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Overcoming chemical challenges in the solid-phase synthesis of high-purity GnRH antagonist Degarelix.

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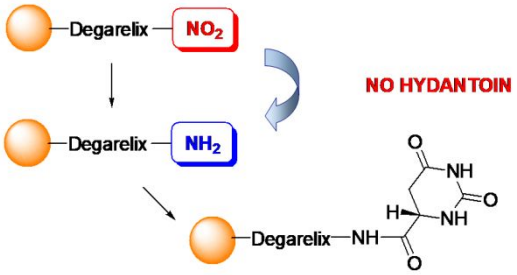
Keywords: degarelix, dihydroorotic acid, Fmoc deprotection, peptide, reduction


Degarelix
NO2
NO HYDANTOIN
Degarelix
NH2
Degarelix
NH
O
NH
O
H
NH
O

ABSTRACT

The highly potent, long-acting, gonadotropin-releasing hormone antagonist Degarelix is known to be very efficient for prostate cancer treatment. The synthesis of the decapeptide Degarelix is complicated because of the presence in its sequence of several unnatural α-amino acids, which are prone to rearrangements and side reactions. In particular, the rearrangement of the dihydroorotic (Hor) moiety with following hydantoin formation in the presence of the bases represents one of the major problems. In this study we describe a novel chemical strategy to overcome this obstacle by the use of the corresponding *p*-nitrophenylalanine derivative, which is reduced on the solid support to *p*-aminophenylalanine and acylated with dihydroorotic acid at the end of the solid-phase synthesis. Thus, the contact of Hor with the bases required for Fmoc deprotection is completely avoided. This approach provides a superior purity of Degarelix when the synthesis is carried out in industrial scale as well.

INTRODUCTION

Cancer is one of the major public health problems worldwide. According to the World Health Organization, globally, nearly 1 in 6 deaths is due to cancer that is responsible for an estimated 9.6 million deaths in 2018.[1] Among other types of malignancies, prostate cancer accounts about 20% of new diagnoses in men and it is the third most common cause of cancer death in US.[2] The treatment of prostate cancer depends on the stage of the disease and includes radical prostatectomy, external beam radiotherapy or brachytherapy in organ-confined case and androgen-deprivation therapy (ADT) at the metastatic stage.[3]

The principal mechanism of ADT is the prevention of androgen receptor signalling, which is involved in the growth of the cancer cells. Generally, it can be achieved by (1) direct blocking of androgen receptors or (2) suppression of circulating testosterone through the action of the gonadotropin-releasing hormone (GnRH) agonists (*via* negative feedback loop) or GnRH antagonists. This class of molecules, blocking the GnRH receptors in the pituitary, prevents the production of the luteinizing hormone (LH) and downstream production of testosterone.[4,5] According to the latter strategy, several GnRH agonists, such as Leuprolide, Goserelin, Triptorelin, and two antagonists (Abarelix and Degarelix) were developed for the prostate cancer treatment.[6-8] The noticeable allergic effects of Abarelix led to its withdrawal from the US market. On the contrary, Degarelix was shown to have a significantly lower risk of histamine release than Abarelix and to induce almost immediate and long-lasting decrease of serum testosterone concentration. After the approval by FDA in 2008, it became widely used in patients with advanced prostate cancer.[9]

Degarelix, formerly known as FE200486, is a synthetic, linear decapeptide chemically described as N-acetyl-3-(2-naphthalenyl)-D-alanyl-4-chloro-D-phenylalanyl-3-(3-pyridinyl)-D-alanyl-L-seryl-4-[[[(*4S*)-hexahydro-2,6-dioxo-4-pyrimidinyl]carbonyl]amino]-L-phenylalanyl-4-[(aminocarbonyl)amino]-D-phenylalanyl-L-leucyl-N6-(1-methylethyl)-L-lysyl-L-prolyl-D-alaninamide (Figure 1).

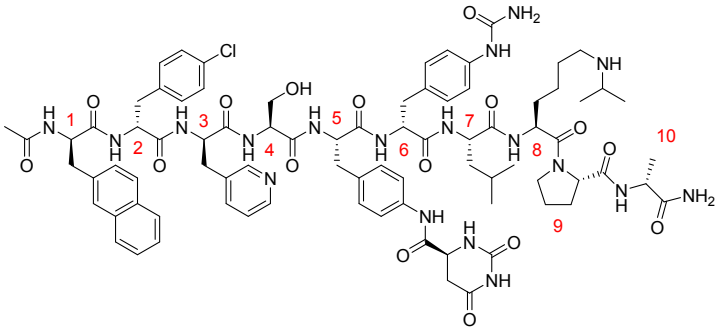


Figure 1. Chemical structure of Degarelix.

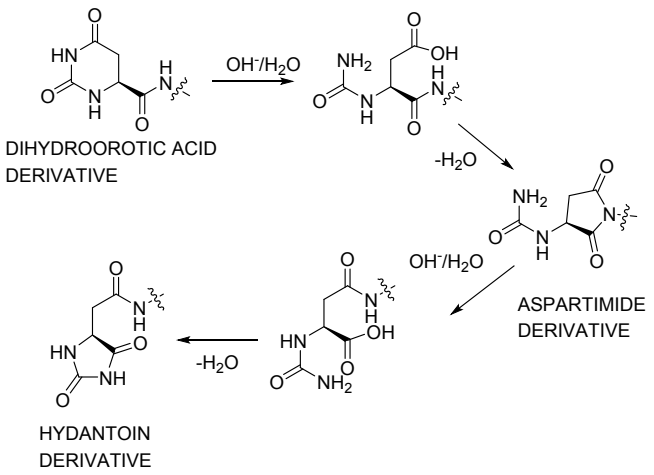
This peptide molecule contains several unnatural α-amino acids, five of which are D-amino acids. It is currently marketed as the acetate salt with the brand name Firmagon (EU and US) or Gonax (Japan). After reconstitution with water and subcutaneous injection, it forms a gel-like depot, which provides sustained release of the drug and ensures its prolonged action.

The high proteolytic stability of Degarelix and the resistance to the fast glomerular clearance are due to the unique combination in its sequence of the hydrophilic and hydrophobic amino acids. The hydrophobic residues allow non-covalent binding to the cell membranes and hydrophobic carrier proteins, such as serum albumin, and protection against proteolytic degradation ("whole body depot effect"). On the contrary, the presence of the hydrophilic residues, in particular, 4-[(aminocarbonyl)amino]-phenylalanine [Aph(Cbm)] and 4-[(dihydroorotyl)amino]-phenylalanine [Aph(Hor)], with their strong ionic and H-bonding interactions, enhances water solubility of Degarelix and reduces its tendency to form gels with respect to other potent GnHR antagonists (such as Acyline).[10] However, these substitutions still permit self-association with the formation of "amyloid" type fibrils, which dissolve over a long period (amyloid $t_{1/2}$ = 15 days).[10,11] For these reasons, Degarelix shows a prolonged bioavailability and duration of action if compared to other derivatives of the same therapeutic class.

The presence of a number of the amino acids with particular properties makes the preparation of Degarelix rather challenging. Aph residues can be introduced into the sequence of Degarelix both

as side-chain modified Aph(Cbm) or Aph(Hor), or as aniline precursors with orthogonal protecting groups. The initial scheme of Degarelix synthesis, developed by the Ferring Research Institute, was based on the *tert*-butyloxycarbonyl (Boc) solid-phase peptide synthesis (SPPS) with the stepwise elongation of the peptide sequence and the final cleavage of the peptide from the resin by the treatment with HF in the presence of anisole as a scavenger.[12,13] All of the amino acids were coupled as ready-to-use derivatives, with the exceptions of Aph(Cbm) and Aph(Hor), which were generated from the corresponding anilines by Fmoc deprotection followed by the reaction with *tert*-butyl isocyanate and Hor, respectively. Though this method allowed obtaining Degarelix in high yields, it was not suitable for the industrial production of the peptide because of the involvement of hazardous and environmentally unfriendly reagents. Therefore, various alternative synthetic schemes were proposed for Degarelix preparation, mostly based on the Fmoc/*t*Bu (*tert*-butyl) strategy, which allows milder reaction conditions.[14-26] In this case, both solid- and liquid-phase approaches, or combination thereof, could be applied. Moreover, the high potency of Degarelix inspired research of the peptide analogs with enhanced activity by substitution of some amino acids and/or side-chain modifications with various success.[27-29]

One of the major challenges in the preparation of Degarelix as an active pharmaceutical ingredient (API) is the high sensitivity of the Hor moiety to the presence of the bases. In this case, a rapid rearrangement of its six-membered ring (with hydrolysis to the *N*-carbamoylaspartyl intermediate) to the five-membered ring hydantoin takes place (Scheme 1).[30-33]



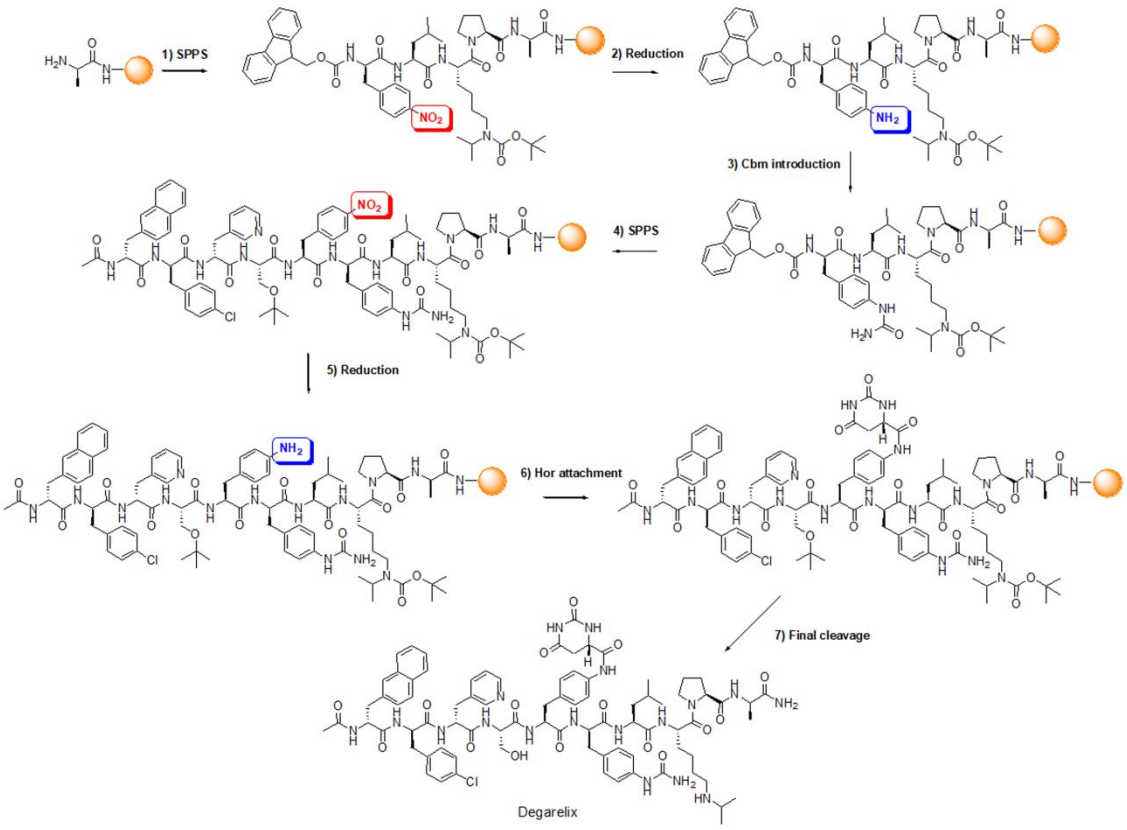
Scheme 1. Rearrangement of the Hor residue in the presence of bases.

Due to the high similarity of the chemical structure of the hydantoin to Hor, the identification of the hydantoin impurity in Degarelix samples is very difficult (*e.g.*, HPLC retention time of the hydantoin impurity is very close to that of Degarelix in almost all of the chromatographic conditions). For the same reason, this impurity noticeably complicates the HPLC purification of the final peptide to get the purity necessary for the release of Degarelix API in the market.[34] This unfortunate situation has to be taken into account for the preparation of Degarelix using the Fmoc-protecting group and the subsequent repetitive deprotection cycles with the organic bases. For example, in the recent patent of Zhang *et al.*[14] it was shown that the treatment of Degarelix with a 2% solution of 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) in DMF, which is often used in SPPS for Fmoc deprotection, resulted in the formation of 1.8% hydantoin. The amount of the impurity increased up to 7%, when 5% of water was added. These results suggest that the use of high-purity reagents is required when the peptide is synthesized by the standard SPPS technique.

The development of methods for the preparation of the crude peptide Degarelix without the hydantoin impurity will significantly lower the cost of production in a large scale with high yields. From this point of view, the formation of the Aph derivatives from the corresponding Phe(*p*-NO_2) residue in SPPS could be a promising approach for introduction of the Aph(Hor) and/or Aph(Cbm)

residues into the Degarelix sequence, in particular for the large-scale production of the peptide. In this case, the introduction of the Hor moiety can be carried out at the very last step of the synthesis, thus avoiding its contact with the bases.[35] The reduction methods of the aromatic nitro group often involve activated metal catalysis or transition metal catalyzed hydrogenation, where other functional groups can be affected.[36,37] Consequently, many of these methods are not applicable in SPPS. Sodium dithionite and stannous chloride showed to be promising alternatives to the catalytic hydrogenation.[38,39] They can function as reducing agents for nitro derivatives on solid supports and allow a complete conversion to the corresponding anilines. However, in the case of sodium dithionite, a phase-transfer catalyst, such as tetrabutylammonium hydrogen sulfate (TBAHS), and a water/organic solvent mixture are needed to ensure the transfer of the inorganic salt to the organic phase, which contains molecule to be reduced attached to the solid support.[39] On the contrary, the reaction with stannous chloride can be carried out with high yields in a homogeneous way in an organic solvent in the presence of DIPEA or DBU.[39,41]

In our effort to discover innovative syntheses of high-purity Degarelix,[42] here we describe a novel approach, where the rearrangement of the Hor moiety of Degarelix is suppressed or absent (Scheme 2). It comprises the introduction of the Hor moiety at the very last step of the synthesis, when the treatment with the bases for Fmoc-deprotection is not required any more. This approach can increase the purity of the crude peptide, thus facilitating its purification and increasing its final yield, which is particularly important for the industrial production. Moreover, it can facilitate the preparation of other active pharmaceutical ingredients characterized by the Hor moiety as well.



Scheme 2. General scheme of the Degarelix synthesis *via* Phe(*p*-NO_2) reduction on the solid support.

RESULTS AND DISCUSSION

In order to find suitable conditions for the reduction of the nitro group on the solid support, two approaches were used: sodium dithionite- and stannous chloride-mediated reductions. In the first case, the phase-transfer reagent (TBAHS) was introduced into the reaction mixture in order to afford the transfer of the salt to the organic phase.[40] The efficiency of the reduction of the Phe(*p*-NO_2) residue was initially investigated using model pentapeptide with the sequences comprising the C-terminal segment of Degarelix (Table 1).

Table 1. Reduction of the Phe(p-NO_2) residue under various conditions

Entry	Chemical structure	Conditions	Nitro group reduction, %	Fmoc deprotection, %
1	Fmoc-D-Phe(p-NO_2)-Leu-Lys(Boc, iPr)-Pro-D-Ala-NH-resin*	0.5 mL of DMF + 0.5 mL of stock solution**, 2 h	0	-
2		0.5 mL of DCM + 0.5 mL of stock solution, 2 h	100	12
3		2.5 mL of $SnCl_2 \cdot 2H_2O$ (1.5 M in DMF) with 1.5 eq of DIPEA (70 µL), 12 h	100	-

*200 mg of peptide resin, loading 0.65 mmol/g.
**Stock solution: $Na_2S_2O_4$ 1050 mg (5 mmol), K_2CO_3 970 mg (7 mmol), TBAHS 170 mg (0.5 mmol) in 5 mL of water.

The reduction of the nitro group with sodium dithionite in DCM-water mixture occurred with high yield in 2 h (Table 1, Entry 2). Nevertheless, a partial Fmoc deprotection was seen due to the presence of the potassium carbonate in the mixture and the alkaline environment. Interestingly, the starting material was completely recovered in the case of DMF-water (Table 1, Entry 1) and no traces of the corresponding Aph were found. On the contrary, during the reduction of the nitro group with stannous chloride, no Fmoc cleavage was observed and the reaction was completed in 12 h (Table 1, Entry 3 and Figure 2). The appearance of p-aminophenylalanine in the amino acid sequence caused the increase of hydrophilicity and the decrease of the retention time of the peptide. This last approach demonstrated the feasibility of the designed reduction strategy of the Phe(p-NO_2) on the solid phase.

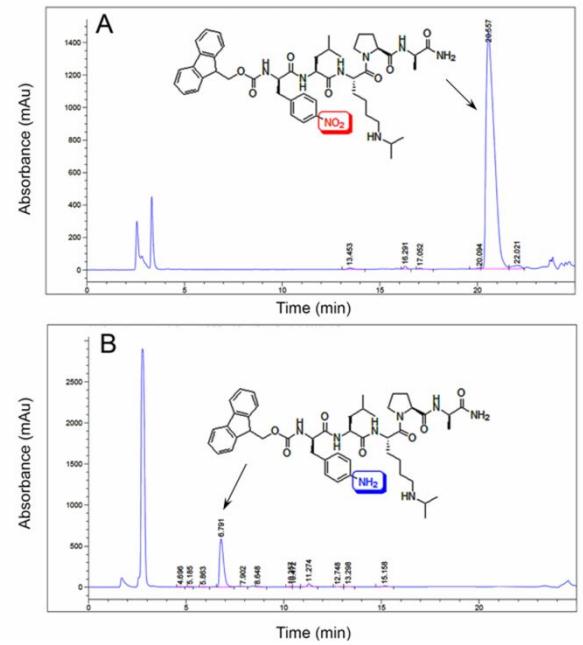


Figure 2. HPLC chromatograms of Fmoc-D-Phe(*p*-NO_2)-Leu-Lys(*i*Pr)-Pro-D-Ala-NH_2 (A), and Fmoc-D-Aph-Leu-Lys(*i*Pr)-Pro-D-Ala-NH_2 (B) (for details, see Experimental Part; analytical method 1).

According to this, we started evaluating also the construction of the Aph(Cbm) residue in the peptide already attached to the solid support (Scheme 2, step 3). In the synthesis proposed by the Ferring Research Institute, the Cbm functionality was introduced by the reaction of the Aph side-chain free amino group with *tert*-butyl isocyanate for 12 h with formation of the corresponding *tert*-butyl protected urea derivative.[12] Nevertheless, upon completion of the synthesis of Degarelix we found that the complete removal of the *tert*-butyl group from the Aph(Cbm) residue required the treatment of the peptide with TFA over a long time, when we observed a partial degradation of the peptide (data not shown). Therefore, we applied another approach, where potassium cyanate is used in the presence of acetic acid to liberate *in situ* cyanic acid, which can react with the free amino group with formation of the Cbm moiety.[43] The use of the solution of potassium cyanate in diluted acetic acid gave unsatisfactory results since, after treatment of the peptide overnight, only about

10% of the Cbm peptide derivative was found in the reaction mixture (Figure S1, A). By performing the same reaction in glacial acetic acid, the conversion was complete but, along with the expected product, a compound with *m/z* Δ_{mass} -1 was found (Figure S1, B). The hypothesis is that the interaction of acetic acid and cyanic acid turned into the formation of the mixed anhydride, leading to acetylation of the amino group.[44] Indeed, the replacement of acetic acid with a 20% solution of phosphoric acid to afford cyanic acid resulted in the formation of the desired peptide in good HPLC purity (Figure S1, C), demonstrating by that the correctness of the hypothesized side reaction and the possibility to build up the Aph(Cbm) amino acid *via* the Phe(*p*-NO_2) intermediate.

Lastly, in order to evaluate the possibility of the construction of the Aph(Hor) moiety during the SPPS protocol, two SPPS syntheses of Degarelix were performed: (1) the reduction of the nitro group followed by the Hor moiety incorporation at the step of the hexapeptide and (2) the reduction and Hor insertion at the end of the synthesis of the peptide chain (Figure 3).

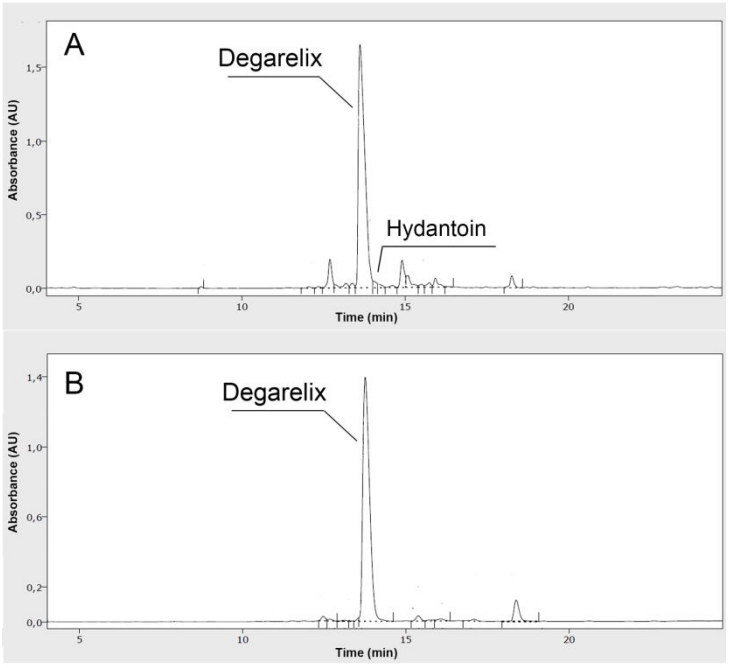


Figure 3. HPLC runs of crude Degarelix, prepared with the nitro group reduction and incorporation of the Hor moiety at the level of the hexapeptide (3 g of Rink amide resin, A) and at the end of

solid-phase synthesis (500 g of Rink amide resin, B) (for details, see Experimental Part; analytical method 2).

The comparison of the HPLC runs reported in Figure 3 shows a higher overall purity of the Degarelix obtained *via* the second approach with respect to the one resulting from the coupling of the dihydroorotic acid before elongation of the Degarelix sequence (87% *vs* 75%) with the amount of hydantoin impurity less than 0.15%. This behavior is related to the sensitivity of the Hor moiety to the SPPS conditions, that enhance the Hor-to-hydantoin isomerism, and to the acetylation of the Hor moiety, when it is present during N-terminal capping. This evidence confirms the validity of the process as a robust manufacturing strategy for the large-scale production of the peptide, especially with insertion of the Hor at the end of the Degarelix synthesis.

An important aspect to be carefully evaluated while using tin(II) chloride for the industrial applications is regulatory requirements. Accordingly to the regulatory agencies new guidance, tin(II) chloride, albeit considered safer respect to other toxic metals, has to be absent or present at low ppm level in the final API.[45] For this reason, the Degarelix API and some its intermediates were analyzed by the inductively coupled plasma mass spectrometry (ICP-MS) to evaluate the amount of residual tin salts. The results showed that tin content is only 0.7% (7000 ppm) in the crude Degarelix and it is below the limit of quantification (0.31 ppb) for the peptide API after HPLC purification (Table 2). Thus, tin(II) chloride excess used at the step of nitro group reduction can be successfully eliminated without the use of scavengers or special purification steps (Table 2).

Table 2. ICP-MS analysis results of Degarelix and its intermediates

Sample	Sn content, % (*w/w*)
Fmoc-Rink amide resin	<LOQ
Fmoc-D-Phe(*p*-NO_2)-Leu-Lys(Boc,*i*Pr)-Pro-D-Ala-Resin	<LOQ
Crude Degarelix	0.700±0.018
Degarelix after HPLC purification	<LOQ

Finally, the identity of Degarelix obtained by the approach reported herein was confirmed by ^{1}H and homo- and heteronuclear NMR analysis by complete assignment of the NMR peaks (Tables S1, S2, Figures S2-8, the data are shown for the API produced with the process comprising the Hor attachment at the last step of the synthesis).

CONCLUSIONS

In summary, this work describes the novel approach for the solid-phase synthesis of the GnRH antagonist Degarelix, which helps to prevent the rearrangement of the Hor moiety in the presence of the organic bases. It comprises the reduction of the nitro group-containing precursor of the peptide and subsequent incorporation of the Hor moiety in the very last step of the synthesis, avoiding by that the contact of Hor with the standard chemicals used in the SPPS. The here described optimized process was successfully scaled-up to 500 g of the starting resin, providing Degarelix API with a very high HPLC purity. This approach can be used for the preparation not only of Degarelix, but also of other peptides of potential pharmaceutical interest containing the Hor moiety in their amino acid sequence.

EXPERIMENTAL PART

Materials and Methods

Iris Biotech: N,N-dimetylformamide (DMF), dichloromethane (DCM), N,N-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), piperidine, Fmoc-protected Rink amide resin; *Sigma Aldrich:* acetonitrile (MeCN) for mass spectrometry (MS) (>99,9%), TFA for MS (>99,9%), methyl *tert*-butyl ether (MTBE), triisopropylsilane (TIS), acetic anhydride, tetrabutylammonium hydrogen sulfate (TBAHS); *Carbosynth*: Ethyl (hydroxyimino)cyanoacetate (Oxyme Pure), N,N′-diisopropylcarbodiimide (DIC); *GL Biochem:* Fmoc-Nal-OH, Fmoc-Cpa-OH, Fmoc-Pal-OH, Fmoc-

Ser(*t*Bu)-OH, Fmoc-Phe(*p*-NO_2)-OH, Fmoc-D-Phe(*p*-NO_2)-OH, Hor, Fmoc-D-Aph(Cbm)-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc, *i*Pr)-OH, Fmoc-Pro-OH, Fmoc-D-Ala-OH, Fmoc-Lys(Boc)-OH.

HPLC and UPLC chromatographies: HPLC and UPLC analyses were performed on Agilent Technologies 1200 and Agilent Technologies 1290 instruments, respectively, using C18 Zorbax Eclipse Plus column (4.6×50 mm, 1.8 μm) for HPLC analyses and Extended C18 Agilent column (4.6×50 mm, 5 μm) for UPLC analyses. Analytical method 1: eluent A, TFA/H_2O 0.1% *v/v*; eluent B: TFA/MeCN 0.07% *v/v*; detection at 224 or 215 nm; gradient mode: 0 min – 25% of eluent B, 20 min – 45% of eluent B. Analytical method 2: gradient mode: 0 min – 25% of eluent B, 30 min – 45% of eluent B. The preparative purification was carried out using Phenomenex Luna C18 250 × 21.2 mm column and the eluent A, TFA/H_2O 0.1% *v/v*; eluent B: water/MeCN 10/90 (*v/v*) with 0.07% TFA. The following gradient elution was applied: 0 min – 17% of eluent B, 5 min – 15% of eluent B, 15 min – 35% of eluent B, 45 min – 50 of eluent B, 47 min – 50 of eluent B.

Mass spectrometry analyses: mass spectra were acquired on a Mariner API-ToF workstation (PerSeptive Biosystems, Framingham, MA) and in the LC-MS mode on Agilent 6530 mass accuracy Q-ToF, operating in the positive mode.

ICP-MS analysis: inductively coupled plasma mass spectrometry analysis (ICP-MS) for the determination of the Sn content was carried out with an Agilent Technologies 7700x ICP-MS (Agilent Technologies International Japan, Ltd., Tokyo, Japan). ICP-MS instrument was equipped with an octopole collision cell operating in kinetic energy discrimination mode used for the removal of polyatomic interferences and argon-based interferences. The optimal performance was obtained by using the collision cell in He mode for Au and He-He mode for Sn.[46] The ICP-MS was tuned daily using a tuning solution containing 1 μg/L ^{140}Ce, ^{7}Li, ^{205}Tl, and ^{89}Y (Agilent Technologies, UK). A 100 μg/L solution (Aristar, BDH, UK) containing ^{115}In, prepared in HNO_3 3.5% was used as internal standard through addition to the sample solution *via* a T-junction.

Multi-element calibration standard CCS-5 "Inorganic ventures" containing B, Ge, Hf, Mo, Nb, P, Re, S, Sb, Si, Sn, Ta, Ti, W, Zr (100 μg/mL) in aqueous HF 1.0 % (v/v) and HNO_3 7.14 % (*v/v*) was used for Sn determination. Multi-element calibration solutions were prepared by gravimetric serial dilution at five different concentrations from 250 ng/L to 100 μg/L using 3.5% HNO_3 solution in MilliQ water. The parameters of the calibration lines were obtained by using the Theil-Sen non-parametric regression technique.[47]

In a typical experiment about 2 mg of the sample were accurately weighed and digested using microwaves in 5 g of 69% solution of nitric acid accordingly to the following procedure: ramp temperature from RT to 220 °C in 5 min, then 220 °C for 5 min, pressure 400 PSI, power 300 W and stirring mode "medium". Then the solutions were diluted with the same solvent used for calibrations and analyzed.

NMR analysis: all NMR experiments were carried out at 298 K in DMSO-d_6 solution (peptide concentration 1.2 mM) using a Bruker AVANCE DRX-400 spectrometer and the TOPSPIN software package. The homonuclear spectra were acquired by collecting 512 experiments consisting of 64–80 scans and 2 K data points. The spin systems of coded amino acid residues were identified using standard DQF-COSY spectra. In the latter case, the spin-lock pulse sequence was 70 ms long. ROESY experiments were utilized for sequence-specific assignment. At the beginning of the experiments the build-up curve of the volumes of NOE cross-peaks as a function of the mixing time (50–500 ms) was obtained in order to avoid the problem of spin diffusion. The mixing time of the ROESY experiment used for interproton distance determination was 150 ms (*i.e.*, in the linear part of the NOE build-up curve). To fully assign all the resonances also heteronuclear experiments, HMQC and HMBC were acquired.

Peptide synthesis

Small scale synthesis. Solid-phase syntheses of the peptides and intermediates in a small scale were carried out using Biotage Syrowave (automated syntheses). In a typical procedure 0.2 g of the Rink amide resin with the loading of 0.65 mmol/g were used. The resin was swollen in 2 mL of DMF for 30 min, Fmoc deprotected with 20% piperidine in DMF (two cycles for 5 and 20 min, respectively), and washed 4 times with 2 mL of DMF. The solid-phase synthesis was carried out using 3 eq of protected amino acids that were preactivated during 3 min with 3 eq of Oxyma Pure and 3 eq of DIC, and subsequentry coupled in 1 h. The unreacted peptide chains were capped with acetic anhydride (10 eq) in the presence of DIPEA (10 eq). The cycles of coupling and deprotection were continued to obtain the desired peptide sequences. At the end of the synthesis, the peptide was cleaved from the resin with 3 mL of the mixture TFA/TIS/water (95:2.5:2.5, *v/v*) and precipitated in 10 mL of MTBE. The product was filtered off and dried.

Synthesis of Degarelix with nitro-group reduction during SPPS. The synthesis was carried out using Biotage MultiSynTech semi-automated synthesizer and 3 g of Rink amide resin (loading 0.65 mmol/g). The resin was swollen in 18 mL of DMF for 30 min, Fmoc deprotected with 20% piperidine in DMF (two cycles for 5 and 20 min) and washed 4 times with 18 mL of DMF. The solid-phase synthesis was carried out using 3 eq of protected amino acids that were preactivated during 3 min with 3 eq of Oxyma Pure and 3 eq of DIC, and subsequentry coupled in 1 h. The cycles of coupling and deprotection were continued to obtain Fmoc-Phe(*p*NO_2)-D-Aph(Cbm)-Leu-Lys(*i*Pr, Boc)-Pro-D-Ala-Rink amide resin. Then the resin was treated with a solution of tin(II) chloride dihydrate (4.4 g, 17.7 mmol) in 14.5 ml of DMF in the presence of DIPEA (0.40 mL, 2.3 mmol). The reaction was carried out overnight under nitrogen bubbling and the resin was washed with DMF (2×18 mL), a solution of 1 eq of DIPEA in 18 mL of DMF and DMF (2×18 mL). The obtained Fmoc-Aph-D-Aph(Cbm)-Leu-Lys(*i*Pr, Boc)-Pro-D-Ala-Rink amide resin was then treated with a solution of Hor-OH (1.5 eq), diisopropylcarbodiimide (1.5 eq) and HOBt (1.5 eq) in 18 mL of DMF. After 1.5 h the solvent was filtered and the resin was washed with DMF (2×18 mL) to have

Fmoc-Aph(Hor)-D-Aph(Cbm)-Leu-Lys(*i*Pr, Boc)-Pro-D-Ala-Rink amide resin. The synthesis was then continued to get H-D-Nal-D-Cpa-D-3-Pal-Ser(*t*Bu)-Aph-D-Aph(Cbm)-Leu-Lys(*i*Pr, Boc)-Pro-D-Ala-Rink resin. The unreacted peptide chains and Nal residue were acetylated with acetic anhydride (10 eq) in the presence of DIPEA (10 eq). At the end of the synthesis, the peptide was cleaved from the resin with 20 mL of the mixture TFA/TIS/water (95:2.5:2.5, *v/v*) and precipitated in 100 mL of MTBE. The product was filtered off, washed several times with MTBE and dried. Yield: 1.53 g (48%). The identity of the intermediates obtained was confirmed by ESI MS analysis after small scale cleavage or complete cleavage for the final product:

H-Phe(*p*NO_2)-D-Aph(Cbm)-Leu-Lys(*i*Pr)-Pro-D-Ala-NH_2: $[M+H]^+_{calc}$ = 866.48, $[M+H]^+_{exp}$ = 866.51

H-Aph-D-Aph(Cbm)-Leu-Lys(*i*Pr)-Pro-D-Ala-NH_2: $[M+H]^+_{calc}$ = 835.51, $[M+H]^+_{exp}$ = 835.46

H-Aph(Hor)-D-Aph(Cbm)-Leu-Lys(*i*Pr)-Pro-D-Ala-NH_2: $[M+H]^+_{calc}$ = 976.53, $[M+H]^+_{exp}$ = 976.47

Degarelix: $[M+H]^+_{calc}$ = 1631.75, $[M+H]^+_{exp}$ = 1631.73

Synthesis of Degarelix with nitro-group reduction at the end of SPPS. The synthesis was carried out using CS-Bio 936 system and 500 g of Rink amide resin (loading 0.65 mmol/g). The resin was swollen in 3 L of DMF for 30 min, Fmoc deprotected with 20% piperidine in DMF (two cycles for 5 and 20 min) and washed 4 times with 3 L of DMF. The solid-phase synthesis was carried out using 3 eq of protected amino acids that were preactivated during 3 min with 3 eq of Oxyma Pure and 3 eq of DIC, and subsequentry coupled in 1 h. The cycles of coupling and deprotection were continued to obtain Ac-D-Nal-D-Cpa-D-3-Pal-Ser(*t*Bu)-Phe(*p*NO_2)-D-Aph(Cbm)-Leu-Lys(*i*Pr,Boc)-Pro-D-Ala-Rink resin, which was treated with a solution of tin(II) chloride dihydrate (733 g, 3.24 mol) in 2.5 L of DMF in the presence of DIPEA (66 mL, 0.37 mol). The reaction was carried out overnight under nitrogen bubbling. The Ac-D-Nal-D-Cpa-D-3-Pal-Ser(*t*Bu)-Aph-D-Aph(Cbm)-Leu-Lys(*i*Pr,Boc)-Pro-D-Ala-Rink resin was then washed with DMF (2×3 L) than with 1 eq of DIPEA in 3 L of DMF and washed with DMF (2x3 L). A solution of Hor-OH (1.5 eq), diisopropylcarbodiimide (1.5 eq)

and HOBt (1.5 eq) in 3 L of DMF was added to the resin and stirred for 1.5 h. The solvent was filtered off and the resin was washed with DMF (2×3 L), DCM (2×3 L) and dried. At the end of the synthesis the peptide was cleaved from the resin with 7.5 L of the mixture TFA/TIS/water (95:2.5:2.5 *v/v*) and precipitated in 22 L of MTBE. The product was filtered off and dried. Yield: 291 g (55%). A part of the crude peptide was purified using preparative HPLC for further ICP-MS and NMR analysis. The identity of the peptides obtained was confirmed by ESI MS after small scale cleavage or complete cleavage for the final product:

Ac-Nal-Cpa-Pal-Ser-Phe(*p*-NO_2)-D-Aph(Cbm)-Leu-Lys(*i*Pr)-Pro-D-Ala-NH_2: $[M+H]^+_{calc}$ = 1521.70, $[M+H]^+_{exp}$ = 1521.72

Ac-Nal-Cpa-Pal-Ser-Aph-D-Aph(Cbm)-Leu-Lys(*i*Pr)-Pro-D-Ala-NH_2: $[M+H]^+_{calc}$ = 1491.73, $[M+H]^+_{exp}$ = 1491.74

Degarelix: $[M+H]^+_{calc}$ = 1631.75, $[M+H]^+_{exp}$ = 1631.73

Reaction of the peptide resin with potassium cyanate. The Fmoc-D-Aph-Leu-Lys(*i*Pr, Boc)-Pro-D-Ala-Rink amide resin was treated with a solution of KOCN (130 mg, 1.6 mmol, 10 eq) in 2.5 mL of 10% acetic acid, glacial acetic acid or a solution of 20% phosphoric acid in DMF. The reaction was carried out overnight and the peptide-resin was washed with DMF (3×2 mL). A sample of the peptide was cleaved using the mixture 1% water/TFA for HPLC analysis. The identity of the peptide obtained was confirmed by ESI MS analysis after small scale cleavage:

Fmoc-D-Aph(Cbm)-Leu-Lys(*i*Pr)-Pro-D-Ala-NH_2: $[M+H]^+_{calc}$ = 896.50, $[M+H]^+_{exp}$ = 896.52

ASSOCIATED CONTENT

Supporting information

HPLC profiles of the reaction of the pentapeptide with potassium cyanate solution and NMR spectra of Degarelix with the tables containing ^1H and ^{13}C chemical shift values of Degarelix molecule.

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