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Automated 'X-Y' robot for peptide synthesis with microwave heating: application to difficult peptide sequences and protein domains

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Precise microwave heating has emerged as a valuable method to aid solid-phase peptide synthesis (SPPS). New methods and reliable protocols, as well as their embodiment in automated instruments, are required to fully use this potential. Here we describe a new automated robotic instrument for SPPS with microwave heating, report protocols for its reliable use and report the application to the synthesis of long sequences, including the β-amyloid 1-42 peptide. The instrument is built around a valve-free robot originally developed for parallel peptide synthesis, where the robotic arm transports reagents instead of pumping reagents via valves. This is the first example of an 'X-Y' robotic microwave-assisted synthesizer developed for the assembly of long peptides. Although the instrument maintains its capability for parallel synthesis at room temperature, in this paper, we focus on sequential peptide synthesis with microwave heating. With this valve-free instrument and the protocols developed for its use, fast and efficient syntheses of long and difficult peptide sequences were achieved.

Supporting information may be found in the online version of this article

Keywords: peptide synthesis; microwave heating; amyloid; LysM

Introduction

Solid-phase peptide synthesis (SPPS) has been tremendously successful in the synthesis of short and medium length peptides because of the development of efficient organic chemistry for amide bond formation as well as for protection of amino groups and side-chain functionalities. One of the current frontiers in peptide science is the assembly of long peptides and small proteins, which has to overcome the accumulation of side reactions and the hurdles posed by so-called difficult sequences. In recent years, precise and fast heating by microwave irradiation has emerged as a new parameter to further optimize SPPS [1–6], especially to enable the synthesis of long and difficult peptide sequences or to improve their purity in general.

During the synthesis of difficult sequences, the peptide chain most likely becomes partially inaccessible typically due to the formation of secondary structures, especially β-sheets [7]. In addition, steric hindrance from β-branched amino acids can be a problem. Methods to suppress intramolecular aggregation have been described and include pseudo-prolines [7], solvent composition [8] and chaotropic salts [9,10]; however, their utility is limited and the efficiency is variable. Intermolecular aggregation could lead to poor solvation of the peptidyl-polymer, but it is less pronounced with low-loading resins having a dynamic structure, such as TentaGel. Heating is likely to reduce both the inter- and intramolecular-derived aggregation and thereby decrease the reaction time and improve the coupling efficiency of bulky and β-branched amino acids, as well as N^{α}-deprotection of sterically hindered peptidyl-polymers. Kappe *et al*. [11] have recently studied microwave-assisted SPPS by comparing it with SPPS using an oil bath and concluded that the observed enhancement effect was of thermal nature. Nonthermal effects were not evident, which contradicts some previous suggestions [12]. Microwave irradiation is an advantageous tool in peptide chemistry because of the rapid and precise elevation of the temperature, and the efficient temperature control during the synthesis. Furthermore, if necessary, it can be combined with precise and reproducible cooling after the reaction, e.g. by pressurized air.

In this paper, the use of a new fully automated robotic instrument with precise microwave heating in SPPS is presented. The instrument is built around a valve-free MultiSynTech instrument, which incorporates an 'X-Y' robot equipped with digital syringe pumps. The MultiSynTech instrument was originally developed for parallel synthesis, where the robotic arm distributes reagents (Figure S1, Supporting Information). Essentially, this mimics the hand movements (i.e. it is chiro-mimic) in manual SPPS and is in contrast to pumping reagents via valves, as in valve-based synthesizers. The robot is combined with a modified Biotage Initiator microwave instrument controlled by a single PC. The instrument

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maintains the capability for parallel synthesis at room temperature (RT); however, in this paper, we focus on sequential peptide synthesis with microwave heating. The reaction vessel, a modified plastic syringe with a filter bottom, is placed in the microwave reactor for the duration of the synthesis (Figure S2). Mixing is crucial during reactions with microwave heating in order to have homogenous heat distribution in the reactor vessel, especially when highly viscous reaction media are being used, as in SPPS. Previously, mixing during microwave heating has been achieved by magnetic stirring or bubbling with nitrogen, with the former not being compatible with resins. However, vortexing has been used extensively for SPPS at RT and has advantages over nitrogen bubbling, as the latter carries a risk of blowing the resin to the top of the reaction vessel. A novel feature of this new instrument described here is that mixing is achieved by efficient vortexing within the microwave cavity. Cooling is simply achieved as the solvents used for washing are at RT.

Materials and Methods

Materials

All organic solvents and N^{α}-Fmoc amino acids were obtained from Iris Biotech GmbH and contained the following side-chain-protecting groups: *tert*-butyl (OtBu, for Glu, Asp, Ser, Thr, Tyr), 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf, for Arg), trityl (Trt, for Asn, Gln, His) and *tert*-butyloxycarbonyl (Boc for Lys). All peptide amides were synthesized on a standard Fmoc-RAM-TG resin from Rapp Polymere GmbH (loading 0.24 mmol/g). Analytical HPLC was performed on a Dionex UltiMate 3000 with Chromeleon 6.80SP3 software. Peptides were analyzed on a Phenomenex Jupiter 300 Å C4 column (5 µm, 4.6 × 150 mm) or a Phenomenex Gemini 110 Å C18 column (3 µm, 4.6 × 50 mm), running a flow of 1.0 ml/min with a linear gradient with increasing amount of buffer for 10–20 min (buffer A: 0.1% formic acid in H_2O; buffer B: 0.1% formic acid in CH_3CN). Mass analyses were performed using ESI-MS (MSQ Plus Mass Spectrometer, Thermo).

Instrument for Robotic SPPS with Microwave Heating

Peptides were assembled on the fully automated solid-phase microwave peptide synthesis system from Biotage AB (Syro *Wave*™). The instrument combines a Syro I synthesis robot from MultiSynTech, with a Biotage Initiator microwave instrument, incorporating a dedicated additional vortex mixer for the microwave cavity (Figure S1). The microwave instrument has an extended microwave guide and is attached on the left side of the Syro I, while the right side maintains the ability for parallel synthesis at RT. The reaction vessel is a modified plastic syringe (with PTFE frit) which is vortexed within the microwave cavity during reactions. The temperature was measured by a calibrated IR sensor. The Initiator heated during coupling reactions and for some syntheses also during Fmoc deprotections. The Initiator can deliver up to 200 W, but here it was programed to provide typically 40–160 W during the ramp-up phase and a cap of max. 60 W was set during the steady-state phase, where the temperature is maintained. For a coupling temperature of 75 °C, the power required to maintain a steady state was ~40 W. The instrument was operated without cooling during microwave irradiation. The total volume of coupling reagents was 2.7 ml, which gave a volume of 3.3 ml with resin. This volume proved efficient for reliable absorption of microwave irradiation for a 10-ml reactor vial. The initial concentration of the activated Fmoc amino acid in the coupling mixture was 0.18 M; however, a concentration of 0.15 M gave almost the same results (data not shown). A complete cycle of deprotection, wash, coupling and wash could be achieved in less than 30 min.

Protocol for Synthesis Using Conventionally RT Methods

Control peptides were prepared conventionally on a fully automated Syro *Wave*™ peptide synthesizer (Biotage AB) using Fmoc-RAM-TG resin (0.433 g). N^{α}-Fmoc deprotection was performed in two stages using solutions: (i) piperidine in DMF (2 : 3) for 3 min and (ii) piperidine in DMF (1 : 4) for 10 min. Coupling reactions were performed using N^{α}-Fmoc amino acids (5.2 equiv., 0.5 M) which were dissolved in a 4 : 1 mixture of HOBt : HOAt (5.2 equiv., 0.5 M), HBTU (5 equiv., 0.43 M) and DIEA (10 equiv., 2 M) in *N*-methyl-2-pyrrolidinone (NMP). After completing synthesis of the peptide sequence, the resin was successively washed with NMP (×3) and DCM (×3) and dried thoroughly. Peptide release was performed using TFA–TES–H_2O (95 : 2.5 : 2.5) for 2 h at RT. The crude product was analyzed by LC-MS.

General Protocol for Synthesis of H-WFTTLISTIM-NH_2 (1)

Peptide **1** was prepared on Fmoc-RAM-TG resin (0.433 g) (Tables 2 and 3, Figure 2). Deprotection was performed in two stages using solutions: (i) piperidine in DMF (2 : 3) and (ii) piperidine in DMF (1 : 4), with the first deprotection step at 60 °C for 2 min and then followed by another deprotection step for 2 min at 60 °C. The resin was then washed with NMP (3 × 45 s). Coupling reactions were performed using N^{α}-Fmoc amino acids (5.2 equiv., 0.5 M), which were dissolved in a 4 : 1 mixture of HOBt : HOAt (5.2 equiv., 0.5 M), HBTU (5 equiv., 0.43 M) and DIEA (10 equiv., 2 M) in NMP. Other coupling reagents were inserted as indicated in Table 3. All couplings were performed for 5 min at 75 °C. Following each coupling step, the resin was washed with NMP (×4). After completing synthesis of the peptide sequence, the resin was successively washed with NMP (×3) and DCM (×3). Peptide release was performed using TFA–TES–H_2O (95 : 2.5 : 2.5) for 2 h at RT. The crude product was analyzed by LC-MS (C18 column) via B gradient elution (0–5 min: 5–37%, 5–8.5 min: 37–38% and 8.5–9.5 min: 38–100%) with an applied flow rate of 1.0 ml/min. Purity 44%. ESI-MS, calculated monoisotopic composition for $C_{58}H_{90}N_{12}O_{14}S$, 1210.6 Da. Found: *m/z* 1211.6 $[M + H]^+$.

Chiral Amino Acid Analysis of Peptide 1 Using COMU as Coupling Reagent

Peptide **1** (Table 3, entry 7, crude products) was submitted to chiral amino acid analysis at C.A.T. GmbH & Co. (Tübingen, Germany) to detect the level of D-enantiomer, which gave the following: L-Threonine (>99.70%), D-Threonine (<0.10%), L-allo Threonine (<0.10%), D-allo Threonine (<0.10%), L-Isoleucine (>99.70%), D-Isoleucine, L-allo Isoleucine (<0.10%), D-allo Isoleucine (<0.10%) Leucine (D-enatiomer 0.10%), Serine (D-enantiomer 0.10%), Methionine (D-enantiomer 0.21%), Phenylalanine (D-enantiomer 0.21%) and Tryptophan (D-enantiomer 0.21%).

General Protocol for Synthesis of H-DAEFRHDSGYEVHHQKL VFFAEDVGSNKGAIIGLMVGGVVIA-NH_2 (2)

Peptide **2** was prepared on Fmoc-RAM-TG resin (0.433 g) (Figure 3). Deprotection was performed in two stages using solutions: (i) piperidine in DMF (2 : 3) and (ii) piperidine in DMF (1 : 4), with

a first deprotection step at 60 °C for 2 min and then followed by another deprotection step for 2 min at 60 °C. Then washed with NMP (3 × 45 s). Coupling reactions were performed using N^{α}-Fmoc amino acids (5.2 equiv., 0.5 M), which were dissolved in a 4 : 1 mixture of HOBt : HOAt (5.2 equiv., 0.5 M), HBTU (5 equiv., 0.43 M) and DIEA (10 equiv., 2 M) in NMP. All couplings were performed for 5 min at 75 °C. Following each coupling step, the resin was washed with NMP (×4). After the incorporation of the 16th amino acid, a double-coupling protocol was performed using the same method as above. After completing synthesis of the peptide sequence, the resin was washed with NMP (×3) and DCM (×3) and dried thoroughly. Peptide release was performed using TFA–TES (97.5 : 2.5) (1 ml) for 2 h at RT, followed by addition of EDT (16 µl) and TMSBr (13 µl) for 15 min. The crude product was analyzed by LC-MS (C4 column) via B gradient elution (0–3.5 min: 5–30%, 3.5–15 min: 30–65% and 15–17 min: 65–100%) with an applied flow rate of 1.0 ml/min. Purity 72%. ESI-MS, calculated average isotopic composition for 4512.3 Da. Found: *m/z* 1129.0 $[M+4H]^{4+}$, 903.5 $[M+5H]^{5+}$, 753.2 $[M+6H]^{6+}$, 645.0 $[M+7H]^{7+}$.

General Protocol for Synthesis of H-YLERELKKLERELKKLSPEELNRYYASLRHYLNLVTRQRY-NH_2 (3)

Peptide **3** was prepared on TentaGel Rink amide resin (loading 0.24 mmol/g, 0.433 g). Deprotection was performed in two stages using solutions: (i) piperidine in DMF (2 : 3) and (ii) piperidine in DMF (1 : 4), With a first deprotection step at RT for 3 min and then followed by another deprotection step for 10 min at RT and washed with NMP (3 × 45 s). Coupling reactions were performed using N^{α}-Fmoc amino acids (5.2 equiv., 0.5 M), which were dissolved in a 4 : 1 mixture of HOBt : HOAt (5.2 equiv., 0.5 M), HBTU (5 equiv., 0.43M) and DIEA (10 equiv., 2 M) in NMP. All couplings were performed for 10 min at 75 °C and double couplings where applied in the entire sequence. Following each coupling, the resin was washed with NMP (×4). After completing synthesis of the peptide sequence, the resin was washed with NMP (×3) and DCM (×3) and dried thoroughly. Peptide release was performed using TFA–TES–H_2O (95 : 2.5 : 2.5) for 2 h at RT. The crude product was analyzed by LC-MS (C4 column) via B gradient elution (0–14 min: 5–100%) with an applied flow rate of 1.0 ml/min. Purity ~25%. ESI-MS, calculated average isotopic composition for $C_{231}H_{374}N_{68}O_{63}$, 5111.8 Da. Found: *m/z* 1279.2 $[M+4H]^{4+}$, 1022.8 $[M+5H]^{5+}$, 853.2 $[M+6H]^{6+}$, 731.1 $[M+7H]^{7+}$.

General Protocol for Synthesis of H-LPERVKVVFPL-NH_2 (4) and H-YENLTNWNIVQAS-NPGVNPYLLPERVKVVFPL-NH_2 (5)

Peptides **4** and **5** were prepared on TentaGel Rink amide resin (loading 0.24 mmol/g, 0.433 g). Deprotection was performed in two stages using solutions: (i) piperidine in DMF (2 : 3) and (ii) piperidine in DMF (1 : 4), with a first deprotection step at RT for 3 min and then followed by another deprotection step for 10 min at RT and washed with NMP (3 × 45 s). Coupling reactions were performed using N^{α}-Fmoc amino acids (5.2 equiv., 0.5 M), which were dissolved in a 4 : 1 mixture of HOBt : HOAt (5.2 equiv., 0.5 M), HBTU (5 equiv., 0.43 M) and DIEA (10 equiv., 2 M) in NMP. All couplings were performed for 10 min at 75 °C and double couplings were applied in the entire sequence. Following each coupling, the resin was washed with NMP (×4). After completing synthesis of the peptide sequence, the resin was washed with NMP (×3) and DCM (×3) and dried thoroughly. Peptide release was performed using TFA–TES–H_2O (95 : 2.5 : 2.5) for 2 h at RT. The crude product was analyzed by LC-MS (C18 column) via B gradient elution (0–9 min: 5%–100%) with an applied flow rate of 1.0 ml/min. The purity of peptide **4** was 75%. The purity of peptide **5** was ~54%. ESI-MS, calculated average isotopic composition for 3682.97 Da. Found: *m/z* 1842.8 $[M+2H]^{2+}$, 1228.8 $[M+3H]^{3+}$, 921.8 $[M+4H]^{4+}$.

Results and Discussion

Couplings (reagents, temperature and time) and N^{α}-deprotection (time and temperature) were optimized using peptide sequence **1** (Table 1), which originates from the *C*-terminus of the MuLV CTL epitope [13]. Carpino *et al.* [14] have reported peptide **1** to be a very difficult sequence. The optimized protocol was then used to synthesize the following peptides (Table 1): human β-amyloid1-42 peptide [15] (peptide **2**), an analog of the peptide hormone PYY3-36 [16,17] (peptide **3**) and a region of the Nfr5 and LysM2 domain (*Lotus japonicus* sequence, peptide **4** and **5**).

Therefore, to evaluate the use of SPPS with microwave heating, four peptides were assembled which in the literature have been reported to be 'difficult sequences' (Table 1). The first sequence investigated was the decapeptide derived from the MuLV CTL epitope [13]. Jung and Redemann had previously reported that the majority of deletion peptides come from the first nine *C*-terminal amino acids, which prompted us to choose this short sequence as a model for the evaluation of peptide synthesis, in particular conventional at RT versus microwave heating. The assembly of peptide **1** using semi-automated solid-phase synthesis has previously been reported to give a HPLC purity of 44% [18]. Here and in the following, yields indicate 'crude purities' after acidolytic release of the peptides into solution. In the semi-automated synthesis, the N^{α}-deprotections were performed at RT for 1 min, followed by 2 min at 60 °C, whereas coupling reactions were at 80 °C for 10 min. Moreover, the semi-automated synthesis was performed using a high-loaded PS-based resin. We build on these results and aimed for a faster and more efficient route for fully automated robotic peptide assembly with microwave heating.

We set out to test different reaction conditions using the fully automated peptide synthesizer (Table 2). As a control, the conventional synthesis of **1** (couplings for 45 min at RT and deprotections at RT for 3 + 10 min) gave a HPLC purity of 35% (Table 2, entry 1). Reducing the coupling time down to 5 min further decreased the purity to 23% (Table 2, entry 2). Using microwave irradiation during the coupling only (10 min at 75 °C) and not the deprotections (3 + 10 min at RT) gave a purity of 40% according to HPLC (Table 2, entry 3). We repeated the optimized conditions from the semi-automated SPPS (couplings at 80 °C for 10 min and deprotections at 60 °C for 2 + 2 min), which afforded a purity of 40% of **1** (Table 2, entry 4). To reduce cycle time further,

Table 1. The five difficult sequences

Peptide	Sequences
1	H-WFTTLISTIM-NH_2
2	H-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA-NH_2
3	H-YLERELKKLERELKKLSPEELNRYYASLRHYLNLVVTRQRY-NH_2
4	H-LPERVKVVFPL-NH_2[a]
5	H-YENLTNWNIVQASNPGVNPYLLPERVKVVFPL-NH_2

[a] Peptide **4** is the first 11 amino acids from the *C*-terminal of peptide **5**.

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Table 2. Different reaction conditions for the synthesis of peptide **1** (H-WFTTLISTIM-NH_2)

Entry	Deprotection	Coupling reagent	Coupling temperature	Coupling time (min)	HPLC purity (%)
1	3 + 10 min, RT	HBTU/(HOBt : HOAt)	RT	1 × 45	35
2	3 + 10 min, RT	HBTU/(HOBt : HOAt)	RT	1 × 5	23
3	3 + 10 min,RT	HBTU/(HOBt : HOAt)	75 °C	1 × 10	40
4	2 + 2 min, 60 °C	HBTU/(HOBt : HOAt)	80 °C	1 × 10	40
5	2 + 2 min, 60 °C	HBTU/(HOBt : HOAt)	75 °C	1 × 5	46

Couplings reagent used was HBTU/(HOBt : HOAt).

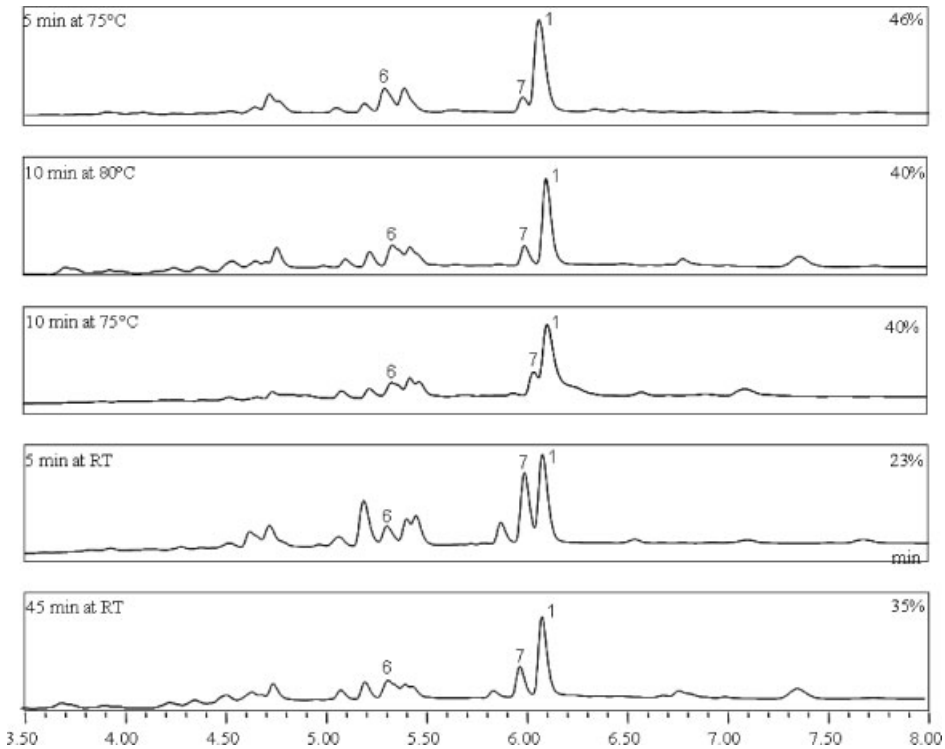

Figure 1. HPLC chromatograms of protocol optimization for peptide **1.** The purity of peptide **1** is given in the right side of each chromatogram. (**1**) des-(Trp), (**2**) des-(Thr) and (**3**) [M+H].

we reduced the coupling time (5 min at 75 °C) and surprisingly a noticeable better purity of 46% was obtained (Table 2, entry 5). The major impurities in all the runs were deletion peptides des-(Trp) and des-(Thr) (Figure 1). We observed that using fresh stock solutions of amino acid and especially new solutions of Trp enhanced the purity by lowering, e.g., des-Trp byproduct formation (data not shown). One of the concerns using elevated temperature during SPPS is the possibility to form epimers. Here, this question was addressed by comparison of previously reported data for this decapeptide with our LC-MS analysis [14]. We saw no indication of epimerization in any of our experiments, which suggested that the heating does not cause significant epimers to form during the synthesis of this peptide.

Thus, when comparing fully automated SPPS of decapeptide **1** under conventional conditions at RT with microwave-assisted assembly, a clear advantage of microwave heating emerged. When applying microwave heating, the peptide was synthesized faster (5 vs 45 min for coupling, 4 vs 13 min for deprotection). The main advantage lies in the dramatic reduction in coupling time, whereas the reduction in time for Fmoc deprotections is less pronounced.

Table 3. Different coupling reagents for the synthesis of peptide **1** (H-WFTTLISTIM-NH_2)

Entry	Coupling reagent	HPLC purity (%)
1	PyBOP/(HOBt : HOAt)	35
2	DIC/(HOBt : HOAt)	38
3	TSTU/(HOBt : HOAt)	40
4	HCTU/(HOBt : HOAt)	42
5	HBTU/(HOBt : HOAt)	44
6	HATU/(HOBt : HOAt)	49
7	COMU/(HOBt : HOAt)	70

In all the experiments, "deprotection was 2 + 2 min at 60 °C and the coupling time was 5 min and the temperature 75 °C".

Additionally, an increase in purity was observed from 35 to 46% using microwave heating.

The overall time for a full cycle of coupling, deprotection and washings is not solely determined by time used for couplings

and deprotections. The time used for washings and for reagent transfer should also be included in the calculation. A cycle time of 30 min for the difficult peptide **1** was obtained, which gives a total synthesis time of 5.5 h (Table S1, Supporting Information). These initial experiments clearly demonstrated that a peptide synthesizer based on an 'X-Y' robot can indeed achieve fast syntheses of peptides.

Next, we evaluated the use of different coupling reagents for the assembly of peptide **1** and their effect on purity. We maintained the optimized microwave heating protocol described above and HOBt/HOAt as nucleophilic additive, but systematically substituted HBTU with other coupling reagents, such as HATU, HCTU, TSTU, DIC, PyBOP and COMU (Table 3). The result showed that TSTU, HCTU and HBTU gave similar purities (Table 3, entry 3–5) and that DIC and PyBOP gave reduced purities compared to HBTU (Table 3, entry 1–2). HATU gave an increase in purity compared with HBTU (Table 3, entry 6). COMU, a relatively new coupling reagent, has been reported to give a significantly higher purity of the Aib-analog of Leu-enkephalin pentapeptide (H-Tyr-Aib-Aib-Phe-Leu-NH_2) than standard coupling reagents, such as HATU and HBTU [19]. We thus tested COMU as a coupling reagent for the synthesis of peptide **1**. This gave the highest HPLC purity (70%), which was significantly better than for other coupling reagents. Analysis of the LC-MS data revealed no epimerization (identical masses for separate HPLC peaks); furthermore, a significant reduction in deletion peptides was observed (Figure 2). Thus, microwave heating with COMU as the coupling reagent gave the best purity of decapeptide **1**.

Peptide **1** (Table 3, entry 7) was taken as a representative example and the crude peptide material was subjected to chiral amino acid analysis. The crude peptide, rather than the purified peptide, was chosen as this provided an analysis of all peptide material in this synthesis and thus avoiding a bias. This showed only insignificant levels of epimerization, which provided additional support for the absence of (or insignificant level of) epimerization under these conditions.

The human β-amyloid1-42 peptide **2** is one of the main constituents in amyloid plaques in the brain of Alzheimer's patients and has been the target of significant attention in recent years; however, its solid-phase assembly is non-trivial. The synthesis of this peptide commenced with a reference experiment in which peptide **2** was synthesized conventionally with an optimized, but very time-consuming protocol at RT (couplings of the first 16 *C*-terminal residues for 45 min and the remaining residues for 2×45 min, with Fmoc deprotection for $3 + 10$ min). Analytical HPLC revealed a peak arising from the Met(O)-35 peptide, however, acidolytic release of the peptide under reducing conditions [20], afforded the crude product in a rewarding yield of 54%. The application of microwave heating during the couplings (first 16 *C*-terminal residues for 5 min and the remaining for 2×5 min), and the same deprotection condition at RT as above, provided comparable yield as for long coupling times at RT (data not shown). Next, microwave irradiation was used during coupling (as above) and deprotection ($2 + 2$ min at 60 °C) this gave an improved purity of 72% (Figure 3). In all syntheses of peptide **2**, a peak arising from the Met(O)-35 peptide was observed when no precautions were taken, therefore all cleavages were performed using the above reductive release protocol. The formation of Met(O)-35 peptide in the synthesis of β-amyloid1-42 peptide has been observed by others [21]. For this particular sequence, human β-amyloid1-42 peptide, heating during Fmoc removal proved advantageous.

The peptide hormone PYY3-36 plays a central role in the regulation of food intake and energy homeostasis [22]. The optimized protocol described above was applied to the synthesis of an analog of PYY3-36 (3) consisting of a helix-loop-helix structure, with both helices being amphipathic (Table 1, entry 3). Although the *C*-terminal helix and the loop were maintained from the native sequence, the *N*-terminal helix was inspired by a structure reported by Vagt *et al*. [23]. In previous experiments, we had observed that this sequence was difficult to synthesize in high yield and purity. First, the synthesis of peptide **3** was attempted using conventional conditions at RT (couplings for 45 min, deprotection for $3 + 12$ min), but this gave no yield. Increasing the coupling times at RT and repeating the couplings gave a good yield and purity (coupling for 2×2 h, deprotection for $3 + 12$ min). Next, the sequence was synthesized on the robotic synthesizer with microwave heating, which required optimization of the protocol, as the initially used 5-min coupling at 75 °C and deprotection $3 + 10$ min at RT gave no product. However, peptide **3** was successfully synthesized using double couplings and microwave heating (2×10 min at 75 °C) and deprotections without heating ($3 + 10$ min at RT). This afforded purity comparable with the much more time-consuming conventionally optimized protocol above. This clearly demonstrated that microwave heating applied to the synthesis of difficult sequences can give dramatically shortened coupling times (coupling for 240 min conventionally vs 20 min using microwave heating).

The LysM domain was predicted to consist of two α-helices and a two-stranded anti-parallel β-sheet in a β-α-α-β structure and has been identified in Nfr5 [24] by sequence alignment of the crystal structure with the LysM domain of *Bacillus subtilis* ykuD [25]. There were significant synthetic challenges arising from the *C*-terminal and the *N*-terminal regions; this is presumably due to the formation of β-sheet-like structures, which is known to pose problems for peptide chain assembly. Thus, peptide **4**, which is derived from the *C*-terminus of the LysM2 domain and contains several β-branched and bulky amino acid residues, was assembled under various conditions. When applying standard SPPS protocols at RT, we were able to synthesize peptide **4** in very low yield, but when using the very slow conventional protocol at RT, the peptide was produced in reasonable yield (couplings for 2×2 h, deprotections for $3 + 10$ min). Then, we turned to the optimized protocol with microwave heating for the synthesis of this sequence (couplings for 2×10 min at 75 °C) and deprotections at RT ($3 + 10$ min), this afforded an excellent HPLC purity of 75% compared with previous work (Figure 4). Again, when comparing conventional and microwave heating protocols, the immense advantage in using microwave heating was seen in the reduction of coupling times (20 min instead of 240 min) and the higher purity obtained by avoiding the formation of several deletion peptides sequences (data not shown). Notably, this was achieved without microwave heating during the deprotections. Finally, we set out to synthesize peptide **5** (continuing from peptide **4**), which is the 32-mer from the *C*-terminus of the LysM2. All couplings were achieved with microwave heating and with the above optimized condition, which afforded the peptide in an acceptable yield 55%.

Conclusion

In conclusion, we have developed reliable protocols for SPPS of difficult and long sequences using a new automated robotic instrument which combines the function of an 'X-Y' laboratory

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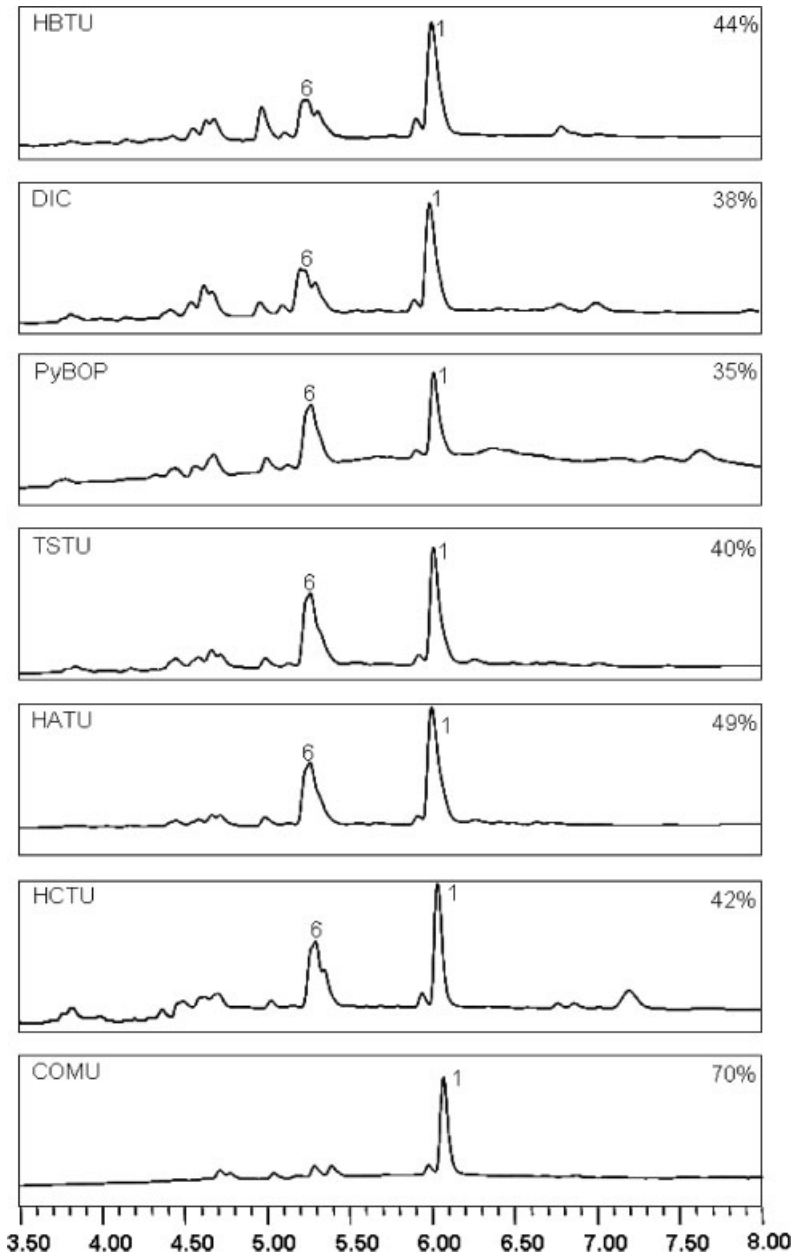

Figure 2. HPLC chromatograms of coupling reagent optimization experiments. The purity of peptide **1** is given in the right side of each chromatogram. (**1**) des-(Trp) and (**3**) [M+H].

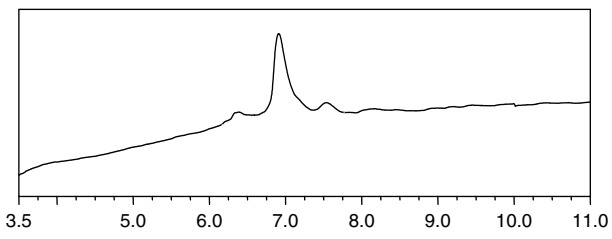

Figure 3. Crude analytical HPLC chromatogram of the β-amyloid1-42 (**2**) after acidolytic release from the support under mild reducing conditions.

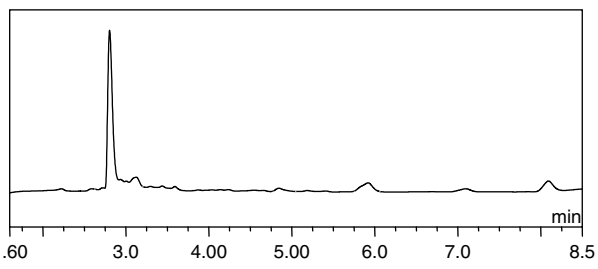

Figure 4. Crude analytical HPLC chromatogram of the *C*-terminal 11mer (**4**) of the LysM2 domain.

robot with microwave heating. Difficult peptides as well as long sequences including the β-amyloid 1–42 peptide and the LysM2 protein were successfully assembled in an excellent crude purity. This study focused on the purities of the synthesized peptides; future studies will also include analyses of the crude yields. In general, syntheses were much faster than with conventional

protocols, where each cycle time with microwave heating often could be efficiently performed in 30 min or less, instead of 3 h or more required for difficult sequences at RT. Microwave heating during N^{α}-Fmoc deprotections proved beneficial for the synthesis of some peptides, but was not required in all cases. In general, all standard coupling reagents gave comparable purities under microwave conditions, with HATU slightly above average and DIC as well as PyBOP slightly below.

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Supporting information

Supporting information may be found in the online version of this article.

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