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

ORGANIC PROCESS RESEARCH & DEVELOPMENT
OPR&D

pubs.acs.org/OPRD

Article

An Optimized Safe Process from Bench to Pilot cGMP Production of API Eptifibatide Using a Multigram-Scale Microwave-Assisted Solid-Phase Peptide Synthesizer

Published as part of the Organic Process Research & Development joint virtual special issue "Process Safety from Bench to Pilot to Plant" in collaboration with ACS Chemical Health & Safety and Journal of Loss Prevention in the Process Industries.

Annunziata D'Ercole,[▽] Lorenzo Pacini,[▽] Giuseppina Sabatino, Matteo Zini, Francesca Nuti, Arianna Ribecai, Alfredo Paio, Paolo Rovero, and Anna Maria Papini*

Cite This: *Org. Process Res. Dev.* 2021, 25, 2754−2771

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: A growing industrial interest toward the peptide drug market fueled the need for the development of effective and cGMP compliant manufacturing methods for these complex molecules. Solid-phase strategies are considered methods of election for medium-length peptide syntheses not only on the research scale but for multigram-scale production, as well. The possibility to use microwave-assisted technology on the multigram scale, recently introduced, prompted us to evaluate the possibility to conveniently set up a safe and fully cGMP-compliant pilot process to produce eptifibatide, a generic peptide active pharmaceutical ingredient. Accordingly, we developed an optimized process on the laboratory scale (1−5 mmol), which was subsequently successfully scaled up to 70 mmol, obtaining all the information required by regulatory agencies to validate the process and qualify the pilot scale plant. The process consists of 5 steps: (1) automated microwave-assisted solid-phase synthesis of eptifibatide linear precursor; (2) cleavage from the resin with concomitant amino acid side-chains deprotection; (3) disulfide-bond formation in solution; (4) purification by flash column chromatography; (5) ion-exchange solid-phase extraction. Since the direct scale-up of a multigram-scale cGMP compliant peptide API production procedure is a challenge that requires an accurate understanding of each involved step, we initially performed a quality management risk assessment, which enabled a smooth and effective achievement of a successful final result.

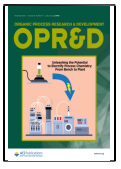
KEYWORDS: *generic peptide drug, peptide production scale-up, multigram-scale microwave-assisted peptide synthesis, technology transfer, peptide production pilot plant*

INTRODUCTION

According to the latest "Transparency Market Research" report, the global peptide therapeutics market was valued in 2018 at ca. 25 billion USD, and it is anticipated to expand until 2027 at a Compound Annual Growth Rate (CAGR) of 7.9%.[1] Moreover, a recent report by Roots Analysis, Business Research and Consulting, expects that the peptide therapeutics market following industry trends and global forecast 2021−2030, will be directly associated with increasing investments in R&D activities, in search of new drug substances for treating infectious diseases, metabolic disorders, diabetes, cancer, and other diseases.[1] Additionally, companies are moving toward the generic peptide drug market that appears to offer a relatively easy and lucrative option, also because of several patents expiring.

Industries that produce generic peptide drugs following cGMP requirements have to cooperate with regulatory authorities, such as the European Medicines Agency (EMA) and US Food and Drug Administration (FDA), to harmonize guidelines on therapeutics production.[2] In this frame, a potential application for EMA and/or FDA approval of a synthetic peptide drug, referred to as a previously approved one, shall be submitted as an Abbreviated New Drug Application (ANDA) of the Federal Food, Drug, and Cosmetic Act (FD&C Act). According to this document, ANDA application is mandatory to demonstrate that the active pharmaceutical ingredient (API) is equivalent to the reference listed drug (RLD). ANDA approval strictly depends on the impurity profile. In particular, a proposed generic synthetic peptide drug cannot contain more than 0.5% of each new specified peptide-related impurity that has to be characterized and demonstrated not affecting safety and effectiveness of the drug.[3−5] Therefore, methods validation and facilities qualification become part and parcel of this demonstrative process, to ensure and preserve the identity, quality, effectiveness, and purity of the generic peptide drug candidate.

Received: September 21, 2021
Published: November 24, 2021



Solid-phase strategies are considered methods of election for medium-length peptide syntheses not only on the research scale but more and more also for multigram-scale production. Since large quantities of hazardous solvents such as DMF, NMP, and/or CH_2Cl_2 are required to perform reactions and washings in this multistep process, innovative synthetic technologies and purification protocols have been proposed with the primary goal to identify greener solvents and procedures. Albericio and co-workers proposed as green solvents 2-MeTHF and Gamma-Valerolactone (GVL), demonstrating good swelling capacity of the solid support (2-chlorotrityl chloride and Wang resins), solubilization capability, and reactivity of amino acid building blocks and coupling reagents, even if demonstrated only on the small scale.[6−8]

Another aspect investigated to improve the efficiency of solid-phase peptide synthesis (SPPS), also from the instrument engineering perspective, is temperature control of the reaction. Pentelute and co-workers reported the development of an automated fast-flow instrument to synthesize up to 164-amino-acid peptide chains via 327 reaction steps, with coupling cycles of 40 s at 90 °C.[9] However, "a potentially limiting factor for the setup is synthesis scale".[10] In fact, the capacity of the reactor currently available allows hosting only 200 mg of resin with a loading of ca. 0.5 mmol/g. To the best of our knowledge, multigram-scale equipment is available only for conventional synthetic approaches in the solid-phase at room temperature (CS-Bio, Gyros).

Consistent with new frontiers of synthesis automation and control engineering is the variable bed flow reactor (VBFR) developed by Seeberger and co-workers. VBFR-SPPS allows monitoring of on-resin aggregation, secondary structure formation, as well as deprotection and coupling reactions that may lead to variation of resin swelling, resulting into decreased coupling efficiency. Although thorough real-time monitoring is pivotal to guaranteeing reproducibility for multigram-scale production, to the best of our knowledge, larger equipment compatible with this technology is still not available.[11]

A different perspective is offered by microwave (MW) technology, which has been demonstrated to overcome possible drawbacks of multigram-scale solid-phase synthesis. As reported by Collins et al., MW irradiation significantly decreases reaction time, increasing crude purity.[12−14] Therefore, MW-SPPS is now considered one of the most important strategies to obtain synthetic peptides. Indeed, among all the innovative technologies above-described, MW irradiation is the unique one currently exploited in a commercially available synthesizer for multigram-scale production. CEM Corporation (Charlotte, NC, U.S.A.) provides three microwave-assisted solid-phase synthesizers allowing the scale-up of a MW-SPPS optimized process.[15] These instruments can perform syntheses from 0.005 to 5 mmol scale (Liberty Prime and Liberty Blue systems) and from 5 to 800 mmol scale (Liberty Pro system). The latter was used within the work reported herein for a cGMP compliant industrial production of a 70 mmol pilot-scale peptide active pharmaceutical ingredient to be commercialized as a generic drug.

Several patents claiming synthetic processes to produce the disulfide-bridged cycloheptapeptide eptifibatide include strategies based on SPPS, liquid-phase peptide-synthesis (LPPS), and hybrid approaches. Among the most remarkable SPPS strategies, Qin et al. disclosed the synthesis of a linear eptifibatide precursor on Sieber resin. Acetamidomethyl (Acm) was used as an orthogonal protecting group of the thiol function both of cysteine and mercaptopropionic acid (MPA). Contemporary Acm removal and disulfide bond formation led to the desired cyclopeptide eptifibatide.[16] Despite the method appearing easy, poor handling of the resin makes it less exploitable, with low yield as an output. On the other hand, Wen et al. proposed the use of different solid supports, such as Rink Amide, Rink Amide AM, and Rink Amide MBHA resins. However, each strategy requires several intermediate steps before isolation of the desired cyclopeptide.[17]

Concerning the strategies used for multigram-scale disulfide bond formation, most of the methods reported in the literature are based on oxidation in solution, after cleavage of the corresponding linear precursor from the resin. Strong oxidizing reagents (such as H_2O_2, DMSO, I_2) are proposed for off-resin oxidation.[18] However, disulfide bridge formation under basic conditions requires accurate reaction optimization conditions, and in particular pH, concentration, and the solvent mixture must be carefully controlled. As recently described, automated MW-assisted solid-phase synthesis significantly decreased production times and waste volumes to obtain up to 5 mmol eptifibatide (Figure 1).[15]

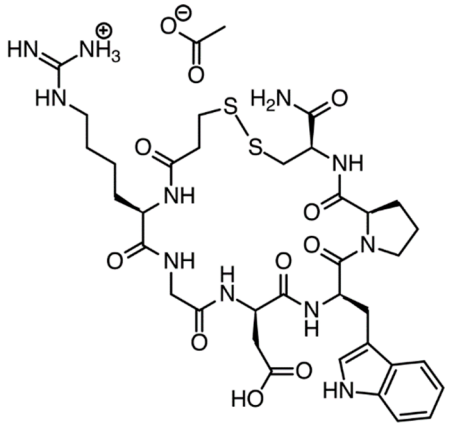


Figure 1. Eptifibatide acetate: N^6-(aminoiminomethyl)-N^2-(3-mercapto-1-oxopropyl)-L-lysylglycyl-L-α-aspartyl-L-tryptophyl-L-prolyl-L-cysteinamide, cyclic (1→6)-disulfide acetate.

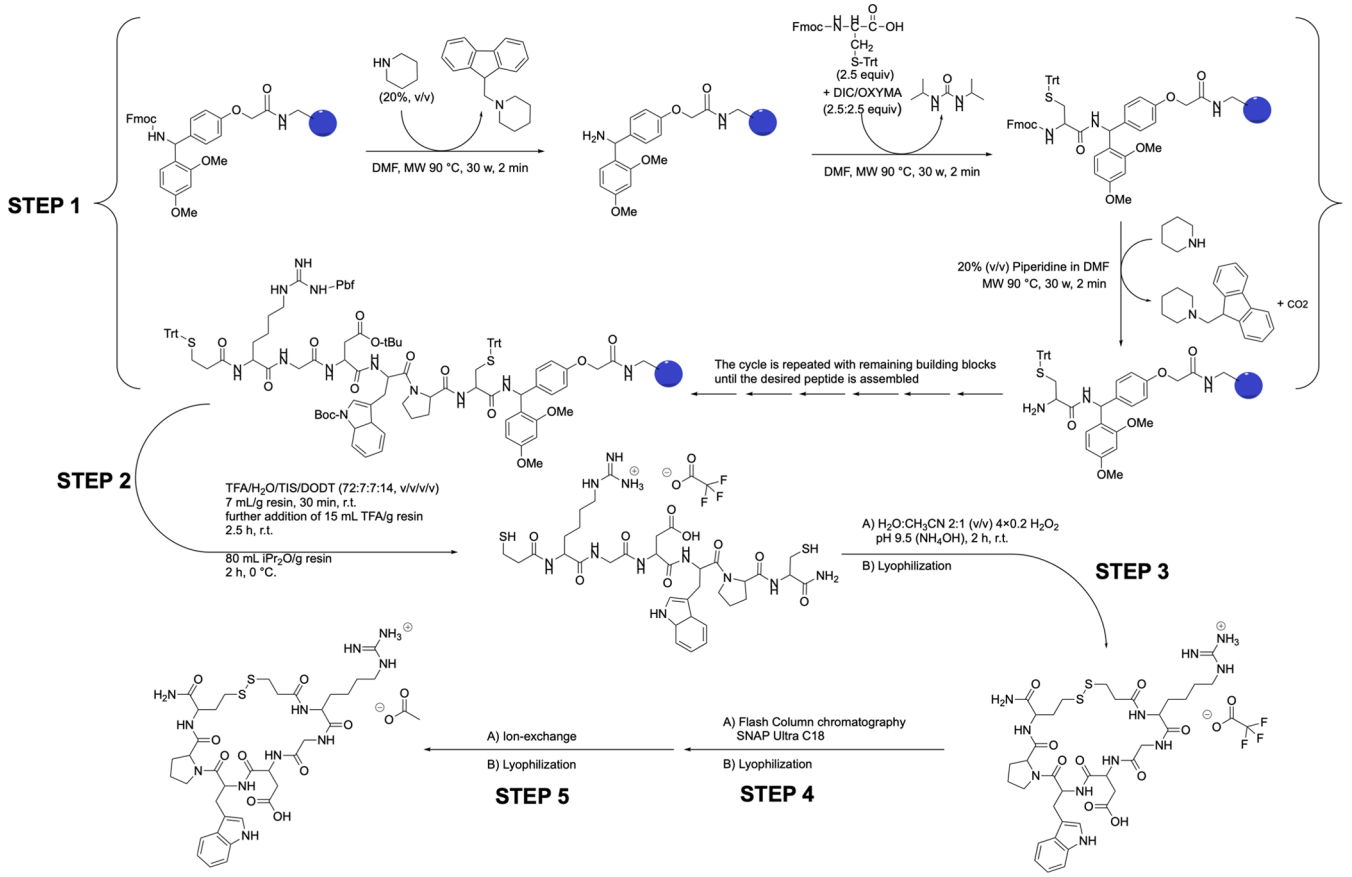
RESULTS AND DISCUSSION

Final aim of the present work is to provide a proof-of-concept that industrial manufacturing of peptide APIs can take advantage from the performances of MW-assisted solid-phase synthesizers available on the market. This approach can be strategic for the scale-up of the synthetic process, particularly for those manufacturers entering the market of peptide generic drugs and requiring scientific knowledge transfer and support in overcoming possible unforeseen events in peptide chemistry.

We report herein the laboratory process optimization (1−5 mmol) of the heterodetic cyclopeptide API eptifibatide acetate (Figure 1) in five steps: (1) eptifibatide linear precursor automated MW-assisted solid-phase synthesis (Liberty Blue, CEM, Charlotte, NC, U.S.A, step 1); (2) cleavage from the resin and amino acid side-chain deprotection (step 2); (3) in solution disulfide-bond formation (step 3); (4) purification by flash column chromatography (step 4), followed by (5) ion-exchange solid-phase extraction (step 5). Different steps are

https://doi.org/10.1021/acs.oprd.1c00368
Org. Process Res. Dev. 2021, 25, 2754−2771

Scheme 1. Synthetic Scheme of Eptifibatide Acetate Production



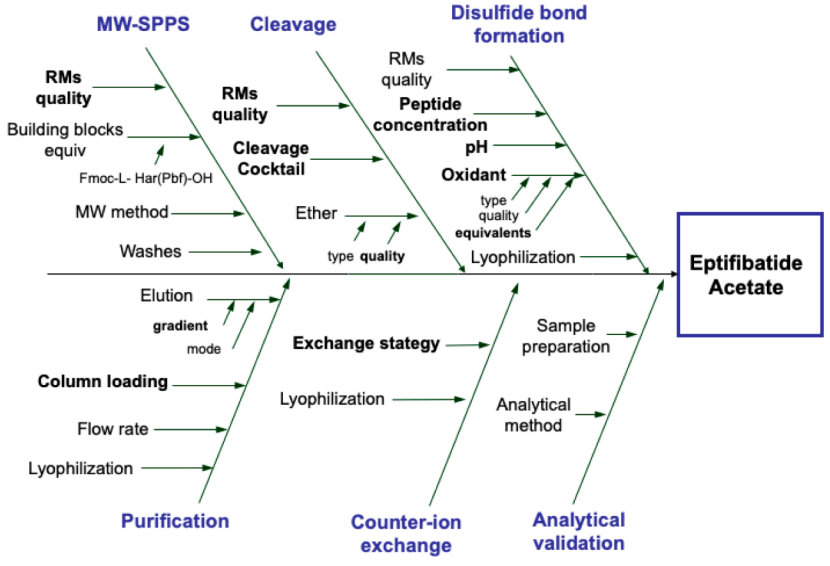


Figure 2. Ishikawa diagram for the development of the cGMP eptifibatide acetate production process. Critical process parameters (CPPs) are reported in **bold**.

defined in Scheme 1. We also demonstrated the scalability of this protocol to an industrial cGMP compliant robust manufacturing process to produce 70 mmol peptide API. To the best of our knowledge, this is the first time that the automated MW-assisted solid-phase multigram-scale peptide synthesizer Liberty Pro (CEM, Charlotte, NC, U.S.A) was used for cGMP production of a peptide API.

In addition, since industrial process optimization shall consider specific requirements such as safety, economic, and, most importantly, regulatory aspects, we examined in depth all those features that we considered critical during the technology transfer from lab- to pilot-scale. These aspects are described in specific paragraphs, titled "scale-up activities", after the description of each step lab-scale optimization.

https://doi.org/10.1021/acs.oprd.1c00368
Org. Process Res. Dev. 2021, 25, 2754−2771

Finally, since the direct scale-up of a kilogram-scale, cGMP compliant peptide API production procedure is a challenge that requires an accurate understanding of each involved step, we initially performed a quality management risk assessment, as described in the following paragraph.

Quality Risk Management: Identification of Critical Quality Attributes and Critical Process Parameters to Be Optimized. Implementation of Abbreviated New Drug Application (ANDA) for eptifibatide approval by regulatory agencies (as for any other peptide generic drug) required a systematic approach to preliminarily define Critical Quality Attributes (CQAs) and understanding the process to follow, identifying critical material attributes (CMAs) and critical process parameters (CPPs). This allowed the establishment of the functional relationships linking CMAs/CPPs to CQAs, according to the International Conference of Harmonization document Q8 (ICH Q8). In this process, profound knowledge of peptide science and therefore of the organic chemistry of the molecule to be produced is pivotal.

In this framework, the Ishikawa diagram was selected to plot the list of the parameters involved in the eptifibatide acetate manufacturing process to identify the critical parameters that have been considered into the optimization process on 1−5 mmol lab-scale (Figure 2).

The present work does not claim to be a detailed description of GMP requirements, rather it would show an example of process optimization and pioneering pilot scale transfer according to cGMP requirements.

We followed the principles of quality risk management to establish a suitably controlled cGMP compliant manufacturing process of an eptifibatide acetate pilot batch across the product life cycle. In particular, we evaluated the risk to quality (thanks to the scientific knowledge of peptide chemistry), and we provided formalities and documentations of the performed experiments commensurate with the level of risk, integrated with facilities, equipment, utilities qualification, materials management, laboratory control, and stability testing.[19] The identification of hazards and the analysis and evaluation of risks associated with exposure to those hazards is a relevant part of risk assessment (EUH019, hazard statement in Classification, Labeling & Packaging regulation).[20]

Step 1. Optimization and Scale-up of Solid-Phase Synthesis of on-Resin Eptifibatide Linear Precursor by Automated MW-Assisted Synthesizers. At the industrial level, cost flow in process costing requires evaluation of direct material costs from the beginning of the process, i.e., reagents and solvents to be used for peptide synthesis, isolation, and purification, including ion-exchange solid-phase extraction. Solid-phase synthesis through stepwise assembly of building blocks on a solid support usually requires large excesses of reagents essential to overcoming limitations due to reactions in the heterogeneous phase. However, this practice strongly affects the cost of the final product.

In this framework, automation is fundamental to getting continuous cycle manufacturing, minimizing intermediates isolation, and preserving both safety and reproducibility.

On-resin fully protected linear eptifibatide precursor MPA-(Trt)-Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(Trt)-Rink Amide AM resin was built via a standard Fmoc/tBu MW-SPPS strategy (see Experimental Section). The coupling system based on N,N′-diisopropylcarbodiimide (DIC) and Oxyma Pure is considered safe, has a low cost, and is suitable for MW-SPPS because of its stability at 90 °C (temperature reached in the MW conditions) and requires lower DMF washing volumes, due to its higher solubility, as compared to more classic coupling reagents.

We describe herein the successful scale-up to 70 mmol of fully protected on-resin eptifibatide linear precursor in 1 day by an automated MW-assisted process, using the Liberty Pro synthesizer, after having optimized the synthesis on a 5 mmol scale on the Liberty Blue synthesizer. The aim is to demonstrate that the MW-assisted process dramatically decreases the time of production, compared to conventional room temperature solid-phase synthesis, usually requiring at least 1 week to obtain similar length peptides. Consequently, labor price, the most relevant cost for industrial manufacturers, will be decreased.

Evaluation of the Minimal Quantity of the High-Cost Building Block Fmoc-L-Har(Pbf)-OH. As underlined above, a careful analysis of the purity profile is pivotal to producing a generic API compliant with cGMP requirements. The on-resin synthesis of the linear eptifibatide precursor requires use of the high cost building block Fmoc-L-Har(Pbf)-OH.

Therefore, optimization of its coupling conditions, minimizing cost flow in process costing, is a challenge. First, we explored the scalability from a 1 to 5 mmol scale investigating (1) single vs double coupling; (2) the use of lower reagents excess; and (3) an increase of coupling time, maintaining the temperature at 90 °C and adapting MW-power to the different scale.

We demonstrated that the first coupling should not be performed with <2.5 equiv of Fmoc-L-Har(Pbf)-OH (Table S1, entries 1−3). This is fundamental to limiting Des-Har^2-linear precursor impurity formation that was observed (Figure S1) possibly because of the high hindrance of the Pbf protecting group and/or δ-lactam formation from the activated Fmoc-Har(Pbf)-OH building block, affecting coupling efficiency.[21] A large excess of activated building blocks or double couplings may overcome this problem. However, in our case, double coupling did not appear to be advantageous in terms of limiting Des-Har^2-linear precursor impurity formation. We succeeded in optimizing the coupling conditions of Fmoc-Har(Pbf)-OH on a 5 mmol scale in 15 min, using 1 × 2.5 equiv, at 90 °C and 17−26 W, obtaining only a 0.6% Des-Har^2-linear eptifibatide impurity (Table S1, entry 5), compared with the 1 mmol scale (Table S1, entry 4). Interestingly, the scale-up to 70 mmol (1 × 2.5 equiv of Fmoc-Har(Pbf)-OH at 90 °C and 1800−800 W, Table S4) did lead in only 40 min to <0.1% of the Des-Har^2-linear eptifibatide impurity (see Experimental Section: IPC by resin cleavage). Therefore, no further investigation was performed of possible δ-lactam formation from the activated Fmoc-Har(Pbf)-OH building block, as recently reported in the case of Fmoc-Arg(Pbf)-OH by de la Torre et al., who observed large quantities of Des-Arg peptides once the temperature was not maintained at <55 °C but without microwaves.[22]

Scaling-up the coupling step from 5 mmol on Liberty Blue to 70 mmol on Liberty Pro (characterized by an optimized dual-mode mixing with overhead mechanical stirring and N_2 bubbling, homogeneously heating resin and diffusing solvents) required a proportional increase of MW power and reaction time to guarantee optimal performances of the synthesis. Increasing the time of coupling was possible because of the stability and the efficiency of the DIC/Oxyma pure coupling system under MW conditions. Reproducibility of the MW-

https://doi.org/10.1021/acs.oprd.1c00368
Org. Process Res. Dev. 2021, 25, 2754−2771

SPPS on the pilot-scale was unequivocally demonstrated (Tables S2, S3, and S4).

Quality Check of Solvents and Reagents in Compliance with Critical Material Attributes (CMAs). Industrial raw materials (RMs) are generally stored in large tanks in the manufacturer's warehouse department and withdrawn as needed.[23,24] The industrial grade material attributes must be demonstrated to be compliant with the analytical ones. Consequently, before starting the 70 mmol pilot scale production, industrial-grade DMF and Oxyma pure quality were tested (use test). First of all, we demonstrated that the reagents were compliant with suppliers' certificate of analysis (CoA). Moreover, their performances were compared on a 5 mmol scale synthesis with a second synthesis performed under the same conditions using analytical-grade reagents. As the UHPLC purity (after cleavage) of the eptifibatide linear precursor crude and Des-Har^2-linear precursor impurity amount resulted as comparable, we considered it acceptable to use industrial-grade DMF and Oxyma pure reagent, decreasing the production cost (Table S5).

Step 2. Cleavage from the Resin and Amino Acid Side-Chains Deprotection to Obtain the Crude Eptifibatide Linear Precursor. The cleavage step, which consists of the concomitant cleavage of the peptide from the resin and side-chain deprotection, can potentially generate impurities that must be precisely characterized to fulfill cGMP requirements for the final regulatory approval.

Identification of the critical quality attributes (CQAs) of the API and critical processing parameters (CPPs) are pivotal to the release of the final peptide batch.[25] Cleavage conditions have to be considered CPPs because of their direct connection to the final purity profile (Figure 2). Deviations and being out of specification (OOS) from the expected behavior may affect the safety and efficacy of the final product, invalidating final approval for human use marketing. Consequently, further investigations to demonstrate the causes, followed by time-consuming corrective actions, are required.

Investigation on the Cleavage Scavenger Mixture Effect on Crude Purity. The mixture TFA/TIS/DODT/H_2O 92.5:2.5:2.5:2.5 (v/v/v/v) was tested first to cleave the eptifibatide linear precursor from the resin (Figures S2 and S3). After cleavage, UHPLC/ESI-MS analysis of the crude product obtained after precipitation in ether showed three main side products: R_t = 5.154 min, $[M + H]^+$ m/z = 890.39 (Imp a); R_t = 5.333 min, $[M + H]^+$ m/z = 890.39 (Imp b), both deviating +56 m/z; R_t = 4.258 min, $[M + H]^+$ m/z = 878.32 (Imp c), deviating +44 m/z, from the calculated mass value $[M + H]^+$ m/z = 834.33 (R_t = 4.333 min) of the eptifibatide linear precursor, respectively (Figures S4, S5, S6, and S7).

Reactions to cleave tBoc and tBu protecting groups from Trp and Asp side chains, respectively, generate *tert*-butyl carbocation species. This highly reactive electrophilic species shall be quenched *in situ* by appropriate nucleophiles added to the TFA cleavage cocktail, i.e., scavengers, in order to prevent reattachment or modification of the deprotected nucleophilic side chains, such as thiol function on cysteine.[26,27]

In the specific case of the eptifibatide linear precursor, cysteine and MPA thiol functions represent possible electrophilic attack acceptors. In this framework, MS/MS analysis of the peak at R_t = 5.154 min revealed the fragment $[M + H]^+$ m/z = 487.3 corresponding to the b_4 fragment with *tert*-butyl carbocation reattachment on MPA thiol function. Therefore, we demonstrated that Imp a is the *tert*-butyl derivative of the eptifibatide linear precursor (Figure S5). Consequently, we can hypothesize that R_t = 5.333 min (Imp b) corresponds to the impurity of the *tert*-butyl derivative on cysteine thiol function.

Cysteine and MPA thiol functions in the crude could be both deprotected from the newly formed StBu-peptide side product only using hazardous reagents, requiring expensive strong acid-resistant equipment. However, since Imp a and Imp b cleaning from the eptifibatide TFA salt after purification (step 4) led to residual amounts compliant with international regulatory standards, we did not consider strong acid treatments.

The best crude UHPLC purity was achieved performing the cleavage in two steps. In the first 30 min, two different cleavage cocktails were tested at 2 mmol, and the data are shown in Table 1. In the second step, a further 230 mL of TFA were added and maintained under stirring for 2.5 h at r.t. to maximize eptifibatide linear precursor crude recovery. Both cleavage conditions led to comparable impurities (Table 1). However, the best crude UHPLC purity was achieved using entry 1 mixture. Therefore, in the final cleavage mixture selected for the multigram-scale synthesis, the TFA/H_2O/TIS/DODT ratio was 91:2.3:2.3:4.4 (v/v/v/v), which corresponds to 0.11 M TIS, 0.33 M DODT, and 1.3 M H_2O.

Table 1. Cleavage Cocktails Tested to Obtain the Highest UHPLC Crude Purity

entry	1st step cleavage mixture volume ratio	UHPLC crude purity,[a] %	Imp a [a] % / Imp b [a] %
1	TFA/H_2O/TIS/DODT	61.5	4.1
	(72:7:7:14)		1.8
2	TFA/H_2O/TIS/DODT	57.1	3.6
	(60:10:10:20)		1.0

[a]Method used to isolate eptifibatide linear precursor crude: Et_2O (80 mL/g resin), r.t., 30 min stirring; 0 °C and 60 min stirring.

Moreover, Imp c was hypothesized to correspond to the intermediate of the eptifibatide linear precursor containing Trp-carbamic acid ($[M + H]^+$ m/z = 878.32).[28,29] In fact, deprotection of the tBoc protecting group from Trp indolyl side-chain occurs via a stepwise pattern of tBu carbocation formation followed by acidolysis of the Trp-carbamic acid intermediate derivative. Interestingly, slow decarboxylation kinetics are distinctly beneficial to avoiding additional impurity formation by electrophilic attack of the tBu carbocation to the indolyl side chain, otherwise favored in the presence of unprotected Trp.

Our hypothesis was demonstrated treating an isolated IPC of the eptifibatide linear precursor crude with 0.1% (v/v) TFA in H_2O/CH_3CN 2:1 (v/v) mixture and analytically monitoring the disappearance of the peak at R_t = 4.258 (completed in 60 min, Figure 3). Therefore, a simple, longer deprotection step should be sufficient for the disappearance of Imp c.

Evaluation of the Optimal Antisolvent to Precipitate Eptifibatide Linear Precursor Crude. Eptifibatide linear precursor crude was precipitated after filtering off the resin from cleavage solution using an antisolvent. Diethyl ether (Et_2O), diisopropyl ether (iPr_2O), methyl-*tert*-butyl ether (MTBE), and cyclopentyl-methyl ether (CPME) were tested, also considering toxicity and safety (Table 2). The high-cost CPME, recently introduced by Albericio and co-workers as a

https://doi.org/10.1021/acs.oprd.1c00368
Org. Process Res. Dev. 2021, 25, 2754−2771

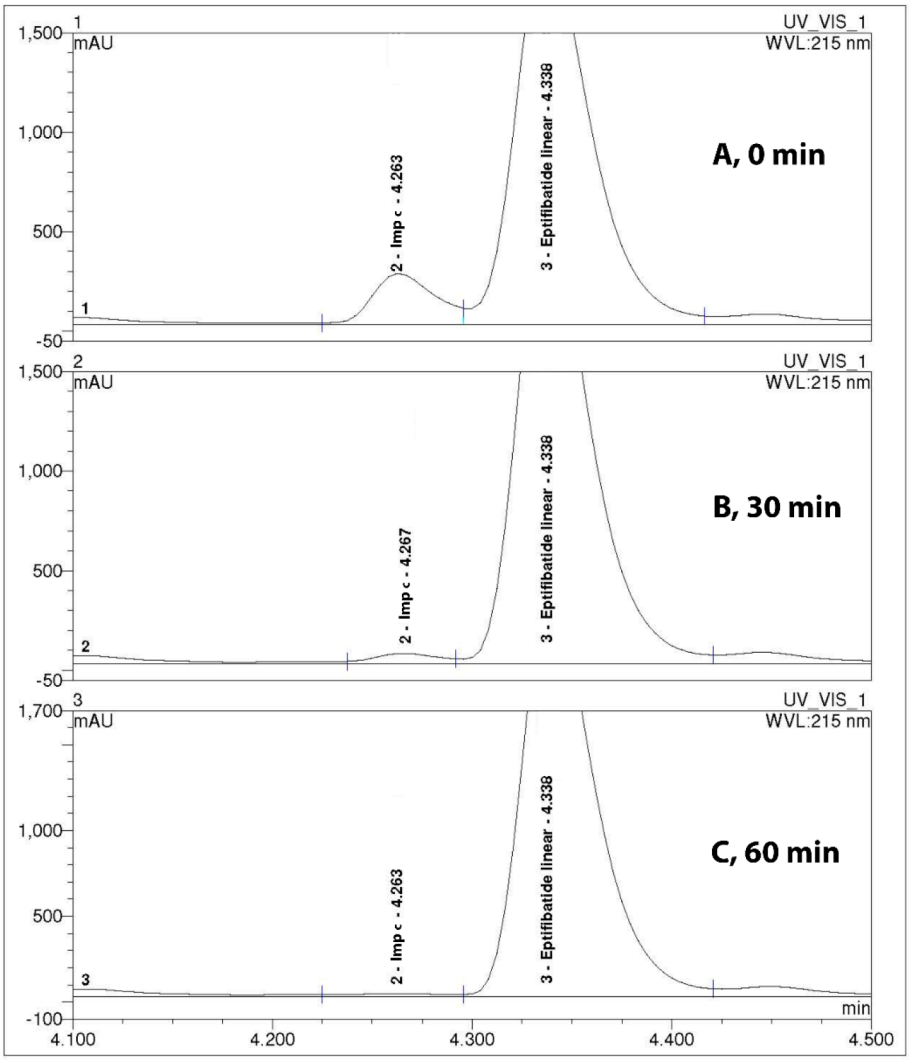


Figure 3. RP-UHPLC traces of eptifibatide linear precursor crude monitoring Trp(Boc) deprotection at different reaction times (zoom 4.1−4.5 min). Panel A, 0 min; panel B, 30 min; panel C, 60 min. C18 column Waters Acquity CSH (130 Å, 1.7 μm, 2.1 × 100 mm); temperature 45 °C; flow, 0.5 mL/min; eluent, 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. R_t = 4.26 ± 0.015 min: Imp c. R_t 4.34 ± 0.01 min: linear eptifibatide precursor.

Table 2. UHPLC Purity Profile, Yield, and Cost of Eptifibatide Linear Precursor Crude Obtained after Resin Cleavage and Precipitation Using Different Solvents

entry	synthesis scale, mmol	antisolvent	UHPLC crude purity,[a] %	Imp a [a]% Imp b [a]%	crude yield,[b] %	cost,[c] €/L
1	0.18	Et_2O	57.0	7.24 2.57	52.3	40−100
2		iPr_2O	58.8	6.85 2.57	73.4	40−50
3		MTBE	55.4	7.18 2.67	73.9	46−65
4		CPME	66.4	4.60 1.63	62.8	100−200
5	1.80	iPr_2O	60.7	6.45 1.05	73.5	40−50

[a]Resin cleavage cocktail: TFA/H_2O/TIS/DODT 92.5:2.5:2.5:2.5 (v/v/v/v), 20 mL/g resin, stirring 3 h at r.t. Method used to isolate eptifibatide linear precursor crude: 80 mL solvent/g resin; r.t., 30 min stirring; 0 °C, 150 min stirring. [b]Yield (%) = $\frac{\text{found weight} \times \left(\frac{\text{peptide content}}{100}\right)}{\text{calcd weight}} \times 100$ (UV average peptide content: 72.0%). [c]Analytical-grade solvent costs for lab-scale production.

https://doi.org/10.1021/acs.oprd.1c00368
Org. Process Res. Dev. 2021, 25, 2754−2771

"green alternative to the hazardous Et_2O and MTBE", did not fulfill industrial needs.[30,31]

Despite the good performances in terms of cost (46−65 €/L), crude precipitate yield (73.9%), and UHPLC purity (55.4%), the antisolvent MTBE (usually considered as an industrial alternative to Et_2O, typically used in lab-scale) has the disadvantage of being classified in the list of substances of very high concern (SVHC) drafted by the European Chemicals Agency (ECHA) as hazardous chemicals for both the environment and human health.[32−34] After evaluation of all factors, iPr_2O was selected as an antisolvent because of low volatility, facilitating large volume management (crude yield 73.4% and UHPLC purity 58.8%) with a competitive cost (40−50 €/L). Moreover, the 10-fold scale-up of crude precipitation conditions maintained the same performance (Table 2, entries 2 and 5).

Scale-up Activities of Cleavage Step (Step 2). The cleavage step process on both the 5 and 70 mmol scale shows that the scale-up produced extremely comparable UHPLC crude purities (5 mmol, 74.1%; 70 mmol, 73.7%) and yields (5 mmol, 84.6%; 70 mmol, 82.2%) as reported in Table 3.

Table 3. Cleavage Step Comparative Analysis at 5 mmol vs 70 mmol Scale, to Obtain Eptifibatide Linear Precursor: UHPLC Crude Purity Profile and Yield

step 2 output[a,b]		70 mmol	5 mmol
eptifibatide linear precursor crude	UHPLC purity (%)	73.7	74.1
	yield[c] (%)	82.2	84.6
impurities	Imp a (%)	5.5	5.3
	Imp b (%)	3.5	2.1

[a]Loading of Rink amide AM resin: 0.97 mmol/g. Coupling conditions: single coupling 1 × 2.5 equiv building block (0.4 M), 1 × 2.5 equiv DIC (3 M), and 1 × 2.5 equiv Oxyma pure (1 M). All reagents were dissolved in DMF. Final resin washing: 9.3 mL/g resin (3 × iPrOH). [b]Resin cleavage cocktail: TFA/H_2O/TIS/DODT 72:7:7:14 (v/v/v/v); 7 mL/g resin, stirring 30 min, r.t.; further addition of 15 mL TFA/g resin, stirring 2.5 h, r.t. Procedure used to isolate eptifibatide linear precursor crude: 80 mL iPr_2O/g resin, 2 h, 0 °C. [c]Yield (%) $= \frac{\text{found weight} \times \left(\frac{\text{peptide content}}{100}\right)}{\text{calcd weight}} \times 100$ (UV average peptide content: 79.0%).

Moreover, both impurity profiles on the 5 mmol scale (Figure S8) and 70 mmol scale show the following: Imp a, ca. 5%; Imp b, ca. 3% (Table 3, Figure 4). Interestingly, no new impurities were detected in the 70 mmol scale crude analysis, demonstrating the successful direct scale-up process (Table S6).

Evaluation of Quality and Risk Assessment of Resin Cleavage Materials: Risk Analysis Associated with Exposure to Volatile Ethers and Peroxide Content, Exothermic Hazard, and TFA Quality (Use Test). Ethers volatility and peroxide-forming tendency are directly related to the fire and explosion propensity. Therefore, peroxide content in iPr_2O was identified as a hazard in risk assessment.[35] Moreover, peroxides can contribute to uncontrolled oxidation of amino acid side chains (such as Cys, Trp), dramatically affecting purity outcomes. Three different industrial-grade iPr_2O with different peroxide content (0, 350, >1000 ppm) were compared with an analytical-grade peroxide-free iPr_2O (Merck KGaA, Darmstadt, Germany; Table S7). A peroxide content of >1000 ppm dramatically affected the purity (32.5% vs >60%) of the crude displaying high retention time broad peaks in the UHPLC profile (oligomer formation). A slight decrease in purity was obtained with iPr_2O containing 350 ppm peroxides. However, undoubtedly, careful control of iPr_2O peroxide content (<350 ppm, ideally peroxide-free) must be performed before precipitation of the eptifibatide linear precursor crude for both safety and purity.

Precipitation of a crude eptifibatide linear precursor from TFA cleavage solution by adding iPr_2O was considered in the risk assessment management because of the exothermic effect during addition. In particular, lab-scale preliminary tests for hazard evaluation to quantitatively assess heat development during the process is fundamental for the final scale-up.[17]

Lab-scale precipitation from the TFA cleavage solution was performed following the optimized conditions described above. The temperature was maintained at <35 °C by ice-bath cooling to limit the temperature increase of the TFA solution during the exothermic addition of iPr_2O at 4 °C. In the 70 mmol pilot scale process, the temperature of the mixture was maintained at <35 °C using a thermostated jacketed reactor under stirring to manage the temperature increase developed during the exothermic iPr_2O addition. This procedure allowed both to

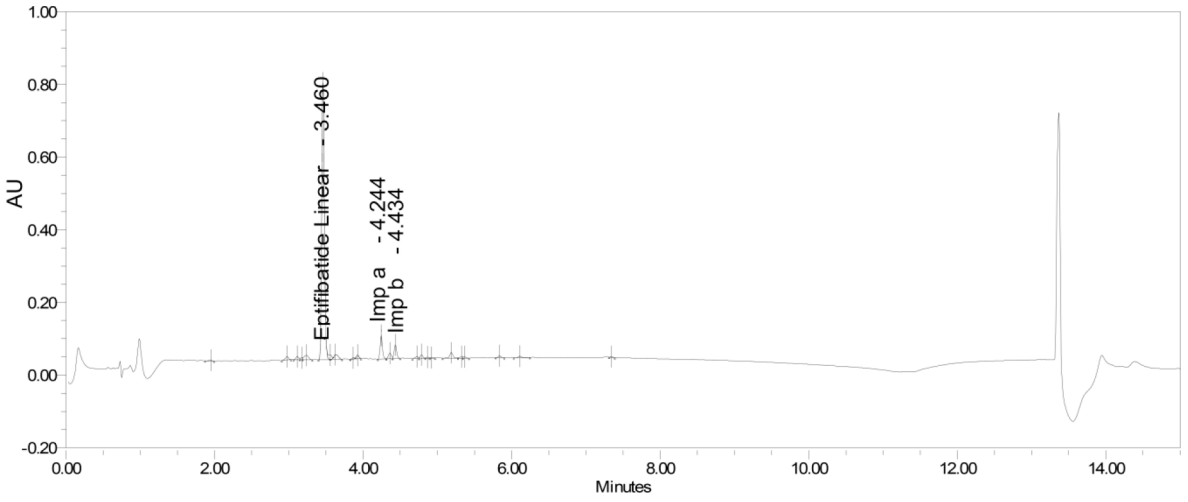
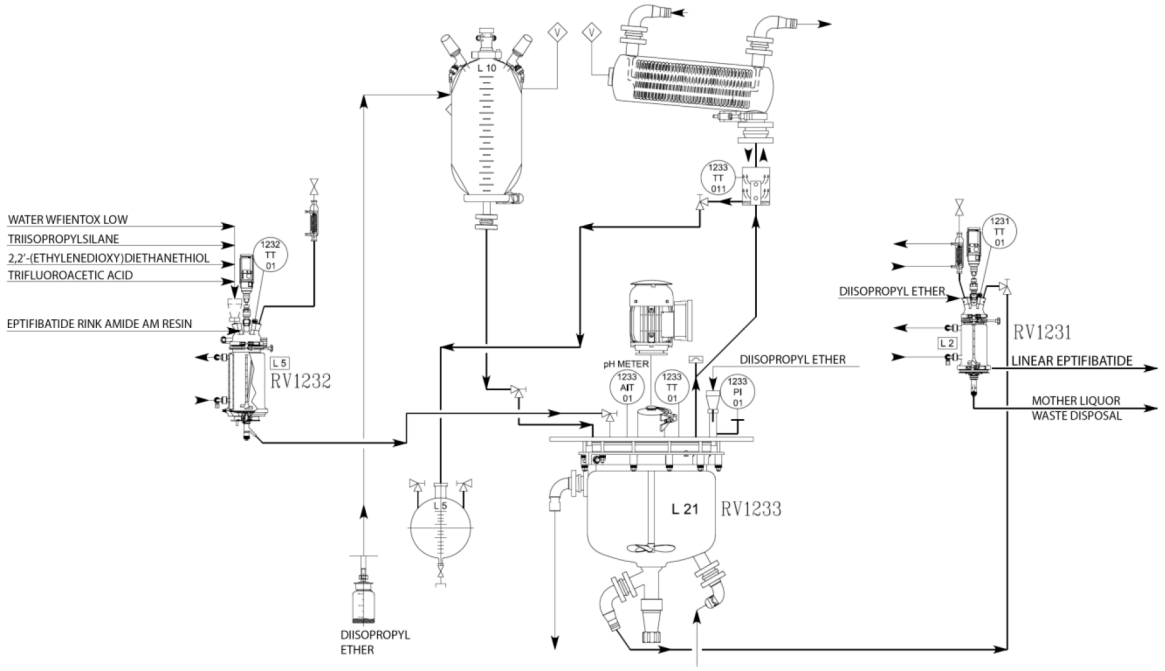


Figure 4. RP-HPLC trace of eptifibatide linear precursor crude (step 2 at 70 mmol scale). R_t = 3.46 min eptifibatide linear precursor; R_t = 4.24 min, Imp a; R_t = 4.43, Imp b (procedure S1).

https://doi.org/10.1021/acs.oprd.1c00368
Org. Process Res. Dev. 2021, 25, 2754−2771

Scheme 2. Industrial Plant Scheme Designed for Resin Cleavage, Side-Chains Deprotection, and Isolation of Crude Eptifibatide Linear Precursor TFA Salt (Table S9)



mitigate workplace safety risks and to guarantee eptifibatide linear precursor crude quality (data not shown).

TFA Quality Check (Use Test). Industrial-grade TFA specifications declared by the supplier (appearance: clear, colorless liquid, free of particulate matter; purity: ≥99.5%; water content: ≤0.1%; UV absorbance λ, 280 nm: ≤0.002; λ, 254 nm: ≤0.005; λ, 230 nm: ≤0.090) were confirmed before use, by the Quality Control Laboratory in FIS - Fabbrica Italiana Sintetici S.p.A. Industrial-grade TFA withdrawn from a storage tank was tested in a cleavage reaction performed on a resin batch obtained on the 5 mmol scale and compared with a second cleavage reaction performed under the same conditions using analytical-grade TFA (Aldrich-grade). Since the UHPLC purity of both eptifibatide linear precursor crudes were comparable (data not shown), the use of industrial-grade TFA was considered acceptable, decreasing the process cost.

Identification of Hold Points in the Scale-up Process. Our industrial production system was organized in batches. The adopted multistep process would need to be stopped before the term to manage any technical unforeseen events or inspection and test plan process (hold points). In particular, during the pilot-scale MW-SPPS (step 1), hold points were identified only after each coupling cycle (including washings and draining), before Fmoc-deprotection of the last building block on the resin. This was to avoid leaving reactive free amino functions on the resin in the reactor.

In addition, resin cleavage and side-chain deprotection under TFA harsh acidic conditions should be performed without delay to preserve purity and yield. The filtered solution containing the peptide crude in TFA was held at room temperature. After 24 h, the crude was precipitated in iPr_2O at 0 °C for 1 h. UHPLC crude purity dramatically decreased compared with the one obtained after immediate precipitation (data not shown). Therefore, the hold point to consider was the precipitation phase. A stability study of eptifibatide linear precursor crude was performed maintaining the suspension after precipitation in iPr_2O at 0 °C for 1, 3, and 24 h. Monitoring UHPLC purity demonstrated crude stability up to 24 h (Table S8). Therefore, we established a TFA cleavage/deprotection treatment time of 0−1 h and a waiting time in the presence of iPr_2O at 0 °C of 3−24 h.

cGMP Equipment and Facilities for the 70 mmol Scale MW-SPPS (Step 1) and Resin Cleavage (Step 2). cGMP peptide API production requires the qualification of manufacturing equipment, proving the suitability of the system. The qualification process consists of four main actions: design, installation, operational, and performance qualifications. The design has to consider final product features, minimizing external contaminations, which could affect product quality. The plant was designed to include a walk-in fume hood (4 m^2) hosting the Liberty Pro MW-assisted synthesizer (CEM, Charlotte, NC, U.S.A) and to allow easy-cleaning procedures both before and after solid-phase peptide manufacturing (step 1). The equipment to perform step 2 was designed to limit both air exposition and operator's handling (Scheme 2).

The amount of starting material and reagents used for both cleavage and side-chain deprotection (step 2) are reported in Table 4.

After the drying session under N_2 inside the Liberty Pro reaction vessel, the linear precursor-resin was transferred into

Table 4. Starting Material and Reagents Used for Both Cleavage and Side-Chain Deprotection (Step 2)

chemical	vol (L)	amount (kg)
eptifibatide linear precursor-resin		0.170
H_2O low endotoxin	0.5	0.085
TIS	0.5	0.066
DODT	1	0.190
TFA	5 (Initial wash) + 22	6.90
iPr_2O	11.8 (Initial wash) + 92	12.68

https://doi.org/10.1021/acs.oprd.1c00368
Org. Process Res. Dev. 2021, 25, 2754−2771

the 5 L jacketed glass filter reactor (RV1232), thermostated at 20 °C. The cleavage cocktail was added from the top of the reactor, and the reaction was carried out under constant stirring. Then, the peptide-cleavage cocktail solution was transferred into the 21 L jacketed glass reactor (RV1233) through the filter on the bottom of the 5 L reactor (RV1232). The transfer to the receiving reactor was performed under vacuum conditions. Furthermore, the 5 L reactor bottom was linked to the head of the 21 L reactor (RV1233) by an inert Teflon tube. The 21 L reactor (RV1233) was thermostated at 0 °C and equipped with two thermometers (one inside the reactor and one immediately before the condenser) and one pHmeter (switched off during this step). The head of the 21 L reactor is linked to a 10 L glass reservoir by an inert Teflon tube and to a double-coil glass condenser (water-cooled) that is linked to a 5 L glass collection flask. Ether addition to the peptide-cleavage cocktail solution was performed while monitoring that the temperature was maintained at <35 °C.

After crude precipitation, the suspension was transferred under vacuum conditions to the 2 L jacketed glass filter reactor (RV1231) by an inert Teflon tube connecting the bottom of the 21 L reactor (RV1233) to the head of the 2 L reactor (RV1231). Final crude filtration was performed at 0 °C. Both transfer of the suspension from the 21 L reactor (RV1233) and eptifibatide linear precursor crude filtration were performed under vacuum conditions. Finally, after a drying session under N_2, the filtering bottom was disassembled from the rest of the 2 L reactor (RV1231), and the crude product was recovered.

Step 3. Disulfide Bond Formation in Solution to Obtain Crude Eptifibatide TFA Salt. Disulfide bond formation in the eptifibatide linear precursor is usually performed by an oxidant reagent. Intramolecular and undesired intermolecular reactions (dimerization/oligomerization) can affect final purity profile if crucial parameters such as, peptide concentration, pH, and type of oxidant reagent are not optimized.

We previously reported a detailed investigation on eptifibatide disulfide bond formation on the 5 mmol scale both in solution (5.3 mM peptide concentration, pH 9.5, in H_2O/CH_3CN, 2:1 (v/v)) by air oxidation over 22 h (98% conversion, 61% purity, and 6.6% dimer) and in the solid-phase. This solid-phase strategy had the advantage of synthesizing and cyclizing eptifibatide in the same reactor.[15] However, in the multigram-scale process developed herein, the use of the less expensive building-block Fmoc-Cys(Trt)-OH (compared to Fmoc-Cys(StBu)-OH used in the previously reported strategy) and the increased HPLC purity yield of eptifibatide crude obtained by air oxidation in solution (65% vs 40.9%) let us further investigate solution disulfide bond formation. Therefore, several oxidants and reaction conditions were tested,[36−40] in order to obtain the highest eptifibatide HPLC crude purity (data not shown). Disulfide bond formation in H_2O_2 aqueous solution was considered a balanced compromise in terms of efficacy, costs, sustainability, and postcleaning procedure of the equipment.

Despite of a fast conversion (<15 min) of the eptifibatide linear precursor (5.3 mM in H_2O/CH_3CN 2:1 v/v) on the 0.5 mmol scale, after the addition of 0.8 equiv of H_2O_2 in one shot at time 0, at pH 9.5 (NH_4OH), a substantial formation of a dimer impurity (>10%) was observed (Figures S9, S10, and S11). Therefore, different reaction conditions were investigated to obtain complete conversion with the highest eptifibatide TFA purity and lowest dimer impurity formation. In particular, the same equivalents of H_2O_2 (0.8 equiv) were added in two or four portions (Table S10), and then the linear precursor concentration was decreased (Table S11). Two additions of 0.44 equiv of H_2O_2 (at 0 and 60 min, respectively, panel 1, Table 5) compared to 4 × 0.22 equiv (at 0, 30, 60, and 90 min, panel 2, Table 5) did not influence either the eptifibatide TFA yield or the dimer impurity formation. However, in the scale-up process, the possibility of four sequential additions of a lower number of H_2O_2 equiv/time allowed a more accurate in process control of the oxidation reaction (Figure 5).

Table 5. Disulfide Bond Formation Comparative Analysis at 5 mmol vs 70 mmol Scale to Obtain Eptifibatide TFA: UHPLC crude Purity Profile and Yield

step 3 output[a]		70 mmol[c]	5 mmol
eptifibatide crude	UHPLC purity (%)	66.4	67.4
	yield (%)[b]	98.3	90.7
impurities	dimer (%)	5.3	5.9

[a]Conditions for disulfide bond formation (70 and 5 mmol): Eptifibatide linear precursor crude (2.1 mM in H_2O:CH_3CN 2:1 v/v) was oxidized adding H_2O_2, pH 9.5 (NH_4OH), 2 h, r.t. [b]Yield (%) $= \frac{\text{Eptifibotide crude weight} \times \left(\frac{\text{peptide contnet}}{100}\right)}{\text{Eptifibotide linear crude weight} \times \left(\frac{\text{peptide content}}{100}\right)} \times 100$. UV eptifibatide crude average peptide content: 71.0%. UV eptifibatide linear precursor crude average peptide content: 79.0%. [c]The procedure was repeated five times on the 10 mmol scale on cGMP compliant glassware.

Therefore, two different concentrations of linear eptifibatide precursor crude (2.1 mM and 1.6 mM) with the addition of 4 × 0.22 H_2O_2 were tested on the 0.5 mmol scale (Figure 6). Lowering the linear precursor concentration from 5.3 mM (entry 2, Table S10) to 1.6 mM (entry 2, Table S11) led to a dramatic decrease of dimer impurity formation (from 8.0% to 3.9%) and an increase in UHPLC purity of eptifibatide TFA (up to 67%).

In conclusion, the identified optimized conditions (eptifibatide linear precursor crude 1.6 mM in H_2O/CH_3CN 2:1 (v/v), pH 9.5 (NH_4OH), 4 × 0.22 equiv H_2O_2 additions at 0, 30, 60, and 90 min) allowed a decrease in reaction time from 22 to 2 h (Figures 5 and 6).

Scale-up Activities of Oxidation in Solution. The above-described optimized conditions for disulfide bond formation were tested on the 10 mmol scale and repeated twice in cGMP compliant glassware. In particular, a comparative analysis of the step 3 process (Table 5) shows that the scale-up produced extremely comparable UHPLC crude purities (5 mmol, 67.4%; 70 mmol, 66.4%) and increased yield (5 mmol, 90.7%; 70 mmol, 98.3%). Moreover, scale-up in process control demonstrates that dimer impurity formation was limited to <6% (Figures S12, S13, and Figure 7).

Concerning step 3 scale-up, a specific cGMP compliant industrial plant was designed both for off-resin disulfide bond formation and isolation of crude eptifibatide TFA salt by freeze-drying (Scheme 3). The amount of starting material and reagents used for pilot disulfide bond formation (step 3) are reported in Table 6.

A 21 L jacketed glass reactor (RV1233) was thermostated at 20 °C. After loading from the top of the reactor, the eptifibatide linear precursor crude was solubilized in the mixture WFI/CH_3CN 2:1 (v/v) previously prepared in a 10 L

https://doi.org/10.1021/acs.oprd.1c00368
Org. Process Res. Dev. 2021, 25, 2754−2771

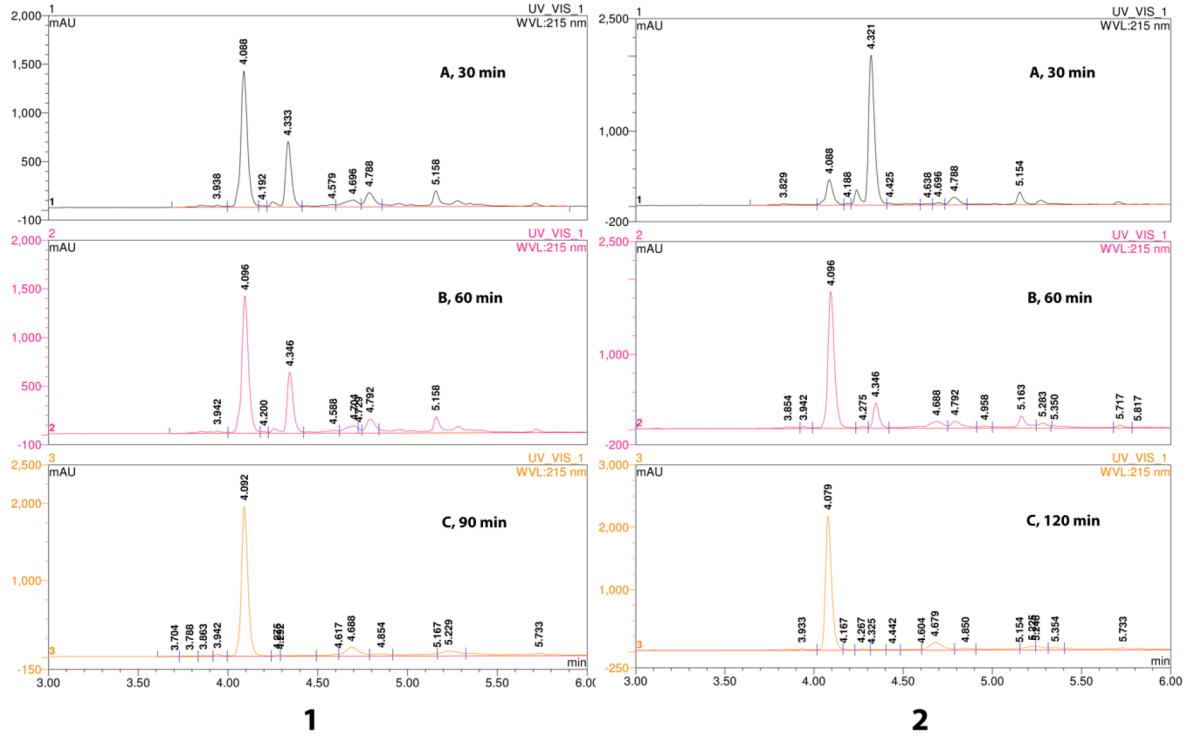


Figure 5. In process control of off-resin disulfide bond formation (0.5 mmol scale): RP-UHPLC traces of eptifibatide crude obtained by eptifibatide linear precursor crude in H_2O/CH_3CN 2:1 (v/v); 5.3 mM, with two (panel 1; entry 1, Table S10) and four (panel 2; entry 2, Table S10) additions of H_2O_2 at different times (A–C) and with pH 9.5, r.t. RP-UHPLC: 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, 5–95% B in 10 min. R_t = 4.085 ± 0.015 min, eptifibatide TFA; R_t = 4.33 ± 0.01 min, eptifibatide linear precursor; R_t = 4.77 ± 0.015 min, dimer impurity.

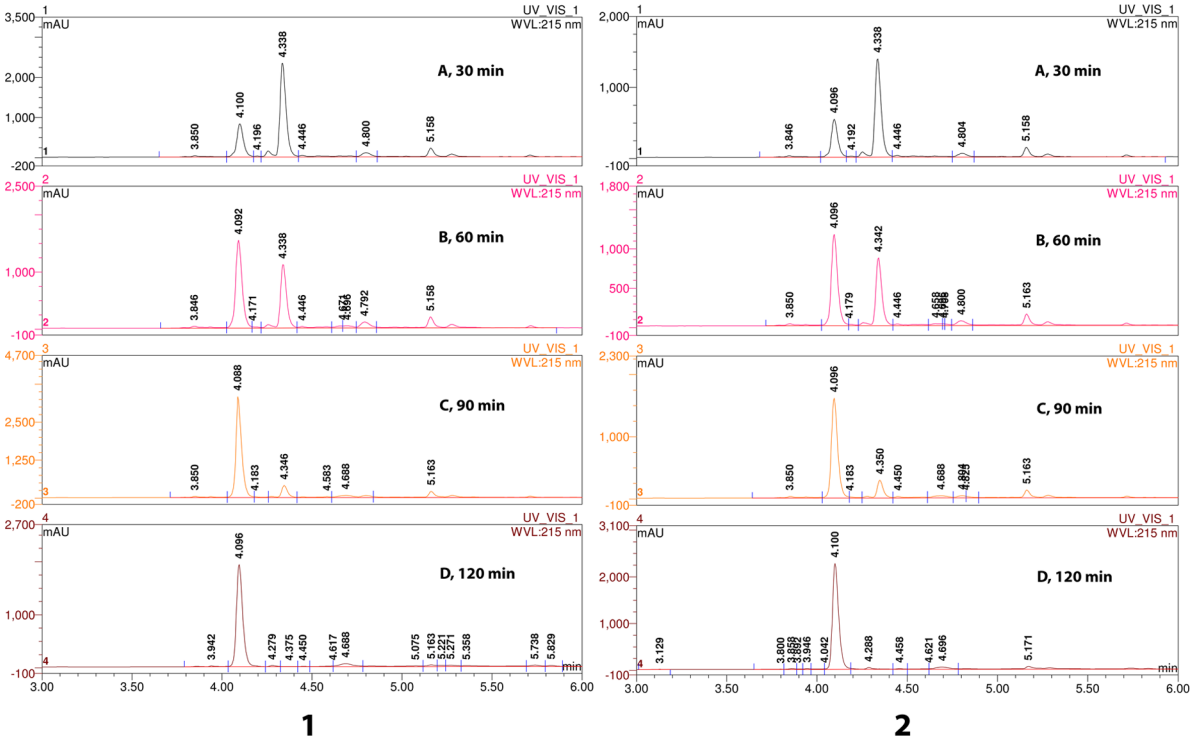


Figure 6. In process control of off-resin disulfide bond formation (0.5 mmol scale): RP-UHPLC traces of eptifibatide crude obtained by eptifibatide linear precursor crude in H_2O/CH_3CN 2:1 (v/v) at concentrations 1 (2.1 mM) and 2 (1.6 mM), with 4 × 0.22 equiv H_2O_2 additions at 0, 30, 60, and 90 min (Table S11); pH 9.5; r.t. RP-UHPLC: 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, 5–95% B in 10 min. R_t = 4.09 ± 0.015 min, eptifibatide TFA; R_t = 4.33 ± 0.01 min, eptifibatide linear precursor; R_t = 4.79 ± 0.015, dimer impurity.

https://doi.org/10.1021/acs.oprd.1c00368
Org. Process Res. Dev. 2021, 25, 2754–2771

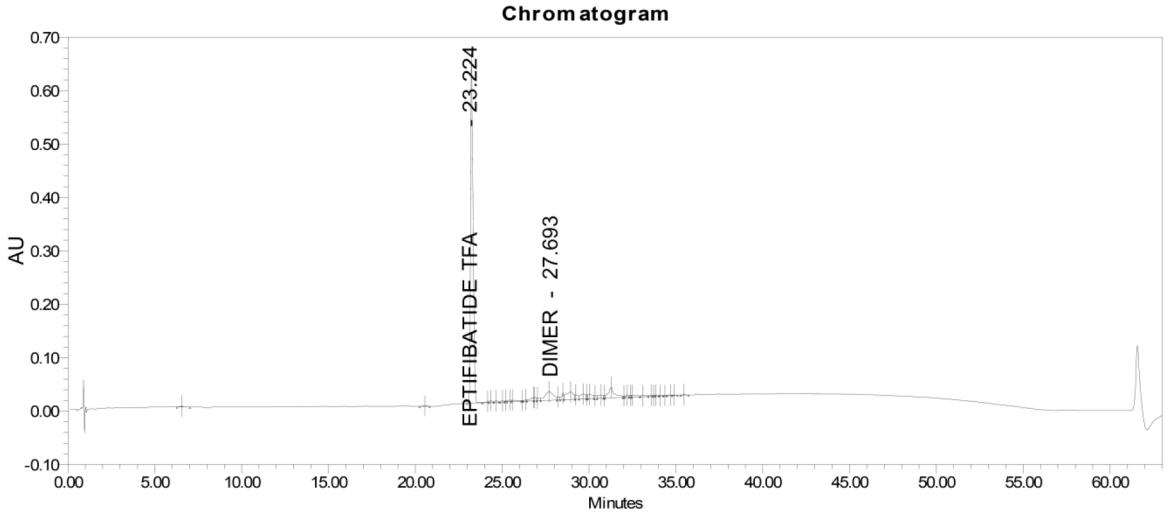


Figure 7. RP-UHPLC trace of eptifibatide TFA (step 3 at 70 mmol scale). R_t = 23.2 min, eptifibatide TFA; R_t = 27.7 min, dimer impurity (procedure S3).

Scheme 3. Industrial Plant Scheme Designed for off-Resin Disulfide Bond Formation to Obtain Crude Eptifibatide TFA Salt (Table S9)

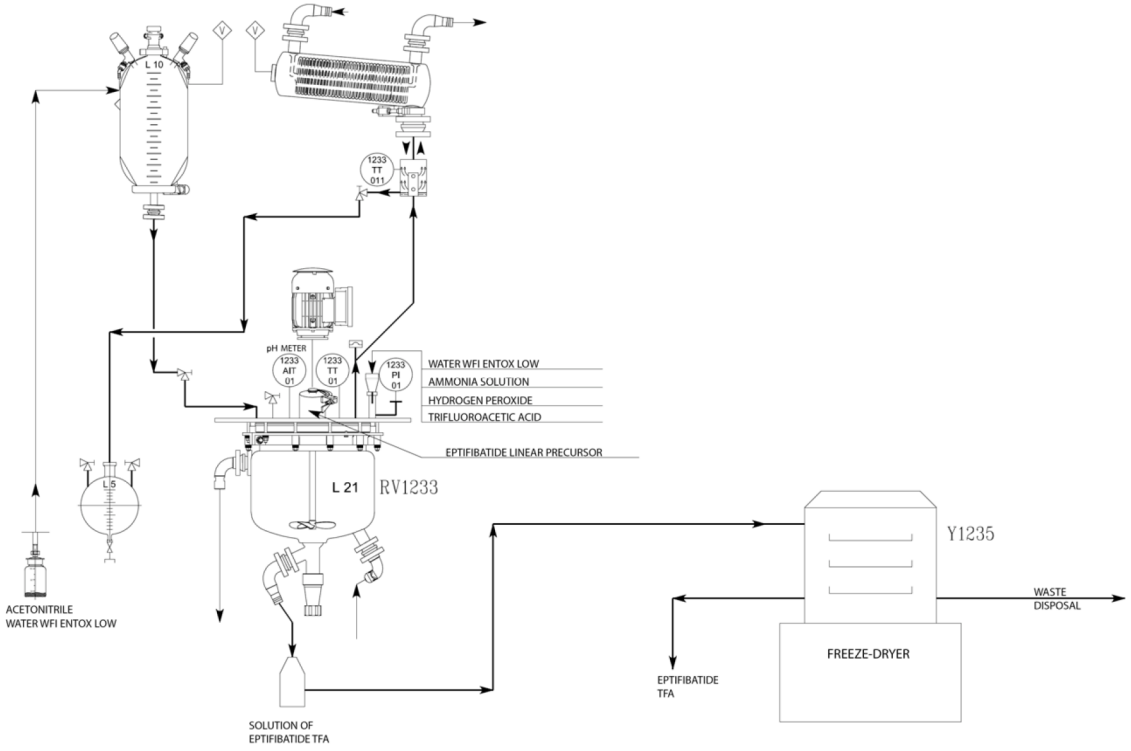


Table 6. Starting Material and Reagents Used for Disulfide Bond Formation (Step 3)

chemical	vol (mL)	amount (g)
eptifibatide linear precursor crude		10.0
CH_3CN	1500.0	1173.0
H_2O low endotoxins	3485.0	3485.0
NH_4OH	0.5	0.45
H_2O_2	3 × 0.2	3 × 0.3
formic acid	15.0	18.3

glass reservoir connected to the head of the reactor by an inert Teflon tube. The pH was adjusted to 9.5 adding dropwise NH_4OH (0.25 M in H_2O) and monitoring by a pHmeter inside the reactor (AIT1233).

The reaction mixture was recovered from the bottom of the reactor and transferred to steel plates into the freeze-dryer after monitoring the complete conversion by IPC via RP-UHPLC. Lyophilization consisted of three phases: reaction mixture freezing (P atm, −60 °C), freeze-drying (0.9 mbar, −60 °C to r.t.), and room temperature drying (0.9 mbar, r.t.). The process continued until water content and residual solvents satisfied critical quality attributes (Table S6).

Step 4. Purification of Crude Eptifibatide: Flash Column Chromatography. RP-UHPLC ESI-MS analysis of crude eptifibatide TFA salt, obtained by off-resin disulfide

https://doi.org/10.1021/acs.oprd.1c00368
Org. Process Res. Dev. 2021, 25, 2754−2771

bond formation in step 3 (Figures S13 and Figure 7), shown on both 5 and 70 mmol scale that the main impurity (5.3% and 5.9%, respectively) was in agreement with the formation of dimers (Table 5). A careful investigation of purification conditions by medium pressure chromatography was performed. In particular, flash reverse-phase chromatography (RPC) was selected as a versatile technology based on an automatic, low-cost, easy-handling purification both for laboratory and pilot scales, such as Isolera One (Biotage, Uppsala, Sweden) equipped with the HP-Sphere C18 column characterized by 25 μm spherical silica particles.[41,42]

The goal was to obtain eptifibatide TFA purity at >98.5%, limiting both eluents consumption and the volume of eluted fractions to freeze-dry. Several parameters were considered: flow rate, eluents composition, column performance, and column cleaning procedure. Moreover, elution mode (linear or step gradient), column loading, and volume to be lyophilized after pooling homogeneous fractions were evaluated. Linear gradient is characterized by an automated gradual increase of strong solvent percentage over time, ensuring result reproducibility and minimizing the possibility of error. Compared to the step gradient method, no elution band broadening occurs. Consequently, fraction volumes to be lyophilized and related costs are decreased.

Starting from 5% eptifibatide TFA crude column loading, two linear gradients were tested: 5−30% (v/v) B in A vs 10−37% (v/v) B in A, both eluted in 12 column volumes (A, 0.1% (v/v) TFA in H_2O; B, 0.1% (v/v) TFA in CH_3CN). While a 5−30% (v/v) B in A linear gradient required 0.8 L of B and 2.4 L of A (Figure S14), the 10−37% (v/v) linear gradient required a slightly lower amount of eluents (0.7 L B and 2.2 L A, Figure S15). On the other hand, yield increased from 24.7% to 34.3% producing the same eluted volume (165 mL, Table 7). We demonstrated that crude loading should be maintained <5% to achieve the highest purity and recovery yield. Despite 8% loading requiring a lower number of purification batches, lower yield was observed (Table 7, 18.1% yield, Figure S16).

Table 7. Recovery, Yield, and Eluate Volume from Eptifibatide TFA Flash Column Chromatography Purification (Step 4)

entry	gradient B in A,[a] %	column loading, %	purity range, %	recovery, g	yield,[b] %	eluate, mL
1	5−30	5	>98.5	1.3	24.7	165
			95−98	1.0		180
			90−92	1.3		405
2	10−37	5	>98.5	1.8	34.3	165
			95−98	0.8		150
			90−92	0.523		300
3	10−37	8	>98.5	1.45	18.1	150
			95−98	1.23		195
			90−92	1.24		390

[a]Flash column chromatography conditions: eptifibatide TFA crude was purified with Biotage Isolera One equipped with a SNAP Ultra C18 120g column (volume 164 mL). Eluents: 0.1% (v/v) TFA in H_2O (A), 0.1% (v/v) TFA in CH_3CN (B); 12 column volumes linear gradients; flow rate, 50 mL/min; λ, 215 nm. [b]Yield (%) $= \frac{\text{Eptifibatide purified weight} \times \left(\frac{\text{peptide content}}{100}\right)}{\text{Eptifibatide crude weight} \times \left(\frac{\text{peptide content}}{100}\right)} \times 100$. UV Eptifibatide TFA average peptide content: 85%. UV Eptifibatide TFA crude average peptide content: 71%.

Fractions containing eptifibatide TFA with 90−98.5% purity (recovery) were collected for further purification to reach the required purity, increasing the final yield (data not shown).

Scale-up Activities of the Flash Column Chromatography Purification. Purification experimental conditions of eptifibatide TFA salt (linear gradient 10−37% (v/v) B in A in 12 column volumes (A, 0.1% (v/v) TFA in H_2O; B, 0.1% (v/v) TFA in CH_3CN; crude loading ≤ 5%) were successfully confirmed at 5 mmol and scaled-up to 10 mmol scale to be repeated five times on cGMP compliant equipment (HPLC purity 5 mmol, 99.2%; 70 mmol, 99.3%; yield 5 mmol, 40.5%; 70 mmol, 41.6%). All the impurities identified in previous steps were observed to be <0.5% after flash column chromatography purification (Table 8, Figure 8, and Figures S17−S19), in agreement with ANDA requirements for eptifibatide, obtained following the cGMP process described herein.

Table 8. Flash Column Chromatography Purification Comparative Analysis at 5 mmol vs 70 mmol Scale, to Obtain Eptifibatide TFA: HPLC Purity Profile and Yield

step 4 output[a]		70 mmol[c]	5 mmol
eptifibatide TFA	HPLC purity (%)	99.3	99.2
	yield[b] (%)	41.6	40.5
impurities	Imp a (%)	<0.1	<0.1
	Imp b (%)	<0.1	<0.1
	Des-Har^2-eptifibatide (%)	<0.1	<0.1
	dimer (%)	<0.1	<0.1
	unknown (%)	<0.5	<0.5

[a]Flash column chromatography conditions: eptifibatide TFA crude was dissolved in H_2O/CH_3CN 1:1(v/v) 0.35M. Column loading: 4% (5 mmol), 2.5% (70 mmol). Eluents: 0.1% (v/v) TFA in H_2O (A) and 0.1% (v/v) TFA in CH_3CN (B). Gradient: 10−37% (v/v) B in A linear gradient in 12 column volumes. [b]Yield (%) $= \frac{\text{Eptifibatide purified weight} \times \left(\frac{\text{peptide content}}{100}\right)}{\text{Eptifibatide crude weight} \times \left(\frac{\text{peptide content}}{100}\right)} \times 100$. UV peptide content of purified eptifibatide, 85%; UV peptide content of eptifibatide crude, 71%. [c]The procedure was repeated five times on the 10 mmol scale on cGMP compliant equipment.

Step 5. Counterion Exchange to Obtain Eptifibatide Acetate Salt. Eptifibatide commercialized under the trade name INTEGRILIN has been approved by the FDA as an acetate salt.[43] Considering that resin cleavage, side-chain deprotection, quenching of the reaction to form a disulfide bond, and final purification, performed in steps 2−4, required the use of harmful trifluoroacetic acid (pK_a = 0), complete counterion exchange of TFA with an acetate anion (pK_a = 4.5) was necessary. Therefore, we performed an SPE on the same equipment used for step 4 (Biotage Isolera, Uppsala, Sweden). In particular, eptifibatide TFA salt presents a charge-to-charge interaction between the carboxylate in TFA and the cation on the homoarginine side chain, the guanidinium group with a pK_a ca. 12.5. However, at pH >8, degradation of the disulfide bond in the drug was observed as previously described.[44] Therefore, the solution of purified eptifibatide TFA salt (output of step 4) in pure H_2O (2.6 mM) at r.t. was adjusted at pH 8 adding an NH_4OH solution (1.5% (v/v) in H_2O). Under these conditions, the peptide was completely soluble, and the resulting disulfide bond was stable (data not shown). Thus, the mixture was loaded on a C18 column. Two isocratic elutions, consisting of (a) 100% H_2O (3 column volumes) and (b) 100% of 0.5% (v/v) AcOH in H_2O (3 column volumes),

https://doi.org/10.1021/acs.oprd.1c00368
Org. Process Res. Dev. 2021, 25, 2754−2771

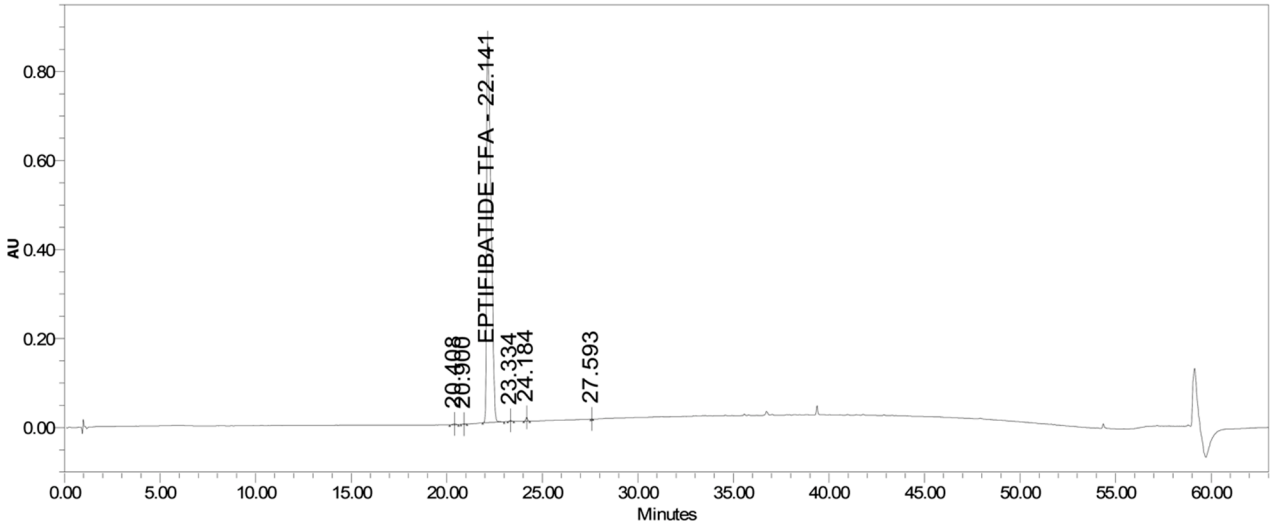


Figure 8. RP-HPLC trace of pure eptifibatide TFA (step 4 at 70 mmol scale). R_t = 22.1 min, pure eptifibatide TFA (procedure S3).

completely removed ammonium trifluoroacetate salt. Finally, the desired eptifibatide acetate solution was eluted with 0.5% (v/v) AcOH in 4:1 H_2O/CH_3CN (v/v); three column volumes. The resulting homogeneous fractions were pooled and freeze-dried to recover eptifibatide acetate.

Scale-up Activities of the Ion Exchange Step. The above-described TFA/acetate counterion exchange strategy was successfully scaled-up from 5 to 70 mmol scale (10 mmol scale repeated 5 times on cGMP compliant equipment) obtaining exactly the same 99.6% HPLC purity and slightly increased yield (5 mmol, 64.6%; 70 mmol, 65.8%, Table 9, Figure S20, and Figure 9). Since TFA residual content is considered a critical quality attribute (CQA) affecting the safety of preclinical and clinical applications, the above-described TFA/acetate exchange procedure was evaluated for its TFA residual content that was demonstrated to be significantly below the cGMP specification limit (5000 ppm). Moreover, acetate content resulted in a 4−10% specification range (Table 9).

Table 9. Ion Exchange Comparative Analysis at 5 mmol vs 70 mmol Scale, to Obtain Eptifibatide Acetate: HPLC Crude Purity Profile and Yield

step 5[a] output		70 mmol[c]	5 mmol
eptifibatide	HPLC purity (%)	99.6	99.6
	yield[b](%)	65.8	64.6
counter Ion	acetate (% w/w)	3.9	4.6
	TFA (ppm)	230	335

[a]Ion exchange conditions: purified eptifibatide TFA was dissolved in H_2O (2.6 mM), column loading 0.8%, pH 8.0 (NH_4OH). Eluents: 0.5% (v/v) AcOH in H_2O (A), 0.5% (v/v) AcOH in CH_3CN (B), H_2O (C), and CH_3CN (D). Elution: a) 100% C, 3 column volumes; b) 100% A, 3 column volumes; c) 20% B in A, 3 column volumes; d) 100% D, 2 column volumes. [b]Yield (%) = $\frac{\text{eptifibatide acetate weight} \times \left(\frac{\text{peptide content}}{100}\right)}{\text{eptifibatide purified TFA weight} \times \left(\frac{\text{peptide content}}{100}\right)} \times 100$. UV eptifibatide acetate peptide content: 87%. UV eptifibatide purified TFA peptide content: 85%. [c]The procedure was repeated seven times at 10 mmol scale on cGMP compliant equipment.

Considering the yield of each step (steps 1−5; Tables 3, 5, 8, and 9), the final total yield of the eptifibatide acetate for the entire production process is 22.1%. All the listed quality attributes (Table 10) that are in agreement with the targets established in module 3 of the Common Technical Document for the Registration of Pharmaceuticals for Human Use (ICH *M4: Common Technical Document*) demonstrate that the above-described production process of eptifibatide acetate allows the release of the final cGMP compliant batch.

Quality attributes of process intermediates and a list of the equipment used to obtain cGMP compliant eptifibatide are summarized in Table S6 and Table 10. This information is required by regulatory agencies to validate the process and qualify the pilot scale plant above-described, to produce cGMP compliant eptifibatide acetate. This is, to the best of our knowledge, the first proof-of-concept of a cGMP compliant generic peptide drug synthesized by a MW-assisted solid-phase synthesizer, on the 70 mmol scale.

CONCLUSIONS

The successful transfer of the lab-scale process (5 mmol) to 70 mmol scale demonstrated the assessment of the quality risk management followed to establish an appropriate controlled manufacturing process of the cGMP eptifibatide acetate pilot batch. Critical attributes that were within CQA limits allowed the batch release of cGMP eptifibatide acetate to receive regulatory agencies' approval (Table 10). The key benefits of the new automated multigram-scale microwave solid-phase peptide synthesizer resulted in rapid production times and the ability to incorporate green chemistry protocols based on MW-SPPS improving peptide purity and minimizing excess reagents. The pilot plant equipped with this technology was qualified by F.I.S.−Fabbrica Italiana Sintetici S.p.A. for cGMP peptide production.

Our results definitely demonstrate that the strong collaboration between an academic facility and a contract development manufacturing organization, for small molecule APIs and intermediate industrial GMP production, is a powerful example of a joint laboratory satisfying the needs of a market entry strategy into peptide API production.

https://doi.org/10.1021/acs.oprd.1c00368
Org. Process Res. Dev. 2021, 25, 2754−2771

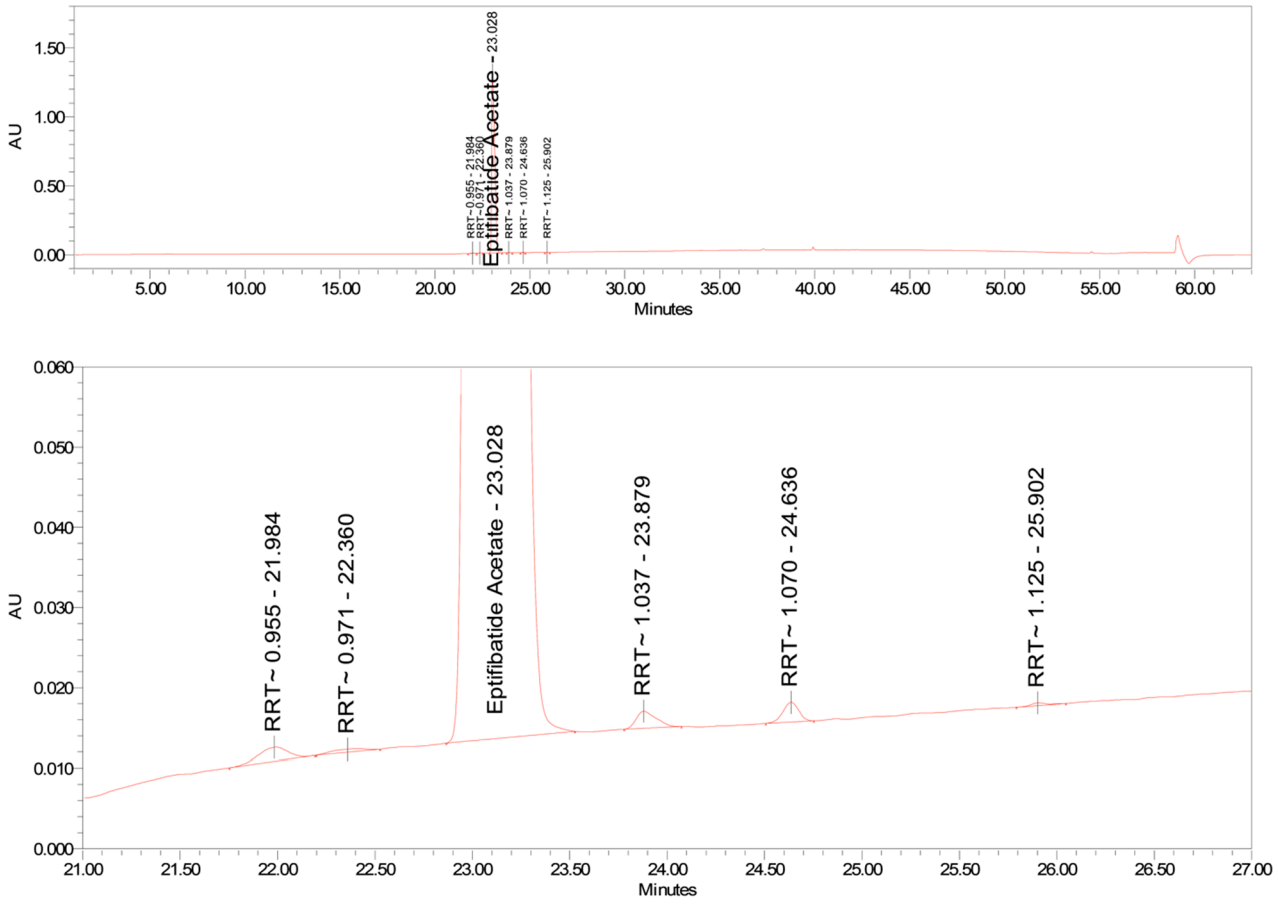


Figure 9. RP-HPLC trace of eptifibatide acetate (step 5 at 70 mmol scale). Top: full chromatogram. Bottom: zoom 21−27 min. R_t = 23.0 min: eptifibatide acetate (procedure S3).

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(Trt)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Pro-OH), aand 3-mercaptopropionic acid (MPA) were purchased from Sigma-Aldrich (Milan, Italy). Rink Amide AM resin was purchased from Sunresin New Materials Co. Ltd., Xi' AN (Shaanxi, China).

Activators *N*,*N*′-diisopropylcarbodiimide (DIC) and Oxima pure were purchased from Sigma-Aldrich (Milan, Italy). Trifluoroacetic acid (TFA), triisopropyl silane (TIS), 2,2′-(ethylenedioxy)diethanethiol (DODT), *N*,*N*-diisopropylethylamine (DIPEA), diisopropyl ether (iPr_2O), diethyl ether (Et_2O), 2-methoxy-2-methylpropane (MTBE), methoxycyclopentane (CPME), 2-propanol, dichloromethane (DCM), acetic acid (99−100%), and HPLC-grade H_2O were purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade acetonitrile (CH_3CN) was purchased from Carlo Erba (Milan, Italy).

Preparation of Eptifibatide Linear Precursor by Fmoc/tBu MW-SPPS. The fully protected eptifibatide linear precursor MPA(Trt)-Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(Trt)-Rink Amide AM resin was obtained starting from 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 orthogonally protected amino acids were added 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, and MPA(Trt)-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 by 30% (v/v) piperidine/DMF (40 equiv, 66 mL); (3) washings with DMF (3 × 50 mL); (4) coupling of 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); and (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 55 °C for cysteine coupling.

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 a vacuum to obtain 15.2 g of MPA(Trt)-Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(Trt)-Rink Amide AM resin.

In Process Control (IPC) Monitoring of the Solid-Phase Reaction Progress. A sample of peptide-resin (25 mg) was washed with DMF (3 × 3 mL) and DCM (3 × 3 mL) and dried under vacuum. Peptide cleavage from the resin and concomitant deprotection of the acid sensitive amino-acid side chains were carried out with the cocktail TFA/TIS/H_2O (1 mL, 96:2:2 (v/v/v/v)). The mixture was maintained for 30

https://doi.org/10.1021/acs.oprd.1c00368
Org. Process Res. Dev. 2021, 25, 2754−2771

Table 10. Eptifibatide Acetate Quality Attributes for cGMP Compliant Batch Release

intermediate/ step	quality attribute	output found	target
eptifibatide acetate step 5	appearance	white powder	white to pink white powder
	HPLC identity[a]	compliant	standard-compliant R_t
	HPLC purity[a]	99.6%	≥98.5%
	HPLC assay[a]	91.2% (w/w)	n.a. reference value
	HPLC total impurities[a]	0.4%	<1.5%
	HPLC single largest impurity[a]	0.1%	<0.5%
	weight	2 g	1−4 g
	water content[b]	6.6% (w/w)	<10%
	acetonitrile content[c]	105 ppm	<410 ppm
	TFA content[d]	230 ppm	5000 ppm
	acetate content[d]	3.9%	<10%
	$[\alpha]_D^{20}$[e]	−85.0°	−94.0 to −79.0°
	MS identification[f]	compliant	$[M + H]^+$ 832.3[f]
	bacterial endotoxins[g]	<1.25 EU/mg	<20 ppm
	Cd[h]	<0.25 ppm	<2 ppm
	Co[h]	<0.25 ppm	<2 ppm
	As[h]	<1 ppm	<10 ppm
	Sb[h]	<1 ppm	<20 ppm
	Ni[h]	<0.5%	<20 ppm
	Cu[h]	15 ppm	<20 ppm
	V[h]	<1 ppm	<10 ppm
	Li[h]	<0.25 ppm	<20 ppm
	Hg[h]	2 ppm	<2 ppm

[a]HPLC analytical methods are described in the Supporting Information (procedures S2, S3, and S5). [b]Water content was quantified by Karl Fisher volumetric titration (USP 921, EP 2.5.12). [c]Acetonitrile content was quantified by headspace gas-chromatography (HS-GC); analytical method is described in the Supporting Information (procedure S7). [d]Ion exchange chromatography (IC); the analytical method is described in the Supporting Information (procedure S8). [e]Specific optical rotation $[\alpha]_D^{20}$ (USP 781, EP 2.2.7). [f]Mass found detected by ESI-MS; the analytical method is described in the Supporting Information (procedure S4). [g]Bacterial endotoxins were quantified by microbiological tests (USP 85, EP 2.6.14). [h]Heavy metals were quantified by inductively coupled plasma mass spectroscopy (ICP-MS, ICH Q3D; USP232, 233, 730; EP 2.4.20).

min at 38 °C under magnetic stirring. The resin was washed with TFA (1 mL) and filtered. The crude product was precipitated with ice-cold Et_2O (4 mL), collected after centrifugation, dissolved in H_2O (1 mL), freeze-dried by a LIO5P DGT lyophilizer (5 Pascal), and analyzed by RP-UHPLC-MS on a Thermo Scientific Ultimate 3000 equipped with a variable wavelength detector and a Thermo Scientific-MSQ PLUS, using a C18 Waters Acquity CSH (130 Å, 1.7 μm, 2.1 × 100 mm; temperature, 45 °C; flow, 0.5 mL/min; eluents, 0.1% (v/v) TFA in H_2O (A) and 0.1% (v/v) TFA in CH_3CN (B); λ, 215 nm).[15]

Cleavage Step. The cleavage, with concomitant deprotection of acid sensitive amino-acid side chains was performed by treating MPA(Trt)-Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(Trt)-Rink Amide AM resin (15.2 g, 5 mmol) with the cocktail TFA/TIS/H_2O/DODT 107 mL, 72:7:7:14 (v/v/v/v) for 30 min at room temperature under mechanical stirring (150 rpm). Then, the mixture was diluted with TFA (230 mL) and stirred mechanically for 2.5 h. The resin was filtered and rinsed with fresh TFA (3 × 30 mL). The cleavage reaction mixture was transferred into a clean round-bottom flask, and the peptide was precipitated by the addition of ice-cold iPr_2O (1216 mL) in 30 min, keeping the temperature below 35 °C. The suspension was stirred for 1 h at 0 °C. The solid crude was filtered, washed with ice-cold iPr_2O (4 × 150 mL), and dried under vacuum. Characterization of the filtered eptifibatide TFA linear precursor crude was performed by analytical RP-HPLC-ESI-MS (Procedures S1, S2, and S4).

The eptifibatide TFA linear precursor crude (5.137 g, 5 mmol) showed 74.1% RP-UHPLC purity (yield 84.6%), R_t 4.3 min, ESI-MS (m/z): $[M + H]^+$ 834.4 (found), 834.9 (calcd; Figures S3 and S8).

Optimized Procedure for off-Resin Disulfide Bond Formation. Eptifibatide TFA linear precursor crude (2.5 g, 2.44 mmol, UHPLC purity 74.1%) was introduced in a 2 L round-bottom flask, dissolved in H_2O/CH_3CN (1:1 (v/v), 750 mL) solution, and maintained under stirring. After 15 min, H_2O (500 mL) was added to the reaction mixture to obtain a final concentration of 5.3 mM. The initial pH of 2.5 was adjusted to 9.5, adding 7.5% NH_4OH (v/v) in H_2O; 5.0 mL. The first aliquot of H_2O_2 (0.05 mL, 0.22 equiv) was added, and the reaction mixture was stirred for 30 min at r.t. Then, HPLC in process control (IPC) was performed to monitor the disulfide bond formation (procedure S6). This addition was repeated every 30 min until complete oxidation. A total of four additions in 2 h was necessary to complete the disulfide bond formation. The reaction was quenched adding TFA 99.9% (v/v); 5 mL, adjusting pH to 2.5. Then, the reaction mixture was lyophilized without further evaporation.

The recovered crude was characterized by analytical RP-UHPLC-ESI-MS (procedures S2, S3, and S4). Crude eptifibatide TFA salt (5.2 g, yield 90.7%) showed 67.4% UHPLC purity, R_t = 4.1 min; ESI-MS (m/z): $[M + H]^+$ 832.5 (found); 831.96 (calcd) (Figures S11 and S13).

Flash Column Chromatography Purification Procedure. Obtained crude eptifibatide TFA salt (4.90 g, 5.2 mmol, UHPLC purity 67.4%), dissolved in H_2O/CH_3CN (1:1 (v/v), 15 mL), was loaded on a SNAP Ultra C18 120g column (Biotage Isolera One, Uppsala, Sweden). Eluents: 0.1% (v/v) TFA in H_2O (A), 0.1% (v/v) TFA in CH_3CN (B). Flow rate: 25 mL/min. λ, 215 nm. Elution method: (a) 100% (v/v) A, 5 column volumes, (b) 10−37% (v/v) B in A gradient, 12 column volumes, (c) 100% (v/v) B, two column volumes. Fractions of 18 mL volume were collected and analyzed by RP-UHPLC-ESI-MS (Procedures S2, S3, and S4). Fractions corresponding to R_t = 4.1 min and UHPLC purity >98.5% were collected, obtaining a total volume of 198 mL that was lyophilized.

Eptifibatide TFA salt (1.69 g, yield 40.5%) was characterized by 99.2% UHPLC purity, R_t = 4.1 min (Figure S19). ESI-MS (m/z): $[M + H]^+$ 832.5 (found); 831.96 (calcd).

Exchange Strategy. Purified eptifibatide TFA salt (4.74 g, 5.0 mmol, UHPLC purity 99.2%) was introduced into a 3 L round-bottom flask and dissolved with pure H_2O (1923 mL, 2.6 mM), and the pH was adjusted to 8 with 1.5% NH_4OH (v/v); 9.6 mL under mechanical stirring (150 rpm). The solution was loaded on a SNAP Ultra C18 120 g column (Biotage Isolera One, Uppsala, Sweden). Eluents: 0.5% (v/v) AcOH in H_2O (A), 0.5% (v/v) AcOH in CH_3CN (B). Elution method: (a) 100% (v/v) H_2O, three column volumes; (b) 100% (v/v) A, three column volumes; (c) 20% (v/v) B in A, three column

https://doi.org/10.1021/acs.oprd.1c00368
Org. Process Res. Dev. 2021, 25, 2754−2771

volumes; (d) 100% (v/v) CH_3CN, two column volumes. Flow rate: 50 mL/min; λ, 215 nm. Fractions of 20 mL volume were collected and analyzed by RP-UHPLC-ESI-MS (procedures S2, S3, and S4). Fractions corresponding to R_t = 4.0 min and UHPLC purity >98.5% were collected, obtaining a total volume of 60 mL that was lyophilized.

Obtained eptifibatide acetate was analyzed by RP-UHPLC-ESI-MS (procedure S2, S3, S4, and S5). TFA and acetate content were quantified by ion exchange chromatography (procedure S8).

Eptifibatide acetate (2.99 g, yield 64.6%) characterized by 99.6% UHPLC purity, R_t = 4.0 min (Figure S20). ESI-MS (m/z): $[M + H]^+$ 832.5 (found); 831.96 (calcd). TFA residual content, 335 ppm; acetate content, 4.6% (w/w).

Overview of Eptifibatide Acetate cGMP Production at 70 mmol Scale. The procedures optimized for each step on a 5 mmol scale (steps 1−5) were scaled up to the pilot scale (70 mmol). MPA(Trt)-Har(Pbf)-Gly-Asp(OtBu)-Trp(Boc)-Pro-Cys(Trt)-Rink Amide AM resin was produced in a multi-gram-scale cGMP qualified MW-assisted solid-phase peptide synthesizer (Liberty Blue, CEM, Matthews, NC, U.S.A.), obtaining 196 g in a single batch. Cleavage from the resin was performed in a single batch, obtaining 51 g of eptifibatide TFA linear precursor crude showing 73.7% of HPLC purity (yield = 82.2%). A disulfide bond in the eptifibatide TFA linear precursor crude was formed in 5 × 10 g batch, obtaining 50 g total of eptifibatide TFA crude with a purity of 66.4% (yield = 98.3%). Eptifibatide TFA crude was purified by flash chromatography in five batches. Fractions with HPLC purity >98.5% (R_t = 23.0 min, Table S12) obtained after purification of each batch were collected, leading to 4 g/batch of pure eptifibatide TFA (99.2% HPLC purity, yield = 41.6%). Each batch of pure eptifibatide TFA salt (4 g) was treated as described in the above-described "exchange strategy" obtaining 2 g/batch of the eptifibatide acetate active pharmaceutical ingredient (API) with a purity of 99.6% (yield = 65.8%). The total yield of the eptifibatide acetate cGMP production process was 22.1%.

Glassware was cleaned following the standard operating procedures (SOP) before and after the manufacturing operations. Moreover, all the personnel involved in the cGMP process was adequately trained in technical operations, safety behavior, personal hygiene, and technical clothing in accordance with the cGMP requirements. The construction criteria of the pilot-scale facility followed the cGMP structural requirements (e.g., air-lock, system air shower, etc.). Monitoring of microbial contamination was duly scheduled as well as facility sanitizations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.oprd.1c00368.

Microwave methods, RP-UHPLC/MS analyses, analytical procedures for 70 mmol scale (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Anna Maria Papini − *MoD&LS Laboratory, University of Florence, 50019 Sesto Fiorentino, Italy; Interdepartmental Research Unit of Peptide and Protein Chemistry and Biology, Department of Chemistry "Ugo Schiff", University of Florence, 50019 Sesto Fiorentino, Italy; CNR-IC Istituto di Cristallografia, 95126 Catania, Italy; PeptLab@UCP Platform of Peptide and Protein Chemistry and Biology, Neuville Campus, CY Cergy Paris Université, 95031 Cergy-Pontoise Cedex, France;* orcid.org/0000-0002-2947-7107; Email: annamaria.papini@unifi.it

Authors

Annunziata D'Ercole − *MoD&LS Laboratory, University of Florence, 50019 Sesto Fiorentino, Italy; Interdepartmental Research Unit of Peptide and Protein Chemistry and Biology, Department of Chemistry "Ugo Schiff", University of Florence, 50019 Sesto Fiorentino, Italy; F.I.S.- Fabbrica Italiana Sintetici S.p.A., 36075 Montecchio Maggiore, Vicenza, Italy*

Lorenzo Pacini − *MoD&LS Laboratory, University of Florence, 50019 Sesto Fiorentino, Italy; Interdepartmental Research Unit of Peptide and Protein Chemistry and Biology, Department of Chemistry "Ugo Schiff", University of Florence, 50019 Sesto Fiorentino, Italy; F.I.S.- Fabbrica Italiana Sintetici S.p.A., 36075 Montecchio Maggiore, Vicenza, Italy*

Giuseppina Sabatino − *MoD&LS Laboratory, University of Florence, 50019 Sesto Fiorentino, Italy; Interdepartmental Research Unit of Peptide and Protein Chemistry and Biology, Department of Chemistry "Ugo Schiff", University of Florence, 50019 Sesto Fiorentino, Italy; CNR-IC Istituto di Cristallografia, 95126 Catania, Italy*

Matteo Zini − *F.I.S.- Fabbrica Italiana Sintetici S.p.A., 36075 Montecchio Maggiore, Vicenza, Italy*

Francesca Nuti − *MoD&LS Laboratory, University of Florence, 50019 Sesto Fiorentino, Italy; Interdepartmental Research Unit of Peptide and Protein Chemistry and Biology, Department of Chemistry "Ugo Schiff", University of Florence, 50019 Sesto Fiorentino, Italy*

Arianna Ribecai − *MoD&LS Laboratory, University of Florence, 50019 Sesto Fiorentino, Italy; F.I.S.- Fabbrica Italiana Sintetici S.p.A., 36075 Montecchio Maggiore, Vicenza, Italy*

Alfredo Paio − *MoD&LS Laboratory, University of Florence, 50019 Sesto Fiorentino, Italy; F.I.S.- Fabbrica Italiana Sintetici S.p.A., 36075 Montecchio Maggiore, Vicenza, Italy*

Paolo Rovero − *MoD&LS Laboratory, University of Florence, 50019 Sesto Fiorentino, Italy; CNR-IC Istituto di Cristallografia, 95126 Catania, Italy; Interdepartmental Research Unit of Peptide and Protein Chemistry and Biology, Department of Neurosciences, Psychology, Drug Research and Child Health—Section of Pharmaceutical Sciences and Nutraceutics, University of Florence, 50019 Sesto Fiorentino, Italy;* orcid.org/0000-0001-9577-5228

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.oprd.1c00368

Author Contributions

$^{\nabla}$A.D. and L.P. equally contributed to the scale-up of the cGMP production process of eptifibatide and its transfer from PeptFarm University of Florence to FIS−Fabbrica Italiana Sintetici S.p.A.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was performed in the context of the University−Industry Joint Laboratory PeptFarm (2017−2019), joining the

Interdepartmental Research Unit of Peptide and Protein Chemistry and Biology of the University of Florence and F.I.S.−Fabbrica Italiana Sintetici S.p.A., which supported in part the project with a specific grant. We gratefully acknowledge Regione Toscana PAR-FAS (2007−2013) for supporting the Laboratory Molecular Diagnostics and Life Sciences (MoD&LS) in the context of the Centre of Competences RISE.

DEDICATION

This paper is dedicated to the memory of Hans-Jürgen Musiol (June 5, 1942 to July 9, 2021), who contributed to building several generations of peptide chemists at the Max-Planck Institute of Biochemistry, Martinsried (Germany).

ABBREVIATIONS

Aa, amino acid; Acm, acetamidomethyl; AcOH, acetic acid; ANDA, abbreviated new drug application; API, active pharmaceutical ingredient; Arg, arginine; Boc, *tert*-butyoxycarbonyl; CAGR, compound annual growth rate; CDMO, contract development manufacturing organization; cGMP, current good manufactoring practices; CH_2Cl_2, dichloromethane; CH_3CN, ACN acetonitrile; CH_3COONH_4, ammonium acetate; CLP, classification labeling and packaging; CPME, methoxycyclopentane; CPP, critical processing parameter; CQA, critical quality attribute; Cys, cysteine; DIC, *N*,*N*′-diisopropylcarbodiimide; Oxyma pure, ethyl cyanohydroxyiminoacetate; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DODT, 2,2′-(ethylenedioxy)diethanethiol; ECHA, European Chemicals Agency; EMA, European Medicines Agency; Et_2O, ethoxyethane; FA, formic acid; FC, flash chromatography; FD&C, Federal Food, Drug, and Cosmetic Act; FDA, Food and Drug Administration; Fmoc, fluorenylmethyloxycarbonyl; FY, financial year; HS-GC, headspace gas chromatography; GDUFA, generic drug user fee act; GVL, 5-methyloxolan-2-one; SPPS, solid-phase peptide synthesis; H_2O_2, hydrogen peroxide; Har, homoarginine; HCl, hydrochrloric acid; HP, high pressure/performance; HPLC, high performance liquid chromatography; IC, ion exchange chromatography; ICP-MS, inductively coupled plasma mass spectroscopy; IEC, ion exchange chromatography; iPr_2O, 2-[(Propan-2-yl)oxy]propane; iPrOH, isopropyl alcohol; KOH, potassium hydroxide; Leu, leucine; LPPS, liquid phase peptide synthesis; MBHA, 4-methylbenzhydrylamine; Met, methionine; MeTHF, 2-methyltetrahydrofuran; Mpa, 3-mercaptopropionic acid; MTBE, 2-methoxy-2-methylpropane; MW, microwave; MW-SPS, microwave solid-phase synthesis; MW-SPPS, microwave solid-phase peptide synthesis; NH_4OH, ammonium hydroxide; NMP, *N*-methyl-2-pyrrolidone dichloromethane; OOS, out of specification; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PS−PEG, polystyrene−polyethylene glycol; R&D, research and development; RLD, reference listed drug; RM, raw material; RP-HPLC, reverse-phase high performance liquid chromatography; RPC, reverse-phase chromatography; SPE, solid-phase extraction; SVHC, substances of very high concern; tBu, *tert*-butyl; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Trp, tryptophan; USD, United States dollars; VBFR, variable bed flow reactor; WFI, water for injection

REFERENCES

(1) *Peptide Therapeutics Market by Type of Peptide (Small, Medium and Large), Route of Administration (Intravenous, Oral, Subcutaneous, Topical and Others), Key Geographical Regions (North America, Europe, Asia-Pacific and Rest of the World) and Key Therapeutic Area (Metabolic Diseases, Oncological Diseases, Endocrine Diseases, Digestive and Gastrointestinal Diseases and Others): Industry Trends and Global Forecasts*; Root Analysis Business Research & Consulting, 2021−2030.

(2) Teixeira, T.; Kweder, S. L.; Saint-Raymond, A. Are European Medicines Agency, US Food and Drug Administration, and Other International Regulators Talking to Each Other? *Clin. Pharmacol. Ther.* **2020**, *107* (3), 507−513.

(3) Branch, S. K. Guidelines from the International Conference on Harmonisation (ICH). *J. Pharm. Biomed. Anal.* **2005**, *38* (5), 798−805.

(4) *Guideline, I. H. T. Impurities in New Drug Substances, ICH Q3A (R2)*; European Medicines Agency: Geneva, Switzerland, 2006; p 25.

(5) *ANDAs for Certain Highly Purified Synthetic Peptide Drug Products That Refer to Listed Drugs of RDNA Origin, Guidance for Industry*; US Food and Drug Administration: Silver Spring, MD, 2017.

(6) Kumar, A.; Jad, Y. E.; El-Faham, A.; de la Torre, B. G.; Albericio, F. Green Solid-Phase Peptide Synthesis 4. γ-Valerolactone and N -Formylmorpholine as Green Solvents for Solid-Phase Peptide Synthesis. *Tetrahedron Lett.* **2017**, *58* (30), 2986−2988.

(7) Jad, Y. E.; Acosta, G. A.; Khattab, S. N.; de la Torre, B. G.; Govender, T.; Kruger, H. G.; El-Faham, A.; Albericio, F. 2-Methyltetrahydrofuran and Cyclopentyl Methyl Ether for Green Solid-Phase Peptide Synthesis. *Amino Acids* **2016**, *48* (2), 419−426.

(8) Jad, Y. E.; Govender, T.; Kruger, H. G.; El-Faham, A.; de la Torre, B. G.; Albericio, F. Green Solid-Phase Peptide Synthesis (GSPPS) 3. Green Solvents for Fmoc Removal in Peptide Chemistry. *Org. Process Res. Dev.* **2017**, *21* (3), 365−369.

(9) Hartrampf, N.; Saebi, A.; Poskus, M.; Gates, Z. P.; Callahan, A. J.; Cowfer, A. E.; Hanna, S.; Antilla, S.; Schissel, C. K.; Quartararo, A. J.; Ye, X.; Mijalis, A. J.; Simon, M. D.; Loas, A.; Liu, S.; Jessen, C.; Nielsen, T. E.; Pentelute, B. L. Synthesis of Proteins by Automated Flow Chemistry. *Science* **2020**, *368* (6494), 980−987.

(10) Mijalis, A. J.; Thomas, D. A.; Simon, M. D.; Adamo, A.; Beaumont, R.; Jensen, K. F.; Pentelute, B. L. A Fully Automated Flow-Based Approach for Accelerated Peptide Synthesis. *Nat. Chem. Biol.* **2017**, *13* (5), 464−466.

(11) Sletten, E. T.; Nuño, M.; Guthrie, D.; Seeberger, P. H. Real-Time Monitoring of Solid-Phase Peptide Synthesis Using a Variable Bed Flow Reactor. *Chem. Commun.* **2019**, *55* (97), 14598−14601.

(12) Collins, J. M.; Singh, S. K.; Vanier, G. S. Microwave Technology for Solid-Phase Peptide Synthesis. *Chem. Today.* **2012**, *30* (2), 26−29.

(13) Carpino, L. A.; Krause, E.; Sferdean, C. D.; Schümann, M.; Fabian, H.; Bienert, M.; Beyermann, M. Synthesis of 'Difficult' Peptide Sequences: Application of a Depsipeptide Technique to the Jung−Redemann 10- and 26-Mers and the Amyloid Peptide Aβ(1−42). *Tetrahedron Lett.* **2004**, *45* (40), 7519−7523.

(14) McNamara, J. F.; Lombardo, H.; Pillai, S. K.; Jensen, I.; Albericio, F.; Kates, S. A. An Efficient Solid-Phase Strategy for the Construction of Chemokines. *J. Pept. Sci.* **2000**, *6* (10), 512−518.

(15) Sabatino, G.; D'Ercole, A.; Pacini, L.; Zini, M.; Ribecai, A.; Paio, A.; Rovero, P.; Papini, A. M. An Optimized Scalable Fully Automated Solid-Phase Microwave-Assisted CGMP-Ready Process for the Preparation of Eptifibatide. *Org. Process Res. Dev.* **2021**, *25* (3), 552−563.

(16) Qin, L.; Yuan, J.; Li, H.; Ma, Y. Method for Preparing Eptifibatide with Solid-Phase Method. CN 101538316 B, September 5, 2012.

(17) Wen, Y.; Zhu, C.; Wang, X.; Han, Y.; Tong, G. Eptifibatide Preparation Method. US 9394341B2, October 22, 2015.

(18) Varray, S.; Werbitzky, O.; Zeiter, T. On-Resin Peptide Cyclization. WO2006045483A2, May 4, 2006.

(19) *Guideline, Quality risk management, ICH Q9*; European Medicines Agency: London, UK, 2015; p 303,

(20) European Regulation (EC) no. 1272/2008.

https://doi.org/10.1021/acs.oprd.1c00368
Org. Process Res. Dev. 2021, 25, 2754−2771

(21) Cezari, M. H.; Juliano, L. Studies on lactam formation during coupling procedures of N alpha-N omega-protected arginine derivatives. *Peptide Res.* **1996**, *9* (2), 88−91.
(22) De la Torre, B. G.; Kumar, A.; Alhassan, M.; Bucher, C.; Albericio, F.; Lopez, J. Successful development of a method for the incorporation of Fmoc-Arg (Pbf)-OH in solid-phase peptide synthesis using N-butylpyrrolidinone (NBP) as solvent. *Green Chem.* **2020**, *22* (10), 3162−3169.
(23) Anderson, N. G.; Burdick, D. C.; Reeve, M. M. Current Practices of Process Validation for Drug Substances and Intermediates. *Org. Process Res. Dev.* **2011**, *15* (1), 162−172.
(24) Martin, V.; Egelund, P. H. G.; Johansson, H.; Thordal Le Quement, S.; Wojcik, F.; Sejer Pedersen, D. Greening the Synthesis of Peptide Therapeutics: An Industrial Perspective. *RSC Adv.* **2020**, *10* (69), 42457−42492.
(25) *Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances, ICH Q6A*; European Medicines Agency: London, UK, 2000.
(26) Buellesbach, E. E.; Danho, W.; Heilbig, H. J.; Zahn, H. *Side Reactions in Peptide Synthesis*; Wroclaw University Press: Wroclaw, 1979; pp 643−646.
(27) Barany, G.; Merrifield, R. B. *The Peptides Analysis, Synthesis, Biology*; Academic Press: New York, 1979; vol *2*, pp 1−298.
(28) Moroder, L.; Musiol, H. J.; Schaschke, N.; Chen, L.; Hargittai, B.; Barany, G. Protection of the Thiol Group. *Synthesis of Peptides and Peptidomimetics*; Georg Thieme, Verlag: Stuttgart, Germany, 2001; vol *E22a*; pp 384−423.
(29) Yang, Y. Peptide Global Deprotection/Scavenger-Induced Side Reactions *Side Reactions in Peptide Synthesis*; Academic Press: Oxford, UK, 2016; vol *3*, pp 43−75.
(30) Al Musaimi, O.; Jad, Y. E.; Kumar, A.; El-Faham, A.; Collins, J. M.; Basso, A.; de la Torre, B. G.; Albericio, F. Greening the Solid-Phase Peptide Synthesis Process. 2-MeTHF for the Incorporation of the First Amino Acid and Precipitation of Peptides after Global Deprotection. *Org. Process Res. Dev.* **2018**, *22* (12), 1809−1816.
(31) Al Musaimi, O.; Jad, Y. E.; Kumar, A.; Collins, J. M.; Basso, A.; de la Torre, B. G.; Albericio, F. Investigating Green Ethers for the Precipitation of Peptides after Global Deprotection in Solid-Phase Peptide Synthesis. *Current Opinion in Green and Sustainable Chemistry.* **2018**, *11*, 99−103.
(32) Alder, C. M.; Hayler, J. D.; Henderson, R. K.; Redman, A. M.; Shukla, L.; Shuster, L. E.; Sneddon, H. F. Updating and Further Expanding GSK's Solvent Sustainability Guide. *Green Chem.* **2016**, *18* (13), 3879−3890.
(33) Prat, D.; Pardigon, O.; Flemming, H.-W.; Letestu, S.; Ducandas, V.; Isnard, P.; Guntrum, E.; Senac, T.; Ruisseau, S.; Cruciani, P.; Hosek, P. Sanofi's Solvent Selection Guide: A Step toward More Sustainable Processes. *Org. Process Res. Dev.* **2013**, *17* (12), 1517−1525.
(34) European Chemicals Agency Candidate List of substances of very high concern for Authorisation. http://echa.europa.eu/candidate-list-table.
(35) Prat, D.; Wells, A.; Hayler, J.; Sneddon, H.; McElroy, C. R.; Abou-Shehada, S.; Dunn, P. J. Correction: CHEM21 Selection Guide of Classical-and Less Classical-Solvents. *Green Chem.* **2015**, *17* (10), 4848−4848.
(36) Andreu, D.; Albericio, F.; Solé, N. A.; Munson, M. C.; Ferrer, M.; Barany, G. Formation of Disulfide Bonds in Synthetic Peptides and Proteins. *Peptide synthesis protocols.* **1994**, *35*, 91−169.
(37) Sidorova, M.; Molokoedov, A.; Az'muko, A.; Kudryavtseva, E.; Krause, E.; Ovchinnikov, M.; Bespalova, Z. D. The Use of Hydrogen Peroxide for Closing Disulfide Bridges in Peptides. *Russ. J. Bioorg. Chem.* **2004**, *30* (2), 101−110.
(38) Calce, E.; Vitale, R. M.; Scaloni, A.; Amodeo, P.; De Luca, S. Air Oxidation Method Employed for the Disulfide Bond Formation of Natural and Synthetic Peptides. *Amino Acids* **2015**, *47* (8), 1507−1515.
(39) Tam, J. P.; Wu, C. R.; Liu, W.; Zhang, J. W. Disulfide Bond Formation in Peptides by Dimethyl Sulfoxide. Scope and Applications. *J. Am. Chem. Soc.* **1991**, *113* (17), 6657−6662.
(40) Song, C.; Sun, J.; Zhao, X.; Huo, S.; Shen, S. A Study to Develop Platinum (Iv) Complex Chemistry for Peptide Disulfide Bond Formation. *Dalton Transactions.* **2020**, *49* (6), 1736−1741.
(41) Insuasty Cepeda, D. S.; Pineda Castañeda, H. M.; Rodríguez Mayor, A. V.; García Castañeda, J. E.; Maldonado Villamil, M.; Fierro Medina, R.; Rivera Monroy, Z. J. Synthetic Peptide Purification via Solid-Phase Extraction with Gradient Elution: A Simple, Economical, Fast, and Efficient Methodology. *Molecules* **2019**, *24* (7), 1215.
(42) Bickler, B. How to choose between linear gradient and step gradients for flash chromatography. Biotage. https://selekt.biotage.com/blog/how-to-choose-between-linear-gradients-and-step-gradients-for-flash-chromatography.
(43) Sikora, K.; Jaśkiewicz, M.; Neubauer, D.; Migoń, D.; Kamysz, W. The Role of Counter-Ions in Peptides—An Overview. *Pharmaceuticals* **2020**, *13* (12), 442−1−29.
(44) Keire, D. A.; Strauss, E.; Guo, W.; Noszal, B.; Rabenstein, D. L. Kinetics and equilibria of thiol/disulfide interchange reactions of selected biological thiols and related molecules with oxidized glutathione. *J. Org. Chem.* **1992**, *57* (1), 123−127.

https://doi.org/10.1021/acs.oprd.1c00368
Org. Process Res. Dev. 2021, 25, 2754−2771