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Development of a novel, automated, robotic system for rapid, high-throughput, parallel, solid-phase peptide synthesis

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ABSTRACT

The development of peptide-based pharmaceutics is a hot topic in the pharmaceutical industry and in basic research. However, from the research and development perspective there is an unmet need for new, alternative, solid-phase peptide synthesizers that are highly efficient, automated, robust, able to synthetize peptides in parallel, inexpensive (to obtain and operate), have potential to be scaled up, and even comply with the principles of green chemistry. Moreover, a peptide synthesizer of this type could also fill the gap in university research, and therefore speed the advancement of peptide-based pharmaceutical options. This paper presents a Tecan add-on peptide synthesizer (TaPSy), which has operational flexibility (coupling time: 15–30 min), can handle all manual synthesis methods, and is economical (solvent use: 34.5 mL/cycle, while handling 0.49 mmol scale/reactor, even with ≤3 equivalents of activated amino acid derivatives). Moreover, it can carry out parallel synthesis of up to 12 different peptides (0.49 mmol scale in each). TaPSy uses no heating or high pressure, while it is still resistant to external influences (operating conditions: atmospheric pressure, room temperature 20–40 ˚C, including high [>70%] relative humidity). The system's solvent can also be switched from DMF to a green and biorenewable solvent, γ-valerolactone (GVL), without further adjustment. The designed TaPSy system can produce peptides with high purity (>70%), even with the green GVL solvent alternative. In this paper we demonstrate the optimization path of a newly developed peptide synthesizer in the context of coupling reagents, reaction time and reagent equivalents applying for a synthesis of a model peptide. We compare the results by analytical characteristics (purity of raw material, crude yield, yield) and calculated overall cost of the syntheses of one mg of crude peptide using a specified set of reaction conditions.

Introduction

Instrumental and automated support of solid-phase peptide synthesis (SPPS) has been used increasingly over the last decades. Pedersen et al. [1] divide the commercially available peptide synthesizers into two main categories: robotic liquid-handling systems, and valve-based reactant-distribution instruments. Both are batch systems, although it should be noted that continuous-flow synthesizers are also available commercially (e.g., variable-bed flow reactor [VBFR], Vapourtec Ltd., Bury St. Edmunds, UK) [2].

The main commercial batch peptide synthesizer instruments can be divided further into categories based on key properties such as heating (microwave or conventional), number of parallel channels, UV monitoring option, and production scale offered by the unit. Some manufacturers cover the whole production scale range (AAPPTec; Louisville, KY, USA [3], CEM; Matthews, NC, USA [4], CSBio; Menlo Park, CA, USA [5]), while others offer instruments for R&D or pilot scale only (Activotec; Cambridge, UK [6], Biotage; Uppsala, Sweden [7], Gyros Protein Technologies; Uppsala, Sweden [8]) Microwave-assisted SPPS is offered by AAPPTec [3], Biotage [7] and CEM [4], and UV monitoring of reactions is either a default feature or an option that can be added to most valve-based instruments. Undoubtedly, the listed manufacturers offer high-quality and high-performance products, but a common feature of these commercially available units is the relatively high price (>$50,000), which can be prohibitive for smaller R&D laboratories with lower budgets. Experiencing this financial hurdle ourselves, we decided to develop an automated system based on an available Tecan Genesis liquid-handling robotic workstation. Our requirements were: (1) significantly lower price than a commercially available unit; (2) parallel syntheses option for up to 12 peptides with robotic reagent handling; (3) higher production volumes than typical R&D units (considering 10 mg pure material as the common scale for first round of in vitro assays); and (4) robust and reliable design.

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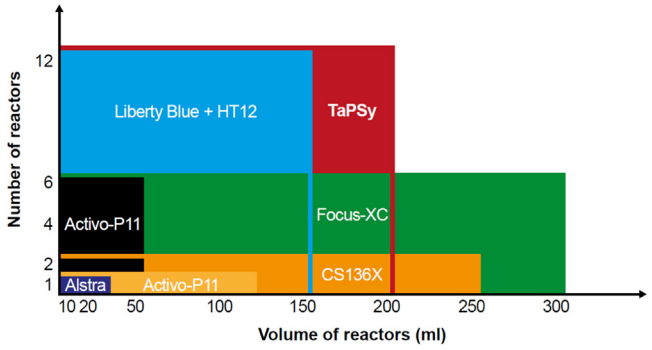

Fig. 1. Characterization of commercially available instruments fitting our workflow. Horizontal axis: volume of the reactor (mL), which correlates with the production scale of the system. Vertical axis: number of parallel reactors.

After evaluating the commercially available synthesizers that could fit into our peptide synthesis workflow, the following systems were considered relevant comparisons for our custom-developed TaPSy instrument: Activo-P11 (Activotec) [9], Focus-XC (AAPPTec) [10], Liberty Blue/HT12 or Liberty PRIME (CEM) [11–13], CSBIO II or CS136X (CS-Bio) [14,15], PurePep Chorus (Gyros Protein Technologies) [16] and Initator+ Alstra (Biotage) [17]. Most manufacturers offer customized synthesizers to fit the customer's needs, which is a constructive and positive attitude, but it also makes instrument evaluation and comparison difficult. It can be clearly seen in Fig. 1, that TaPSy opens a new space in peptide synthesis parameter design, as the high number of independent reactors (12) is combined with a relatively high production scale (≤200 mL per reactor).

Our aim in this work is to demonstrate the capability our new instrument, finding optimal working conditions in the context of coupling reagents, reaction time and reagent equivalents. For optimization of TaPSy, a medium-difficulty sequence was selected for synthesis. The selected sequence was ipAoa-LRRY-VHLFYLT-NH_2, which contains a non-standard AA type of modification. We compare the results by analytical characteristics (purity of raw material, crude yield, yield) and calculated overall cost of the synthesis of one mg of crude peptide using a certain set of reaction conditions.

Materials

Fmoc-protected amino acid derivatives (AAs) and Oxyma Pure (ethyl cyano(hydroxyimino)acetate) as coupling agent were purchased from Bachem AG (Bubendorf, Switzerland), TentaGel S RAM resin from Rapp Polymere GmbH (Tuebingen, Germany), Rink Amide MBHA resin from Merck KGaA (Darmstadt, Germany), and *N,N′*-diisopropylcarbodiimide (DIC) and DMF from VWR International Kft. (Debrecen, Hungary). Biorenewable γ-valerolactone (GVL) and all other reagents and solvents were purchased from Merck KGaA. EZPak flash cartridges (100 g) with polyethylene frits (pore size: 20–25 μm) were purchased from BGB Analytik AG (Böckten, Switzerland). Isopropylidene-protected aminooxyacetic acid derivative was prepared in our laboratory as described by Enyedi et al. [18].

TaPSy

The liquid handling was carried out with a 2-meter long Tecan Genesis worktable (Tecan Group Ltd, Männedorf, Switzerland). The worktable layout used for peptide synthesis contains standard plate racks, disposable tip stations, vial racks and custom carriers, racks, and tools; a schematic representation (**Fig. S1**) and image of the populated worktable (**Fig. S2**) are provided in the Supporting Information.

The Tecan add-on peptide synthesizer (TaPSy) was custom-developed and self-made by the authors at Tavanta Therapeutics Hungary Zrt. (Budapest, Hungary). The equipment consists of the following components (A more comprehensive layout and component description can be seen in the **Supporting Information; Fig. S3–4** and **Table S1**):

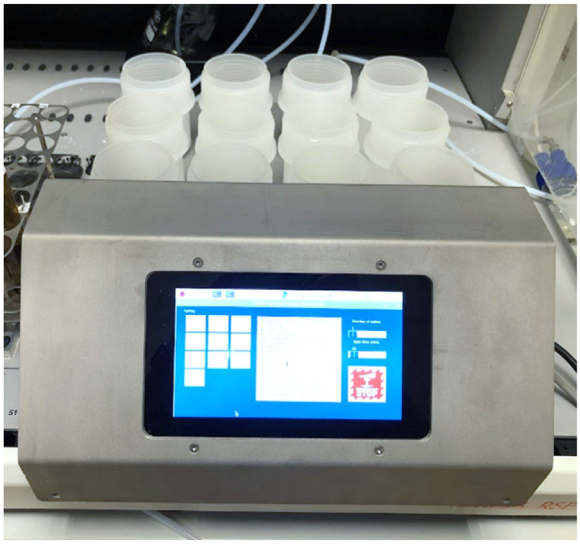

Fig. 2. TaPSy unit with 12 vessels.

- Vacuum pump for gentle solvent removal through frits (KNF N96 series; KNF Neuberger Inc., Trenton, NJ, USA)
- Waste removal pump (KNF NF1.60 series; KNF Neuberger Inc.)
- Solenoid valves (ASCO SCG225 series; Emerson Electric Co., St. Louis, MO, USA, and Bürkert 6427 series; Bürkert Austria GmbH, Mödling, Austria)
- Raspberry Pi 4 equipped with a Touch Display (Farnell s.r.o., Prague, Czech Republic).

As shown in Fig. 2, TaPSy was built to handle 12 vessels. Each of the vessels are open flash chromatography cartridges with a polyethylene frit at the base. Mixing is done by a nitrogen stream (1–2 bars [100–200 kPa] incoming gas flow, fine-tuned with a needle valve).

The vessels are mounted on a large plate that has four key functions: integration of the vessels with (1) a fluid flushing system and (2) a nitrogen stream; (3) separation of the flushing and nitrogen lines from the vessels using integrated solenoid valves; and (4) secure mounting of the vessels. The 12 reaction tubes held by the plate are accessible by the Tecan liquid-handling arm, with reagents added using 1000 μL disposable tips to avoid any cross-contamination. DMF was added as a system liquid (therefore, the limiting volume is no longer the disposable tips, but the syringe pump volume), thus faster handling time was achievable, even with larger volumes. The system used PTFE tubing (outer diameter: 6 mm, inner diameter: 4 mm, Pneucom Bt., Budapest, Hungary) because of its high chemical resistance.

By design, our instrument has inlet only of pure solvent, all other reactants are located external to the machine on the Tecan worktable in standard glassware. Liquid handling steps of the solution of any reactants (AA solution in reaction solvent, deprotection agents, coupling agent solutions) are done by disposable tips by Tecan liquid handling arm. After every synthetic step an extensive washing step is applied. Because of these facts, we never faced with any blockage in the tubing of the instrument after several hundreds of peptide syntheses already done. The after-synthesis cleaning process consists of using regular laboratory glassware washing of the reactant containers and the reaction tubes with the option to exchange the polyethylene frits in it.

Due to the limitations associated with the programming capabilities of the Tecan Gemini robot-handling software (e.g., the user cannot create procedures and functions, limiting user interactions), Tavanta created a Visual Basic/C#/C++ application with the desired menu and

Table 1
Protocols used by TaPSy.

Step	Procedure	Solvent usage (mL)	Time (min)
0	Resin swelling	8	30–60
(Initialization)	4 × Fmoc deprotection	4 × 3.5	4 × 1 (4 × 2 if 20% piperidine used)
	6 × wash after deprotection	6 × 4	6 × 1
1	AA coupling	3.5	5–30
2	3 × wash after coupling	3 × 4	3 × 1
3	2 × Fmoc deprotection	2 × 3.5	2 × 1 (2 × 2 if 5% or 20% piperidine used)
4	6 × wash after deprotection	6 × 4	6 × 1

form-based graphical user interface (GUI). This provided the required program logic to create the Gemini program code and execute our internally generated code to robotically synthesize peptides. The process of software development is discussed in more detail in the **Supporting Information (Figs. S5–9)**.

Protocols

Peptides were prepared by SPPS using the standard Fmoc/tert-butyl procedure on TentaGel S RAM and on Rink Amide MBHA resins. The protocols used by the TaPSy are shown in Table 1. Isopropylidene-protected aminooxyacetic acid (ipAoa) was attached to the *N*-terminus of the built peptide chain with the same protocol as the AAs. For the liquid handling system, the required AA and the ipAoa were dissolved in DMF (or GVL) with a final concentration of 0.1 M. The peptides were cleaved from the resin at the end of the synthesis using a 95% TFA, 2.5% triisopropylsilane (TIS), and 2.5% water (V/V/V) mixture (room temperature, stirring, 1.5 h). The crude peptides were precipitated in cold ether then centrifuged (3800 g for 6 min). After centrifugation, the ether was decantated from the crude peptides, then the precipitate was dissolved in 10% V/V acetic acid. Finally, the crude peptides were lyophilized on a bench-top apparatus (CoolSafe 4L; LaboGene A/S, Lillerød, Denmark) at -110 ˚C. The final crude peptides were analyzed by reversed phase-HPLC (RP-HPLC) coupled with ESI-MS (Electrospray Ionization Mass Spectrometer).

Liquid-chromatography mass spectrometry (LC-MS)

Liquid-chromatography mass spectrometry (LC-MS) analyses were performed on a single quadrupole mass spectrometer (Shimadzu LCMS-2020; Shimadzu Europe GmbH, Duisburg, Germany) using online RP-HPLC coupling. RP-HPLC separation was performed on a Shimadzu Prominence HPLC system (DGU-20A5, LC-20AD, SIL-20AC, SPD-M20A, CTO-20AC) using a Kinetex EVO C18 column (Phenomenex; Torrance, CA, USA; 5 μm, 250 mm × 4.6 mm). Linear gradient elution (0 min 5% B, 5 min 5% B, 31 min 60% B, 32 min 90% B, 35 min 90% B, 37 min 5% B, 50 min 5% B) with eluent A (0.05% TFA in water, V/V) and eluent B (0.05% TFA in acetonitrile, V/V) was used at a flow rate of 0.5 mL/min at 40 ˚C. The mass spectra were acquired in the 200–1300 m/z range, while the UV-visible spectra were analyzed at λ=220 nm.

The final purity from crude samples were established with a comparison to a calibration curve, to eliminate/minimalize external effects. Furthermore, the calibration and crude peptide samples were prepared the same and the injection volume was also generalized. The calibration curve was determined from purified peptide (1, 0.75, 0.5, 0.25 mg) in 1 mL ACN/H_2O solution (70/30 V/V%) and the injected volume was 5 μL in all cases (**Fig. S10**). The crude peptides were analyzed from a 1 mg/mL solution (solvent was the same as the before 70/30 V/V% ACN/H_2O) and the injected volume was 5 μL in all cases. LC-MS data were analyzed by LabSolutions software (Shimadzu).

Results and discussion

For optimization of TaPSy, a medium-difficulty sequence was selected for synthesis. The selected sequence was ipAoa-LRRY-VHLFYLT-NH_2, which contains a non-standard AA type of modification. According to Peptide Companion (which is an Excel macro [19] based on the work of Krchňák et al. [20]) three AA coupling is almost 1.2 on the synthesis difficulty scale (coupling reaction is considered difficult if score >1.2). Furthermore, due to it is 12 AA long, with a non-standard AA the authors consider it as a moderately difficult sequence. Moreover, this sequence had been prepared by the authors previously with a manual SPPS technique, so the sequence and potential difficulties of synthesis were well known, and the LC-MS spectra straightforward to analyze. [21]

First, the modified oligopeptide was synthesized on a low capacity (0.4 mmol/g) TentaGel S RAM resin, with the commonly used coupling reagents, HATU/DIPEA, plus a standard 3 equivalents coupling reagent and AAs. As discussed by Alberico et al. [22], aminium salt-derived coupling reagents (HATU, HBTU, TATU, etc.) are not recommended for use with excess DIPEA as they increase the chance of a reaction between the aminium salt and the *N*-terminal amino component. Such a reaction will lead to a guanidine derivative, and thus peptide chain elongation will be terminated. The TentaGel type of resin is commonly used in automatic SPPS techniques, mainly when the resin must withstand high pressure. Moreover, the low resin capacity makes synthesis easier because there is less chance of steric hindrance due to its good swelling ability, and so the coupling time can be lowered. However, one main drawback of the polyethylene glycol (PEG)-based TentaGel S RAM resin, in which PEG is not cross-linked, is that during cleavage PEG may appear as a contaminant in the crude peptide.

As shown in Fig. 3, the final material (purity: 21.5%) contained threonine-, arginine-, and leucine-deficient sequences (these types of deletion are usual in the selected sequence). Moreover, the crude peptide was highly contaminated with PEG from the resin, while the crude yield (25.4%) was also quite low. As a consequence, the TentaGel S RAM resin was replaced with Rink Amide MBHA resin in the subsequent experiments.

Rink Amide MBHA resin is a commonly used resin in manual peptide synthesis. It has a lower swelling ability than TentaGel S RAM and is not designed to withstand high pressure. However, as TaPSy does not apply pressure, and as one of the main goals was to provide a good one-to-one robotized SPPS alternative to manual synthesis, the Rink Amide MBHA appeared to be a good resin candidate. Moreover, a higher capacity Rink Amide MBHA resin (0.65 mmol/g) was selected, therefore the yield could easily be increased. In addition, these types of resins do not produce resin linker(s) contamination of the crude peptides (**Fig. S11**).

In the first round of the experimental matrix, we wanted to determine the ideal coupling time and potential minimalization of the reactants, while using the HATU/DIPEA coupling reagents. The same protocol was used as in the earlier experiments (Table 1), with the coupling changed accordingly. The results are shown in Table 2 and **Figs. S11–22**.

As shown in Table 2, the purity obtained with the 30-min and 15-min coupling times and 3 equivalents reagent usage are similar, however

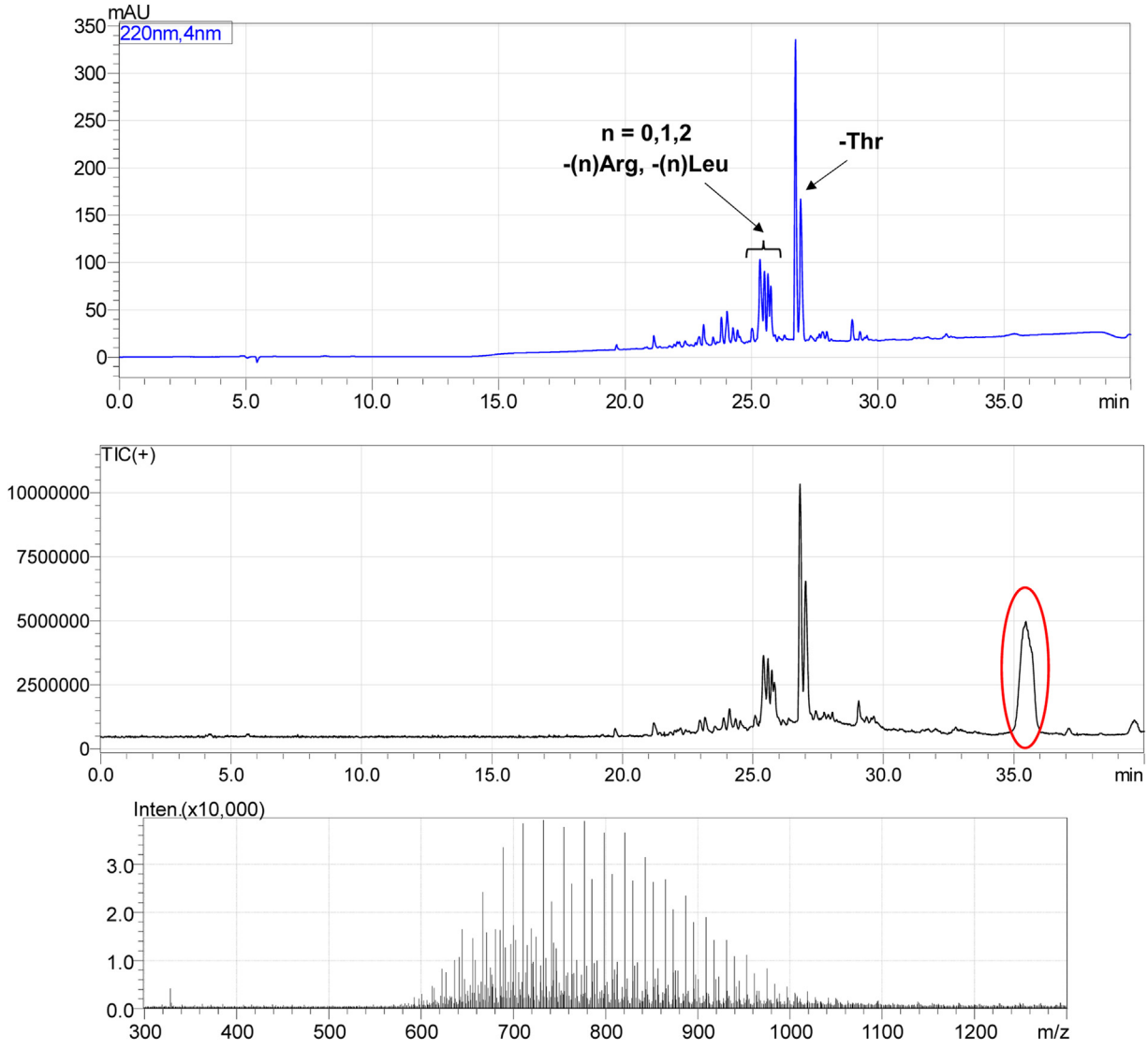

Fig. 3. Purity of the crude peptide (ipAoa-LRRY-VHLFYLT-NH2) and PEG contamination, as synthesized on the TentaGel S RAM resin (0.4 mmol/g); coupling time: 30 min; coupling reagents: HATU/DIPEA 3/3 equivalents; AA: 3 equivalents; Solvent: DMF. (From above: LC chromatogram, TIC chromatogram, MS spectra of the PEG contamination circled in red on the TIC chromatogram)

Table 2
Crude yield and purity with various parameters using Rink Amide MBHA (0.65 mmol/g) resin – experimental space and testing of coupling times.

Coupling reagents	Coupling time (min)	Coupling reagent (equivalents)	Amino acid (equivalents)	Crude yield (%)[a]	Purity (%)[b]	Yield (%)[c]
HATU/DIPEA	30	3/3	3	80.2	21.7	17.4
		2/2	2	79.8	22.9	18.2
		1.2/1.2	1.2	78.5	16.4	12.9
	15	3/3	3	74.4	23.9	17.8
		2/2	2	66.3	12.6	8.4
		1.2/1.2	1.2	62.4	16.3	10.2
	5	3/3	3	39.4	8.0	3.2
		2/2	2	63.2	14.0	8.8
		1.2/1.2	1.2	30.3	6.3	1.9
	2	3/3	3	47.0	14.4	6.7
		2/2	2	52.0	14.5	7.5
		1.2/1.2	1.2	35.6	9.6	3.4

[a] Based on the theoretical yield according to resin capacity.
[b] According to HPLC chromatograms and calibration curve (**Fig. S10**).
[c] % Yield was calculated from the crude yield and purity.

Table 3
Crude yield and purity with various parameters using Rink Amide MBHA (0.65 mmol/g) resin – experimental space and testing of alternative solvents.

Coupling reagents	Solvent	Coupling time (min)	Coupling reagent (eq.[a])	Amino acid (eq.[a])	Crude yield (%)[b]	Purity (%)[c]	Yield (%)[d]
HATU/DIPEA	DMF	15	3/3	3	74.4	23.9	17.8
			2/2	2	66.3	12.6	8.4
			1.2/1.2	1.2	62.4	16.3	10.2
	NMP/DMF (50:50 V/V%)	15	3/3	3	59.3	14.2	8.4
			2/2	2	41.6	14.8	6.2
			1.2/1.2	1.2	39.4	11.5	4.5

[a] eq. - equivalents
[b] Based on the theoretical yield according to resin capacity.
[c] According to HPLC chromatograms and calibration curve (**Fig. S10**).
[d] % Yield was calculated from the crude yield and purity.

the shorter coupling time resulted in a poorer crude yield. Interestingly, lowering the reactant equivalents (coupling reagents and AA) did not cause a significant change in crude yield with the 30-min coupling time. With short coupling times (5 min and 2 min) the corresponding peptide was successfully synthesized, however the random factors (combinatorial chemistry like behavior with several side product peaks on the chromatograms) was quite high and these inconsistent data suggest that the TaPSy cannot operate reliably with a coupling time of less than 15 min.

In the next step, aiming to bring the results of the 15-min coupling closer to those obtained with 30-min coupling, we changed the solvent to a 50:50 V/V% DMF:NMP mixture – potentially to increase the resin's swelling and general solvation, and to disturb potential peptide aggregation. As shown in Table 3 and **Figs. S23–S25**, the change of solvent did not improve crude yield or purity. In fact, more interestingly, the change in solvent produced worse results overall. As the added 50% NMP did not improve the yield or purity, the authors saw no justification to change to 100% NMP, due to economic and financial considerations.

After the ideal time range had been determined (15–30 min), we introduced two other commonly used coupling reagents (Oxyma/DIC and PyAOP/DIPEA, both in 3/6 equivalents), to assess how they influenced crude yield and purity. Oxyma-based coupling reagents are popular at present as they are a good alternative to the Class 1 explosive benzotriazole-based HOBt family (HOBt, HOAt etc.).[23, 24] PyAOP is a phosphonium salt derivative of HOAt, and is used when hindered AAs have to be incorporated, or during synthesis of difficult sequences. Moreover, unlike uronium/aminium salts (HATU, HBTU, TATU, etc.), excess PyAOP does not undergo side reactions which could block the amino terminus and hinder further chain assembly. [25] For better data comparison the HATU/DIPEA experiments were repeated with higher base excess. Results obtained with these coupling reagents are presented in Table 4 and **Figs. S26–43**.

Interestingly the HATU/DIPEA coupling reagent with raised excess DIPEA (6 equivalents instead of 3 equivalents) shown no advantage compared to the standard 3 equivalents Moreover, the data suggest as the coupling time grows in time the purity lessen, meanwhile the crude yield will increase. These data support Alberico's [22] suggestion that aminium salt-derived coupling reagents (HATU, HBTU, TATU, etc.) are not recommended for use with excess DIPEA as they increase the chance of a reaction between the aminium salt and the *N*-terminal amino component.

The results suggest that with Oxyma/DIC, there is no possibility of decreasing coupling time, nor reagent or AA usage, as this markedly decreased purity and yield. However, PyAOP/DIPEA displayed the highest range of yield and purity, with decreases in coupling time, reagent and AA usage having little effect– similar results were observed with 30-min 3/6/3 equivalents, 30 min 2/4/2 equivalents, and 15 min 3/6/3 equivalents parameters. With this optimization step the overall yield was increased to the 70+% overall yield range.

To create a 'golden mean' the 30-min experimental protocol was modified to include coupling reagents (Oxyma/DIC and PyAOP/DIPEA) fixed at 3/6 equivalents, and AA lowered to 1.2 equivalents. As shown in Table 5 and **Figs. S44** and **S45**, Oxyma/DIC 3/6 did not work well as the coupling reagent with a lowered AA (1.2 equivalents). However, the PyAOP/DIPEA 3/6 coupling reagent provided the opportunity to lower the AA requirement as far as 1.2 equivalents, therefore presenting a greener, more cost-effective approach.

To further improve the green chemistry approach, TaPSy and three of the best parameters were tested with a green solvent alternative to DMF: γ-valerolactone (GVL). [26–28] GVL was successfully implemented by Kumar et al. [26] on a CEM Liberty Blue system [11]. However due to the sensitivity of GVL to polymerization and ring opening, 2-2-96% DBU/piperidine/DMF could not be used as a deprotection mixture, as DBU is a characteristically harsh base. Therefore, the alternative deprotection agent – 20% piperidine – was used. [29] Another potential limitation of the solvent change to GVL, is that the Fmoc deprotection solution must be made daily or in situ, due to GVL-associated ring opening in the presence of piperidine, as discussed by Kumar et al. [30] However, as TaPSy makes the deprotection mixture in situ, this is not a limitation of GVL usage that is encountered with the TaPSy system. Moreover, due to kinetic reasons mentioned by Ralhan et al. [31] the deprotection time was increased from 1 min to 2 min.

As shown in Table 6 and **Figs. S46–49**, in the green solvent trial the results with PyAOP/DIPEA were worse than with Oxyma/DIC, which was not the case in the standard DMF solvent tests. This was probably due to the sensitivity of GVL to ring opening and polymerization, meaning basic coupling reagents must be avoided. To further promote the greener approach the best run from these batch was repeated where the deprotection agent (20% piperidine) was decreased to 5% piperidine as there are reports that the piperidine could be used successfully in this percentage. [32] However, the results suggest that 5% piperidine is insufficient for deprotection as shown by the decreased purity and yield.

Overall, the results indicate that if there is a need to change to a greener approach, TaPSy is capable and up to the challenge.

The best results achieved with TaPSy in our analyses are on a par with those of a recent HPLC-based synthesizer (FlowPep_ELTE) with newly developed protocols by Farkas et al. [33], which produced a 14-mer peptide with 70–95% purity and 70–98% crude yield (depending on the protocol used). The fastest protocol (total synthesis time 2.9 h) combined with the best coupling reagents produced a yield of 80.4%, which is comparable with our best result of a 75.8% yield.

To provide a better basis for comparison between the two peptide synthesizers (by thought experiment), if both machines synthesized a 15-mer peptide, the total run time of the Farkas et al. [33] flow SPPS device (using the fastest protocol) would be ~112 min (~1.9 h), with a total solvent usage of 90 mL. With TaPSy (using a 15-min coupling time), the total run time would be 750 min (12.5 h) using 690 mL of solvent (each parameter contains the resin pretreatment – resin swelling and initial Fmoc deprotection). Thus, there is no doubt that the HPLC-based apparatus has a great advantage in terms of speed in the synthesis of one sequence. However, if seven or more different peptides have to

Table 4
Crude yield and purity with various parameters using Rink Amide MBHA (0.65 mmol/g) resin – experimental space and testing of alternate coupling reagents.

Coupling reagents	Coupling time (min)	Coupling reagent (equivalents)	Amino acid (equivalents)	Crude yield (%)[a]	Purity (%)[b]	Yield (%)[c]
HATU/DIPEA	30	3/6	3	88.6	25.4	22.5
		2/4	2	67.9	24.8	16.9
		1.2/2.4	1.2	25.0	1.41	0.4
	15	3/6	3	35.9	38.9	14.0
		2/4	2	54.7	35.6	19.5
		1.2/2.4	1.2	45.2	12.6	5.7
Oxyma/DIC	30	3/6	3	82.4	77.2	63.5
		2/4	2	78.5	40.1	31.5
		1.2/2.4	1.2	52.3	11.9	6.2
	15	3/6	3	66.3	44.1	29.3
		2/4	2	38.9	38.7	15.0
		1.2/2.4	1.2	15.3	-	-
PyAOP/DIPEA	30	3/6	3	91.2	81.2	74.0
		2/4	2	96.6	78.5	75.8
		1.2/2.4	1.2	80.3	43.6	35.0
	15	3/6	3	90.0	76.5	68.9
		2/4	2	86.5	53.2	46.0
		1.2/2.4	1.2	55.5	24.3	13.5

[a] Based on the theoretical yield according to resin capacity.
[b] According to HPLC chromatograms and calibration curve (**Fig. S10**).
[c] % Yield was calculated from the crude yield and purity.

Table 5
The crude yield and purity with various parameters using Rink Amide MBHA (0.65 mmol/g) resin – experimental space and testing of reduced amino acid usage.

Coupling reagents	Coupling time (min)	Coupling reagent (equivalents)	Amino acid (equivalents)	Crude yield (%)[a]	Purity (%)[b]	Yield (%)[c]
Oxyma/DIC	30	3/6	3	82.4	77.2	63.5
			1.2	62.5	25.8	16.1
PyAOP/DIPEA	30	3/6	3	91.2	81.2	74.0
			1.2	89.0	74.4	66.2

[a] Based on the theoretical yield according to resin capacity.
[b] According to HPLC chromatograms and calibration curve (**Fig. S10**).
[c] % Yield was calculated from the crude yield and purity.

Table 6
Crude yield and purity with various parameters using Rink Amide MBHA (0.65 mmol/g) resin – experimental space and testing of solvent change to the green GVL.

Coupling reagents	Coupling time (min)	Fmoc deprot.	Coupling reagent (equivalents)	Amino acid (equivalents)	Crude yield (%)[a]	Purity (%)[b]	Yield (%)[c]
Oxyma/DIC	30	20%	3/6	3	61.5	72.0	44.3
PyAOP/DIPEA	30	pip.	3/6	3	28.0	35.7	10.0
PyAOP/DIPEA	30		3/6	1.2	26.2	29.6	7.8
Oxyma/DIC	30	5% pip.	3/6	3	50.0	4.4	2.2

[a] Based on the theoretical yield according to resin capacity.
[b] According to HPLC chromatograms and calibration curve (**Fig. S10**).
[c] % Yield was calculated from the crude yield and purity.

Table 7
Comparison between various SPPS synthesizers [33–35] and TaPSy.

	Coupling time	Cycle time	Maximum theoretical yield	Used solvent/cycle		Reagents used	Crude yield	Purity	Yield
	(sec)	(min)	(μmol)	(mL)	Weighted with max. theoretical yield [a] (mL/mmol)	(eq. [b])	(%)	(%)	(%)
TaPSy	900-1800	50	487	47	96.5	1.2-6	96.6	78.5	75.8
FlowPep_ELTE	100	7	59	6	101.7	≤3-6	94	95	98.3
AFPS	7	0.7	100	50	500.0	6	60	N/D	-
HTFSP	10	3	71	12	169.0	2-6	40	94	37.6

[a] Lower value is better, greener.
[b] eq. – equivalents.

Table 8
Price comparison between the top 10 test results with TaPSy (The row highlighted in yellow indicate the test that used the lowered AA (1.2 equivalents) amount; while the row highlighted in green indicate the test that used GVL as a solvent.).

Coupling reagents	Coupling time (min)	Coupling reagent (equivalents)	AA (equivalents)	Solvent	Crude yield (%) [a]	Purity (%) [b]	Yield (%) [c]	Price with solvent cost (€/mg peptide)
PyAOP/DIPEA	30	2/4	2	DMF	96.6	78.5	75.8	0.14
Oxyma/DIC	30	3/6	3	DMF	82.4	77.2	63.5	0.14
PyAOP/DIPEA	30	3/6	3	DMF	91.2	81.2	74.0	0.18
PyAOP/DIPEA	30	3/6	**1.2**	DMF	89.0	74.4	66.2	0.18
PyAOP/DIPEA	15	3/6	3	DMF	90.0	76.5	68.9	0.19
PyAOP/DIPEA	15	2/4	2	DMF	86.5	53.2	46.0	0.23
Oxyma/DIC	30	2/4	2	DMF	78.5	40.1	31.5	0.25
PyAOP/DIPEA	30	1.2/2.4	1.2	DMF	80.3	43.5	35.0	0.29
Oxyma/DIC	15	3/6	3	DMF	66.3	44.1	29.2	0.30
Oxyma/DIC	30	3/6	3	GVL (20% pip)	61.5	72.0	44.3	0.47

[a] Based on the theoretical yield according to resin capacity.
[b] According to HPLC chromatograms and calibration curve (**Fig. S10**).
[c] % Yield was calculated from the crude yield and purity.

be made, the TaPSy will be comparable, or even faster, in terms of total synthesis time per peptide, as TaPSy can operate in parallel mode while the flow system works in sequential mode. For a fairer comparison of solvent usage, we first must examine the amount and capacity of the resins used. For the HPLC-based SPPS the highest theoretical yield could be achieved with 112 mg of Hypogel200 (Iris Biotech GmbH, Marktredwitz, Germany) with a resin capacity of 0.53 mmol/g, whereas the TaPSy could process 750 mg of Rink Amide MBHA resin with a resin capacity of 0.65 mmol/g. This means that the maximum theoretical yield would be 59.4 μmol with the HPLC-based SPPS, and 487.5 μmol with TaPSy – a difference of more than eightfold in favor of TaPSy. Thus, for the HPLC-based synthesizer to generate 487.5 μmol of peptide, its solvent usage of 90 mL (determined above) must be multiplied by eight, as the total capacity is much lower. In theory it has to use 720 mL of solvent, while the TaPSy uses 690 mL. Consequently, in cases of larger scale peptide synthesis the TaPSy could be faster and/or greener than the recent HPLC-based synthesizer with newly developed protocols. Moreover, the TaPSy is already prepared for implementation of a green and biorenewable solvent with GVL, while the flow system and its protocol are still in development for replacement of DMF and/or NMP as a solvent.

Another flow based SPPS reactor was made by Mijalis et al. [34], this device (AFPS – automated flow-based peptide synthesis) is automated as the TaPSy, however the maximum theoretical yield is 100 μmol (200 mg of ChemMatrix PEG Rink-Amide resin; capacity: 0.5 mmol/g), while the TaPSy is capable up to 487.5 μmol (750 mg RinkAmide MBHA resin; capacity: 0.65 mmol/g). To compare the solvent usage for one cycle the AFPS uses 50 mL solvent while the TaPSy uses 46.5 mL, meanwhile the flow-based system handles 100 μmol "capacity" while the TaPSy 487.5 μmol. The AFPS uses 6 equivalents of AA and coupling reagent, while the TaPSy could operate 1.2–6 equivalents AA and coupling reagent range. Meanwhile the AFPS highest crude yields are maximum 60%, while the TaPSy's crude yield could achieve 90+%. The purity capabilities could not be compared as Mijalis et al. did not provide purity values. Undeniably, the AFPS is very fast (only 40 s one cycle) compared to the TaPSy (50 min/cycle). If we use the same thought experiment as before – synthesis of 12 different 15-mer peptide – the AFPS could synthesize them in 120 min, while the TaPSy would finish in 750 min.

Naoum et al. [35] shows a new stirring based SPPS synthesizer (HTFSP - High-Temperature Fast-Stirring Peptide Synthesis), where they achieved very low coupling times (10 s), and cycle times (3 min). 12–18 mL solvent usage, highest crude yield was 40% and the highest purity was 94% (therefore the yield is 38%). The HTFSP's discussed maximum theoretical yield is 71 μmol (100 mg RinkAmide MBHA resin; capacity: 0.71 mmol/g), meanwhile the TaPSy could operate with nearly 7-fold scale (487.5 μmol). For the maximum theoretical yield, the TaPSy only use 46.5 mL solvent, while the HTFSP use a quarter of TaPSy's with 12 mL (overall the TaPSy uses less solvent/μmol). While the TaPSy's cycle time is much slower, the highest crude yield was 90+% and the purity was 75+% (yield 70+%), which results in twice as much overall yield, than the HTFSP.

Finally, the main advantage of the TaPSy compared to the previously discussed devices, that it can run 12 different peptides automated, without human intervention, while the HTFSP [35] is not automated, therefore it is highly labor consuming, and the time limiting factor (currently) in that solution is manual labor. Meanwhile, the two flow-based systems (FlowPep_ELTE [33], AFPS [34]) are automated, however between two production runs, the reactors needed to be changed by the operators. Therefore, in an industrial or R&D setup the TaPSy could operate 24/7 without manual labor while the two flow-based systems cannot. Moreover, the TaPSy could be beneficial in universities and R&D departments workflow as the TaPSy could do the workload overnight, then during working hours the synthesized peptides could be processed comfortably by the faculties.

Finally, a minor analysis compared the production costs of the 12-mer model peptide synthesis with various setups. The price was calculated for 1 mg purified peptide, including raw material and exposable costs, excluding personnel, equipment amortization and other overhead expenses. The top 10 results are presented in Table 8 (the full results could be seen in **Table S3**), detailing the synthesis parameters with the cost and product quality. In these calculations, the production price (expressed in €/mg model peptide; 12-mer) includes the cost of every reagent and solvent used during the synthesis process with TaPSy.

Highlighting the relationship between production yield and cost, Fig. 4 clearly shows that most effort increasing the yield is not only beneficial by technical means, but in financial aspect too.

Conclusion

In this work, a novel Tecan add-on peptide synthesizer – TaPSy – was developed. The device opens a new space in synthesis parameter design, as it can accommodate 12 independent reactors, combined with a relatively high production scale in 200 mL vessels (Fig. 1). The capability of TaPSy was tested by synthesizing a 12-mer peptide under different reaction conditions (different resins, coupling times, reagent types, reagent excesses, solvents, and AA equivalents), with final products analyzed by LC-MS. The results show that the TaPSy system is highly robust, successfully producing the selected 12-mer peptide in all cases. However, the 'sweet spot' in terms of coupling time was 30 min, and the best results were obtained with the PyAOP/DIPEA coupling reagents (~70% yield). In addition, results showed that in case of the PyAOP/DIPEA coupling reagents, there is an opportunity to lower the excess reagent from 3 to 2

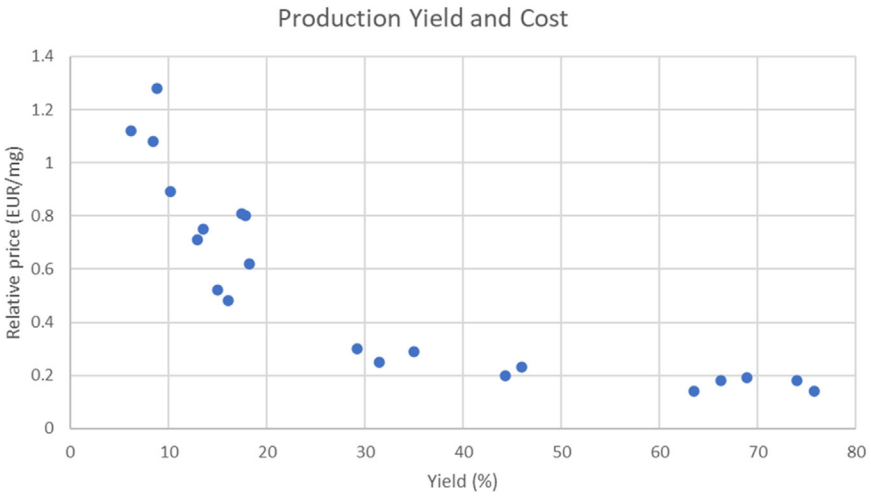

Fig. 4. Visualization of decreasing production cost with increasing yield.

or even 1.2 equivalents, thus creating the possibility of a greener chemistry approach and a reduced cost of production. Moreover, to further reinforce the green chemistry approach, our work also demonstrated that TaPSy could carry out peptide synthesis with the green solvent, GVL. Overall, results showed that the new TaPSy device is highly efficient, automated, able to synthetize peptides in parallel mode (up to 12 different peptide sequences), inexpensive (to obtain and to operate), robust, and complies with the principles of green chemistry. Moreover, TaPSy has the potential to be scaled up further, and used under pilot-scale conditions.

Non-standard abbreviations

AA, amino acid; DIC, *N,N′*-diisopropylcarbodiimide; DIPEA, *N,N*-diisopropylethylamine; ESI-MS, Electrospray ionization mass spectrometry; GVL, γ-valerolactone; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate; HBTU, O-(7-azabenzotriazole-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, hydroxybenzotriazole; ipAoa, isopropylidene-protected aminooxyacetic acid; LC-MS, liquid-chromatography mass spectrometry; MBHA, 4-methylbenzhydrylamine; PEG, polyethylene glycol; PyAOP, (7-azabenzotriazol-1-yloxy)-tripyrrolidinophosphonium hexafluorophosphate; PTFE, polytetrafluoroethylene; RP-HPLC, reversed-phase high-performance liquid chromatography; SPPS, solid-phase peptide synthesis; TaPSy, Tecan add-on peptide synthesizer; TATU, O-(7-azabenzotriazole-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate; TIS, triisopropylsilane.

Author contributions

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Notes

The authors declare no competing financial interest.

SUPPORTING INFORMATION

Additional details of Tecan add-on peptide synthesizer (TaPSy) worktable layout, software development and methodology, and LC-MS analysis of peptide products.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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References

[1] Pedersen SL, Jensen KJ. Instruments for automated peptide synthesis. In: Jensen KJ, Tofteng Shelton P, Pedersen SL, editors. Peptide synthesis and applications. Methods in molecular biology. Totowa, NJ: Humana Press; 2013. p. 215–24. doi:10.1007/978-1-62703-544-6_15.
[2] Variable bed flow reactor (VBFR) https://www.vapourtec.com/peptide-synthesis-reactor (accessed Mar 21, 2022).
[3] AAPPTec. Peptide Synthesizers https://www.peptide.com/peptide-synthesizers/(accessed Mar 21, 2022).
[4] CEM. Peptide Synthesis https://cem.com/uk/peptide-synthesis (accessed Mar 21, 2022).
[5] CSBio. Peptide Synthesizers https://www.csbio.com/synthesizer.html (accessed Mar 21, 2022).
[6] Activotec. Synthesizers https://www.activotec.com/product-category/peptidesynthesizers/(accessed Mar 21, 2022).
[7] Biotage. Peptide Synthesis https://www.biotage.com/peptide-synthesis (accessed Mar 21, 2022).
[8] Gyros Protein Technologies. Instruments for Automated Peptide Synthesis https://www.gyrosproteintechnologies.com/peptide-synthesizers (accessed Mar 21, 2022).
[9] Activotec. Activo-P11 Automated Peptide Synthesizer https://www.activotec.com/product/activo-p11-automated-peptide/.
[10] AAPPTec. Focus XC https://www.peptide.com/peptide-synthesizers/focus-xc/ (accessed Mar 21, 2022).
[11] CEM. Liberty Blue https://cem.com/liberty-blue/(accessed Mar 21, 2022).
[12] CEM. Liberty Blue HT12 https://cem.com/liberty-blue-ht12/(accessed Mar 21, 2022).
[13] CEM. Liberty PRIME https://cem.com/liberty-prime/(accessed Mar 21, 2022).
[14] CSBio. CSBio II https://www.csbio.com/csbioii.html (accessed Mar 21, 2022).
[15] CSBio. CS136X https://www.csbio.com/136x.html (accessed Mar 21, 2022).
[16] Gyros Protein Technologies. PurePep® Chorus https://www.gyrosproteintechnologies.com/purepep-chorus-peptide-synthesizer-modular-design-that-can-grow-with-your-chemistry-needs (accessed Mar 21, 2022).
[17] Biotage. Biotage® Initiator+ AlstraTM https://www.biotage.com/alstra-peptide-synthesis (accessed Mar 21, 2022).

[18] Enyedi KN, Tóth S, Szakács G, Mező G. NGR-peptide–drug conjugates with dual targeting properties. PLoS One 2017;12(6):e0178632. doi:10.1371/journal.pone.0178632.
[19] Peptide Companion http://www.spyderinstitute.com/Prediction.xlsm.
[20] Krchňák V, Flegelová Z, Vágner J. Aggregation of resin-bound peptides during solid-phase peptide synthesis. Int J Pept Protein Res 1993;42(5):450–4. doi:10.1111/j.1399-3011.1993.tb00153.x.
[21] Kiss K, Biri-Kovács B, Szabó R, Ranđelović I, Enyedi KN, Schlosser G, Orosz Á, Kapuvári B, Tóvári J, Mező G. Sequence modification of heptapeptide selected by phage display as homing device for HT-29 colon cancer cells to improve the anti-tumour activity of drug delivery systems. Eur J Med Chem 2019;176:105–16. doi:10.1016/j.ejmech.2019.05.016.
[22] Albericio F, Bofill JM, El-Faham A, Kates SA. Use of onium salt-based coupling reagents in peptide synthesis. J Org Chem 1998;63(26):9678–83. doi:10.1021/jo980807y.
[23] Subirós-Funosas R, Prohens R, Barbas R, El-Faham A, Albericio F. Oxyma: an efficient additive for peptide synthesis to replace the benzotriazole-based HOBt and HOAt with a lower risk of explosion[1]. Chem – A Eur J 2009;15(37):9394–403. doi:10.1002/chem.200900614.
[24] Khattab SN. Sulfonate esters of 1-hydroxypyridin-2(1H)-one and ethyl 2-cyano-2-(hydroxyimino)acetate (oxyma) as effective peptide coupling reagents to replace 1-hydroxybenzotriazole and 1-hydroxy-7-azabenzotriazole. Chem Pharm Bull 2010;58(4):501–6. doi:10.1248/cpb.58.501.
[25] Albericio F, Cases M, Alsina J, Triolo SA, Carpino LA, Kates SA. On the use of PyAOP, a phosphonium salt derived from HOAt, in solid-phase peptide synthesis. Tetrahedron Lett 1997;38(27):4853–6. doi:10.1016/S0040-4039(97)01011-3.
[26] Kumar A, Jad Y, Collins J, Albericio F, la torre B. Microwave-assisted green solid-phase peptide synthesis using γ-valerolactone (GVL) as solvent. ACS Sustain Chem Eng 2018;6. doi:10.1021/acssuschemeng.8b01531.
[27] Al Musaimi O, El-Faham A, Basso A, la torre B, Albericio F. γ-Valerolactone (GVL): an eco-friendly anchoring solvent for solid-phase peptide synthesis. Tetrahedron Lett 2019;60. doi:10.1016/j.tetlet.2019.151058.
[28] Ferrazzano L, Catani M, Cavazzini A, Martelli G, Corbisiero D, Cantelmi P, Fantoni T, Mattellone A, De Luca C, Felletti S, Cabri W, Tolomelli A. Sustainability in peptide chemistry: current synthesis and purification technologies and future challenges. Green Chem 2022;24(3):975–1020. doi:10.1039/D1GC04387K.
[29] Thomas C, Peruch F, Bibal B. Ring-opening polymerization of lactones using supramolecular organocatalysts under simple conditions. RSC Adv 2012;2(33):12851–6. doi:10.1039/C2RA22535B.
[30] Kumar A, Sharma A, de la Torre BG, Albericio F. Scope and limitations of γ-valerolactone (GVL) as a green solvent to be used with base for fmoc removal in solid phase peptide synthesis. Molecules 2019;24(21). doi:10.3390/molecules24214004.
[31] Ralhan K, KrishnaKumar VG, Gupta S. Piperazine and DBU: a safer alternative for rapid and efficient fmoc deprotection in solid phase peptide synthesis. RSC Adv 2015;5(126):104417–25. doi:10.1039/C5RA23441G.
[32] Zinieris N, Leondiadis L, Ferderigos N. Nα-fmoc removal from resin-bound amino acids by 5% piperidine solution. J Comb Chem 2005;7(1):4–6. doi:10.1021/cc049872d.
[33] Farkas V, Ferentzi K, Horváti K, Perczel A. Cost-effective flow peptide synthesis: metamorphosis of HPLC. Org Process Res Dev 2021;25(2):182–91. doi:10.1021/acs.oprd.0c00178.
[34] Mijalis AJ, Thomas DA 3rd, Simon MD, Adamo A, Beaumont R, Jensen KF, Pentelute BL. A fully automated flow-based approach for accelerated peptide synthesis. Nat Chem Biol 2017;13(5):464–6. doi:10.1038/nchembio.2318.
[35] Naoum JN, Alshanski I, Mayer G, Strauss P, Hurevich M. Stirring peptide synthesis to a new level of efficiency. Org Process Res Dev 2022;26(1):129–36. doi:10.1021/acs.oprd.1c00304.