those previously reported in the literature, including patents (Table S1) in the management of the disulfide bridge formation critical step. This step was optimized not only in the most common solution conditions (off-resin), but mainly, in the interest of API producers, on-resin, by a fully automatic protocol, directly in the microwave solid-phase synthesizer.

Five-Millimolar Scale Synthesis of Eptifibatide by Fmoc/tBu MW-SPPS of the Linear Precursor and Off-Resin Disulfide Bridge Formation. First of all we optimized the microwave protocol for a 5 mmol scale fully automated Fmoc/tBu solid-phase synthesis of the linear Eptifibatide precursor MPA(Trt)-Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(Trt)-Rink Amide AM resin (Table S2). The peptide was assembled via standard Fmoc/tBu MW-SPPS strategy (see Experimental Section), using as coupling reagents *N*,*N*′-diisopropylcarbodiimide (DIC) and Oxyma Pure, which is nonexplosive, compared with 1-hydroxybenzotriazole (HOBt), used for years for amide bond formation, but that is no more accepted at the industrial level.[25] The coupling system based on DIC/Oxyma, in addition to being safe and low cost, does not require the use of a base unlike the most popular onium coupling methods, such as 3-[bis(dimethylamino)-

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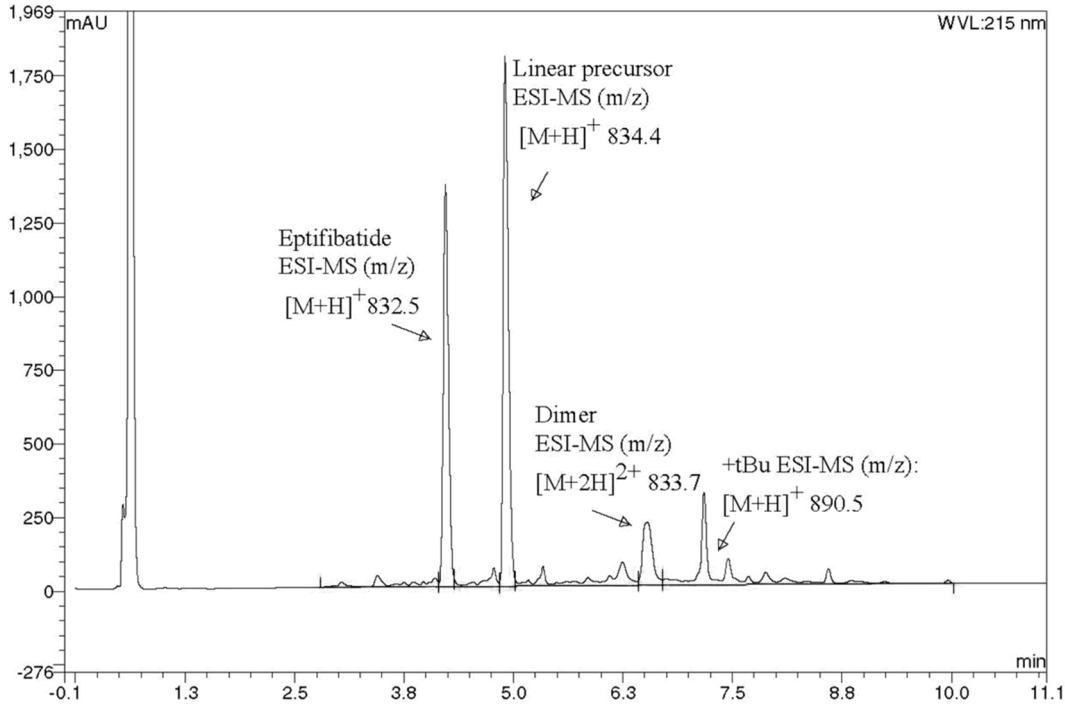

Figure 2. RP-UHPLC monitoring of Eptifibatide disulfide bridge formation by off-resin strategy (pH 8, H_2O/CH_3CN 2:1, 5.3 mM, 22 h at rt). RP-UHPLC-MS: C18 column Waters Acquity CSH (130 Å, 1.7 μm, 2.1 × 100 mm); temperature 45 °C; flow: 0.5 mL/min; eluent: 0.1% (v/v) TFA in H_2O (A) and 0.1% (v/v) TFA in CH_3CN (B), λ 215 nm, gradient: 12−45% B in 10 min.

methyliumyl]-3*H*-benzotriazol-1-oxide hexafluorophosphate (HBTU), requiring high base quantities (e.g., *N,N*-diisopropylethylamine, DIPEA). Moreover, DIC is convenient in terms of time and solvent consumption because it is stable at 90 °C (temperature reached in the microwave conditions) and requires lower DMF washing volumes because of its higher solubility. The acid-labile Trt group, protecting Cys and MPA thiol functions, was cleaved simultaneously after cleavage of the linear peptide precursor from the resin (Supporting Information). Then the disulfide bridge between MPA at position 1 and Cys at position 7 was formed in solution starting from the free thiol-containing linear precursor MPA-Har-Gly-Asp-Trp-Pro-Cys-NH_2 under very mild basic conditions in the presence of atmospheric oxygen. Air oxidation of unprotected linear peptides is accepted as the cleanest and safest strategy,[26] occurring in eco-friendly buffered aqueous solution. However, in these conditions peptides are prone to uncontrolled intermolecular disulfide arrangements, typically occurring in basic conditions. Therefore, high dilution conditions (0.1−1 mM) are generally used for selective air oxidation in 0.1 M NH_4HCO_3 aqueous solution at pH 7−8 for 24 h. However, these conditions require large solvent volumes, dramatically increasing the final industrial process costs. In our strategy, we successfully used 10 times higher concentration solutions (2.1−10.6 mM) limiting side-products formation, with clear cost−benefit advantages.[26] In particular, optimization of the air-oxidation conditions of the crude linear Eptifibatide precursor was accomplished by fine-tuning the following critical parameters: (i) buffer (NH_4HCO_3 vs NH_4OH); (ii) pH (7.5−9.5 range); (iii) concentration (2.1−10.6 mM); (iv) solvent (different ratio of H_2O/CH_3CN, considering the low solubility in water of both the linear precursor and Eptifibatide itself). Moreover, monitoring of the possible intermolecular disulfide bond side-products formation was an essential part of the study. Therefore, we used HPLC-ESI-MS to monitor the formation of Eptifibatide (cyclic oxidized form) and the concomitant consumption of the precursor (linear reduced form), in order to determine the conversion rate at different conditions, paying particular attention to the appearance of additional chromatographic peaks, attributed to dimers and oligomers formed by interchain disulfide bridge(s), which however in our 5 mmol-scale procedure was limited (Figure 2, Figures S1 and S2).

We initially tested the different conditions fixing the reaction time at 22 h, as summarized in Table 1. It is clear that simultaneously increasing the pH (from 7.5 to 9.5) and decreasing the concentration (from 10.6 to 2.1 mM) enabled us to obtain a complete conversion limiting interchain disulfide bridged side-products formation.

Table 1. Evaluation of Reaction Parameters 1; Effect of pH and Concentration at a Fixed Reaction Time of 22 h on Off-Resin Disulfide Bond Formation in Eptifibatide

entry	concentration (mM)	pH	−SH to S−S conversion (%)	dimer (%)
1	10.6	7.5	12.0	4.7
2	5.5	8.0	40.2	8.0
3	3.2	8.5	57.5	4.9
4	2.1	9.5	85.0	4.6

At pH 7.5, the oxidation reaction was slow: only 12% Eptifibatide was formed after 22 h. On the other hand, an increase of pH from 8 to 9.5 allowed obtaining 85% of crude Eptifibatide at the same reaction time. Therefore, efficiency of the oxidation reaction was tested at pH 9.5, focusing on the reaction rate and solution concentration. Data are reported in Figure 3.

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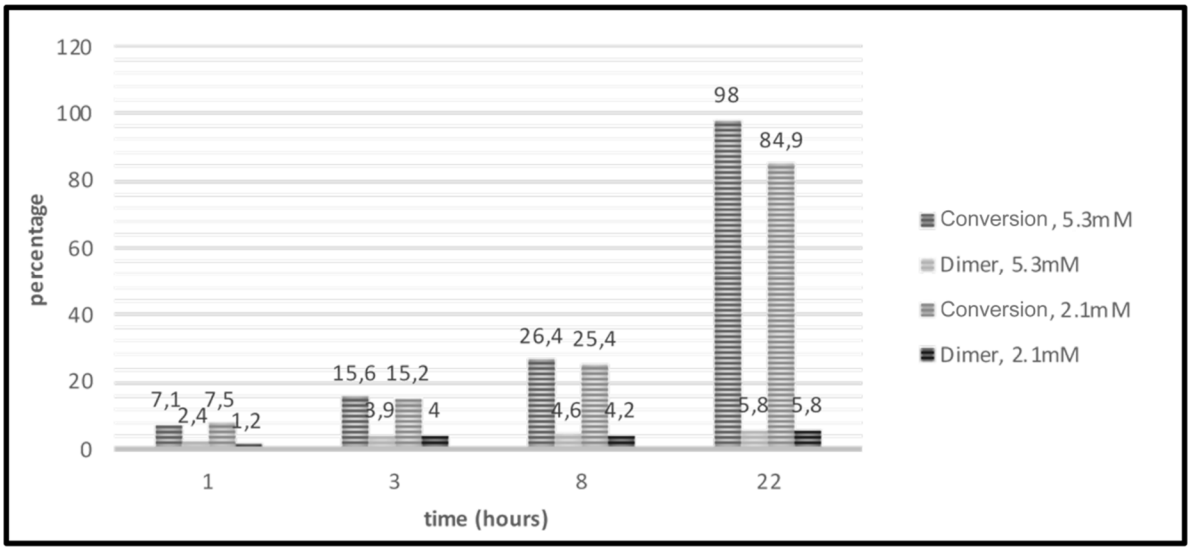

Figure 3. Evaluation of reaction parameters 2. Effect of reaction time and concentration at fixed pH 9.5 on off-resin disulfide bond formation in Eptifibatide.

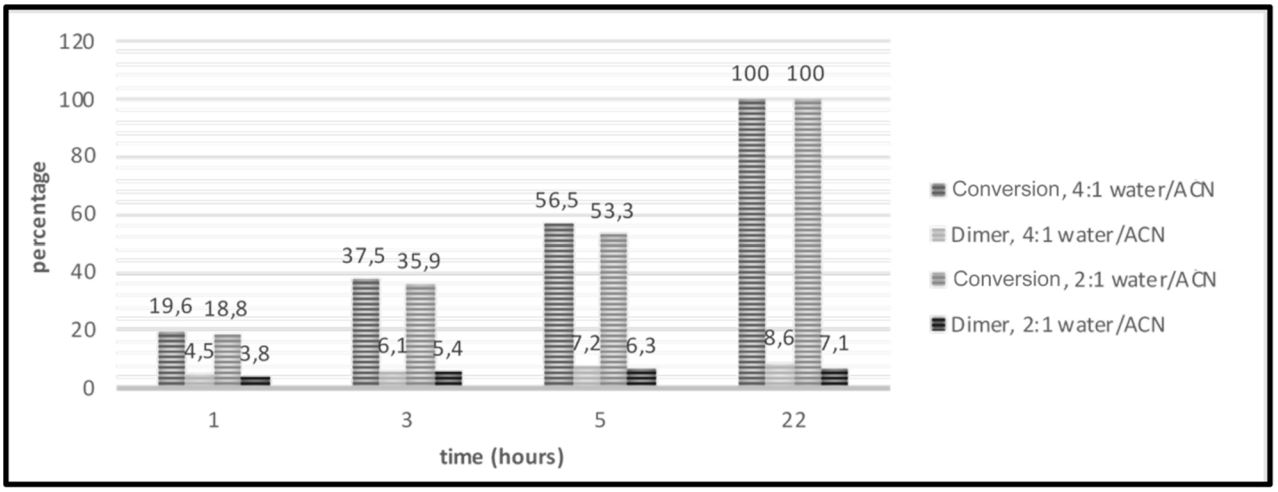

Figure 4. Evaluation of reaction parameters 3. Effect of solvent and reaction time at fixed pH 9.5 and concentration (5.3 mM) on off-resin disulfide bond formation in Eptifibatide.

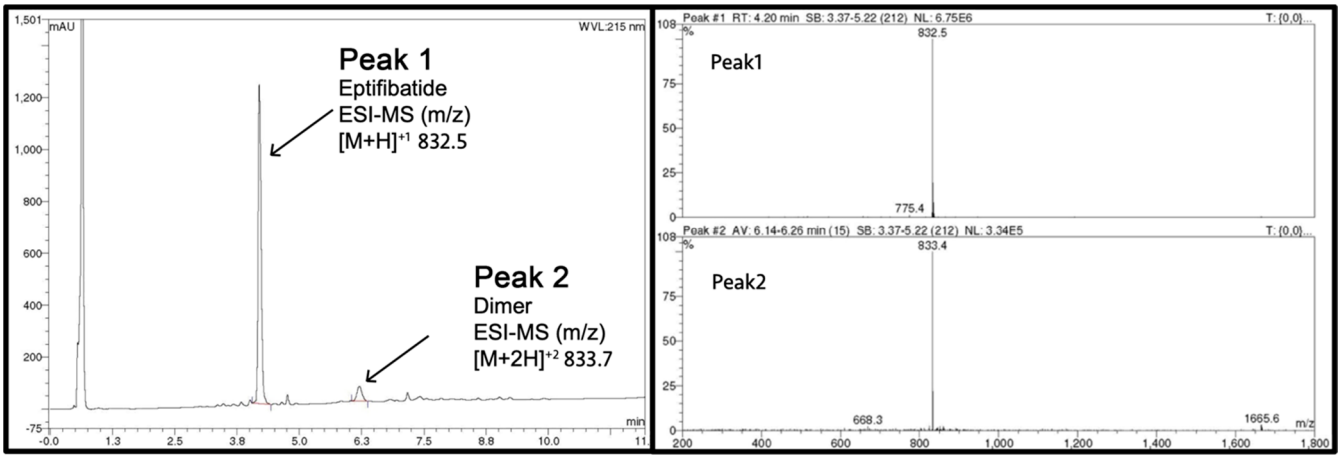

Figure 5. RP-UHPLC-ESI-MS traces of Eptifibatide crude obtained by off-resin oxidation reaction (5.3 mM Eptifibatide linear precursor in H_2O/CH_3CN, 2:1; r.t.; pH 9.5; 22h). RP-UHPLC-ESI-MS: C18 column Waters Acquity CSH (130 Å, 1.7 μm, 2.1 × 100 mm); temperature 45 °C; flow: 0.5 mL/min; eluent: 0.1% (v/v) TFA in H_2O (A) and 0.1% (v/v) TFA in CH_3CN (B), λ 215 nm, gradient: 12−45% B in 10 min. Rt 4.196 min: Eptifibatide (Peak 1) and Rt 6.200 min: Dimer (Peak 2).

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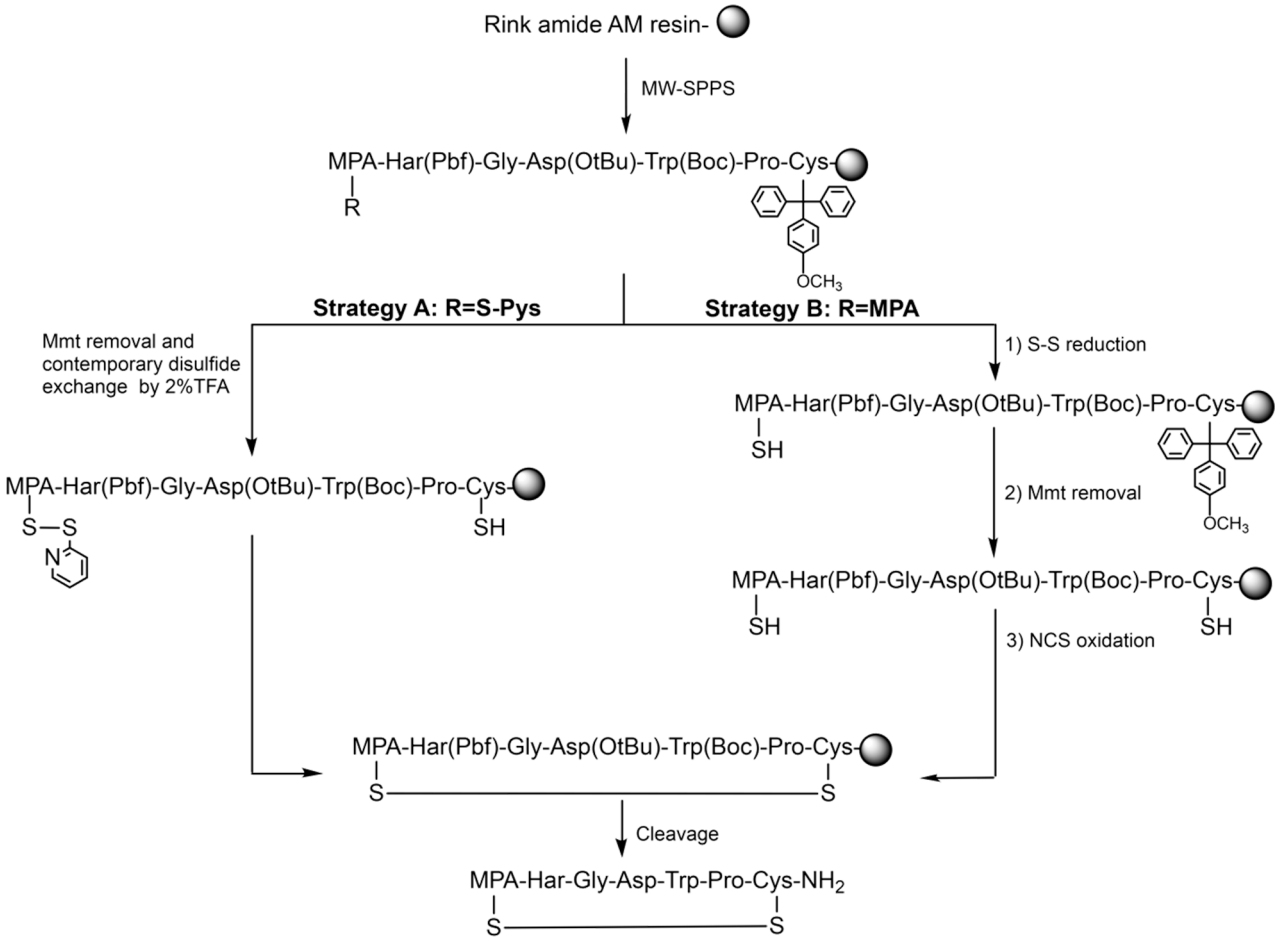
Scheme 1. Fully Automated Microwave-Assisted Synthesis of Eptifibatide Performing On-Resin Disulfide Bond Formation: Strategies A and B



At these pH conditions, maximum oxidation occurred in 22 h. Moreover, we observed that when the concentration was decreased (from 5.3 to 2.1 mM), the conversion at 22 h rate decreased without any advantage in terms of side-products formed. Generally speaking, the possibility to work at higher concentration (5.3 mM) is preferred because the volume to be handled is lower.

Moreover, no oxidation improvement was observed by changing the composition of the solvent mixture H_2O/CH_3CN from 4:1 to 2:1 (Figure 4).

In conclusion, optimal conditions to oxidize the linear Eptifibatide precursor by off-resin disulfide formation were estimated to be 5.3 mM concentration in H_2O/CH_3CN (2:1); pH 9.5; 22 h reaction time at room temperature. At these conditions, 98% conversion of the linear precursor was obtained, leading to crude Eptifibatide with an HPLC purity 61% (Figures 5 and S3a).

Selection of the less green and more expensive solvent mixture ratio H_2O/CH_3CN (2:1), compared with H_2O/CH_3CN (4:1), was forced by the lower dimer side-product formation in higher acetonitrile content mixture. In particular, in the higher water content mixture (4:1), a precipitate formation limited reaction monitoring and the putative side-product aggregate, sticking to the glass vessel, affected postsynthesis cleaning procedures. Moreover, off-resin disulfide bond formation suffers from the typical carbocation nucleophile reactivity, toward the free amino acid side chains formed during the resin cleavage, affecting the final yield because of possible irreversible impurities (Figures S1, S2, and S3a).

Flash chromatography purification of 4.6 g crude Eptifibatide was carried out using Biotage Isolera One equipped with SNAP Ultra 120 g C18 column and H_2O/CH_3CN as eluent system. Eptifibatide (1.32 g, 1.88 mmol, 28.7% yield) showed a 99.2% RP-UHPLC purity, with 0.8% total impurities, and each impurity <0.5%, in agreement with cGMP requirements (Figure S3b).

Fully Automated Microwave-Assisted Synthesis of Eptifibatide: Comparison of Four Different On-Resin Disulfide Bond Formation Strategies. In order to overcome the above-described difficulties inherent to oxidation in solution of the Eptifibatide linear precursor, we deeply explored the possibility to develop a full on-resin, possibly single reactor process. With the idea in mind of developing a completely automatic synthetic procedure to be scaled in the Liberty PRO solid-phase peptide synthesizer, we investigated four on-resin different approaches (Strategies **A**–**D**) on a Liberty Blue instrumentation without isolating linear Eptifibatide precursor (Schemes 1 and 2).

The final aim of optimizing a fully automated synthesis of Eptifibatide is to provide a solid-phase single reactor process managing three-dimensional orthogonal protections on on-resin peptide residues involved in disulfide bridge formation (MPA1 and Cys7).

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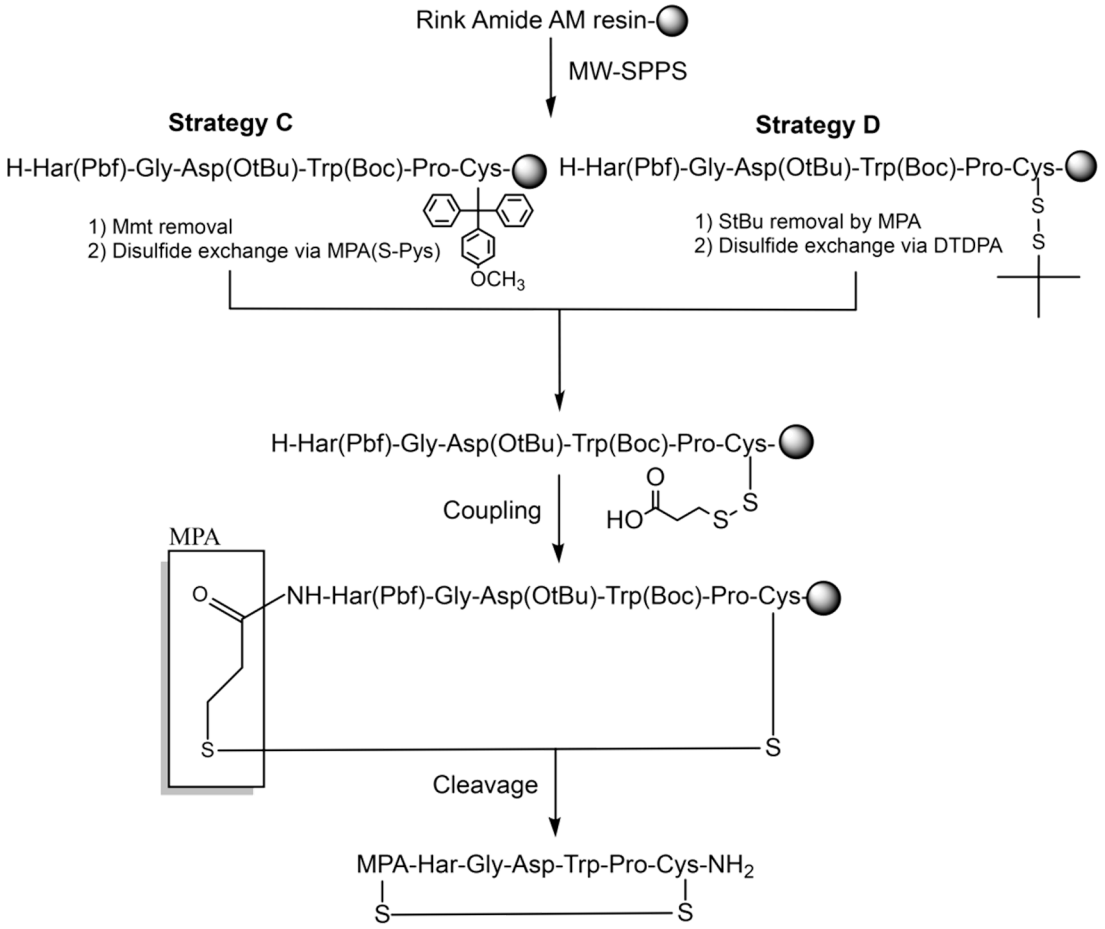
Scheme 2. Fully Automated Microwave-Assisted Synthesis of Eptifibatide Performing On-Resin Disulfide Bond Formation: Strategies C and D



Performing all steps in an automated instrument single reactor simplify the respect of cGMP compliances, cutting the financial and time costs related to multiple equipment qualification.

Moreover, a fully automated process is particularly appealing in the context of industrial production where limiting workforce and safety requirements are key challenges.

Strategies **A** and **B** (Scheme 1) require the Fmoc/tBu MW-assisted synthesis of the common peptide-resin fragment Fmoc-Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(Mmt)-Rink Amide AM Resin.

The selection of the three-dimensional 4-methoxytrityl (Mmt) protecting group on Cys7 thiol side-chain was dictated both by its easy removal in mild acidic conditions and commercial availability of Fmoc-Cys(Mmt)-OH building block. The use of the most expensive Fmoc-Cys(Mmt)-OH, compared with Fmoc-Cys(Mtt)-OH, is justified by the milder acidic conditions required for its removal without affecting peptide stability to the Rink linker resin.

The possibility to use the same Mmt protecting group on thiol function in MPA is limited by commercial unavailability of MPA(Mmt). Therefore, we tested the efficiency of the commercially available MPA(PyS) in Strategy **A** and 3,3′-dithiodipropionic acid (DTDPA) in Strategy **B**, linked respectively to the N-terminal amino function on Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(Mmt)-Rink Amide AM Resin, for further on-resin disulfide bridge formation (Scheme 1).

Strategy A. In particular in Strategy **A**, after coupling MPA(PyS) in the MW reactor to Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(Mmt)-Rink Amide AM Resin, mild acidic conditions were used to cleave the Mmt protecting group on Cys and favoring the contemporary disulfide-exchange reaction between free thiol on Cys side-chain and the pyridyne-2-thiol group (PyS) on MPA.[27−29] This reaction occurring in 25 min at r.t., led to the desired disulfide bond in crude Eptifibatide that, after resin cleavage, was recovered in 60% yield with 34.9% HPLC purity (Figure S4). The main disadvantage of Strategy **A** is the high cost of the building blocks Fmoc-Cys(Mmt)-OH and MPA(PyS).

Strategy B. However, in Strategy **B**, after MW-assisted amide bond formation between DTDPA and the N-terminal amino function on Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(Mmt)-Rink Amide AM Resin and DTT-mediated reduction of the S−S bond in DTDPA linked to the peptide-resin,[30] we took advantage of N-chlorosuccinimide (NCS) oxidative properties, leading to the disulfide bond formation by the highly reactive sulfenyl chloride formation toward thiol in Cys after Mmt removal with a rapid, clean, and efficient reaction. As previously demonstrated by Albericio et al.,[31] also in our experience, this reaction was strongly affected in terms of final crude purity, by the possible use of NCS reagent excess (data not shown). A more important drawback of Strategy **B** is the use of the harmful NCS reagent requiring management of the resin out of the microwave reactor of the synthesizer to avoid dramatic and unsafe instrument

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contaminations, requiring cleaning procedures that will increase energy and water costs, a prime concern for manufacturers. Additionally, a big advantage (compared with the previously described Strategy **A**) is the use of the harmless, inexpensive, and greener DTDPA that is essentially the MPA S−S bridged dimer, that is, $(MPA)_2$ instead of the more expensive MPA(PyS) building block.

After resin cleavage, crude Eptifibatide was recovered in 82% yield with 42% HPLC purity (Figure S5).

Strategy C. The Strategy **C** starts from the same peptide-resin fragment above-described, i.e. Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(Mmt)-Rink Amide AM Resin, used in Strategies **A** and **B**. After Mmt deprotection, S−S bond is formed on Cys7 free thiol function by MPA(PyS) mediated disulfide-exchange to obtain Cys(MPA). Then by PyBop/DIPEA activation (5:7 equiv) of the carboxylic function on Cys(MPA) at r.t. for 16 h,[32] the head to MPA on cysteine side-chain cyclization occurs by nucleophilic attack of the N-terminal Har on the peptide-resin (Scheme 2).

Optimization of Cys(MPA) formation on the resin was achieved performing the reaction directly in the MW reactor (90W, 50 °C, 30 min) as reported in Table S3, and as observed by IPC of Har-Gly-Asp-Trp-Pro-Cys(MPA)-NH_2 cleaved from the resin, using the protocol reported in the Supporting Information (Figure S6).

After resin cleavage, crude Eptifibatide was recovered in 61% yield with 56.5% HPLC purity (Figure S7). In conclusion, the peculiarity of Strategy **C** is the on-resin formation of S−S bond by disulfide-exchange between MPA(PyS) and thiol function in Mmt-cleaved cysteine. Then, the carboxylic function of MPA linked on cysteine by S−S bridge, that is, Cys(MPA), after appropriate activation could form the amide bond with the N-terminal amino function of Har(Pbf) on the peptide-resin. This strategy has the advantage that in the peptide industry, the formation of the amide bond is pivotal and among the more important transformations in the design of synthetic plants. Moreover, amide bond formation is optimized in solid-phase synthesis in particular in microwave-assisted peptide synthesizer.

Strategy D. The Strategy **D** (Scheme 2) takes advantage of the interesting step in Strategy **C** above-described (to form the disulfide bond directly on cysteine before closing the cyclopeptide by amide formation), using the less expensive building block Cys(StBu) compared with Cys(Mmt). Therefore, starting from the peptide-resin fragment Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(StBu)-Rink Amide AM resin, the first issue of the Strategy **D** is to propose a greener, safer, and low-cost reducing agent for orthogonal deprotection conditions of Cys(StBu) at position 7.

DTT was immediately excluded because of its high cost in the industrialization process. Although DTT was widely reported in the literature as an efficient reducing agent also for functional groups in peptide sequences,[33,34] as well as demonstrated in the case of Strategy **B**.

Several assays with different reagents were performed with the aim to provide a scalable fully automated solid-phase microwave-assisted cGMP-ready process for Eptifibatide (see Supporting Information).

In particular, 20% (v/v) β-mercaptoethanol (βME) in basic conditions (0.1 M NMM or 0.05 M DIPEA) in DMF[33,34] allowed removing StBu at r.t., but it required a long reaction time. In the case of the starting peptide-resin used in Strategy **D**, quantitative reduction was reached with the same reaction cocktail, in 15 min in microwave conditions (75 °C, 45 W), (see Supporting Information).[33] However, the high toxicity and difficult cleaning procedures of the reaction vessel in the MW-synthesizer, make βME inconvenient for a kilogram-scale on-resin production.

Moreover, 10% (v/v) 2-aminoethane-1-thiol (2-MEA) in DMF at r.t. allowed quantitative reduction in 16 h. An increase of 2-MEA reagent up to 20% (v/v) did not correspond to an improved reaction rate, but to the on-resin formation of Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(2-MEA)-Rink Amide AM resin as the result of a side-reaction involving the thiol functional group on Cys after StBu deprotection (IPC, Figure S8). Considering that MPA is the moiety that in Eptifibatide is linked by an amide bond to Har and that in biological systems has been reported to act as a reducing agent,[34] we used MPA for our optimized fully single reactor on-resin disulfide bond formation in Eptifibatide. To the best of our knowledge, we were the first to propose MPA to deprotect StBu on cysteine. In particular the peptide-resin Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(StBu)-Rink Amide AM was treated with MPA/DIPEA (40:41 equiv) in DMF for 24 h at r.t. in the Liberty Blue instrument. We obtained an acceptable StBu deprotection with only 2.2% residual Cys(StBu) containing peptide but no disulfide-exchange on the free thiol on cysteine (IPC not shown). Therefore, the desired disulfide-exchange to link MPA on cysteine was successfully achieved treating the resin directly in the reaction vessel into the instrument, with a DMF solution of the MPA dimer (DTDPA) and DIPEA (40:1.2 equiv) at r.t. for 21 h. Then, the peptide-resin was finally ready for head to MPA on cysteine side-chain cyclization by amide bond formation between the carboxylic function of MPA on Cys and N-terminal Har, by MW-assisted coupling with DIC and Oxyma Pure (see Experimental section). After resin cleavage, crude Eptifibatide was recovered in 60% yield with 40.9% HPLC purity (Figure S9).

The relevance of Strategy **D** is essentially based on the selection of the StBu orthogonal protection on cysteine that is easily removed by MPA acting as a novel reducing agent for Cys(StBu) deprotection. Moreover, all Strategy **D** operations (amino acid couplings, orthogonal side-chain protecting groups removal, and final head to MPA on cysteine side-chain cyclization) can be really carried out in a single reactor on-resin condition. In fact, easy washings of MPA excess and removal of StBu deprotection byproduct compared with reagents and protecting groups used in the other strategies can be performed by fully automated process in the instrumentation.

To the best of our knowledge, this is a unique strategy performing all the processes including disulfide bond formation to prepare Eptifibatide in a single reactor.

CONCLUSIONS

We investigated several strategies for the preparation of Eptifibatide, scalable to kilogram-scale, having in common the use of the MW-SPPS procedure, which is now available not only at R&D level but also for the large-scale manufacturing of peptides. Following the very fast microwave-assisted Fmoc/tBu synthesis of the Eptifibatide linear precursor by a DIC/Oxyma Pure coupling protocol at 90 °C, we explored both the solution (off-resin) and the solid-phase (on-resin) disulfide bond formation.

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Concerning the oxidation in solution, we focused our attention on the mild disulfide formation procedure based on the use of air and in particular, we studied the key factors (pH, concentration, reaction time, and solvent mixture composition) that affect the purity of the final product and the overall yield. In our hands, the best conditions for the off-resin oxidation of Eptifibatide linear precursor are the following: 5.3 mM concentration in H_2O/CH_3CN (2:1); pH 9.5; 22 h reaction time at room temperature. At these conditions, 98% conversion of the linear precursor was obtained on a 5 mmol scale, leading to crude Eptifibatide with a 61% HPLC purity. These overall satisfactory results are hampered in part by some drawbacks, particularly relevant for the large-scale production of this peptide, such as the formation of unwanted oxidation byproducts, mainly dimers, or the use of large volumes of an environmentally unfriendly solvent (CH_3CN).

In order to overcome these difficulties, we studied four different on-resin Strategies (**A**−**D**), with the final aim to develop a fully automated, single reactor procedure. To achieve the on-resin thiol deprotection we chose Mmt (**A**−**C**) or StBu (**D**) for the protection of the C-terminal Cys residue, while the N-terminal MPA moiety was introduced as activated SPyS (**A**) or as DTDPA, the MPA dimer (**B**). On the other hand, in Strategies **C**−**D**, the MPA moiety is introduced via S−S bond formation on cysteine after orthogonal deprotection. Therefore, the main difference among these strategies is the final cyclization step, obtained by direct formation of an S−S disulfide bridge (Strategies **A**−**B**) or via head to MPA on cysteine side-chain amide bond formation (Strategies **C**−**D**).

The need to select inexpensive raw material led us to perform a careful search for the most suitable reducing agent to deprotect StBu. We identified the *β*ME (30 equiv in 20% v/v in DMF) in 0.05 M DIPEA with microwave assistance at 75 °C for 15 min as the best reduction condition. However, toxicity and difficult microwave-synthesizer cleaning from this thiol reagent makes its use not perfectly suitable for a kilogram-scale production. So, we switched to the safer 2-MEA (40 equiv in 10% v/v in DMF and 4 equiv NMM), obtaining complete StBu deprotection in 16 h at room temperature. Interestingly, we also investigated the use of MPA as a reducing reagent and the best oxidation condition identified has been 40 equiv of MPA and 41 equiv of DIPEA. MPA represents a simple replacement of the reducing agent with the advantage of using an unexpensive raw material of the moiety present in Eptifibatide.

In summary, Strategies **A**, **B**, and **C** lead us to identify a limit: repeated washes with weakly acid solution to remove Mmt can cleave part of the peptide from the resin, leading to a variable, but unacceptable loss of yield. This limit has been overcome replacing the Mmt protecting group with Cys(StBu), as described in Strategy D, a method whose peculiarity is the use of MPA as reducing agent to perform at the same time the reduction of Cys(StBu) and its oxidation to Cys−S−S−MPA.

In conclusion, Strategy **D** represents an optimized scalable fully automated solid-phase microwave-assisted cGMP-ready process to prepare Eptifibatide.

EXPERIMENTAL SECTION

Materials. Peptide-grade *N*,*N*-dimethylformamide (DMF), all Fmoc protected amino acids (Fmoc-Gly-OH, Fmoc-Har(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Cys(StBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Cys(Mmt)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Pro-OH), 3-mercaptopropionic acid (MPA), and 3,3′-dithiodipropionic acid (DTDPA) were purchased from Sigma-Aldrich (Milan, Italy). Rink Amide AM resin was purchased from Sunresin New Materials Co. Ltd., Xi' AN (Shaanxi, China).

3-(2-Pyridinyldithio)propanoic acid MPA(PyS) was purchased from Carbosynth (Compton, U.K.).

Activators *N*,*N*′-diisopropylcarbodiimide (DIC), Oxyma Pure and (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) were purchased from Sigma-Aldrich (Milan, Italy). *N*-Chlorosuccinimide (NCS), trifluoroacetic acid (TFA), triisopropylsilane (TIS), and 2,2′-(ethylenedioxy)diethanethiol (DODT), *N*,*N*-diisopropylethylamine (DIPEA), diisopropyl ether (iPr_2O), diethyl ether (Et_2O), 2-propanol, dichloromethane (DCM), and HPLC Plus Water were purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade acetonitrile (CH_3CN) was purchased from Carlo Erba (Milan, Italy).

Fully Automated Synthetic Strategy of the Linear Precursor of Eptifibatide and Off-Resin Disulfide Bond Formation. *Preparation of the Linear Precursor of Eptifibatide by Fmoc/tBu MW-SPPS.* The fully protected linear precursor of Eptifibatide MPA(Trt)-Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(Trt)-Rink Amide AM resin was obtained starting from the Rink Amide AM resin (loading 0.93 mmol/g, 5.4 g, 5 mmol). Sequence elongation was performed on a microwave-assisted solid-phase peptide synthesizer Liberty Blue (CEM, Matthews, NC, U.S.A.) following the Fmoc/tBu strategy. Reaction temperatures were monitored by an internal fiber-optic sensor. Both deprotection and coupling reactions were performed in a Teflon vessel applying microwave energy under nitrogen bubbling. After the first Fmoc-deprotection, the following amino acids orthogonally protected were added automatically from C- to N-terminal: Fmoc-Cys(Trt)-OH, Fmoc-Pro-OH, Fmoc-Trp(Boc)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Har(Pbf)-OH, MPA(Trt)-OH, in the presence of the coupling reagents DIC and Oxyma Pure. The Fmoc/tBu MW-SPPS cycle consisted in (1) swelling in DMF (50 mL) for 30 min; (2) Fmoc-deprotection by 30% (v/v) piperidine/DMF (40 equiv, 66 mL); (3) washings with DMF (3 × 50 mL); (4) coupling with the Fmoc-protected amino acids (2.5 equiv, 0.4 M in DMF), Oxyma Pure (2.5 equiv, 1 M in DMF), and DIC (2.5 equiv, 3 M in DMF); (5), washings with DMF (3 × 50 mL). Peptide elongation was performed by repeating the MW-SPPS cycle for each amino acid. Both deprotection and coupling reactions were performed reaching 90 °C except 50 °C for Cys coupling (Table S2).

After all amino acids were coupled, the resin was filtered, washed with DMF (3 × 50 mL) and 2-propanol (3 × 50 mL), and dried under vacuum to obtain 14.1 g of peptide-resin. The linear crude Eptifibatide precursor was cleaved from the resin following the procedure described in Supporting Information. The crude linear precursor of Eptifibatide (4.7 g, 5 mmol) was characterized with a 71% RP-UHPLC purity (yield 99%), R_t 4.9 min, gradient 12−45% B in 10 min. ESI-MS (m/z): $[M + H]^+$ 834.4 (found), 834.9 (calcd).

Optimized Synthetic Procedure for Off-Resin Disulfide Bond Formation in Eptifibatide. The crude linear Eptifibatide precursor (4.7 g, 5 mmol, HPLC purity 71%) was introduced in a 2 L round-bottom flask, and a mixture of water and CH_3CN (1:1, 630 mL) was added and then stirred for about 15 min. After complete dissolution, additional water (315 mL)

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was added to the reaction mixture to obtain a final concentration of 5.3 mM. Initially the measured pH, being 2.5, was adjusted to 9.5 adding NH_4OH 7.5% (9.4 mL). After the mixture was mechanically stirred for 22 h at 350 rpm, at room temperature, the reaction was quenched by adding TFA (3.7 mL), adjusting the pH to 2.5. Then the reaction mixture was lyophilized without further evaporation.

The crude Eptifibatide was obtained with 61% HPLC purity (4.6 g, yield 98%). R_t 4.2 min; ESI-MS (m/z): $[M + H]^+$ 832.5 (found); 831.96 (calcd) (Figure S3a).

The above-described procedure was optimized after performing the experiments in the conditions described in Table 1, Figure 3, and Figure 4, each one on 5 mmol of crude linear Eptifibatide precursor.

Fully Automated Synthetic Strategies of Eptifibatide Including on-Resin Disulfide Bond Formation (A−D). *Preparation of the Peptide-Resin Precursor Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(Mmt)-Rink Amide AM Resin by Fmoc/tBu MW-SPPS (Strategies **A**, **B**, and **C**).* The fully protected linear precursor Har(Pbf)-Gly-Asp(OtBu)-Trp-(Boc)-Pro-Cys(Mmt)-Rink Amide AM resin was obtained starting from the Rink Amide AM resin (loading 0.93 mmol/g, 5.4 g, 5 mmol). Sequence elongation was performed on a microwave-assisted solid-phase peptide synthesizer Liberty Blue (CEM, Matthews, NC, U.S.A.) following the Fmoc/tBu strategy. Reaction temperatures were monitored by an internal fiber-optic sensor. Both deprotection and coupling reactions were performed in a Teflon vessel applying microwave energy under nitrogen bubbling. After the first Fmoc-deprotection, the following amino acids orthogonally protected were added automatically from C- to N-terminal: Fmoc-Cys(Mmt)-OH, Fmoc-Pro-OH, Fmoc-Trp(Boc)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Har(Pbf)-OH, in the presence of the coupling reagents DIC and Oxyma Pure. The Fmoc/tBu MW-SPPS cycle consisted of (1) swelling in DMF (50 mL) for 30 min; (2) Fmoc-deprotection in 30% (v/v) piperidine/DMF (40 equiv, 66 mL); (3) washings with DMF (3 × 50 mL); (4) coupling with the Fmoc-protected amino acids (2.5 equiv, 0.4 M in DMF), Oxyma Pure (2.5 equiv, 1 M in DMF), and DIC (2.5 equiv, 3 M in DMF); (5), washings with DMF (3 × 50 mL). Peptide elongation was performed by repeating the MW cycle for each amino acid coupling and deprotections as reported in Table S2 of the Supporting Information. After all amino acids were coupled, the resin was filtered, washed with DMF (3 × 50 mL) and with 2-propanol (3 × 50 mL), and dried under vacuum to obtain 12.3 g of peptide-resin. The linear Eptifibatide precursor on the resin was divided into three aliquots for subsequent on-resin disulfide bond formation using Strategies **A**, **B**, and **C**.

*Strategy **A** for On-Resin Disulfide Bond Formation in Eptifibatide.* MPA(PyS) (216 mg, 10 equiv) was coupled to the on-resin protected linear peptide precursor Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(Mmt)-Rink Amide AM resin (230 mg, 0.1 mmol, 0.43 mmol/g) by a microwave-assisted protocol (75 °C, 35 W, 5 min) using DIC (0.154 mL, 10 equiv) and Oxyma Pure (142 mg, 10 equiv) as coupling reagents in DMF (5 mL). Then, the peptide-resin was filtered and washed with DMF (3 × 2 mL) and DCM (3 × 2 mL). 4-Methoxytriphenylmethyl (Mmt) cysteine deprotection and contemporary formation of the disulfide bridge via disulfide-exchange reaction with the head terminal MPA(PyS) were achieved by treating the resin with a mixture of TFA/TIS/DCM (10 mL, 3:5:92) for 25 min at r.t. Following the cleavage procedure described in the Supporting Information, we performed the final cleavage with the cocktail TFA/H_2O/TIS (10 mL, 95:2.5:2.5).

Crude Eptifibatide (50 mg, 60% yield) was obtained with a 34.9% HPLC purity (Figure S4).

*Strategy **B** for On-Resin Disulfide Bond Formation in Eptifibatide.* 3,3′-Dithiodipropionic acid (DTDPA) (2.63 g, 5 equiv) was coupled to the peptide-resin Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(Mmt)-Rink Amide AM resin (2.5 mmol, 7.3 g, 0.34 mmol/g) by a microwave-assisted protocol at 75 °C (35 W, 5 min) using DIC (1.93 mL, 5 equiv) and Oxima Pure (1.77 g, 5 equiv) in DMF (40 mL). The coupling reaction was IPC monitored. The peptide-resin was filtered and washed with DMF (3 × 20 mL) and DCM (3 × 20 mL). DTDPA-Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys-(Mmt)-Rink Amide AM resin was treated with dithiothreitol (DTT, 3 g, 19 mmol) and 0.1 M 4-methylmorpholine (NMM) in DMF (60 mL) to reduce the S−S bond in DTDPA forming MPA. After 12 h, IPC monitoring confirmed 93% conversion (data not shown), the resin was washed with DMF (3 × 60 mL), Mmt deprotection was performed in a cocktail of TFA/TIS/DCM (8 × 20 mL, 2:5:93) until the yellow color disappeared and the deprotection solution turned colorless. Oxidation was performed treating the supported protected linear peptide MPA-Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys-Rink Amide AM resin (2.1 g, 0.75 mmol) with a solution of *N*-chlorosuccinimide (120 mg, 1.2 equiv) in DMF (24 mL) for 10 min at r.t. After washing the resin with DCM (3 × 25 mL), the cleavage was performed following the procedure described in Supporting Information using a mixture of TFA/H_2O/TIS (40 mL, 95:2.5:2.5).

Crude Eptifibatide (590 mg, 82% yield) was obtained with a 42.4% HPLC purity (Figure S5).

*Strategy **C** for On-Resin Disulfide Bond Formation in Eptifibatide.* The peptide-resin Har(Pbf)-Gly-Asp(OtBu)-Trp-(Boc)-Pro-Cys(Mmt)-Rink Amide AM resin (200 mg, 0.087 mmol) was treated with a cocktail of TFA/TIS/DCM (10 mL, 3:5:92) for 25 min at r.t. to deprotect the cysteine residue. On-resin disulfide-exchange between the activated MPA(PyS) (187 mg, 10 equiv) in DMF (5 mL) and DIPEA (45 μL, 3 equiv) and the free cysteine thiol function on the peptide-resin was performed in the microwave module of the Liberty Blue to form the disulfide bond (50 °C, 90 W, 30 min). The resin was filtered, washed with DMF (3 × 3 mL) and DCM (3 × 3 mL), and dried under vacuum. The IPC showed 82% of desired linear Eptifibatide derivative Har-Gly-Asp-Trp-Pro-Cys-(MPA)-NH_2 (Figure S6). The head to MPA on cysteine side-chain cyclization (required to obtain Eptifibatide), was achieved by PyBop (226 mg, 5 equiv) and DIPEA (105 μL, 7 equiv) in DMF (3 mL) at r.t. in 16 h. The final cleavage from the resin was performed following the procedure described in the Supporting Information using a cocktail of TFA/H_2O/TIS (5 mL, 95:2.5:2.5).

Crude Eptifibatide (50 mg, 61% yield) was obtained with a 56.5% HPLC purity (Figure S7).

*Preparation of the Peptide-Resin Precursor Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(StBu)-Rink Amide AM by Fmoc/tBu MW-SPPS (Strategy **D**).* The fully protected linear precursor Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys-(StBu)-Rink Amide AM resin was obtained starting from the Rink Amide AM resin (loading 0.93 mmol/g, 1.1 g, 1 mmol). Sequence elongation was performed on a microwave-assisted solid-phase peptide synthesizer Liberty Blue (CEM, Matthews,

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NC, U.S.A.) following the Fmoc/tBu strategy. Reaction temperatures were monitored by an internal fiber-optic sensor. Both deprotection and coupling reactions were performed in a Teflon vessel applying microwave energy under nitrogen bubbling. After the first Fmoc-deprotection, the following amino acids orthogonally protected were added automatically from C- to N-terminal: Fmoc-Cys(StBu)-OH, Fmoc-Pro-OH, Fmoc-Trp(Boc)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Har(Pbf)-OH, in the presence of the coupling reagents DIC and Oxyma Pure. The Fmoc/tBu MW-SPPS cycle consisted of: (1) swelling in DMF (15 mL) for 30 min; (2) Fmoc-deprotection by 30% (v/v) piperidine/DMF (20 mL, 60 equiv); (3) washings with DMF (3 × 15 mL); (4) coupling with the Fmoc-protected amino acids (5 equiv, 0.4 M in DMF), Oxyma Pure (5 equiv, 1 M in DMF), and DIC (5 equiv, 3 M in DMF); (5), washings with DMF (3 × 15 mL). Peptide elongation was performed by repeating the MW cycle for each amino acid coupling and deprotections as reported in Table S2 of the Supporting Information. After all amino acids were coupled, the resin was filtered, washed with DMF (3 × 15 mL) and with 2-propanol (3 × 15 mL), and dried under vacuum to obtain 2.5 g of peptide-resin Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(StBu)-Rink Amide AM.

*Strategy **D** for Optimized Fully Single Reactor On-Resin Disulfide Bond Formation in Eptifibatide.* The StBu protecting group was removed from cysteine on the linear peptide-resin Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys-(StBu)-Rink Amide AM (loading 0.43 mmol/g, 1.6 g, 0.7 mmol), treating the resin with a solution of MPA (2.44 mL, 40 equiv) and DIPEA (5 mL, 41 equiv) in DMF (25 mL). After 24 h at r.t. (without removing the resin from the Liberty Blue instrument), the IPC monitoring the reaction progress in solid-phase, showed an almost complete cysteine deprotection. The resin was filtered, washed with DMF (3 × 25 mL) and DCM (3 × 25 mL), and added with a solution of DTDPA (5.9 g, 40 equiv) and DIPEA (146.3 μL, 1.2 equiv) in DMF (25 mL), and maintained for 21 h at r.t. under N_2 bubbling in the instrument. The IPC showed that Cys(MPA) was correctly formed on the peptide-resin by disulfide-exchange (data not shown). Therefore, after filtration and washings with DMF (3 × 25 mL), the peptide-resin was added with DIC (130 μL, 1.2 equiv) and Oxyma Pure (120 mg, 1.2 equiv) under two consecutive MW cycles, refreshing the solution of the reagents (70 °C, 150 W in 30 s and then 90 °C, 30 W in 10 min) to form the head to MPA on cysteine side-chain amide-bond cyclization including the disulfide bridge of Eptifibatide. Finally, the resin was filtered and washed with DMF (3 × 25 mL) and DCM (3 × 25 mL), then dried under vacuum. The final cleavage was performed following the procedure described in the Supporting Information using the cocktail TFA/H_2O/TIS (20 mL, 95:2.5:2.5).

Crude Eptifibatide (400 mg, 60% yield) was obtained with a 40% HPLC purity (Figure S9).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.oprd.0c00490.

Table of patent landscape, microwave methods, and RP-UHPLC/MS analyses (PDF)

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Author Contributions

¶G.S., A.D., and L.P. equally contributed to the optimization of Eptifibatide process in the context of PeptFarm, University of Florence.

Notes

The authors declare no competing financial interest.

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DEDICATION

Dedicated to Prof. Dr. Luis Moroder on the occasion of his 80th birthday.

ABBREVIATIONS

CMOs = contract manufacturing organizations; MW-SPPS = microwave -assisted solid-phase peptide synthesis; R&D = research and development; APIs = active pharmaceutical ingredients; cGMP = current good manufacturing practice; SPPS = solid-phase peptide synthesis; Fmoc = 9-fluorenylmethyloxycarbonyl; tBu = tert-butyl; FDA = Food and Drug Administration; NMP = N-methyl-2-pyrrolidone; REACH = European Regulation on Registration, Evaluation, Authorization, and Restriction of Chemicals; GSPPS = green solid-phase peptide synthesis; DMSO = dimethilsulfoxide; MPA = 3-mercaptopropionic acid; HOBt = 1-hydroxybenzotriazole; HBTU = 3-[bis(dimethylamino)methyliumyl]-3*H*-benzotriazol-1-oxide hexafluorophosphate; MW = microwave; MTBE = methyl-tert-butyl ether; CPME = cyclopentyl methyl ether; DMF = *N*,*N*-dimethylformamide; DTDPA or MPA_2 = 3,3′-dithiodipropionic acid; MPA(PyS) = 3-(pyridyne-2-thiol)-propanoic acid; DIC = *N*,*N*′-diisopropylcarbodiimide; PyBOP = (benzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate; NCS = *N*-Chlorosuccinimide; TFA = trifluoroacetic acid; TIS = triisopropylsilane; DODT = 2,2′-(ethylenedioxy)diethanethiol; DIPEA = *N*,*N*-diisopropylethylamine; iPr_2O = diisopropyl ether; Et_2O = diethyl ether; DCM = dichloromethane; CH_3CN = acetonitrile; NMM = 4-methylmorpholine; βME = β-mercaptoethanol; 2-MEA = 2-aminoethane-1-thiol; NPyS = 3-nitro-2-pyridynesulfenyl; Mmt = 4-methoxytrityl; PyS = pyridine-2-thiol; IPC = in process control; R_t = retention time; calcd = calculated; ESI-MS = electrospray ionization mass spectrometry; RP-UHPLC-MS = reversed-phase ultrahigh performance liquid chromatography mass spectrometry; DTT = 1,4-dithiothreitol; NH_4OH = ammonium hydroxide; NH_4HCO_3 = Ammonium bicarbonate; StBu = S-tert-butylthio; Trt = triphenylmethyl; Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; Boc = tert-butyloxycarbonyl

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