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Steps towards sustainable solid phase peptide synthesis: use and recovery of *N*-octyl pyrrolidone†

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The investigation of new green biogenic pyrrolidinones as alternative solvents to *N,N*-dimethylformamide (DMF) for solid phase peptide synthesis (SPPS) led to the identification of *N*-octyl pyrrolidone (NOP) as the best candidate. NOP showed good performances in terms of swelling, coupling efficiency and low isomerization generating peptides with very high purity. A mixture of NOP with 20% dimethyl carbonate (DMC) allowed a decrease in solvent viscosity, making the mixture suitable for the automated solid-phase protocol. Aib-enkephalin and linear octreotide were successfully used to test the methodologies. It is worth noting that NOP, DMC and the piperidine used in the deprotection step could be easily recovered by direct distillation from the process waste mixture. The process mass intensity (PMI), being reduced by 63–66%, achieved an outstanding value representing a clear step forward in achieving green SPPS.

Introduction

The development of solid-phase peptide synthesis (SPPS) that originates from the seminal work of Bruce Merrifield back in the sixties[1] gave access to previously unavailable long peptides *via* the iterative formation of amide bonds.[2] Thanks to SPPS availability and its automation, polypeptides became a target for medicinal chemists.

This modality is playing a major role in modern therapeutics,[3] with more than 70 peptides on the market and about >100 in clinical trial advanced stages.

The different synthetic strategies for peptide synthesis have been clearly described by Albericio in 2009.[4] Since then, only two novel industrial technologies have been reported, the enzymatic approach by Enzypep[5] and the hiving technology by Jitsubo.[6] However, SPPS is still the core technology for peptide synthesis and several research groups are now focusing their attention on the optimization of every single step to increase process greenness.[7–9]

Almost complete conversions are achieved thanks to the use of reagents in excess that can be removed by extensive washing and filtration of the solid support anchored growing peptide. Therefore, considering process greening, a fundamental role is played by the solvents.[10,11]

N,N-Dimethylformamide (DMF) is technically the perfect solvent for SPPS. However, its toxicological profile[12] is a serious matter of concern in the pharmaceutical industry. In fact, DMF and its most common substitute *N*-methylpyrrolidone (NMP)[13] are reprotoxic and were labeled as hazardous; accordingly, their replacement has been considered "advisable or requested" by the ACS Green Chemistry Institute® Pharmaceutical Round Table (GCIPR)[14] that defined this goal as one of the hot topics in the list of the 12 green chemistry key research areas (KRAs). The development of innovative and general methods for sustainable amide bond formation and peptide synthesis have also been reported in the updated version of the list of priorities.

On the basis of these considerations, the selection of greener alternatives needs to take into consideration the sustainability and the toxicity to the natural environment and human health in the whole solvent lifecycle.

Several solvents have been reported to be suitable for a greener SPPS (GSPPS), such as 2-methyl tetrahydrofuran (2-MeTHF),[15] acetonitrile (ACN) and THF,[16] 2-MeTHF and cyclopentyl methyl ether (CPME),[17] γ-valerolactone (GVL)[18] and *N*-formylmorpholine (NFM),[19] propylene carbonate (PC),[20] dimethylisosorbide (DMIE),[21] and *N*-butyl pyrrolidone (NBP).[22]

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Green solvent mixtures affording suitable physicochemical properties for all the steps of solid phase processes, Cyrene©/diethyl carbonate (DEC), sulfolane/DEC, anisole/dimethyl carbonate (DMC),[23] dimethyl sulfoxide (DMSO)/ethyl acetate (EtOAc)[24] and NFM/1,3-dioxolane (DOL),[25] have also been explored. However, some of these solvents like EtOAc, 1,3-dioxolane or 2-MeTHF do not have the optimal characteristics for industrial application because of their low flash point.

In this context, the family of *N*-alkylpyrrolidones may offer new opportunities since, despite having a similar structure to NMP, they display a completely different metabolic profile. They are not toxic or reprotoxic and they still retain the main features of the parent dipolar aprotic solvent. NBP has recently been applied by both academia and industrial researchers to SPPS on polystyrene-based resins, affording crude peptides with comparable quality to those synthesized in DMF. In addition, NBP was also employed in a mixture with ethyl acetate providing interesting results.[26]

We have explored herein the characteristics of *N*-alkylpyrrolidones and their use in SPPS, focusing our attention on the safest members of this class such as *N*-octyl-2-pyrrolidone (NOP), *N*-cyclohexyl-2-pyrrolidone (NCP) and *N*-benzyl-2-pyrrolidone (NBnP).

Aiming to the widest application, we have defined protocols for manual or automatic SPPS, decreasing the PMI (product mass intensity) by solvent recycling.

Results and discussion

N-Alkylpyrrolidone chemical–physical parameters, toxicological data and *in vitro* metabolism

In the selection of suitable candidates for DMF or NMP replacement for GSPPS, chemical–physical parameters are the first characteristics to be evaluated (Table 1).

N-Alkylpyrrolidones share as a common feature the lack of acid protons, while the elongation of the alkyl chain consistently decreases their polarity moving from NMP to NBnP.

The high boiling point of this family of compounds together with their high flash point temperatures (always >100 °C, except for NMP) are interesting characteristics for industrial application since there are no safety concerns and solvents can be recovered by distillation.

Table 1 physicochemical parameters of *N*-alkylpyrrolidones[a]

Solvent	Viscosity at 25 °C (mPa s)	Flash point (°C)	Boiling point °C (pressure mmHg)
DMF	0.8	58	36 (1)
NMP	1.65	91	68 (4)
NBP	4.0	108	80–85 (0.5)
NOP	6.6	142	114–115 (0.8)
NCP	11.5	141	94–97 (0.5)
NBnP	21.4	113	124–125 (0.8)

[a] Viscosity and flash point data were obtained from ECHA database and boiling points from Scifinder database. See also ref. 22*a* and 27.

Concerning *N*-alkylpyrrolidones' viscosity, the observed values may represent a challenge for large-scale application in automated syntheses, since difficulties in transfer and purging may occur. Anyway, a viscosity close to 4 mPA s is considered acceptable, if the efficiency of all the steps of SPPS (swelling, coupling, and protecting group cleavage) is maintained.[9a] In this context, a simple addition of a low viscosity green cosolvent is a viable solution to overcome this specific issue.

From the perspective of industrial application of new SPPS protocols, low cost, bulk availability and reliability of the supply chain of the solvent also have to be ensured. Among the other pyrrolidones, NOP is also known as Surfadone™ LP-100 [28] and is widely used as a low-foaming non-ionic rapid wetting agent and a dynamic surface tension modulator in the cosmetic industry, the pigment industry, cleaning detergent production and polymer manufacturing. For this reason, its price is comparable to those of the most common solvents (about 1–2$ per kg). The industrial preparation of NMP predominantly involves the reaction of γ-butyrolactone (GBL) with monomethylamine.[29] Similarly, other *N*-alkylpyrrolidones can be prepared using the corresponding amines. Although GBL is commonly prepared *via* the petrochemical route from succinic anhydride or succinic acid, it could also be obtained by the fermentation of sugar, and hence be transformed from a fossil-based reagent to a renewable source.[30] However, the cost of fermentation and the difficulties in treating the complex biomass-derived matrices are still challenges to be tackled[31] and, even if the research on biorefineries is advanced, technological implementation is hampered by low profitability.[32] Commercially available pyrrolidones are currently not prepared from biogenic GBL, but with prospective novel economically viable biomass treatment protocols, they might be included in the future in the list of biogenic solvents, displaying a high potential for more sustainable chemical approaches. In this context, since it will become inevitable to move away from a petroleum-based society, scientific and technological efforts to turn these sustainable alternatives into industrially feasible solutions are mandatory.

Data reported in Table 2 clearly show that NOP, among the *N*-alkyl pyrrolidones, is the safest one, being not reprotoxic and with very high tolerability when administered orally or intradermally.

The metabolism of these products generally involves oxidation at the 5' position of the lactam ring and on the first carbon of the *N*-alkyl side chain. Through this pathway, NMP generated consistent quantities of the masked formaldehyde.[34]

When NOP was incubated in the presence of rat (RLM) and human liver microsomes (HLM), it was rapidly cleared with an average half-life ($t_{1/2}$) of 11.5 (±2.0) min in RLM and 23.8 (±5.6) min in HLM (Fig. 1).

The *in vitro* metabolism of NOP in RLM and HLM was very similar and led to the formation of several metabolites (M1–M11, Fig. 2, Fig. S1 and Table S1, ESI†). The major one was **M1** (RT = 3.19 min; Fig. 2) having an *m*/*z* value of 212.1642, with a mass shift of 14 Da with respect to NOP. As shown in Fig. 2

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Table 2 Toxicity parameters of solvents evaluated in this study in comparison to DMF and NMP[33]

Solvent	Acute oral toxicity (mg per kg bw)	Acute inhalation toxicity	Acute dermal toxicity (mg per kg bw)	Reproductive toxicity
DMF	3010	>5.8 mg L^{-1} air	>3160	Yes
NMP	150	>5.1 mg L^{-1} air	>5000	Yes
NBP	300	>5.1 mg L^{-1} air	>2000	**No**
NOP	>2200	n.a.	>4000	**No**
NCP	370	n.a.	1600	**No**
NBnP	n.a.	n.a.	n.a.	n.a.

n.a.: not available.

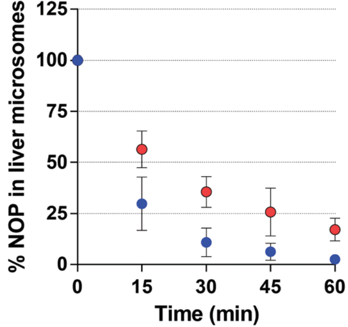

Fig. 1 *In vitro* metabolic stability of *N*-octyl pyrrolidone (NOP). Time course of NOP stability in rat (blue circles) and human (red circles) liver microsomes.

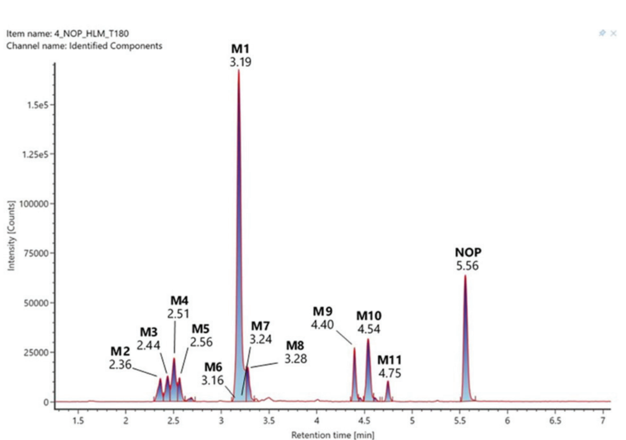

Fig. 2 HPLC-HRMS trace of a HLM incubation of *N*-octyl pyrrolidone (NOP) at t = 3 h. Parent compound NOP and metabolites **M1–M11** are reported together with their RT. The most abundant metabolite (**M1**, with a mass shift of +14 with respect to NOP) and two minor metabolites (**M6** and **M7**, with a mass shift of +16 with respect to NOP) co-elute. Co-eluting metabolites are detectable as Extracted Ion Chromatograms in Fig. S3, ESI.†

and Table S1,† besides **M1**, other minor metabolites were observed. A cluster of metabolites (**M6**, **M7**, and **M9–11**) displaying a mass shift M + 16 with respect to NOP suggests a hydroxylation reaction (+O) while keto metabolites (**M1, M2, M3, M4, M5**, and **M8**) displaying a mass shift M + 14 (+O-2H) are derived as second-generation metabolites from the dehydrogenation of hydroxylated ones. This is compatible with the oxidation of a methylene group to a keto group involving either the 2-pyrrolidone ring or the *N*-octyl side chain of NOP. The low and high energy mass spectra related to either keto or mono-hydroxylated metabolites **M1–M11** are shown in the ESI, Fig. S4–S14.†

Effect of *N*-alkylpyrrolidones as solvents in the key steps of SPPS

Solvents for SPPS need to fulfill a series of requirements at the same time: a good swelling efficacy for different resins, ability to completely solubilize amino acids, coupling reagents and related by-products, and promotion of both coupling and deprotection steps. Therefore, before testing pyrrolidones in SPPS protocols, we firstly evaluated separately their efficiency in all these steps.

Solubility tests

To evaluate the suitability of the selected solvents in SPPS, their ability to solubilize commonly employed protected amino acids and coupling reagents was investigated. Fmoc-Val-OH was initially selected as the reference amino acid, demonstrating perfect solubilisation in all the tested solvents (NOP, NCP and NBnP) at a standard 0.2 M concentration (Table 3). The same dissolution protocol was applied to the main coupling reagent combinations: OxymaPure®/DIC (A), COMU/DIPEA (B), PyBOP/DIPEA (C), PyOxyma/DIPEA (D), HOBt/DIC (E), and HBTU (F) (Table 4). Optimal results were generally achieved, except for HBTU (F), which scarcely dissolved in all the solvents and was hence excluded from further studies. The solubility screening was then extended to Fmoc-protected natural and non-natural amino acids (Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Aib-OH, Fmoc-Phe-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Pro-OH and Fmoc-Thr(*t*Bu)-OH) that were dissolved at a 0.2 M concentration, both alone and in the presence of the aforementioned additives after a 5 minute pre-activation (Table 3 and Table S2†).

Solubility issues were detected in the case of Fmoc-Cys(Trt)-OH, but upon decreasing the concentration to 0.1 M, a slightly lower value than that of the standard used in SPPS, good solubility was achieved in NCP, even if not in NBnP (Table 3). When the amino acids were mixed with the coupling additives, only in sporadic cases the solubility was incomplete, requiring

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Table 3 Solubilisation efficacy of Fmoc-AA(PG)-OH amino acids in *N*-alkypyrrolidones[a]

Fmoc-AA(PG)-OH										
Solvent	Val	Gly	Ala	Aib	Phe	Pro	Tyr(*t*Bu)	Thr(*t*Bu)	Lys(Boc)	Cys(Trt)
NOP	green	green	green	green	green	green	green	green	green	green
NCP	green	green	green	green	green	green	green	green	green	green, 0.1 M
NBnP	green	green	green	green	green	green	green	green	green	red, 0.1 M

[a] Solubilisation monitored at 0.2 M concentration unless 0.1 M is specified. Legend: green = soluble; yellow = moderately soluble; red = insoluble. PG: protecting groups.

Table 4 Solubilisation efficacy of coupling reagents **A–F** and Fmoc-Val-OH with **A–E** in *N*-alkylpyrrolidones[a]

	Coupling reagents						Mixture Fmoc-Val-OH + coupling reagents (**A–E**)				
Solvent	OxymaPure®/DIC **A**	COMU/ DIPEA **B**	PyBop/ DIPEA **C**	PyOxyma/ DIPEA **D**	HOBt/DIC **E**	HBTU **F**	Val + **A**	Val + **B**	Val + **C**	Val + **D**	Val + **E**
NOP	green	green	yellow	green	green	red	green	yellow	green	green	green
NCP	green	green	green	green	green	red	green	green	green	green	green
NBnP	green	green	green	green	green	red	green	green	green	green	green

[a] Legend: green = soluble; yellow = moderately soluble; red = insoluble.

an extra-dilution to observe clear solutions. Fmoc-Cys(Trt)-OH demonstrated in this case good solubility when combined with OxymaPure®/DIC (A) and COMU/DIPEA (B) in NOP and NCP, overcoming the above-reported limited solubility of the pure Fmoc-protected amino acid. On the other hand, NBnP was not able to dissolve this amino acid, even in combination with coupling reagents or in more dilute solutions (Table S2†).

Overall, OxymaPure®/DIC and COMU/DIPEA appeared to be the best coupling systems because of their general good results with all solvents and amino acids (see Fig. S16–S18 in the ESI†).

Note that OxymaPure®/DIC has been reported over the last ten years as a highly efficient coupling agent able to reduce racemization with performances overcoming those displayed by most of the common analogues.[35]

Swelling tests

The first key step in SPPS is the choice of the resin and its swelling. A suitable solvent should be able to properly swell different commercially available resins, demonstrating the broadest applicability. Polystyrene based resins (PS-resins) cross-linked with 1–2% of divinylbenzene are most commonly used due to their mechanical and chemical stability as well as for their low cost and bulk availability, also TentaGel-based resins, made of a polystyrene (PS)/polyethylene glycol (PEG) network are often used for their increased compatibility with polar solvents. PEG-based resins, commonly known as ChemMatrix resins (CM), have recently received great attention for their good performances reported in the synthesis of long peptides. On these bases, cross-linked polystyrene (PS – Merrifield), PEG-PS (TentaGel – TG) and pure PEG-based (ChemMatrix – CM) resins were considered for swelling with *N*-alkylpyrrolidones. Moreover, different acid-sensitive linkers were considered for each matrix: Wang and Trt-Cl linkers for obtaining the final peptide as a C-terminal acid and Rink Amide linker for a C-terminal amide peptide. Details on bead size, loading and cross-linking of the selected resins are reported in Table S3.†

Recently, Amadi-Kamalu *et al.*[36] developed a model to predict resin swelling in a given solvent, highlighting how the solvent–resin interaction is a complex process, especially as swelling could be related to physical parameters such as bead size and degree of cross-linking. However, experimental evaluation of swelling still represents the most reliable method and reported protocols were applied for all the solvent/resin combinations.[20,37]

Briefly, a dry resin sample in the desired solvent was allowed to mildly shake in a graduated syringe for 30 minutes; after 5 minutes of re-equilibration, the solvent was removed under vacuum and the volume increase of the resin was measured (mL g^{-1}).

In general, solvents giving resin swellings higher than 4 mL g^{-1} are considered good, values between 2 and 4 mL g^{-1} correspond to moderately good solvents and swelling lower than 2 mL g^{-1} are identified as poor candidates.

Noteworthily, the degree of swelling should not exceed 4 mL g^{-1} since a large volume of solvent would lead to reagent dilution. However, the swelling is considered acceptable if it corresponds to ±30% of the measured volume for DMF.[22]

The measured swelling values of pyrrolidones and DMF are shown in Table 5 as a mean value of analyses in triplicate.

NOP and NCP afforded excellent swelling of PS-based resins, commonly employed for industrial purposes and are comparable to DMF in swelling the Wang linker. Moreover,

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Table 5 Swelling[a] of resins in DMF and pyrrolidones at RT after 30 min

Resin	DMF ($mL\ g^{-1}$)	NOP ($mL\ g^{-1}$)	NCP ($mL\ g^{-1}$)	NBnP ($mL\ g^{-1}$)
PS-Wang	5.6	5.5	5.1	2.3
PS-Trt-Cl	3.2	3.4	3.6	1.6
PS-RinkAmide	3.2	1.6	1.6	1.6
TG-Wang	6.0	1.7	2.5	1.3
TG-RinkAmide	6.2	4.3	3.4	4.0
CM-Wang	4.4	1.4	1.6	1.6
CM-RinkAmide	7.8	3.4	6.1	6.7

[a] Data reported are the average of triplicate measurements, see Table S4 in the ESI.†

better results were observed with the Trt-Cl resin. This behaviour suggests that these solvents display a higher affinity to less polar resin matrices. Concerning 100% PEG-based resins, acceptable results were found for CM-RinkAmide when NCP and NBnP were used, with the latter affording a 15% lower swelling compared to DMF. Overall, *N*-alkylpyrrolidones cover a satisfying range of applicability since acid terminating peptides can be prepared using PS-Wang or PS Trt-Cl resin with NOP and NCP while amide peptides may be synthesized using CM-RA resin with NCP or NBnP.

Coupling reaction in the solution phase

The efficiency of pyrrolidones in promoting the coupling step was first investigated in the liquid phase. Accordingly, the synthesis of model dipeptide[17] Z-Phg-Pro-NH_2 was performed in the selected solvents (NOP, NCP, and NBnP) and in DMF as a benchmark reaction, screening different coupling reagent combinations, and checking both the conversion and the racemization degree (Table 6).

Measuring the racemization ratio is fundamental to predict the efficiency of the solvent in SPPS couplings, and its inhibition is necessary to avoid further purifications and low final yields. Dipeptide Z-Phg-Pro-NH_2 is particularly prone to racemization due to the acidity of the benzylic α-proton in Phg.[38] Moreover, proline reacts slower than other amino acids and achieving a complete conversion represents the main hurdle.

All the couplings were performed using (L)-amino acids and the crude was analysed by HPLC-MS injection after 3 h, in order to determine the dipeptide conversion and the racemization degree. The couplings were performed with equimolar amounts of the two amino acids and with a concentration of 0.125 M. The conversions were generally good in under all conditions; moreover, the DIC/OxymaPure® system provided only about 1% racemization in pyrrolidones (Table 6, entries 6, 11 and 16), which is less than that observed in DMF. The PyBOP/HOBT/DIPEA and HOBT/DIC combination afforded a high level of racemization in all the solvents tested, including DMF. COMU/DIPEA and PyOxyma/DIPEA showed better values than the latter two combinations, but are generally less effective than DIC/OxymaPure®.

Considering these data, together with the solubilisation screening of coupling reagents and activated amino acids,

Table 6 Conversion (%) and racemization ratio (%) in the synthesis of Z-Phg-Pro-NH_2[a]

Coupling reagent **A-E**, 3h, 0°C to rt, solvent
A: OxymaPure®/DIC
B: COMU/DIPEA
C: PyBOP/HOBt/DIPEA
D: PyOxyma/DIPEA
E: HOBt/DIC

Entry	Solvent	Coupling reagent	DL[b] (%)	Conversion (%)
1	DMF	A	1.9	92
2		B	0.9	99
3		C	24.1	98
4		D	1.0	87
5		E	13.2	85
6	NOP	A	0.9	95
7		B	6.6	82
8		C	17.1	94
9		D	2.5	96
10		E	11.7	99
11	NCP	A	1.1	96
12		B	2.2	82
13		C	21.6	92
14		D	2.3	83
15		E	13.2	99
16	NBnP	A	1.4	99
17		B	1.6	99
18		C	32.1	99
19		D	0.7	95
20		E	10.0	99

[a] All the reactions were performed by pre-activating (3 min) Z-Phg-OH. Conversion and racemization ratios were calculated by HPLC. [b] Defined as (Z-D-Phg-Pro-NH_2)/(Z-L-Phg-Pro-NH_2) × 100. The DL epimer was identified after the synthesis of Z-D-Phg-Pro-NH_2 where Z-D-Phg-OH was used as a starting material.

DIC/OxymaPure® was considered as the best system for further investigations. This choice was supported also by their very low cost, which is an essential requirement for SPPS use on the industrial scale.

Deprotection kinetic tests

To develop a total DMF-free method, a solvent displaying excellent behaviour in the deprotection step is a fundamental tool. In this context, the removal of the Fmoc group was investigated in the liquid phase to evaluate the effect of the solvent and to avoid the possible interference of resin swelling or peptide aggregation issues that could occur in the solid phase.[39] Piperidine is the base of choice in Fmoc SPPS, due to its role as a fast deprotecting agent and a dibenzofulvene scavenger. Accordingly, the tests were conducted using a 20% piperidine solution to investigate the deprotection kinetics, as commonly reported in SPPS protocols.[9a]

Qualitative kinetic tests on Fmoc-Phe-OH deprotection were carried out according to the method developed by Jad *et al.*[40] and the deprotection rates were monitored by HPLC-MS at fixed time intervals.

Fmoc-Phe-OH deprotection was also tested in DMF and NBP for comparison. The reaction was considered complete upon the disappearance of the HPLC peak corresponding to Fmoc-Phe-OH; notably, the reactions performed in all investi-

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gated pyrrolidones revealed complete Fmoc removal in 2 minutes (see Fig. S31 and S32 in the ESI†), perfectly in line with DMF performances.

SPPS in environmentally favourable solvents

After investigating the effects of *N*-alkylpyrrolidones as solvents in liquid phase couplings, the study was extended to the SPPS of Aib-enkephalin pentapeptide (H-Tyr-Aib-Aib-Phe-Leu-COOH/COONH$_2$). Aib residue insertion represents a hard step because of its steric hindrance that often leads to slow-rate coupling reactions and consequently to partial mis-incorporation.[15] The amount of the des-Aib sequences provides a measure of the efficacy of each pyrrolidone compared to DMF or standard alternatives.

Aib-enkephalin was manually synthesized on different resin/solvent combinations according to the best swelling results: (i) PS-Wang and PS-Trt-Cl resins with NOP and NCP and (ii) ChemMatrix-RinkAmide (CM-RA) resin with NBnP. Treatment with TFA-based cleavage cocktails afforded the peptide as acid at the C-terminal in case (i) and as amide in case (ii). These conditions also allowed the simultaneous removal of the *tert*-butyl group in the Tyr side chain. The peptides were assembled on Fmoc-Leu-preloaded resins using 20% piperidine as the Fmoc deprotection agent and DIC/OxymaPure® as the coupling reagent. *N*-Alkylpyrrolidones were used in all the synthetic steps, including washing. Double Fmoc-Aib-OH couplings were performed in all syntheses, according to the common literature procedures.[15–17] HPLC purities of the crude products after cleavage are reported in Table 7.

When the reaction was performed using NOP, excellent purities were observed, providing about 98% of the target pentapeptide and <1% of des-Aib byproduct on Wang resin (entry 1). Less than 3% impurity was generated on TrtCl-resin (entry 2). Interestingly, these results are consistently better than those obtained in the reference solvents DMF and NBP, which provided a des-Aib amount >11% and >8%, respectively (entries 5, 6 and 7, 8). NCP showed comparable results to DMF in terms of pentapeptide purity, but lower amounts of des-Aib were detected (entries 3 and 4). Instead, NBnP demonstrated a very low efficiency as only 4.3% of Aib-enkephalin was detected, while des-Aib tetrapeptide (16%) and other incomplete sequences corresponding to the remaining 80% of the total were identified in the crude (entry 9).

Table 7 HPLC purities of Aib-enkephalin pentapeptide assembled on different resins and solvents

Entry	Solvent	Resin[a]	Pentapeptide[b] (%)	Des-Aib (%)	Other (%)
1	NOP	PS-Wang	97.7	0.8	1.5
2		PS-Trt-Cl	97.4	2.6	—
3	NCP	PS-Wang	88.5	10.5	1.0
4		PS-Trt-Cl	89.9	10.0	1.1
5	DMF	PS-Wang	85.8	14.2	—
6		PS-Trt-Cl	88.1	11.9	—
7	NBP	PS-Wang	91.1	8.9	—
8		PS-Trt-Cl	91.6	8.4	—
9	NBnP	CM-RA	4.3	16.0	79.7
10[c]	DMF	CM-RA	53.0	47.0	—

[a] Pre-loaded Fmoc-Leu-resins were used. [b] Double Fmoc-Aib-OH couplings. [c] From ref. 16.

As a consequence, NCP and NBnP were not further studied while NOP was instead selected as an ideal greener alternative to common solvents.

Consequently, more sustainable conditions for SPPS were applied for the synthesis of linear octreotide (H-D-Phe^1-Cys^2-Phe^3-D-Trp^4-Lys^5-Thr^6-Cys^7-Thr^8-ol) and the final intermediate was obtained by the solid-phase synthesis method via industrial manufacturing of the API octreotide.

This peptide was one of the first biologically stable somatostatin analogues to be synthesized and used in oncology.[41] The entire protocol was performed with NOP as the solvent and compared with reference syntheses in NBP and DMF. Preloaded Fmoc-Thr(*t*Bu)-ol-Trt-PS resin was used, which is highly suitable for the synthesis in NOP. Although Octreotide is a medium-length peptide, frequent mis-incorporation of the Cys^7 residue occurs and for this reason the coupling step to introduce this residue was repeated twice in all the synthetic sequences. Standard 20% piperidine solution and DIC/OxymaPure® reagents were employed for the deprotection and coupling steps, respectively. The HPLC purities of the crude products after cleavage are reported in Table 8. It is important to mention that all the detected species are related to linear octreotide and hence the precursors of the final cyclic API. Des-Cys^7 or other truncated sequences were never observed.

The excellent results obtained using NOP both in the synthesis of Aib-enkephalin and linear octreotide showed that its high viscosity value (6.6 mPa s for NOP *vs.* 4.0 mPa s for NBP and 0.8 mPa s for DMF) does not affect the SPPS efficiency. The issue of viscosity anyway has to be considered when moving from manual synthesis to automatic synthesizers. Heating the reaction system had been previously applied as a solution in SPPS in NBP.[34] Instead, in order to decrease NOP viscosity and to perform the reaction in an automatic synthesizer without heating, DMC (0.59 mPa s) was selected as the co-solvent. In fact, DMC proved to be a good cosolvent for SPPS.[23] The addition of 20% DMC to NOP allowed decreasing the viscosity down to 3.94 mPa s (see Table S5†), thus allowing the use of automated systems. After having evaluated the swelling degree of NOP/DMC 80 : 20 in the PS resins (see Table S4†), the synthesis of the linear octreotide intermediate was repeated using this sustainable solvent mixture both with manual and automated protocols (Table 8). In both SPPS protocols, the final purity was comparable to the corresponding synthesis carried out in DMF, NBP and NOP alone.

Solvent and base recycling

Although NOP and NOP/DMC proved to be greener alternatives to DMF, in order to achieve a truly sustainable protocol, the Process Mass Intensity (PMI) should be decreased by recovering the main source of waste, namely the solvents (NOP or NOP/DMC) and the piperidine used in the deprotection step. NOP offers several alternatives for the recovery process, being

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Table 8 HPLC purity of linear octreotide in NOP, NOP/DMC, DMF and NBP

Compound	RRT	NOP	NOP/DMC 80:20 Manual SPPS	NOP/DMC 80:20 Automated SPPS	DMF	NBP
Cyclized N,O shift	0.83	—	—	—	1.4	—
Cyclized	0.88	0.7	—	—	5.8	2.4
TM-N,O shift 1	0.92	1.0	1.4	—	—	0.7
TM-N,O shift 2	0.95	5.2	2.4	2.0	—	4.2
TM + CO_2	0.97	—	21.6	—	5.1	4.8
TM	1.00	77.6	65.1	86.0	76.8	77.7
TM + Boc	1.10	—	1.7	—	—	—
TM + *t*Bu	1.14	11.7	5.2	10.0	9.0	7.2
TM + Boc2	1.18	—	0.8	—	—	—
TM + *t*Bu2	1.26	3.8	1.8	2.0	1.9	3.0
Product purity[a] (%)		100	100	100	100	100

[a] HPLC purity calculated as the sum of all target product adducts; RRT = relative retention time.

very lipophilic. The final mixtures can be washed with water and then NOP can be recovered by distillation or it can be directly distilled in order to recycle all the volatile chemicals. DIC cannot be recycled as it is unstable and almost completely transforms into 1,3-diisopropylurea during the coupling step.

Coupling streams and deprotection streams should be recovered separately in order to avoid piperidine consumption by excess Fmoc-amino acids used in the coupling steps. When the mixture of NOP/DMC is used, due to the small difference between piperidine and DMC boiling points (106 °C *vs.* 90 °C at 760 mmHg), these two components are recovered in the same fraction and reused in the deprotection steps in further SPPS processes, after rebalancing the required relative ratio.

The results obtained by applying these protocols to the SPPS of linear octreotide in comparison to the DMF standard process are reported in Table 9.

The percentage of waste related to the solvents involved in the swelling, coupling, deprotection and washing steps is very similar in all different protocols (NOP 67.8%, NOP/DMC 69.0%, DMF 68.4%, see Tables S7 and S8†). Concerning the synthesis performed in NOP (entry 1), piperidine was easily recovered in 95% yield, considering the maximum amount that could be collected. NOP was recovered in the second fraction of the condensed liquid in 85% yield. Under these conditions, a PMI value for the global process was more than halved (268 with solvent/base recovery *vs.* 722 without).

When the green mixture of NOP/DMC was used for the same synthesis (entry 2), distillation of the deprotection streams under vacuum allowed us to obtain, at 25 mmHg, DMC and piperidine together (95% each) in a 1 : 0.86 ratio (V/V), as confirmed by ^{1}H NMR (see the ESI†). Furthermore, a vacuum increase to 0.25 mmHg allowed the recovery of NOP in 85% yield. Finally, distillation under vacuum of the coupling wastes allowed the recovery in high yield of both DMC (95%) and NOP (85%), decreasing the total PMI from 748 (without recovery) to 256. The final waste related to the solvent is limited to 10% for the process with NOP and to 8.7% when DMC/NOP was used.

OxymaPure® could be isolated from the solid residue by following the protocol reported by Rasmussen.[24] However, this additional recovery procedure increased the PMI and allowed us to isolate OxymaPure® in 38% yield; therefore, in our protocol, we did not consider coupling agent recovery.

A full evaluation of NOP compared to other solvents commonly used in SPPS should comprise the complete lifecycle perspective of the solvent impact. A quantitative comparison concerning the carbon footprint between SPPS protocols in DMF and NOP cannot be performed due to the lack of literature data on NOP. Anyway, for a qualitative estimation, since all the reactants and procedures in the two SPPS protocols are the same, attention can be exclusively focused on the solvent carbon intensity (contribution to CO_2 emission), considering

Table 9 Process mass intensity (PMI) and recovery in GSPPS

Entry	Solvent[a]	PMI	Waste stream	Recovery (yield %)	PMI after recovery
1	NOP	722	Depr	NOP (85) Pip[b] (95)	**268**
			Coupling	NOP (85)	
2	NOP/DMC	748	Depr	NOP (85) DMC/Pip[b](95)	**256**
	NOP/DMC		Coupling	DMC (95) NOP (85)	
3	DMF	735	—	—	—

[a] The wastes coming from the coupling steps and from deprotection steps were distilled separately. [b] Piperidine (Pip) involved in the formation of the DBF-piperidine adduct was subtracted from the total piperidine volume.

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the primary production and the disposal by incineration or recycling. The production technologies of NMP and NOP are very similar. Thus, the carbon footprint can be calculated from that of NMP (4.22 kg CO_2 per kg)[42] by considering a 20–30% increase in carbon content per kg due to the octyl chain and the higher boiling point. Therefore, the estimated range could be between 5.06– and 5.49 kg CO_2 per kg. The carbon footprint related to DMF production has been reported to be lower than that of NMP (3.57 kg CO_2 per kg).[43]

However, NMP recovery by double distillation decreases the carbon footprint by 40%.[42] On the other hand, NOP can be recovered by single distillation and the contribution of this process to carbon footprint is consistently lower than the advantage arising from the reduced amount of virgin solvent required. We can expect to have an impact of NOP recycling on the carbon footprint similar to the one of NMP (>40%).

These considerations allow to rebalance the apparent disadvantages of NOP in comparison to DMF. The comparable carbon footprint combined with the health, environmental and safety data and the future prospects of sustainable production of NOP from biomasses make this potential solvent a credible sustainable alternative.

Conclusions

The evaluation of alternative green and safe pyrrolidones allowed us to identify NOP and NOP/DMC as the best solvents in terms of yield and selectivity for SPPS. To test the solvent performances, Aib-enkephalin and linear Octreotide were synthesized by SPPS producing the final products in excellent purities using both manual and automated procedures. The solvents and the piperidine could be easily recovered by decreasing the process PMI by 63% for the NOP protocol and 66% for the NOP/DMC one. Selecting green alternatives to dipolar solvents for SPPS is an extensively investigated issue, but the development of a methodology including solvent and reagent recovery and recycling represents a step forward in the achievement of a really green SPPS.

Experimental procedures

General methods

Unless otherwise stated, all materials, solvents and reagents were obtained from commercial suppliers, of the best grade, and used without further purification. The solvents used were of high-performance liquid chromatography (HPLC) reagent grade. In particular, the Fmoc amino acids and resins were supplied by Iris Biotech, Merck or Fluorochem. The coupling reagents were purchased from Merck or Novabiochem. Piperidine was supplied by Merck. DMF, other organic solvents, and HPLC-grade acetonitrile (CH_3CN) were purchased from Merck. Milli-Q water was used for RP-HPLC analyses. NOP was purchased from Merck.

The solid-phase synthesis of the peptides was carried out manually or using a CSBio-CS136X peptide synthesizer (automated syntheses).

HPLC-MS analyses were performed on an Agilent 1260 Infinity II system coupled to an electrospray ionization mass spectrometer (positive-ion mode, m/z = 100–3000 amu, fragmentor 30 V), using Agilent Zorbax-SB-C18 5 μm, 250 × 4.6 mm or Phenomenex Luna C18 5 μm, 250 × 4.6 mm columns; 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), flow: 0.5 mL min^{-1} or 1.0 mL min^{-1}. Chemstation software was used for data processing.

The ^1H NMR spectra were recorded using an INOVA 400 MHz instrument with a 5 mm probe. All chemical shifts were quoted relative to the deuterated solvent signals.

Distillations were performed with an Edwards RV3 vacuum pump.

The viscosity was measured using a CS10 Bohlin (Malvern) rheometer in a titanium cylinder/steel cup (Bob-Cup C25) configuration at a temperature of 25 °C controlled by a Julabo MP thermostat. The calibration of the instrument was previously verified with an appropriate standard, namely 2,2,4-trimethyl-1,3-pentanediol monoisobutyrate (tabulated viscosity at 30 °C = 9.96 mPa s).

In vitro metabolism of NOP in rat and human liver microsomes

A NADPH-generating solution (10 mM glucose-6-phosphate, 2 mM $NADP^+$, 5 mM $MgCl_2$ and 0.4 U mL^{-1} glucose-6-phosphate-dehydrogenase) was prepared in 100 mM phosphate buffered saline (PBS) of pH 7.4. 15 μL of RLM or HLM preparation (final protein concentration = 1 mg mL^{-1}) were added to 60 μL of a co-factor mix and 222 μL of PBS buffer. The samples were pre-incubated under continuous stirring at 37 °C for 5 min and then 3 μL of stock solution of NOP was added to start the reaction. The compound was incubated at a final concentration of 10 or 100 μM. Aliquots of RLM and HLM incubation solutions were collected at the beginning of the experiment (t = 0 min) and at t = 3 h and the enzymatic reaction was quenched by the addition of a double volume of CH_3CN. The samples were centrifuged (16 000g, 10 min, and 4 °C) and the supernatant was directly injected into an HPLC-HRMS system for metabolite identification.

UPLC-HRMS conditions for *in vitro* NOP metabolite profiling

A Waters Acquity UPLC I-Class (Waters, USA) coupled to a Waters V-Ion IMS qToF was employed for ion mobility-enabled acquisitions in the metabolite ID workflow. The V-Ion IMS qToF was calibrated daily employing a Waters Major Mix (Waters, USA) and the system performance was checked daily for accurate mass and Collisional Cross Section (CCS) accuracy and precision by injecting a system suitability test (SST) mixture composed of nine QC standards. For UPLC separation, mobile phases A and B were CH_3CN and ultra-pure water both with 0.1% v/v formic acid and the column used was an Acquity

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UPLC CSH C18 (2.1 × 100 mm, 1.7 μm; Waters, USA). The linear gradient was: 0 min: 5%A; 0–15 min: 5–95%A; 15–16 min: 95% A; 16–17 min: 95–5%A; and 17–20 min: 5%A. Total run time: 20 min; flow rate: 0.5 mL min^{-1}; injection volume: 2 μL; column temperature: 50 °C. Acquisition range: m/z = 50–300 amu. Instrumental parameters were set as follows: source temperature: 120 °C; desolvation temperature: 500 °C; source gas flow: 20 L h^{-1}; desolvation gas flow: 800 L h^{-1}; capillary voltage: 0.8 kV (ESI+); cone voltage: 20 V; collision energy: low energy: 4 eV; high energy: 10–45 eV; and reference mass: leucine enkephalin $[M + H]^+$ m/z = 556.27658. The software UNIFI v.1.8.2 (Waters, USA) was used for data acquisition and processing.

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Solubility tests

1 mL of the desired pyrrolidone was added to 0.2 mmol of either the protected amino acid/coupling reagent (pair of reagents) or both protected amino acid and coupling reagent (s), and stirred at room temperature until a clear solution was observed. In the case of incomplete dissolution (see red boxes in Tables 3,4 and S2†), an extra solvent was added to reach a concentration of 0.1 M and to achieve total solubilisation, unless otherwise specified.

Resin swelling

Weighed resin (0.1 g) was introduced into a 5 mL syringe fitted with a polypropylene fritted disc (the void volume of the tip and the syringe was estimated to be 0.2 mL). The desired solvent or mixture of solvents (2 mL) was added to the resin, which was shaken for 30 minutes at room temperature. The swollen resin was allowed to stand for another 5 minutes and then the solvent was removed under vacuum. The volume of the dry resin was then recorded and the swelling value was calculated by the following formula:

$$\text{Degree of swelling } (\text{mL g}^{-1}) = 100 \times (\text{volume of the swelled resin} - 0.2\text{ mL})/0.1\text{ g}$$

All the measurements were performed in triplicate and the data were reported as the medium value. Triplicate measurements with the related standard deviation values are reported in Table S4.†

Fmoc cleavage kinetics in the solution phase

Fmoc-Phe-OH (0.1 mmol) was dissolved in the desired pyrrolidone. Piperidine was added to the suspension in order to achieve the desired concentration (20% base solution) in the final 1 mL deprotection mixture total volume. The reaction mixture was stirred at room temperature and the samples of the solution (20 μL) were taken at t = 0 (before base addition) and after 2, 4, 6, 8, 10, and 15 minutes. The samples were diluted with 1.5 mL of CH_3CN/TFA (1% v/v) and injected into an HPLC-UV system. The reaction was considered complete after the disappearance of the HPLC peak corresponding to Fmoc-Phe-OH. All kinetics showed a complete reaction after 2 minutes from the start of the reaction, independently from the employed solvent. The selected chromatograms are reported in the ESI.†

For HPLC separation, mobile phases A and B were H_2O + 0.08%TFA and CH_3CN + 0.08%TFA and the column used was an Agilent Zorbax-SB-C18 5 μm, 250 × 4.6 mm. The linear gradient was: 0 min: 95%A; 0–15 min: 95–5%A; 15–20 min: 5–95%A; and 20–22 min: 95%A. Total run time: 22 min. Flow rate: 1 mL min^{-1}.

Coupling in the solution phase: synthesis of Z-Phg-Pro-NH_2

Z-Phg-OH (0.2 mmol) was dissolved in a glass vial with 2.5 mL of DMF or the selected pyrrolidone (NCP, NOP or NBnP). The desired coupling reagent combination (0.3 mmol) was then added (reagents **A–E** in Table 6: OxymaPure/DIC, COMU/DIPEA, PyBOP/HOBt/DIPEA, PyOxyma/DIPEA, and HOBt/DIC). After 5 minutes of preactivation, H-Pro-NH_2 (0.2 mmol) was added. The solution was stirred at 0 °C for 1 h and then at room temperature. After 3 h from the beginning of the reaction, an aliquot (80 μL) of the solution was diluted with 0.5 mL of a 1 : 2 CH_3CN/H_2O mixture and injected into an HPLC-MS system, in order to monitor the conversion and the racemization ratio. The selected chromatograms are reported in the ESI.†

For HPLC separation, mobile phases A and B were H_2O + 0.08%TFA and CH_3CN + 0.08%TFA and the column used was an Agilent Zorbax-SB-C18 5 μm, 250 × 4.6 mm. The linear gradient was: 0 min: 80%A; 0–15 min: 80–60%A; 15–20 min: 60% A; and 20–30 min: 60–80%A. Total run time: 30 min. Flow rate: 1 mL min^{-1}.

Solid phase synthesis of H-Tyr-Aib-Aib-Phe-Leu-OH/H-Tyr-Aib-Aib-Phe-Leu-NH_2 (Aib enkephalin)

Aib-enkephalin manual syntheses were carried out at room temperature in glass syringes fitted with a polyethylene porous disc and connected to a vacuum source to remove excess reagents and solvents, by using 0.2 g of preloaded Fmoc-Leu-resins. Specifically, preloaded PS-Wang and PS-Trt-Cl resins (loading 1.1 mmol g^{-1}) were used for synthesis with NBP, NOP and NCP, while preloaded ChemMatrix-RinkAmide resin (loading 0.5 mmol g^{-1}) was used with NBnP. After the swelling of the resin in 2 mL of the selected solvent (DMF, NBP, NCP, NOP or NBnP), the Fmoc protective group was removed by 20% piperidine in the selected solvent (2 times × 2 mL, 15 min each) and then the resin was washed with the selected solvent (3 times × 1.5 mL, 1 min each). Fmoc-Phe-OH, Fmoc-Aib-OH, Fmoc-Aib-OH, and Fmoc-Tyr(*t*Bu)-OH (three-fold excess with respect to the loading of the resin) were pre-activated by OxymaPure® and DIC (three-fold excess of the reagents with respect to the loading of the resin) for 3 minutes and coupled to the resin for 60 minutes. In the case of both Aib residues in the sequence, the coupling of Fmoc-Aib-OH was repeated a second time. After each coupling step, the Fmoc protective group was removed by treating the peptide resin with 20% piperidine in the selected solvent (2 times × 2 mL, 15 min each) and the resin was washed with the selected solvent (3 times × 1.5 mL, 1 min each). After the Fmoc cleavage of the N-terminal amino group, the peptide resin was further washed with DCM (3 times × 2 mL, 1 min each) and dried under a vacuum for 12 hours. The dry peptide resin was suspended in

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5 mL of the TFA/TIS/H_2O (95/2.5/2.5 v/v/v) mixture and stirred for 2 h. The resin was filtered off, washed with TFA (1 time × 1 mL, 1 min) and diisopropylether (25 mL) cooled to 4 °C was added to the solution dropwise. The peptide was filtered and dried *in vacuo* to obtain crude Aib-enkephalin that was directly analysed by HPLC-MS (Table 7). The related chromatograms and MS spectra are reported in the ESI.†

For HPLC separation, the mobile phases A and B were H_2O + 0.08%TFA and CH_3CN + 0.08%TFA and the column used was a Phenomenex Luna C18 5 µm, 250 × 4.6 mm. The linear gradient was: 0 min: 80%A; 0–15 min: 80–60%A; 15–20 min: 60%A; and 20–30 min: 60–80%A. Total run time: 30 min. Flow rate: 0.5 mL min^{-1}.

Solid phase synthesis of H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol (linear octreotide)

Linear octreotide manual syntheses were carried out at room temperature by using Fmoc-Thr(*t*Bu)-ol-Trt-PS resin (0.2 g, loading 1.1 mmol g^{-1}) in glass syringes fitted with a polyethylene porous disc and connected to a vacuum source to remove excess reagents and solvents. After the swelling of the resin in 2 mL of the selected solvent (DMF, NBP, NOP or NOP/DMC), the Fmoc protective group was removed by 20% piperidine in the selected solvent (2 times × 2 mL, 15 min each) and then the resin was washed (3 times × 1.5 mL, 1 min each). Fmoc-Cys(Trt)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-D-Trp(Boc)-OH, Fmoc-Phe-OH, Fmoc-Cys(Trt)-OH, and Fmoc-D-Phe-OH (three-fold excess with respect to the loading of the resin) were pre-activated by OxymaPure® and DIC (three-fold excess of the reagents with respect to the loading of the resin) for 3 minutes and coupled to the resin for 60 minutes. In the case of the first insertion of 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 20% piperidine in the selected solvent (2 times × 2 mL, 15 min each) and the resin was washed (3 times × 1.5 mL, 1 min each). After the Fmoc cleavage of the N-terminal amino group, the peptide resin was further washed with DCM (3 times × 2 mL, 1 min each) and dried under vacuum for 12 hours. The dry peptide resin was suspended in 5 mL of the TFA/TIS/1-dodecanethiol (90/5/5 v/v/v) mixture and stirred for 4 h. The resin was filtered off, washed with TFA (1 time × 1 mL, 1 min) and diisopropylether (25 mL) cooled to 4 °C was added to the solution dropwise. The peptide was filtered and dried *in vacuo* to obtain crude linear octreotide. The HPLC purities calculated as the sum of all target molecule adducts are reported in Table 8.

Automated syntheses of linear octreotide were performed at room temperature using a CSBio-CS136X peptide synthesizer under the same conditions as those reported for the manual protocol with the only exception that the NOP/DMC 80 : 20 mixture was used as the solvent through all the steps (Fmoc cleavage, coupling, and washings).

The final global deprotection/cleavage on dry resin was carried out analogously to the manual synthesis. The related chromatograms and MS spectra are reported in the ESI.†

For HPLC separation, the mobile phases A and B were H_2O + 0.08%TFA and CH_3CN + 0.08%TFA and the column used was a Phenomenex Luna C18 5 µm, 250 × 4.6 mm. The linear gradient was: 0 min: 80%A; 0–15 min: 80–60%A; 15–20 min: 60%A; and 20–30 min: 60–80%A. Total run time: 30 min. Flow rate: 0.5 mL min^{-1}.

Solvent/base recycling

The deprotection stream waste (including washings) and coupling stream waste (including swelling and washings) were collected separately and directly distilled under vacuum. When the SPPS of linear octreotide was performed in NOP alone, the recovery of the deprotection waste was obtained by initially setting the vacuum to 25 mmHg and temperature at 40 °C to recover piperidine (95% yield) and then it was increased to 0.25 mmHg (temperature 130 °C) to collect NOP (85% yield). The coupling waste was directly distilled under high vacuum since only NOP was recovered (85%).

When the SPPS of linear octreotide was performed in NOP/DMC 80 : 20, the recovery of the deprotection waste was obtained by initially setting the vacuum to 25 mmHg and the temperature to 40 °C to recover piperidine (95% yield) and DMC (95% yield) together and then it was increased to 0.25 mmHg (temperature 130 °C) to collect NOP (85% yield). In the same way, the coupling waste was distilled in two steps allowing the recovery of DMC first (95%) and then that of NOP (85%) under higher vacuum.

Author contributions

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

Conflicts of interest

There are no conflicts to declare.

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