Replacing piperidine in Solid Phase Peptide Synthesis: effective Fmoc removal by alternative bases

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Replacing piperidine in Solid Phase Peptide Synthesis: effective Fmoc removal by greener alternative bases

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Solid Phase Peptide Synthesis (SPPS) is a key technology for the production of pharmaceutical grade peptides, despite it represents the worst modality in the pharma segment if considering its Process Mass Intensity (PMI). Consequently, academic and industrial research teams focused their attention on greening SPPS protocols by introducing more sustainable alternatives to the most common reagents and solvents. In this context, 3-(diethylamino)propylamine (DEAPA) was identified to be a viable alternative to piperidine for Fmoc removal. In addition, the use of DEAPA in *N*-octyl-pyrrolidone (manual synthesis) or *N*-octyl pyrrolidone/dimethyl carbonate 8/2 v/v (automated synthesis) was proved to be able in minimizing the formation of side products, like diastereoisomers and aspartimide-containing derivatives.

Introduction

The success of peptides in several therapeutic areas like diabetes, cancer and rare diseases has boosted their market.[1] This success is directly related to the availability of reliable synthetic techniques that allowed medicinal chemists to better explore this molecular space.[2] Nowadays, several technologies are available for the synthesis of pharmaceutical grade polypeptides,[3] as for instance recombinant production via fusion proteins, liquid-phase peptide synthesis (LPPS), peptide-anchored liquid-phase peptide synthesis (PA-LPPS), solid-phase peptide synthesis (SPPS) and the chemo-enzymatic peptide synthesis (CEPS).

The use of recombinant technologies can be applied only to the production of long peptides made of proteinogenic amino acids.[3c,4] In nowadays drug discovery, the pharmacological profile is improved, in terms of half-life, drug delivery, and conformational induction, via the introduction of fatty side chains, unnatural amino acids and cyclic architectures. The recombinant technology can be used also for peptides where the number of modification respect to the natural sequence is limited and the final peptide can be produced with late stage chemical reactions. Typical examples are the synthesis of Liraglutide, Semaglutide or new-insulin analogues.[5] LPPS chemistry is very efficient in limiting the reagents excess,[6] however, dilution and process efficiency remained a serious drawback for long peptides due to product solubility. The use of anchors in PA-LPPS was introduced with the aim to solve these issues,[7] but the different technologies developed so far have some pitfalls in terms of greenness, suitability to long peptides and process efficiency. There is currently growing interest in this area and the main issues will be probably fixed in the next future. On the other hand, the access to these innovative technologies is often limited by intellectual property.

SPPS, originated from the seminal work by Merrifield back in 1964,[8] is still playing a central role for the synthesis of pharmaceutical grade polypeptides. This is even more important for the production of peptides with improved resistance against metabolism, since the introduction of non-proteinogenic amino acids is not compatible with the production via fully recombinant fermentation processes. In addition, the development of greener technologies like CEPS, is entirely integrated with the Fmoc based SPPS technology.[9] The development of Green, or at least greener, Solid Phase Peptide Synthesis (GSPPS) has to face several issues, and the primary one is the selection of the proper solvent. DMF, being technically perfect in all the steps of the protocol, is the industrial solvent of choice for SPPS, even if it is toxic and reprotoxic. Since DMF is by far the main component of the PMI in the upstream process, several groups worldwide focused their efforts on the development of protocols based on the use of greener and/or sustainable and potentially recyclable solvents.[10] Suitable alternatives to DMF, able to afford proper resin swelling, complete solubility and stability of reagents and easy removal of byproducts by washing, have been reported. In this context, we recently proposed the introduction of several solvent mixtures with the target to increase greenness, maintaining reaction performances also in automated synthesis.[11] However, in spite of the excellent results showed by different teams using greener solvents/solvent mixtures, (see the list of solvents in references 10 and 11) the industry is still reluctant in changing DMF moving towards GSPPS when the DMF-technology has been optimized and consolidated in decades.

Anyway, other key components of SPPS, as the base and the coupling reagent, still need to be considered in completing GSPPS puzzle. The modification and optimization of coupling reagents, mainly driven in the last decades by Albericio and coworkers, was critical to increase coupling efficiency thus limiting the impact on the final purification processes.[12] Moving to Fmoc-removal, since piperidine is still the

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base of choice, the improvement of SPPS was also devoted to the identification of greener and recyclable bases.[13]

In Figure 1, the chronological order of discovery of alternative deprotecting bases and their use in green solvents is reported. In fact, piperidine is an efficient base, but it is also a substance under strict regulation being used for the illegal production of phenylcyclohexyl piperidine (PCP), even known as "angel dust", which is a powerful hallucinogen.[14] Moreover, the presence of genotoxic nitrosamines is an issue in the pharma segment being the required limit very low (ppb). The presence of nitrosamines in peptide synthesis is not generally a threat. However, the fate of the waste should be considered. Piperidine, being a secondary amine, can rapidly and directly generate the corresponding nitrosamine if exposed to nitrites in the environment.[15]

We have investigated herein the use of alternative bases with good greenness scores that are able to efficiently deprotect the Fmoc moiety without interfering with the SPPS of the growing peptide. Moreover, since regulatory agencies are decreasing the impurity limits to avoid any potential immunogenic side effect,[16] we decided to explore the impact of the base on the formation of a variety of typical process related impurities in order to define a reliable and robust SPPS protocol.

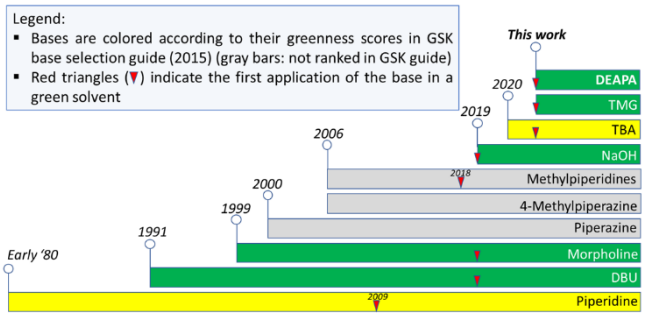


Figure 1. Chronology of bases introduction in SPPS.[13,17]

Results and Discussion

The Fmoc removal efficiency of *tert*-butyl amine (TBA), *N,N,N',N'*-tetramethyl guanidine (TMG) and 3-(diethylamino)propylamine (DEAPA) was compared to that of piperidine.

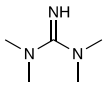
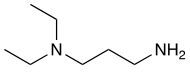
The general characteristics of the four bases are reported in Table 1. TMG and DEAPA were selected because of the lower oral acute toxicity and the consistently higher flash point compared with both piperidine and TBA. In fact, the GSK's greenness score of these two bases was better than that of piperidine and TBA, being 7 *vs* 5.[17] In addition, DEAPA is a less toxic analogue of tris(2-aminoethyl)amine introduced in nineties by Carpino et al. for the same purpose, taking advantage of the presence of primary and tertiary amine moieties in the structure.[18] Recently, Guryanov et al. described the removal of Fmoc using several alternative bases and TBA was chosen for the synthesis of Degarelix.[13h,i] TBA was surprisingly efficient taking into consideration its bulkiness and for this reason its performances were further explored in this study. Concerning nucleophilicity, as a balance of steric and electronic effects, the Mayr's parameter gave a ballpark estimation of the different bases. While TBA and TMG have a consistently lower nucleophilicity in respect to piperidine, DEAPA should have a behaviour similar to primary bases.

Concerning the environmental issue of nitrosamine formation, primary amines like TBA/DEAPA are generally not considered problematic. On the other hand, tertiary amines as DEAPA or TMG should undergo to strong acid induced decomposition in order to generate the corresponding secondary amines as good substrate for nitrosation. The bases we have considered are less prone to generate genotoxic nitrosamines in comparison with piperidine.[15b,c]

Fmoc removal kinetics

The alternative bases were firstly tested in qualitative solution experiments, with the target to determine Fmoc removal efficiency on a single amino acid. Fmoc-Phe-OH deprotection was evaluated in *N*-butyl pyrrolidone (NBP), *N*-octyl pyrrolidone (NOP) and DMF and monitored by HPLC-UV at different reaction times. NBP and NOP

Table 1. Properties of selected bases[a]

Entry	Base	Pka	Density (g/mL)	Mayr Parameter[b] (N)	LD50[a] mg/kg	Flash point °C	bp °C	GSK greenness scale[c]
1	 ***Piperidine***	11.2	0.862	17.35 (MeCN)	740	7.5	107	5
2	 ***TBA***	10.6	0.696	12.35 (MeCN)	316-514	-38	45.2	5
3	 ***TMG***	13.6	0.919	13.58 (DCM)	835	55	158.4	7
4	 ***DEAPA***	10.5	0.824	15.11 (MeCN)[d]	830	51.5	170	7

[a] All data are from the ECHA website. [b] From Mayr's database of reactivity parameters. [c] From ref. 17. [d] The N parameter for DEAPA is not available, therefore the nucleophilicity of its primary amine was considered similar to that of propylamine.

Table 2. Time (min) required to obtain a complete Fmoc removal in solution with different bases % and solvents

	DMF			NBP			NOP		
	Base w/w								
Base	5%	10%	20%	5%	10%	20%	5%	10%	20%
	Deprotection time, min								
Piperidine	6	2	2	8	2	2	6	4	2
TBA	>15	>15	6	>15	>15	6	>15	>15	10
TMG	8	4	2	8	2	2	6	2	2
DEAPA	>15	10	4	>15	15	4	>15	10	4

were evaluated since they are credible greener alternatives to DMF for SPPS, and their viscosity can be mitigated generating mixtures with other solvents like dimethyl carbonate (DMC 20%) or by increasing the reaction temperature. The selected bases were tested in a range between 5% and 20% (v/v) in order to estimate the minimum amount required for an effective deprotection during SPPS. Reaction times, corresponding to the complete disappearance of the HPLC peak relative to Fmoc-Phe-OH, are reported in Table 2. Interestingly, the deprotection kinetics in solution are almost independent from the used solvent. TMG and DEAPA were able to remove the Fmoc group in a range of time comparable to that commonly observed using piperidine at room temperature, while TBA induced deprotections were consistently slower. The reaction performed with TMG demonstrated fast deprotection kinetics even at low percentages of the base (6-8 minutes at 5%), while using TBA and DEAPA longer deprotection times were required. This trend was quite predictable, being consistent with the pK_a values of the four amines, as outlined in Table 1. In particular, a 10% TBA solution did not guarantee the deprotection within 15 minutes, independently from the chosen solvent, but its doubled amount was able to complete the reaction in 6-10 minutes in all cases.

Analogously, 10% DEAPA furnished satisfactory results, deprotecting Fmoc-Phe-OH in 10 minutes when tested in DMF or NOP, or in 15 minutes when tested in NBP.

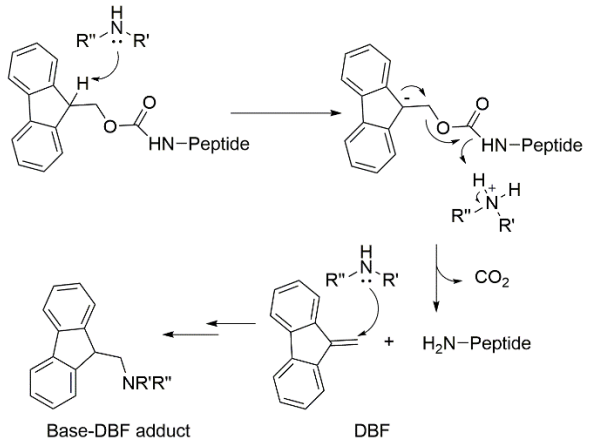
In order to define the optimal deprotection conditions with the new bases, the lower percentage leading to complete conversion in at least 15 minutes was considered acceptable. This choice depends on the standard SPPS protocols, where deprotection step is normally stopped after 15 minutes and is often repeated twice.[19] Therefore, using NOP as solvent, the best conditions to be applied are 5% of TMG, 20% of TBA or 10% of DEAPA. These conditions were also employed for further investigations.

Deprotection mechanism

The general mechanism of Fmoc removal was described in Scheme 1. It is well known that a secondary base like piperidine, after the formation of dibenzofulvene (DBF), is able to attack the double bond preventing the formation of impurities coming from the reaction of DBF with the free just formed alpha-amino group or with any other reactive species which might be present on the growing peptide bound to the resin.

However, when TBA, TMG and DEAPA were used, DBF did not generate any of these side products nor insoluble compounds coming from DBF oligomerization.

To evaluate the reaction mechanism of Fmoc removal, a standard amount of dry resin Fmoc-Gly-Trt-PS was treated with a 20% solution of each base in DMF-d_6 for 30 minutes, thus simulating a standard deprotection (2 x 15 minutes). The resin was then filtered and the filtrate was immediately analysed by ^{1}H NMR spectroscopy in order to reveal the presence of dibenzofulvene (DBF) and, eventually, the formation of the base-DBF adduct, deriving from the mechanism commonly observed for piperidine (Scheme 1).



Scheme 1. Mechanism of Fmoc group removal.

^{1}H NMR analysis demonstrated that TBA and TMG are not nucleophilic enough to react with DBF while DEAPA is able to generate the corresponding DEAPA-DBF side product (^{1}H NMR spectra reported in Supplementary Information). This behaviour is in agreement with the already discussed Mayr's parameter N.

The ability of piperidine and DEAPA to react with DBF was explored in DMF, NBP and NOP and monitored by HPLC after 15 min reaction (Table 3). The solvent showed a minor influence on the reaction

Table 3. Formation of base-DBF adduct in the studied solvents

Entry	Base (%)	Solvent	Base-DBF adduct:DBF[a]
1	Piperidine (20)	DMF	96:4
2	Piperidine (20)	NBP	96:4
3	Piperidine (20)	NOP	96:4
4	Piperidine (10)	NOP	90:10
5	DEAPA (10)	DMF	14:86
6	DEAPA (10)	NBP	10:90
7	DEAPA (10)	NOP	13:87

[a] Calculated on the basis of HPLC signal integration of crude samples after 15 min deprotection, after correction with Relative Response Factor (RRF=0.8, see ESI)

outcome, while the amount and the nature of the base played a key role.

The piperidine-DBF/DBF ratio using 20% v/v of piperidine was 96/4 independently from the solvent (entries 1-3), slightly decreased with 10% piperidine in NOP (entry 4), while the corresponding deprotection carried out with DEAPA generated a ~10/90 ratio (entries 5-7). The analysis was performed after 15 min from the base addition, however, very similar data were observed after only 6 minutes (see SI). Anyway, the presence of DBF did not pose any synthetic problem, since in all the syntheses we have performed, no side reactions with the growing peptide were detected. In fact, after cleavage, the possible presence of DBF-capped truncated sequences was always checked by HPLC-MS. In addition, it has been recently reported by Takahashi et al.[7d] that 2-mercaptopropionic acid or thiomalic acid can be added as DBF scavenger in case of need.

Racemization tests

To assess the usefulness of the proposed green bases in SPPS, the effect on racemization has to be considered. Undesired epimerization is known to occur during coupling steps but also during basic treatments for deprotection.

This phenomenon affects in particular residues with electron-withdrawing groups on side chains that are able to stabilize the negative charge on Cα.[20] This side reaction should be minimized as much as possible to avoid diastereomeric impurities of the final target compound that could be very difficult to eliminate by the final chromatographic purification.

Cysteine is known to be extraordinarily prone to undergo racemization during peptide synthesis. Many studies were recently reported for evaluating the degree of racemization in tripeptides containing Cys as a susceptible residue. In most cases, the epimerization was investigated during coupling steps, by evaluating the effect of different reagent combinations, solvents or Cys side chain protecting groups.[21]

H-Phe-L-Cys-Gly-OH and H-Phe-D-Cys-Gly-OH were prepared as standards, and HPLC conditions were optimized to obtain a good separation of the diastereoisomers (see Experimental Section). Then, full NOP-mediated SPPS of H-Phe-L-Cys-Gly-OH was performed in parallel using piperidine, TBA, TMG and DEAPA as deprotecting agents in 30% v/v concentration for 60 minutes. These extreme conditions were adopted to emphasize the eventual effect of the base in promoting the racemization, and specifically to simulate a longer peptide sequence where Cys would be subjected to repeated exposure to basic treatments. The experiments were repeated using the green mixture NOP/DMC 8/2, already reported by us,[11b] to further verify that the addition of a cosolvent does not affect the level of isomerization in racemization-prone peptides.

Table 4. Racemization ratio (%) in the synthesis of H-Phe-Cys-Gly-OH in NOP and NOP/DMC 8/2 with different % bases[a]

Entry	Base	w/w %	DL (%) in NOP[b]	DL (%) in NOP/DMC 8/2[b]
1	Piperidine	30	< 0.1	< 0.1
2	TBA	30	< 0.1	< 0.1
3	TMG	30	2.4	2.2
4	DEAPA	30	< 0.1	< 0.1

[a] Racemization ratio were determined by HPLC; [b] defined as (H–Phe–D-Cys–Gly–OH)/(H–Phe–L-Cys–Gly–OH) × 100

Even if the acceptable level of racemization to "safely" introduce cysteine is generally <1%,[21b,c] our target was to minimize the formation of the D-Cys diastereoisomer to a 0.1% threshold for the synthesis of pharmaceutical grade peptides, in agreement with the new provisions introduced by the regulatory authorities for the limitation of impurities.

Surprisingly, no substantial differences were observed among TBA, DEAPA and piperidine (Table 4, entries 1,2,4) being the racemization ratio below 0.1% in all experiments.

On the other hand, the more basic TMG revealed a higher tendency to promote the cysteine isomerization (entry 3); anyway, by performing the deprotection with 5% v/v of base, which does not strongly impact on the deprotection speed, the target 0.1% isomerization level was achieved (See Supplementary Information).

Aspartimide formation

Aspartimide formation is a common side reaction in SPPS to be considered when developing new protocols.[22]

The cyclization occurs following the nucleophilic attack on the Asp Cγ by the proximal NH amide. The aspartimide ring may then be opened with formation of further unwanted chemical species. Specifically, aspartimide could undergo nucleophilic attacks from water or from a base on carbonyl groups in two positions, leading to the corresponding α/β peptides or side base-adducts, respectively (Scheme 2). The alpha-peptide may correspond to the target peptide, although racemization at Asp might occur.

Basic conditions, which are mandatory for Fmoc group cleavage, may also favor the formation of unwanted aspartimide derivatives during Fmoc-based SPPS and their occurrence generally increases with the number of Fmoc cleavage cycles after the introduction of an aspartic acid residue (Asp) in the peptide chain. In addition, aspartimide formation is directly related to the nature of the Asp side chain protective group, and the adjacent amino acid.[23]

Albericio et al. introduced the use of modulatory agents, namely OxymaPure®, in order to hamper aspartimide formation reducing the base tendency to react on the amide Nα vicinal to Asp. Moreover, some recent studies describe the use of novel backbone amide or carboxylic acid protecting groups in order to improve the synthesis of aspartimide-prone peptides.[24]

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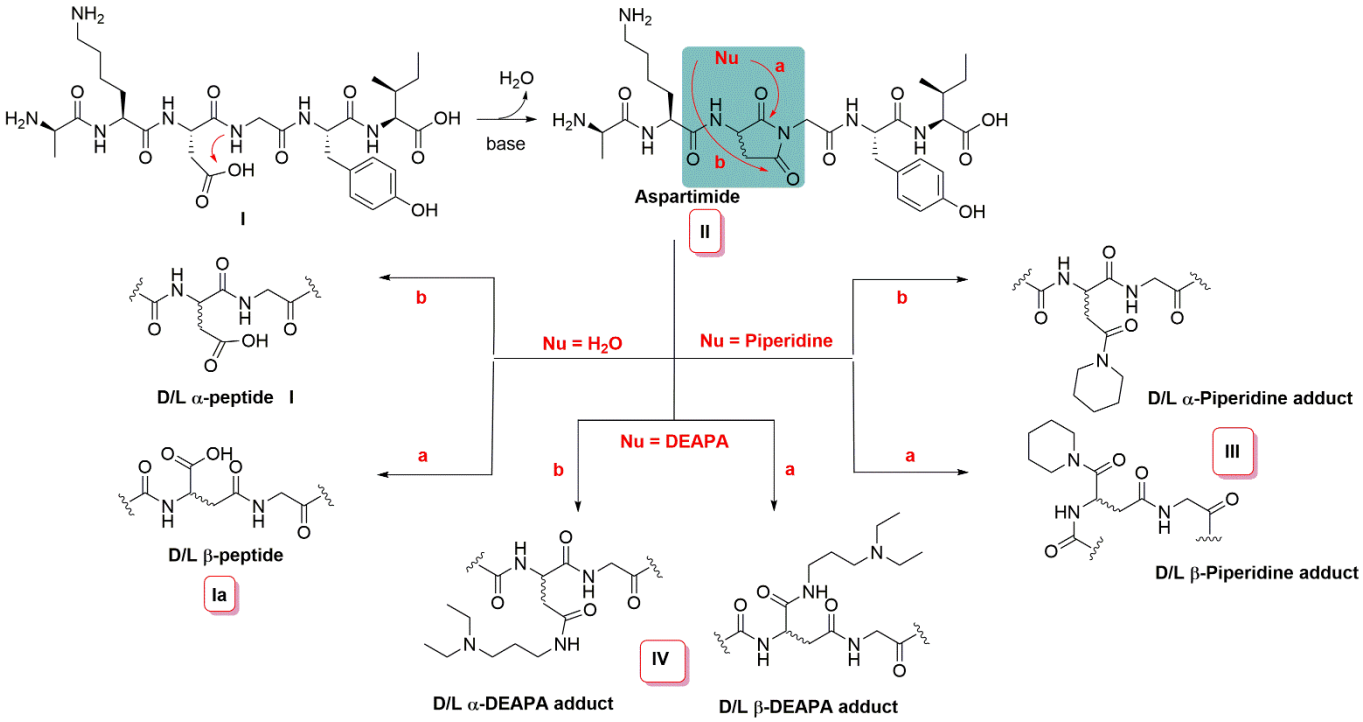

Scheme 2. Mechanism of aspartimide derivatives formation in the presence of TMG, TBA, DEAPA or piperidine.

In addition, it was recently discovered that the solvent can impact this side reaction, with less polar ones minimizing aspartimide formation, both at room temperature and at 50°C. NBP was in fact reported to have less tendency to produce this side reaction, if compared to DMF.[25] In this context, we have evaluated the behaviour of the new bases in DMF, NBP and NOP in inducing aspartimide and related side-products formation in peptides containing an Asp residue. In particular, we used as a model the hexapeptide H-Ala-Lys-Asp-Gly-Tyr-Ile-OH **I** (Scheme 2).[25] Peptide **I** was prepared by full-SPPS in DMF using standard deprotection and coupling conditions (piperidine 20%; OxymaPure®/DIC) on Fmoc-Ile-Trt-PS resin. Fmoc-Asp(OtBu)-OH was employed since *tert*-butyl protecting group does not hinder aspartimide formation; moreover, the presence of -Asp-Gly- in the sequence should favour the progress of the side reaction. The content of aspartimide impurity II in the final compound was determined by HPLC through a mini-cleavage of the functionalized resin, allowing to set a reference value of the incidence of **II** naturally formed after standard synthesis (2.6% indicated as "starting point" in Table 5). The model peptide **I** was then exposed to basic conditions for 4 hours in order to simulate repeated α-amino deprotection cycles. Resin-bound H-Ala-Lys-Asp-Gly-Tyr-Ile-OH **I** (still bearing side-chain protections) was treated at 25°C with 20% TBA, 5% TMG or 10% DEAPA solutions in DMF, NBP or NOP. The deprotections using 20% piperidine solution at 25°C and 40°C were included in the study as comparison (Table 5). As expected, there are two general trends, since aspartimide **II** increases with the temperature and with solvent polarity.

As shown in Scheme 2, the formation of the aspartimide **II** may be followed by nucleophilic attack by the base itself or by water with formation of further impurities. TMG and TBA, being less nucleophilic (see Mayr's N parameter in Table 1), did not display any direct reaction with aspartimide **II**. On the contrary, piperidine that is a very good nucleophile generated consistent quantities of piperidides **III** (Scheme 2 and Table 5 entries 1-4,6). The only exception was the reaction in NOP at room temperature (entry 5). On the other hand, when DEAPA was used, the corresponding amine-adducts **IV** were detected only at 40°C, however in spite of our efforts we have not been able to exactly determine the amount, since these by-products co-elute with target hexapeptide **I**.

Unfortunately, TBA (entries 7-9) and TMG (entries 10-12) did not provide a good outcome and proved not to be compatible with the presence of an Asp residue in the sequence. TMG showed the worst performances even if the use of NOP greatly minimized the formation of **II** (entry 12). Furthermore, when TMG was used, the formation of an isomer partially co-eluting with **I** was observed in DMF and NBP (entries 10 and 11), identified as the beta-peptide **Ia** according to Kumar et al.[25]

Table 5. HPLC purity of H-Ala-Lys-Asp-Gly-Tyr-Ile-OH after stress tests with different bases and solvents.[a]

Entry	Base (%)	Solvent	T °C	Hexapeptide **I** %	Aspartimide **II** %	Base adduct (%)	Δ Asp %
Starting point				97.4	2.6	-	
1	Piperidine (20)	DMF	25	83.9	8.7	**III** (7.4)	13.5
2	Piperidine (20)	DMF	40	36.0	27.3	**III** (36.7)	61.4
3	Piperidine (20)	NBP	25	90.0	7.1	**III** (2.9)	7.4
4	Piperidine (20)	NBP	40	60.5	22.3	**III** (17.2)	36.9
5	Piperidine (20)	NOP	25	94.9	5.1	-	2.5
6	Piperidine (20)	NOP	40	77.0	10.2	**III** (12.8)	20.4
7	TBA (20)	DMF	25	70.7	29.3	-	26.7
8	TBA (20)	NBP	25	79.5	20.5	-	17.9
9	TBA (20)	NOP	25	94.5	5.5	-	2.9
10	TMG (5)	DMF	25	14.5[b]	85.5	-	82.9
11	TMG (5)	NBP	25	27.9[b]	72.1	-	69.5
12	TMG (5)	NOP	25	93.0	7.0	-	4.4
13	DEAPA (10)	DMF	25	89.9	10.1	-	7.5
14	DEAPA (10)	NBP	25	95.7	4.3	-	1.7
15	DEAPA (10)	NOP	25	96.6	3.4	-	0.8
16	DEAPA (10)	NOP	40	88.6	6.4	**IV** (5.0)	8.8
17	DEAPA (10)	NOP/DMC 8/2	25	95.8	4.2	-	1.6

[a] The peptide was treated for 4h with the base solution and the amount of the different species was determined by HPLC assuming a RRF of 1. [b]Data include also a partially co-eluting isomer of **I**,identified as β-peptide **Ia**

Looking at the data from a different perspective, the synthesis of aspartimide **II** is a challenge from a synthetic point of view. Impurity standards are necessary for analytical method validation and safety assessment that comprise immunogenic in vitro studies.[16] Accordingly, aspartimides can be easily generated by exposing the peptide to TMG.

It is worth noting that the 10% solutions of DEAPA for Fmoc removal showed the best performances in terms of aspartimide **II** minimization, in all solvents, with NOP being the best one generating only 0.8% of the impurity at 25°C (entries 13-15). Interestingly, DEAPA was able to contain the amount of aspartimide **II** at 40°C (entry 16), and at 25°C with the mixture NOP/DMC 8/2 (entry 17). The NOP/DMC mixture was used to decrease the viscosity to 3.9 mPa·s, below the 4 mPa·s value that has been reported as the maximum tolerable threshold for pilot plant manufacturing by Martin et al.[10j]

(L)-dihydroorotic fragment isomerization

Degarelix **V** is a decapeptide having sequence Ac-D-Nal-D-Cpa-D-Pal-Ser-Aph(Hor)-D-Aph(Cbm)-Leu-Lys(iPr)-Pro-D-Ala-NH_2 (Figure 2) that is approved as a medicinal product for the treatment of patients with advanced prostate cancer and marketed under the trade name Firmagon®, as a third-generation gonadotropin releasing hormone (GnRH) receptor antagonist. One of the main problems in the preparation of Degarelix is the high sensitivity of the (L)-dihydroorotic acid (indicated as Hor) moiety of the Aph(Hor) residue in position 5 of the sequence in the presence of an aqueous basic solution. Under these conditions, a rapid rearrangement of the 6-membered Hor ring occurs, with formation of a 5-membered hydantoin ring (Figure 2). The stability of Degarelix **V** to hydantoin rearrangement was performed, according to Zhang and coworkers,[26] by treating Degarelix for 24 hours in a DMF solution with 10% of base, namely piperidine, DEAPA, TMG and TBA. The samples were

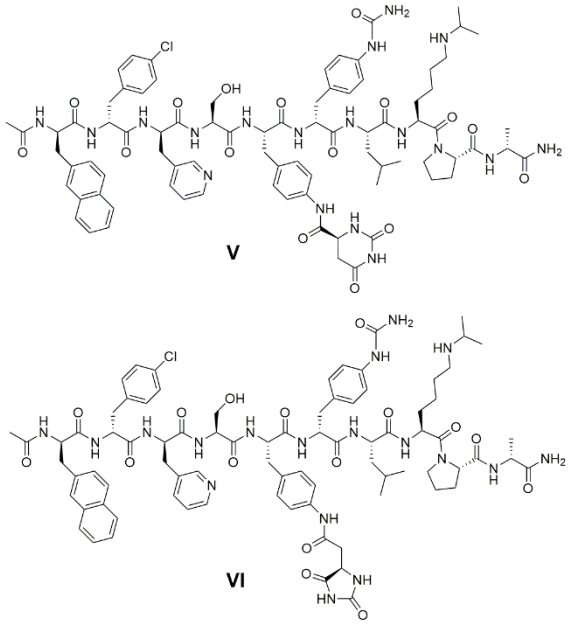

Figure 2. Structures of Degarelix **V** and related hydantoin impurity **VI**

Table 6. HPLC purity of Degarelix after 24 h stability tests in base solutions

Entry	Base solution	Degarelix V[a] %	Hydantoin VI[a] %
t=0	-	99.97	0.03
1	10% Piperidine/DMF	99.96	0.04
2	10% TMG/DMF	98.7	1.3
3	10% TBA/DMF	99.96	0.04
4	10% DEAPA/DMF	99.97	0.03

[a] Determined by HPLC.

analysed by HPLC after 24 hours to estimate the amount of the hydantoin analogue; data are reported in Table 6.

The experiment could not be carried out in NOP or NOP/DMC for solubility issues. However, since DMF is a more polar solvent and magnifies basicity, the selected condition can be considered the worst-case scenario.

TMG is promoting the isomerization process showing the formation of 1.3% of hydantoin (entry 2) while in the presence of all the other bases the (L)-dihydroorotic fragment was stable. The results observed for the reaction with piperidine and TBA (entries 1,3) are in line with the literature data;[13h,i,26] interestingly, also using DEAPA the formation of the hydantoin impurity **VI** was suppressed (entry 4).

Aib-Enkephalin SPPS in NOP with alternative bases

Full SPPS of Aib-Enkephalin (H-Tyr-Aib-Aib-Phe-Leu-OH) were performed to evaluate the effect of different bases in Fmoc removal. This peptide does not contain base sensitive amino acids and allows to evaluate the reaction performances with the steric demanding Aib residue. The syntheses were performed starting from Fmoc-Leu-Wang-PS preloaded resin using DIC/OxymaPure® for the coupling in NOP as solvent. Fmoc removals were performed using the best conditions for each base, 20% piperidine as reference,[11b] 20% TBA, 5% TMG and 10% DEAPA (Table 7). All syntheses confirmed the high efficacy of this solvent in SPPS and in minimizing the des-Aib impurity, especially if compared to DMF, as we recently reported.[11b] Additionally, the different bases showed comparable results with 20% piperidine (entries 1-5).

Table 7. HPLC purities for Aib-Enkephalin pentapeptide assembled in NOP with different bases and in NOP/DMC 8/2 with DEAPA.

Entry	Base (%)	Pentapeptide [b] %	Des-Aib %	Other %
1[a]	Piperidine (20)	97.8	0.8	1.4
2	TBA (20)	97.9	0.8	1.3
3	TMG (5)	97.8	1.1	1.1
4	DEAPA (10)	97.1	2.6	0.3
5[c]	DEAPA (10)	98.6	1.0	0.4
6[d]	DEAPA (10)	97.5	2.1	0.4

[a]From Ref. 11b; [b] Double Fmoc-Aib-OH couplings[10a-c]; [c]Deprotections pre Fmoc-Aib-OH insertion were performed at 40°C; [d]The synthesis was performed in NOP/DMC 8/2 using the same conditions of entry 4.

In all cases the target pentapeptide purity was superior to 97%. SPPS with 10% DEAPA was repeated performing the deprotection steps previous to Aib residues at 40°C: in this case the final peptide purity was superior to 98.5%. The results of the synthesis performed in the NOP/DMC 8/2 mixture, using the automated system, was almost identical to the corresponding reaction performed, manually, in NOP (entries 4 and 6).

SPPS of linear Octreotide in NOP with DEAPA

Based on the previous results, DEAPA was selected as the best alternative to piperidine for SPPS. The synthesis of linear Octreotide (H_2N-D-Phe-Cys^2-Phe-D-Trp-Lys-Thr-Cys^7-Thr-ol) was performed in DMF, NOP and NOP/DMC 8/2 as solvents, with 10% DEAPA as standard deprotection mixture. Preloaded Fmoc-Thr(tBu)-ol-Trt-PS resin and DIC/OxymaPure® coupling reagents were employed. As previously reported,[11] the coupling of the first inserted Fmoc-Cys(Trt)-OH (Cys^7 in the final sequence) was repeated twice. The linear octreotide SPPS in DMF and NOP at 25°C generated similar data in terms of final peptide mixture quality (Table 8). The synthesis in NOP required an initial longer swelling time (4 hours) to completely suppress the deletion of Cys^7.

Interestingly, the process performances at 40°C were very similar in terms of purity but only 1.5 equivalents of Fmoc-AA-OH, DIC and OxymaPure® were required to reach complete conversions, both in NOP and NOP/DMC 8/2.

The automated synthesis that was then performed at 25°C using NOP/DMC 8/2 as solvents mixture,[11b] generated similar results, even if it was necessary to perform swelling and first deprotection at 40°C to completely avoid the insurgence of des-Cys^7 side product. In summary, the data reported in Table 8 showed the flexibility of DEAPA in manual and automated SPPS. In particular, the combination of DEAPA and NOP proved to contain the side products formation, thus allowing also to perform the complete SPPS process at 40°C decreasing the amino acid excess necessary to achieve complete conversion.

Table 8. HPLC Purity of Linear Octreotide in DMF, NOP, NOP/DMC 8/2 with 10% DEAPA solvent and temperature effect.

			Solvent				
		DMF	NOP	NOP[a]	NOP (40°C)	NOP/DMC 8/2 (25°C)[b]	NOP/DMC 8/2 (40°C)
Compound	**RRT**						
TM-N,O shift 1	0.92		0.5				1.7
TM-N,O shift 2	0.95	3.6	5.9	5.6	3.8	7.5	8.1
TM+CO_2	0.97				4.4		
Des Cys[7]	0.98		8.3				
TM	1.00	83.6	72.2	78.1	78.2	81.9	81.1
TM+t-Bu	1.14	9.7	9.0	12.3	10.2	8.5	9.1
TM+2(t-Bu)	1.26	3.1	4.0	4.0	3.4	2.1	

[a]4 hours initial swelling time; [b]Swelling and first Fmoc removal were performed at 40°C.

Table 9. Process Mass Intensity (PMI) and recovery in GSPPS.

Entry	Solvent (T °C)[a]	PMI	Waste Stream	Recovery (yield %)	PMIr
1	NOP (25)	730	Depr	NOP (85)	264
				DEAPA (92)	
			Coupling	NOP (85)	
2	NOP (40)[b]	719	Depr	NOP (85)	255
				DEAPA (92)	
			Coupling	NOP (85)	
3	NOP/DMC 8/2 (25)	757	Depr	NOP (85)	255
				DMC (95)	
				DEAPA (92)	
			Coupling	DMC (95)	
				NOP (85)	
4	NOP/DMC 8/2 (40)[b]	747	Depr	NOP (85)	245
				DMC (95)	
				DEAPA (92)	
			Coupling	DMC (95)	
				NOP (85)	
5	NOP (25)[c]	722	Depr	NOP (85)	268
				Pip (92)	
			Coupling	NOP (85)	

[a] The wastes coming from coupling steps and from deprotection steps were distilled separately; [b] SPPS at 40°C were conducted with 1.5 equivalents of coupling reagents and Fmoc-AA-OH; [c] from Ref. 11b

Process Mass Intensity

Among the green metrics, PMI is the most rapid and efficient method to evaluate the sustainability of a synthetic process.[27] In Table 9 we have reported the PMI and the PMI after recovery (PMIr) of the octreotide upstream process described in Table 8. We decided to evaluate DEAPA involving processes only in green solvents. The coupling and deprotection waste streams were kept and distilled separately in order to recover NOP (or NOP and DMC) from the coupling waste stream and NOP (or NOP and DMC) and DEAPA from the deprotection waste stream. DMC, NOP and DEAPA were isolated separately and reused in SPPS. On the other hand, when the same protocol was applied to the waste streams of reactions performed with piperidine, the base co-distilled with DMC and the recovered solutions were reused in SPPS, after proper adjustment of the volume ratios. Independently from the reaction temperature or the solvent used, the PMI values after recovery of solvents and DEAPA were very similar and efficient (see entries 1-6). These data are comparable to the ones described by us using piperidine as deprotecting base.[11b]

Conclusions

The main components of SPPS wastes in the upstream process are the solvents and the base used for Fmoc removal. In this work, the performances of potential alternative bases (TBA, TMG and DEAPA) have been compared.

DEAPA proved to be an efficient base for Fmoc removal in SPPS upstream processes. Although this base is not able to trap DBF efficiently, no traces of truncated DBF-capped sequences were observed in any experiment. On the other hand, the simple addition of scavengers, like thiols, can be applied in case of need. DEAPA is an alternative to the highly regulated piperidine, generating similar results, being not regulated, less toxic and able to better control aspartimide related side reactions. Accordingly, it ranked in the first position of our screening aimed at selecting a base with a better green score for SPPS.

DEAPA was used in combination with NOP or NOP/DMC mixture, already explored by our group as SPPS suitable environmentally friendly solvents. This protocol allowed to achieve results comparable to those obtained with piperidine, using both manual

and automated synthesis. In our opinion, DEAPA may be introduced as an alternative to piperidine in the development and optimization of peptide synthesis, especially when aspartimide issues have to be overcome.

Experimental procedures

General methods

Unless otherwise specified, all solvents and reagents were obtained from commercial suppliers, of the best grade, and used without further purification. Specifically, Fmoc amino acids and resins were supplied by Iris Biotech, Alfa Aesar, Merck or Fluorochem. Coupling reagents were purchased from Merck or Novabiochem. Piperidine, TBA, TMG and DEAPA were supplied by Merck or TCI (purity >99%). DMF, *N*-octyl-pyrrolidone, *N*-butyl-pyrrolidone, dimethyl carbonate and HPLC-quality acetonitrile (CH_3CN) were purchased from Merck. Milli-Q water was used for RP-HPLC analyses. Automated solid-phase peptide syntheses were carried out manually or using CSBio-CS136X peptide synthesizer. SPPS at 40°C were performed with a Minichiller 300 from Huber. Unless otherwise specified, HPLC-MS analyses were performed on Agilent 1260 Infinity II system coupled to an electrospray ionization mass spectrometer (positive-ion mode, m/z = 100–3000 amu, fragmentor 30 V), using columns Agilent Zorbax-SB-C18 5 µm, 250 x 4.6 mm or Phenomenex Luna C18 5 µm, 250 x 4.6 mm; temperature: 25°C; injection volume: 10 µL, UV: 220 nm, elution phases: H_2O+0.08%TFA (mobile phase A) and CH_3CN+0.08%TFA (mobile phase B) in gradient mode, flow: 0.5 mL/min or 1.0 mL/min. Chemstation software was used for data processing. ^1H NMR spectra were recorded with an INOVA 400 MHz instrument with a 5 mm probe. All chemical shifts were quoted relative to deuterated solvent signals. Distillations were performed with an Edwards RV3 vacuum pump. Samples of lyophilized Degarelix used for stability tests were provided by Fresenius kabi.

Fmoc removal kinetics

Fmoc-Phe-OH (0.1 mmol) was dissolved in the desired solvent (NOP, DMF or NBP). The selected base (piperidine, DEAPA, TMG or TBA) was added to the solution in order to achieve the desired concentration (in a 5-20% range solution) in the final 1 mL deprotection mixture total volume. The reaction mixture was stirred at room temperature and samples of the solution (20 µL) were taken at t=0 (before base addition) and after 2, 4, 6, 8, 10, 15 minutes. The samples were diluted with 1.5 mL of ACN/TFA (1% v/v) and injected in HPLC-UV. Reaction was considered complete at disappearance of the HPLC peak corresponding to Fmoc-Phe-OH. Times corresponding to complete kinetic reactions are reported in Table 2. Relative chromatograms are reported in Figures S1-S15.

Gradient HPLC analysis: 5-95% (mobile phase B) in 15 minutes, 95-5% from 15 to 20 minutes, flow: 1.0 mL/min.

Monitoring of DBF-amine adduct formation

50 mg of dry resin Fmoc-Gly-Trt-PS were swelled in 2 mL of DMF for 30 minutes. The resin was filtered and 0.75 mL 20% base solution (piperidine, DEAPA, TBA or TMG) in DMF-d_6 was added on the resin and stirred for 30 minutes. The resin was filtered and the filtrate was straight analysed by ^1H NMR spectroscopy in order to reveal the presence of DBF alone or the formation of the DBF-amine adduct (Scheme 1 and Figures S20-S23).

The presence of base-DBF adduct was also monitored by Fmoc cleavage kinetics in liquid phase and calculated on the basis on HPLC signal integration of crude samples after RRF correction at t=6 min deprotection (results reported in Table S1) or after t=15 min deprotection (results reported in Table 3). Selected relative chromatograms are reported in Figures S27-S34.

Gradient HPLC analysis: 5-95% (mobile phase B) in 15 minutes, 95-5% from 15 to 20 minutes, flow: 1.0 mL/min.

SPPS of H-Phe-L-Cys-Gly-OH and H-Phe-D-Cys-Gly-OH in DMF as reference compounds for Cys racemization tests

The synthesis was carried out by using Fmoc-Gly-Trt-PS resin (200 mg, loading 1.1 mmol/g). After swelling of the resin in 2 mL of DMF, Fmoc protective group was removed by 20% piperidine in DMF (2×2 mL, 15 min each) and the resin was washed with DMF (3×2 mL). Fmoc-L-Cys(Trt)-OH (or Fmoc-D-Cys(Trt)-OH) and Fmoc-Phe-OH (three-fold excess with respect to the loading of the resin) were diluted in DMF (2,5 mL), pre-activated by DIC and OxymaPure® (three-fold excess of the reagents with respect to the loading of the resin) for 3 min and coupled to the resin in 60 min. After each coupling step the Fmoc protective group was removed by treating the peptide resin with a 20% piperidine solution in DMF (2×2 mL, 15 min each), and the resin was washed with DMF (3×2 mL). After Fmoc-cleavage of *N*-terminal alpha-amino group the peptide resin was washed with DMF (3×2 mL) and DCM (3×2 mL). Dry peptide resin was suspended in 5 mL of the mixture TFA/TIS/H_2O/1-dodecanethiol (92.5/2.5/2.5/2.5 *v/v/v/v*) and stirred for 2 h. The resin was filtered off and diisopropylether (20 mL) cooled to 4°C was added to the solution. The peptide was filtered and dried *in vacuo* to obtain crude H-Phe-L-Cys-Gly-OH or H-Phe-D-Cys-Gly-OH as reference compounds for racemization tests. Relative chromatograms are reported in Figures S35-S37.

Gradient HPLC analysis: 0-60% mobile phase B in 30 minutes, flow: 0.5 mL/min.

Cys racemization tests during SPPS of H-Phe-L-Cys-Gly-OH

Full Fmoc-based SPPS of H-Phe-L-Cys-Gly-OH were conducted as reported above, but the synthesis was carried out using NOP or NOP/DMC 8/2 for all steps and the following conditions for Fmoc group cleavage in parallel: 30% DEAPA, 30% piperidine, 30% TBA and 30% TMG in NOP for 60 minutes. In case of TMG, SPPS of H-Phe-L-Cys-Gly-OH was conducted also with the following conditions for Fmoc group cleavage: 20% TMG, 15% TMG, 10% TMG and 5% TMG in NOP for 60 minutes.

Racemization ratio (D/L %) in the preparation of H-Phe-Cys-Gly-OH was determined by HPLC % areas of the two diastereoisomers, and calculated as (H–Phe–D-Cys–Gly–OH A%)/(H–Phe–L-Cys–Gly–OH A%) × 100, as reported in Table 4. Relative chromatograms are reported in Figures S38-S52.

Gradient HPLC analysis: 0-60% mobile phase B in 30 minutes, flow: 0.5 mL/min.

SPPS of H-Ala-Lys-Asp-Gly-Tyr-Ile-OH in DMF as reference compound for aspartimide formation detection

The synthesis was carried out by using Fmoc-Ile-Trt-PS resin (800 mg, loading 1.1 mmol/g). After swelling of the resin in 6 mL of DMF, Fmoc protective group was removed by 20% piperidine in DMF (2×4 mL, 15 min each) and the resin was washed with DMF (3×4 mL). Fmoc-Tyr(tBu)-OH, Fmoc-Gly-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ala-OH (three-fold excess with respect to the loading of the

resin) were diluted in DMF (6 mL), pre-activated by DIC and OxymaPure® (three-fold excess of the reagents with respect to the loading of the resin) for 3 min and coupled to the resin for 60 min. After each coupling step the Fmoc protective group was removed by treating the peptide resin with 20% piperidine in DMF (2×4 mL, 15 min each), and the resin was washed with DMF (3×4 mL). After Fmoc cleavage of *N*-terminal amino group the peptide resin was washed with DMF (3×4 mL) and DCM (3×4 mL). 100 mg of dry peptide-bound resin were suspended in 3 mL of the mixture TFA/TIS/H_2O (90/5/5 *v*/*v*/*v*) and stirred for 2 h. The resin was filtered off and diisopropylether (10 mL) cooled to 4°C was added to the solution. The peptide was filtered and dried *in vacuo* to obtain crude H-Ala-Lys-Asp-Gly-Tyr-Ile-OH. HPLC purities of the crude after cleavage are reported in Table 5 (97.4% hexapeptide **I** and 2.6% aspartimide impurity **II**. Relative chromatogram is reported in Figure S53.

Gradient HPLC analysis: 10-40% mobile phase B in 30 minutes, flow: 0.5 mL/min.

Stress stability tests of H-Ala-Lys-Asp-Gly-Tyr-Ile-OH

100 mg of dry peptide-bound H-Ala-Lys-Asp-Gly-Tyr-Ile-Trt-PS-resin (prepared as described above) were swelled in 2 mL of the desired solvent (NOP, NOP/DMC 8/2, DMF or NBP) for 30 minutes. The resin was filtered and then subjected to the following stress test conditions in parallel: a 2 mL solution of 10% DEAPA, or 20% piperidine, or 20% TBA or 5% TMG in NOP, DMF or NBP was added to the resin and stirred for 4 hours at RT; alternatively a 2 mL solution of 10% DEAPA in NOP/DMC 8/2 was stirred for 4 hours at RT, or a 2 mL solution of 20% piperidine in NOP, DMF or NBP and 10% DEAPA in NOP was stirred for 4 hours at 40°C, according to what reported in Table 5. The resin was filtered, washed with NOP, NOP/DMC 8/2, DMF or NBP (3x2 mL) and with DCM (3x2 mL). Dry peptide resin was suspended in 3 mL of the mixture TFA/TIS/H_2O (90/5/5 *v*/*v*/*v*) and stirred for 2 h. The resin was filtered off and diisopropylether (10 mL) cooled to 4°C was added to the solution. The peptide was filtered and dried *in vacuo* to obtain crude H-Ala-Lys-Asp-Gly-Tyr-Ile-OH **I**, aspartimide impurity **II** and eventually base adduct impurities **III** or **IV** in different ratios according to the used conditions (results reported in Table 5). Relative chromatograms are reported in Figures S54-S70.

Gradient HPLC analysis: 10-40% mobile phase B in 30 minutes, flow: 0.5 mL/min.

(L)-dihydroorotic fragment isomerization

A sample of Degarelix, as a lyophilized powder of 99.61% of purity, was dissolved in DMF with 10% of piperidine, TMG, TBA and DEAPA at a concentration of 17 g/L. The stability at RT was followed for 24 h and monitored by HPLC-UV, as reported in Table 6. Relative chromatograms are reported in Figures S75-S79. HPLC analyses were performed on Agilent 1260 Infinity II system, using a column Waters Xterra Shield RP18, 3.5 µm, 4.6 x 150 mm; temperature: 25°C; injection volume: 10 µL, UV: 245 nm; elution phase: 65:35, 40 mM Ammonium Acetate Buffer pH 10.0: Acetonitrile (mobile phase A). Isocratic HPLC analysis: 100% mobile phase A from 0 to 30 minutes.

SPPS of H-Tyr-Aib-Aib-Phe-Leu-OH (Aib-Enkephalin) in NOP or NOP/DMC 8/2.

The synthesis was carried out by using Fmoc-Leu-Wang-PS resin (200 mg, loading 1.2 mmol/g). After swelling of the resin in 2 mL of NOP, Fmoc protective group was removed by the following conditions: 10% DEAPA, 20% TBA or 5% TMG in NOP (2×2 mL, 15 min each) and the resin was washed with NOP (3×2 mL). Fmoc-Phe-OH, Fmoc-Aib-OH, Fmoc-Aib-OH, Fmoc-Tyr(tBu)-OH (three-fold excess with respect to the loading of the resin) were diluted in NOP (2,5 mL), pre-activated by DIC and OxymaPure® (three-fold excess of the reagents with respect to the loading of the resin) for 3 min and coupled to the resin in 60 min. In case of Fmoc-Aib-OH the coupling was repeated a second time. After each coupling step the Fmoc protective group was removed by treating the peptide resin with the following conditions: 10% DEAPA, 20% TBA or 5% TMG in NOP (2×2 mL, 15 min each), and the resin was washed with NOP (3×2 mL). In a selected case (Table 7 entry 5), the deprotection steps before insertion of Fmoc-Aib-OH were performed at 40°C. In another case (Table 7 entry 6), the full SPPS was repeated using NOP/DMC 8/2 as solvent mixture and 10% DEAPA as deprotecting agent with an automatic synthesizer. After Fmoc cleavage of the *N*-terminal amino group the peptide resin was washed with NOP (3×2 mL) and DCM (3×2 mL). Dry peptide resin was suspended in 5 mL of the mixture TFA/TIS/H_2O (90/5/5 *v*/*v*/*v*) and stirred for 2h. The resin was filtered off and diisopropylether (20 mL) cooled to 4°C was added to the solution. The peptide was filtered and dried *in vacuo* to obtain crude Aib-Enkephalin. HPLC purities of the crude after cleavage are reported in Table 7. Relative chromatograms are reported in Figures S80-S84.

Gradient HPLC analysis: 20-40% mobile phase B in 15 minutes,40% mobile phase B from 15 to 20 minutes, 40-20% mobile phase B from 15 to 20 minutes, flow: 0.5 mL/min.

SPPS of H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol (Linear Octreotide)

The synthesis was carried out by using Fmoc-Thr(tBu)-ol-Trt-PS resin (200 mg, loading 1.1 mmol/g) at rt or at 40°C (full synthesis or selected steps), according to what specified in Table 8. After swelling of the resin in 2 mL of DMF, NOP or NOP/DMC 8/2 (30 minutes or 4 hours), Fmoc protective group was removed by 10% DEAPA in DMF, NOP or NOP/DMC 8/2 (2×2 mL, 15 min each) and the resin was washed with DMF, NOP or NOP/DMC 8/2 (3×1.5 mL). Fmoc-Cys(Trt)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-D-Trp(Boc)-OH, Fmoc-Phe-OH, Fmoc-Cys(Trt)-OH, Fmoc-D-Phe-OH (three-fold excess with respect to the loading of the resin) were diluted in the chosen solvent (2,5 mL), pre-activated by DIC and OxymaPure® (three-fold excess of the reagents with respect to the loading of the resin) for 3 min and coupled to the resin in 60 min. In case of the first inserted Fmoc-Cys(Trt)-OH (Cys^7 in the final sequence) the coupling was repeated a second time. After each coupling step the Fmoc protective group was removed by treating the peptide resin with 10% DEAPA in DMF, NOP or NOP/DMC 8/2 (2×2 mL, 15 min each), and the resin was washed with DMF, NOP or NOP/DMC 8/2 (3×1.5 mL). After Fmoc cleavage of the *N*-terminal amino group the peptide resin was washed with DMF or NOP or NOP/DMC 8/2 (3×1.5 mL) and DCM (3×2 mL). Dry peptide resin was suspended in 5 mL of the mixture TFA/TIS/1-dodecanethiol (90/5/5 *v*/*v*/*v*) and stirred for 4 h. The resin was filtered off and diisopropylether (25 mL) cooled to 4°C was added to the solution. The peptide was filtered and dried *in vacuo* to obtain crude linear octreotide. HPLC purities calculated as sum of all target molecule adducts are reported in Table 8. Relative chromatograms are reported in Figures S90-S95.

Gradient HPLC analysis: 20-40% mobile phase B in 15 minutes,40% mobile phase B from 15 to 20 minutes, 40-20% mobile phase B from 15 to 20 minutes, flow: 0.5 mL/min.

Process Mass Intensity

Process Mass Intensity calculations were performed considering SPPS of linear Octreotide (see Table 8) pre- and post- solvent and

base recovery, as reported in Table 9. When the syntheses were fully performed at 40°C, half amount of coupling reagents (Fmoc-AA-OH, DIC and OxymaPure®) were employed. The deprotection stream waste (including washings) and the coupling stream waste (including swelling and washings) were collected separately and directly distilled under vacuum. Concerning the SPPS in NOP alone, in the deprotection stream DEAPA was initially distilled (25 mmHg at 60 °C) and recovered in 92% yield. The vacuum was then increased to 0.25 mmHg (temperature 130 °C) to collect NOP (85% yield). The coupling waste was instead directly distilled under high vacuum (0.25 mmHg at 130°C) to recover NOP (85% yield). Concerning the SPPS in NOP/DMC 8/2, the deprotection waste was distilled in three consequent steps using a gradual vacuum setting: DMC (25 mmHg at 40°C) was recovered (95%) in a first fraction, DEAPA (25 mmHg at 60°C) in a second fraction (92%), and finally NOP (0.25 mmHg at 130 °C) in a third fraction (85%). Similarly, the coupling waste was distilled in two fractions recovering DMC first (95%) and then NOP (85%) under higher vacuum. Detailed PMI calculations are reported in the Supporting Information (Tables S2-S9)

Contributions

G.M., C.P. and P.C. equally contributed to the research. A.T., W.C., A.R. and M.M. designed research; G.M., P.C., A.M., L.F., D.C. and T.F. performed experiments; A.T. and W.C. analyzed data and all authors contributed to writing the manuscript.

Conflicts of interest

"There are no conflicts to declare".

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