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Microwave-assisted TFA cleavage of peptides from Merrifield resin

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Microwave-assisted (MW) reactions are of special interest to the chemical community due to faster reaction times, cleaner reactions and higher product yields. The adaptation of MW to solid phase peptide synthesis resulted in spectacular syntheses of difficult peptides. In the case of Merrifield support, used frequently in synthesis of special peptides, the conditions used in product cleavage are not compatible with off-resin monitoring of the reaction progress. The application of MW irradiation in product removal from Merrifield resin using trifluoroacetic acid (TFA) was investigated using model tetrapeptides and the effects were compared with standard trifluoromethanesulphonic acid (TFMSA) cleavage using elemental analysis as well as chromatographic (HPLC) and spectroscopic (IR) methods. The deprotection of benzyloxycarbonyl and benzyl groups in synthetic bioactive peptides was analyzed using LC-MS and MS/MS experiments. In a 5 min microwave-assisted TFA reaction at low temperature, the majority of product is released from the resin, making the analytical scale MW-assisted procedure a method of choice in monitoring the reactions carried out on Merrifield resin due to the short reaction time and compatibility with HPLC and ESI-MS conditions. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: microwave-assisted synthesis; SPPS; Merrifield resin; cleavage; protecting groups

Introduction

Since 1992, when Wang described the use of the kitchen microwave oven in SPPS [1], a number of reports have been published that show advantages of microwave irradiation in organic chemistry, including peptide synthesis (loading of the resin [2–6], coupling of amino acids [7,8] or in cleavage of peptides from solid support [9]).

The application of microwaves in cleavage of peptides from solid support attracts a lot of attention; however, the number of available reports is limited, and in most cases the product is removed from the resin in a classical way [8,10–14]. A typical procedure was recently described by Harris *et al*. [15], who synthesized peptides on Wang and Rink amide polystyrene (PS) resin and released the products from the resin by treatment with TFA/TIS/H_2O/EDT (94 : 1 : 2.5 : 2.5, v/v/v/v) either at room temperature (rt) for 2–3 h or under microwave irradiation (18 min, 10 W, maximum temperature 35 °C). Microwave irradiation was also applied to the cleavage of highly lipidated peptide from 2-chlorotrityl support with TFA/H_2O (1 : 1, v/v; 150 W, 20 min at 80 °C) [16]. It was found that the cleavage of the peptide from the resin proceeds well using low-power microwave irradiation. Brandt *et al*. [17] compared three different resins: PEGA (poly(ethylene glycol)polyacrylamide), PS, TG (tentagel) and three handles: rink amide and two kinds of BAL (backbone amide linker) in the microwave-assisted synthesis of the phosphorylated 15-mer peptide. To release the product from the resin, a small sample of resin was treated with TFA/H_2O (19 : 1) or with TFA/TFMSA (9 : 1) for 2 h at rt, and also under microwave irradiation for 5 min at 60–80 °C. It was demonstrated that all resins performed well when heated with strong acids and only the PEGA resin started to degrade at 60 °C in TFA/TFMSA.

Despite the advantages of Fmoc peptide synthesis strategy on Wang resin, the Merrifield resin and Boc/Bzl method is still in use, especially in the synthesis of peptide conjugates when nonstandard reaction conditions are often required [18–22]. The standard acidolysis of the Merrifield linker requires strong acids such as liquid HF, TFMSA or HBr/TFA [23]. As these acids have to be removed before the analysis of the product, the procedure is inconvenient for reaction monitoring or pilot syntheses on a small scale (10–50 mg of the resin). The TFA : DCM (1 : 1) mixture was used by Stadler and Kappe [24] as the cleavage reagent in microwave irradiation (500 W, 30 min) to remove a number of carboxylic acids from the Merrifield resin. No degradation of the polymer support was detected, but the high reaction temperature (120 °C) seems to be not compatible with SPPS because of the possibility of peptide degradation [25].

In our research on peptide–heterocycle conjugates [26,27] we used microwave-assisted cleavage from Wang resin to monitor the heterocycle formation. The product cleavage was complete after five irradiation steps (1 min) in TFA/H_2O (95 : 5) mixture, which was confirmed by incubation of the same resin sample in TFA for further 2 h and HPLC analysis of the evaporated filtrate. Owing to high absorbance of the quinoxaline–peptide conjugate, the HPLC-UV is a very sensitive method of product detection (A. Kluczyk, unpublished results). We have found that, after the microwave-assisted cleavage, there is no product left on the resin; therefore, the release of product from Wang resin is complete in 5 min at 20 W irradiation energy, which is in agreement with literature data [15].

However, there are classes of peptide conjugates, which require a more stable linker to the solid support for a successful synthesis.

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In our research on solid-phase imidazole-containing peptides, we used the Merrifield resin, and, to monitor the reaction progress, we attempted to use the microwave-assisted TFA cleavage from this resin.

We investigated the efficiency of the method using model peptides with various *C*-terminal amino acids to determine the influence of the steric hindrance on product release from Merrifield resin (*R*):

Boc-Gly-Phe-Ala-Gly-*R*	[peptide 1]
Boc-Gly-Phe-Ala-Ile-*R*	[peptide 2]
Boc-Gly-Ile-Ala-Phe-*R*	[peptide 3]
Boc-Gly-Phe-Ala-Val-*R*	[peptide 4]

All the peptides contain the Phe residue to facilitate the monitoring of the cleavage progress by HPLC with UV detection. The sequences were also designed to avoid the possible incomplete side-chain protection removal interference in product analysis.

To determine the effect of microwave-assisted TFA cleavage on protecting groups, we selected four peptidyl resins used previously for the synthesis of IL-1 inhibitors (peptides 5 and 8) [28], TGFβ fragments (peptide 6) [29] and tuftsin analogs (peptide 7) [30]:

Boc-Pro-Gly-Gly-Gly-Val-Thr(Bzl)-Lys(Z)-Phe-Tyr(Bzl)-Phe-*R*	[peptide 5]
Boc-Arg(NO_2)-Thr(Bzl)-Pro-Lys(Z)-Val-*R*	[peptide 6]
Ac-Lys(Z)-Arg(NO_2)-Pro-*R*	[peptide 7]
Boc-Val-Thr(Bzl)-Lys(Z)-Phe-Tyr(Bzl)-Phe-Ile-Thr(Bzl)-Gly-Ser(Bzl)-Glu(OBzl)-*R*	[peptide 8]

In this work, we present the study on the microwave-assisted method of peptide cleavage from Merrifield resin using TFA, which could be used not only for the final release of the product but also in a fast off-resin method of reaction monitoring compatible with ESI-MS and HPLC.

Materials and Methods

Reagents

All solvents and reagents were used as supplied, except for TFMSA, which was distilled at atmospheric pressure prior to use. Boc-amino acid derivatives and Boc-amino acid–loaded resins were synthesized in-house. The Merrifield support (2% cross-linked chloromethylated copolymer of styrene – divinyl benzene) was purchased from Reanal, Hungary and loaded using the cesium carbonate procedure [31]; the loading was calculated according to the mass difference and was in the range of 0.6–0.7 mmol g^{-1}. Boc-Ile attached to Merrifield resin (loading 0.52 mmol g^{-1}), used for synthesis of peptide 2a, was purchased from Amino Tech. *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU) and TFA were purchased from Iris Biotech.

All the microwave-assisted reactions were carried out in a monomode microwave Discover CEM apparatus, equipped with IR temperature sensor and gas cooling system. The reactor was used in Power-Time mode (a set power value was applied for a specific time, with the temperature safety limit of 50°).

Peptide Synthesis

Synthesis of peptides 1–4

SPPS was performed manually in polypropylene syringe reactors (Intavis AG) equipped with polyethylene filters, according to standard Boc/Bzl procedure. As the preliminary results indicated a very low MW-assisted cleavage from peptidyl resin 2, we repeated this synthesis using commercially available Boc-Ile-Merrifield resin (peptide 2a).

Synthesis of peptides 5–8

The syntheses were published elsewhere [28–30].

Cleavage from Merrifield resin

Standard procedure (room temperature)

A sample of resin (50 mg) was placed in polypropylene syringe reactor and was treated with TFA/TFMSA/cresol (25 : 3 : 2, 1 ml) for 2.5 h at rt. The resin was removed by filtration and rinsed with TFA. The combined filtrates were added to cold diethyl ether and the precipitated peptide was collected by centrifugation.

Microwave-assisted procedure

A sample of resin (50 mg) in a 2-ml polypropylene syringe reactor was treated with TFA/TIS/H_2O (95 : 2.5 : 2.5, 1 ml) for 15 min at rt. Then the reactor was transferred into microwave synthesizer cavity and subjected to microwave irradiation with gas cooling (pressure of 20 psi was maintained during irradiation) for a total time of 15 min (15 × 1 min) at 30 W with magnetic stirring, and a temperature limit of 50 °C. Between irradiations, the sample was cooled in an ice-water bath for 2 min. Finally, the solution was removed and the resin was washed with TFA. The combined filtrates were evaporated in a stream of nitrogen. In all the experiments, the 2-ml syringe reactors with perforated Parafilm® caps were used.

For UV-HPLC experiments, the total reaction time was prolonged to 30 min, with the cleavage mixture replaced every five steps (5 irradiations by 1 min).

When using different types of reaction vessels (standard CEM glass microwave vials, 5-ml syringe reactors, etc.), it might be necessary to modify the reaction conditions to keep the reaction temperature below 50 °C (shorter reaction time, more efficient cooling, etc.).

Standard procedure (elevated temperature, 45 °C)

A sample of resin (50 mg) was treated with TFA/TIS/H_2O (95 : 2.5 : 2.5, 1 ml) for 15 min at rt, then for 30 min at 45 °C in a water bath with occasional shaking. The solution was separated and the resin was washed with TFA. The combined filtrates were evaporated in a stream of nitrogen.

After peptide cleavage, the deprotected resins were immediately prepared for further analysis by consecutive washing with DCM, TEA/DCM (1 : 9), DCM, DCM/MeOH (1 : 1) and MeOH and drying *in vacuo*.

Peptide Analysis

HPLC

Prior to HPLC analysis, all peptides were dissolved in MeOH : water (1 : 1) solution. The analysis was performed using a Thermo Separation HPLC system with UV detection (210 nm) on a Vydac Protein RP C18 column (250 × 4.6 mm, 5 µm), with gradient elution of 0–80% B in A (A = 0.1% TFA in water; B = 0.1% TFA in acetonitrile/H_2O, 4 : 1) over 45 min (flow rate 1 ml min^{-1}, rt).

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Elemental analysis

The carbon, nitrogen and hydrogen content of the deprotected resins was established using VarioEL III CHNS analyzer (Elementar Analysensysteme, Germany) in the Laboratory of Elemental Analysis and Environmental Studies at the Faculty of Chemistry, University of Wroclaw.

Infrared spectroscopy

The samples of deprotected resins were ground and mixed with KBr. The IR spectra for KBr tablets were recorded on an FTIR IFS 66/ S spectrometer (Bruker, Germany) in Laboratory of Infrared Spectroscopy at the Faculty of Chemistry, University of Wroclaw.

Mass spectrometry

Mass spectra were recorded on a Bruker micrOTOF-Q mass spectrometer and apex ultra 7T FT-ICR mass spectrometer with electrospray ionization in the Laboratory of Mass Spectrometry at the Faculty of Chemistry, University of Wroclaw. The samples were dissolved in a 50 : 50 acetonitrile–water mixture containing 0.1% HCOOH.

The LC–MS analysis was performed using Agilent 1200 HPLC system coupled to micrOTOF-Q mass spectrometer. For separation, a RP C18 Zorbax (50 × 2.1 mm, 3.5 μm) column was used, with gradient elution of 0–100% B in A (A = 0.1% HCOOH in water; B = 0.1% HCOOH in acetonitrile) over 30 min (flow rate 0.1 ml min^{-1}, room temperature).

Results and Discussion

We investigated the efficiency of the product release from the Merrifield resin by analyzing the changes in resin weight and elemental composition, as well as the changes in IR spectra. To concentrate on the peptide removal from Merrifield support using the microwave-assisted TFA procedure, we used model peptides 1–4, which did not require protection of side chains. The resins deprotected in TFA with microwave irradiation were compared with other portions of the same batch, subjected to standard cleavage (TFA/TFMSA/cresol). Both samples were neutralized, washed and dried to reduce the interference from acids used in deprotection.

The model tetrapeptides were synthesized on Merrifield resin using a standard Boc procedure. The resins loaded with longer fully protected peptides were selected from the research group repository [28–30].

Optimization of the Protocols of MW-assisted Cleavage

In the earlier experiments, we investigated the influence of microwave power on cleavage efficiency. Samples of peptide 3 were removed from the Merrifield support during 5-min microwave irradiations of 20, 30, 35 and 40 W. The HPLC analysis of cleaved peptide indicated that the increase in MW power by over 30 W does not result in higher removal yield, but causes overheating of the mixture. Therefore, we used the 30-W microwave power setting to release peptides from the resin in further experiments.

We also optimized the time of MW irradiation. A sample of peptidyl resin 3 was suspended in TFA/H_2O and irradiated with 30 W MW for 5 min. Then the cleavage solution was filtered,

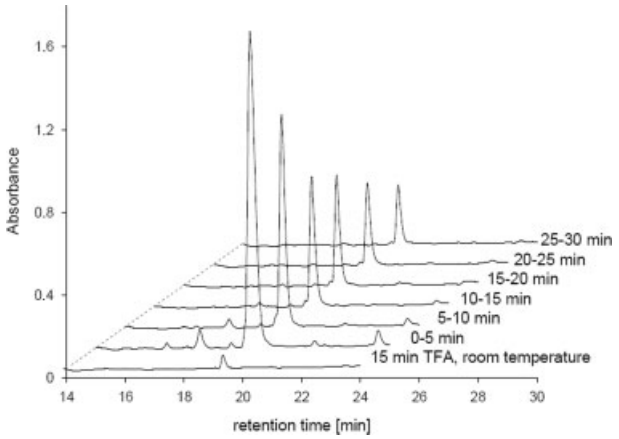

Figure 1. The HPLC chromatograms obtained after cleavage of peptide 3 (main peak) from Merrifield resin using presoaking in TFA and consecutive 5-min MW 30-W irradiations.

the resin was rinsed with TFA/H_2O and again irradiated using a fresh portion of TFA/H_2O. The procedure was repeated six times. The respective filtrates (from irradiation stage and washing step) were evaporated and subjected to HPLC analysis. As shown in Figure 1, the intensity of peptide signal decreased in samples from consecutive experiments, but the product signal did not disappear even after 30 min of irradiation. The same tendency was observed for other model peptides.

The initial experiments showed the increase in peptide content during the second irradiation, which indicated that a time period is required for the proper swelling of the resin (a dry sample was used in these experiments). To allow proper penetration of the resin by TFA and to improve the yield in the next experiments, we incubated the resin prior to the first irradiation step in the cleavage mixture for 15 min at rt. As indicated by the '15-min TFA' chromatogram in Figure 1, during this time the product is practically not released from the support. The soaking period also allows for a safe release of gaseous products of TFA-induced Boc group decomposition.

The temperature of the reaction has to be controlled, both by reactor IR temperature probe and the external metering, because TFA and its solutions strongly absorb the microwave energy. In a separate experiment, after each irradiation step, a protected digital temperature probe was inserted into the reaction mixture. The conditions (microwave power, time) were adjusted to keep the reaction temperature below 50 °C. Before the next irradiation step, the syringe reactor was placed into ice-water bath for 2 min to readjust the initial reaction conditions. A similar approach (intermediate cooling step) was used previously by Bacsa *et al*. [10] for synthesis of model peptides.

MW-assisted Cleavage *Versus* Standard TFMSA Reaction

To compare the yields of cleavage of the peptides from Merrifield support with and without MW radiation, we evaluated the amount of released peptides using the previously established UV-HPLC procedure, and the level of resin deprotection, using elemental analysis, weight comparison and IR spectroscopy. We selected peptides of different lengths (from tripeptide to undecapeptide) and sequences to investigate the influence of the *C*-terminal residue on cleavage efficiency (peptides 5–8).

The standard reaction used the TFA/TFMSA/cresol mixture, with a reaction time of 2.5 h. The product was precipitated in cold diethyl

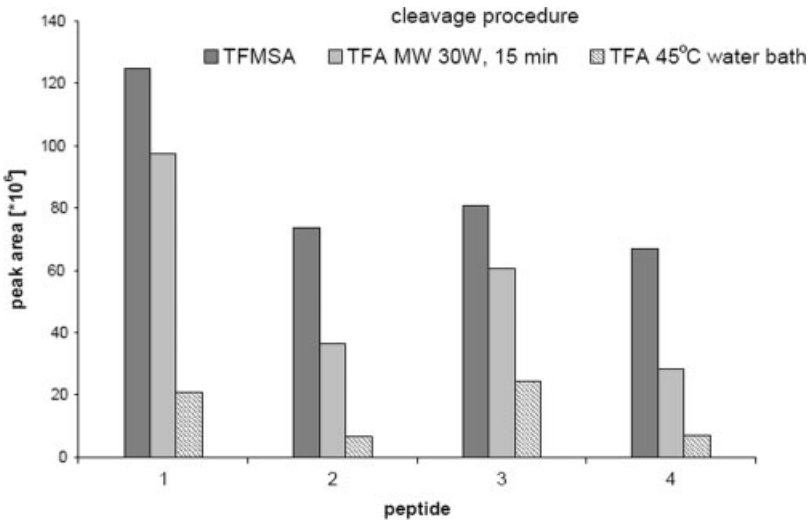

Figure 2. The comparison of HPLC peak areas of peptides 1–4 cleaved from the Merrifield resin using standard TFMSA procedure, TFA with microwave irradiation (30 W, 15 × 1 min) and TFA at elevated temperature 45 °C (water bath). Fifty-milligram resin samples from the same synthetic batch were used in each case.

ether and separated by centrifugation. In the microwave-assisted procedure, the resins were treated with TFA/TIS/H_2O solution for 15 min at rt and then for 15 min (15 × 1 min, with cooling between irradiations) at 30 W. Afterward, the cleavage mixture was evaporated. All the peptides released from Merrifield resin were analyzed by HPLC with UV detection and mass spectrometry (MS).

In case of peptides 5–8, we observed that not all protecting groups were removed at the same time and to the same extent, which resulted in several signals in HPLC chromatograms and was confirmed by MS analysis. Model peptides 1–4 were designed specially for UV-based analytical procedure; hence, a direct comparison of chromatographic peak intensity was possible.

As shown in Figure 2, the TFMSA treatment releases more product from the Merrifield resin than in the MW-assisted TFA reaction; however, in the case of peptide 1, with *C*-terminal Gly residue, the difference is minimal. The branched *C*-terminal residues (Ile in case of peptide 2 and Val in peptide 4) seem to be more resistant to microwave-assisted TFA cleavage.

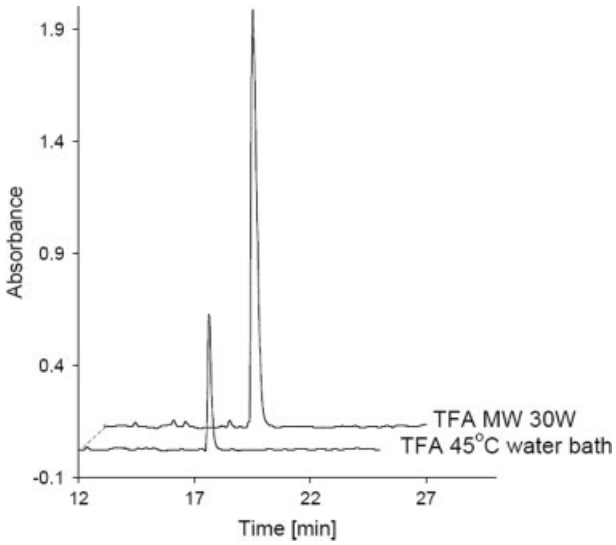

Figure 3. The HPLC profiles of peptide 4 removed from the Merrifield resin using TFA in water bath at elevated temperature and in MW reactor.

Comparison of the TFA Cleavage at 45 °C and the MW Procedure

To investigate the influence of thermal effects on TFA cleavage of the product from Merrifield resin, the reactions were run without MW irradiation. All samples were treated with the cleavage mixture at rt for 15 min to achieve proper swelling. The peptide resins were subjected to the reaction conditions (TFA/H_2O/TIS mixture) for 30 min in thermostated water bath at 45 °C, whereas another set of resin samples underwent the microwave-assisted procedure (30 W 15 × 1 min, cooling between irradiations). For the thermal reaction, the 30-min period was selected to compensate for the prolonged exposition of the microwave sample to TFA during the cooling steps. In the microwave-assisted procedure, the temperature did not exceed 50 °C (measured after irradiation).

Figure 3 shows the HPLC profiles of the crude peptide 4 (H-Gly-Phe-Ala-Val-OH) obtained from the resins incubated with TFA in a water bath and with MW irradiation. It is evident that the yield of product is much higher for the MW-assisted reaction than in the case of conventional heating (water bath). We compared the results obtained for four model tetrapeptides with different sequences, and in each case the microwave-assisted cleavage resulted in more product release (Figure 2). The observed differences in reaction yield under microwave irradiation as compared to conventional heating (water bath) are in agreement with several reports on possible nonthermal effects in microwave-assisted reactions [24,32–35].

Elemental Composition and Weight Analysis of the Resin

Taking into account the partial deprotection of side-chain groups (peptides 5–8) as well as the solubility of hydrophilic model peptides in diethyl ether, we investigated the resin left after the cleavage to determine the completeness of peptide removal.

The elemental composition of all resin samples was analyzed and the difference in nitrogen content was determined, as in the peptidyl Merrifield resin the synthesized peptide is the only

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Table 1. Nitrogen loss and weight difference in peptidyl resins deprotected using standard TFMSA and microwave-assisted (MW) TFA (30 W, 15 × 1 min) procedures

C-terminal residue	Peptide	Nitrogen loss (%)		Mass difference between peptidyl resin before and after cleavage (mg)		
		TFMSA	MW	TFMSA	MW	Theory[a]
Gly	1	93.3	65.5	12.8	15.9	10.3
Ile	2	87.7	10.9	18.6	12.7	12.8
	2a	94.3	8.8	19.4	13.5	10.1
Phe	3	93.9	43.7	18.3	15.4	14.1
	5	71.4	24.2	14.2	18.8	26.1
Val	4	100.0	12.8	18.4	13.5	12.5
	6	81.3	26.0	17.8	14.4	18.7
Pro	7	18.4	19.3	5.9	11.5	14.8
Glu	8	35.0	21.1	21.1	26.3	25.4

Fifty-milligram resin samples from the same synthetic batch were used in each case.
[a] Calculated according to the loading of the resin and peptide sequence.

component containing nitrogen. The weight difference between the loaded and deprotected resins was measured (50 mg samples were used). The theoretical weight loss was calculated according to the resin loading and the peptide sequence (Table 1).

The composition analysis shows that the MW-assisted TFA reaction leaves more peptide on the resin than the TFMSA procedure; however, the difference depends strongly on the *C*-terminal residue, for example, peptides with *C*-terminal Val and Ile residues are more resistant to MW-assisted cleavage, whereas peptide 7 (Ac-Lys-Arg-Pro-OH) in general shows poor cleavage efficiency. The results obtained for peptide 2, significantly lower than for other peptides, prompted us to repeat the synthesis using a commercial Boc-Ile-Merrifield resin (loading 0.52 mmol g^{-1}, peptide 2a). As shown in Table 1, the results obtained for these independently synthesized peptides are practically identical, confirming that the *C*-terminal Ile residue is responsible for the poor cleavage efficiency in the MW-assisted procedure.

The weight difference between the peptidyl resin and the resin after the cleavage is less pronounced in the analyzed samples. For the pairs of samples that contain *C*-terminal Ile and Val, the weight loss is significantly higher after TFMSA treatment, whereas in other cases the MW-assisted procedure is more efficient. The theoretical loss values were calculated according to the loading of the resins, whereas the samples after cleavage taken from the same synthetic batch and neutralized and dried by the same procedure after the cleavage show the real difference between the methods. Actually, in the case of longer peptides, both the weight difference and the elemental analysis results may be affected by the fact that the protective groups could be removed from the peptide when it is still attached to the resin. This may be responsible for the results for peptides 5 and 8, the longest in the series, where the nitrogen loss is relatively small, but the weight difference does not differ much from the other samples.

The results show that the efficiency of the cleavage reaction depends on the *C*-terminal amino acid and the length of peptide. The MW-assisted reaction is affected by the amino acid residues with sterical hindrance. It is visible that the peptide is removed from the resin by TFA with microwave assistance, and the prolongation of the reaction time should increase the yield to the level of TFMSA cleavage.

Infrared Analysis of the Resin

All the resins, after removing peptides with TFMSA method or MW irradiation, were analyzed using IR spectroscopy. The spectra of respective peptidyl resins were used as references. The IR spectroscopy was frequently applied to the monitoring of solid-phase reactions [3,36–38].

The bands, both at 1450 and 1943 cm^{-1}, were attributed to aromatic bonds vibrations of Merrifield resin [36] and could be treated as a baseline. The band at 1680 cm^{-1} corresponds to absorption of amide carbonyl group and another characteristic band at 1740 cm^{-1} is attributed to the ester carbonyl group vibrations. The selection was confirmed after comparing the IR spectra of commercially available free Merrifield resin and the resin loaded with Boc-Ile. Figure 4 shows that the carbonyl bands have the largest intensity for peptide resin and practically disappear in samples after cleavage both with the classical method and MW irradiation. These bands are more intensive for resin after microwave-assisted cleavage than after classical method, which suggests that MW-assisted release of the peptides from Merrifield resin is less efficient; however, both methods remove a significant

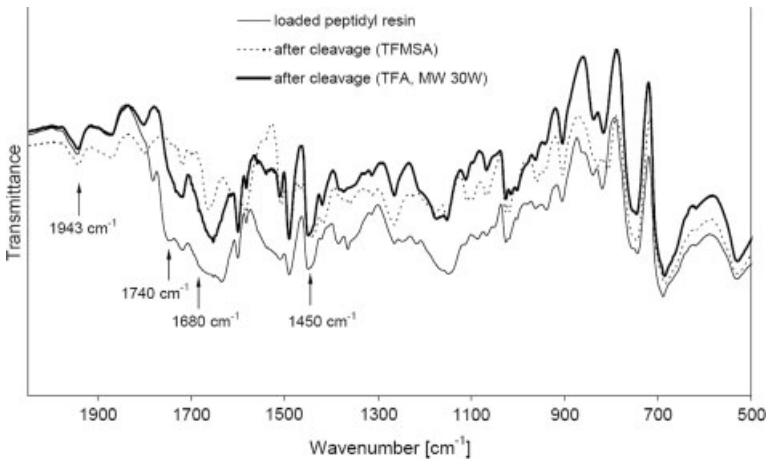

Figure 4. The comparison of IR spectra: resin loaded with the peptide 1 (thin line), the resin after TFMSA deprotection (dotted line) and TFA–MW deprotection (thick line).

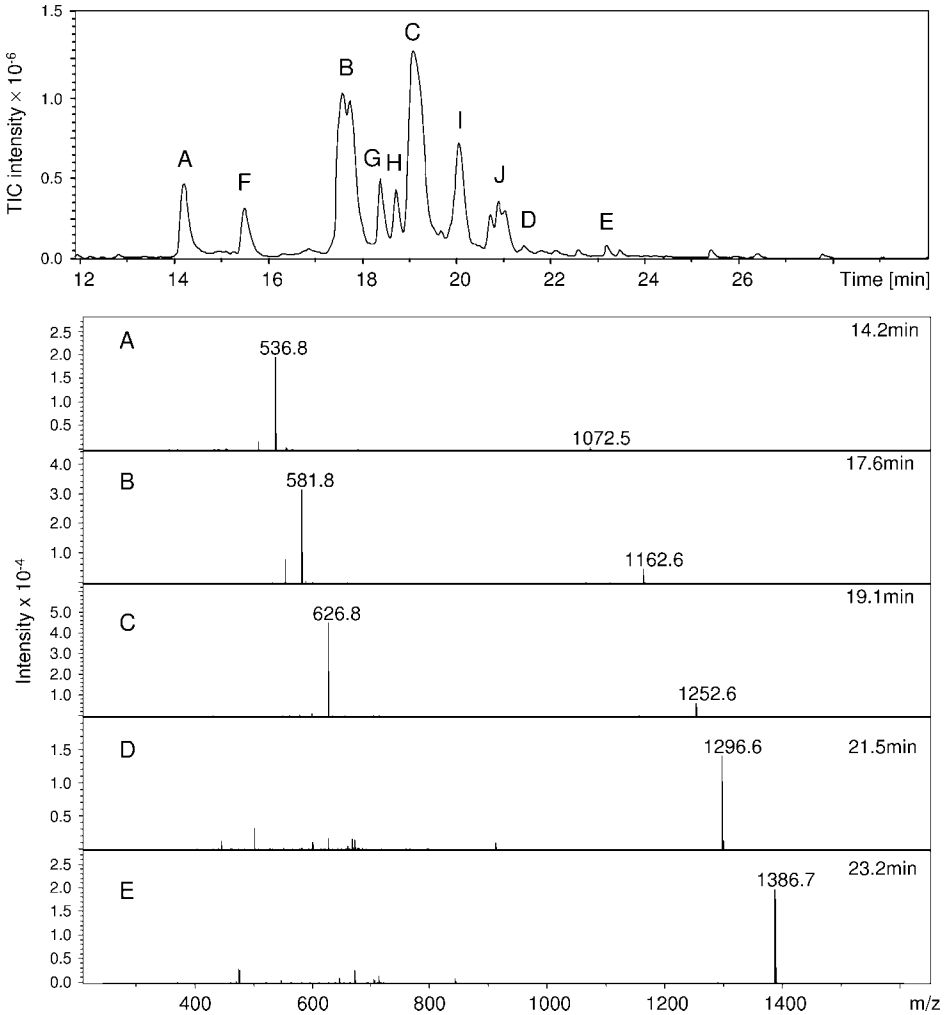

Figure 5. Top panel: the LC–MS chromatogram of crude peptide 5 obtained after 15-min MW-assisted TFA cleavage from Merrifield resin (total ion current, positive ion mode). Chromatographic peaks A–E correspond to forms of peptide 5 with various protection level, peaks F–J represent methylated forms of compounds A–E. Panels A–E: mass spectra of peaks of deprotected (A) and partially protected (B–E) forms of peptide 5.

amount of the product. A similar conclusion was obtained after the analysis of IR spectra of other resin pairs, with samples 5, 7 and 8 showing the smallest changes, which is in agreement with the results of weight analysis.

Although MW irradiation is not superior to the TFMSA cleavage in terms of yield, the advantage of using microwave-assisted TFA protocol for Merrifield resin lies in the possibility of working with a minute amount of the resin (few milligrams), removing the solvent by evaporation and analyzing the product, which allows for monitoring of reaction progress. The standard precipitation of product after TFMSA cleavage in diethyl ether is not suitable for very small samples and hydrophobic products, frequently soluble in ether. The results show that the 5-min irradiation at 30 W removes most of the product, and the sample could be used after dilution for direct electrospray mass spectrometry analysis. Such a procedure could accompany the classical Kaiser test or even replace it if reactions do not involve the amino group. A similar effect could be obtained through the HF procedure, which, however, requires complicated equipment and special handling due to safety reasons.

The increase in the amount of peptidyl resin is possible, but requires a very strict temperature control with shorter irradiation steps to avoid overheating of the reaction mixture. The procedure involving the transfer of the sample from microwave reactor to the cooling bath is tedious, and the commercially available microwave synthesizers with internal thermostating (like the CoolMate system introduced recently by CEM) might be of use in this application.

MW-assisted Removal of the Protecting Groups

We also investigated the release of the amino acid protecting groups used in Boc/Bzl peptide synthesis strategy during MW-assisted cleavage of the peptide from Merrifield resin. The reactions were performed using small samples of peptidyl resin 5 (Boc-Pro-Gly-Gly-Gly-Val-Thr(Bzl)-Lys(Z)-Phe-Tyr(Bzl)-Phe-R), which was selected because of the presence of Z (benzyloxycarbonyl) and benzyl ether (both aromatic and aliphatic type) protecting groups. The Boc (*t*-butoxycarbonyl) protecting group is removed by TFA during the 15-min preincubation period [39]. The analysis of the HPLC profiles of the products after TFMSA and MW-assisted cleavage indicated that after the MW procedure the peaks observed in the chromatogram appear at much longer retention times than those from the TFMSA cleavage sample, suggesting a higher hydrophobicity caused by the not-complete deprotection.

The LC–MS analysis performed after 15 min of irradiation revealed a small amount of the expected product (peak A in

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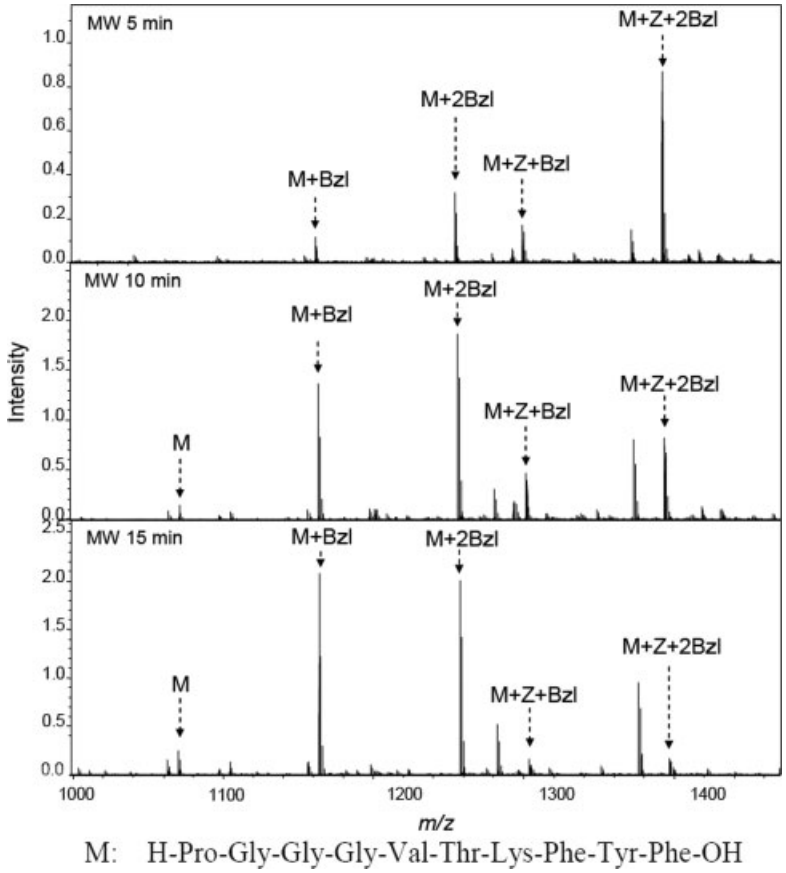

Figure 6. The mass spectra of peptide 5 after MW-assisted TFA cleavage from Merrifield resin after 5, 10 and 15 min of irradiation (negative ion mode).

Table 2. Calculated monoisotopic masses and *m/z* values for the possible partially protected forms of peptide 5

Signal	M (g mol^{-1})	*m/z*	Protecting groups removed	Protecting groups left
A	1071.5	358.2 $[M+3H]^{3+}$ 536.8 $[M+2H]^{2+}$ 1072.5 $[M+H]^{+}$	Z and 2Bzl	–
B	1161.6	581.8 $[M+2H]^{2+}$ 1162.6 $[M+H]^{+}$	Z and Bzl	Bzl
C	1251.7	418.3 $[M+3H]^{3+}$ 626.8 $[M+2H]^{2+}$ 1252.7 $[M+H]^{+}$	Z	2Bzl
D	1295.7	1296.7 $[M+H]^{+}$	Bzl	Z and Bzl
E	1385.7	1386.7 $[M+H]^{+}$	–	Z and 2Bzl

The letters A–E indicate identified signals in LC–MS chromatogram (Figure 5).

Figure 5) and most of the possible forms containing combinations of protecting groups (Table 2 and Figure 5). The partially separated peak B represents two isomeric peptides with a single benzyl protection left (on Thr or Tyr residues). The most abundant peak C corresponded to the peptide containing two benzyl groups, whereas the peptide with one benzyl group removed and the fully protected form were found in peaks D and E, respectively.

In the mass spectrum extracted from peak I signals characteristic for peptide containing two benzyl groups could be observed; however, additional signals corresponding to the methyl ester of the peptide were also present. The *m/z* value of the fully deprotected peptide with an additional methyl group was also evident in peak F (*m/z* 543.8). Peaks G and H represent the two possible combinations of benzyl ether and methyl ester. The methylated products are probably artifacts, formed when the sample was dissolved in methanol before LC–MS analysis.

To confirm the order of removal of the protecting groups, we investigated the samples collected in separate experiments after 5, 10 and 15 min of the MW irradiation, and analyzed them in negative ion mode to avoid the differences in ionization efficiency of Nα and Nε amino groups, as well as to simplify the spectrum due to the lack of multiple charge ions (Figure 6).

We found that during MW-assisted TFA cleavage of the peptide from the Merrifield support the removal of the protecting groups is incomplete. After 5 min of 30-W microwave irradiation, the dominant signal in MS spectrum represents the peptide with all side-chain protecting groups (Figure 6). During the next stage of the removal procedure, the blocking groups are gradually released from the peptide, with the Z group being the most labile group, which could be concluded from the relative intensity of the signals corresponding to peptides without the Z or one Bzl group. The fully deprotected peptide appeared in the sample after 10 min of MW irradiation, but its abundance was low and did not increase much in the sample irradiated for a further 5 min (Figure 6).

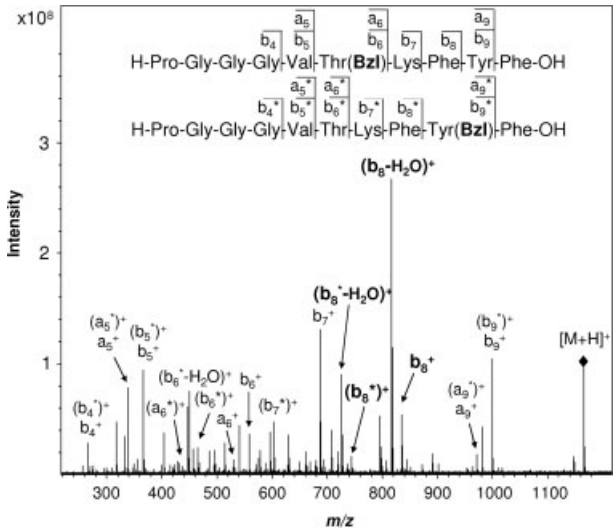

Figure 7. The MS/MS spectrum of the ion $m/z = 1162.6$, corresponding to peptide 5 with one protective benzyl group preserved (FT-ICR, collision energy of 30 eV). The asterisks indicate the daughter ion series produced when the benzyl group is located on Tyr residue.

The intensity of the signal of peptide with only one Bzl group left increases significantly with the irradiation time. There are two possible locations of that benzyl group – in the side chain of threonine or tyrosine residues. It seems that the remaining group is quite stable, because of a gradual increase in M+Bzl signal, but there are practically no changes in abundance of the completely deprotected peptide (signal M) after prolonged MW irradiation. As phenolic ethers are more sensitive to acidic environment [39], we suppose that the Bzl group is removed from tyrosine.

In order to confirm this hypothesis, we investigated the collision-induced dissociation (CID) of the ion $m/z = 1162.6$ using a high-resolution FT-ICR mass spectrometer. To facilitate the fragment identification, the experiment was performed in positive ion mode. Figure 7 shows the fragmentation spectra obtained by CID-MS/MS of the singly charged precursor ion $m/z = 1162.6$, corresponding to peptide 5 with one remaining benzyl group. The relative abundance of b_8 and b_8-H_2O daughter ions, containing the Thr(Bzl) fragments, could be compared to that of respective ions formed from peptide with Tyr(Bzl) (marked with asterisks). The results indicate that the signal at $m/z = 1162.6$ comes from the mixture of two isomeric ions:

H-Pro-Gly-Gly-Gly-Val-Thr(Bzl)-Lys-Phe-Tyr-Phe-OH
H-Pro-Gly-Gly-Gly-Val-Thr-Lys-Phe-Tyr(Bzl)-Phe-OH

The population of peptides with benzyl group still present on the Thr residue is much bigger; however, the removal is not completely specific.

Peptide 8, which contains five benzyl protecting groups as well as the Z group, provided us with the opportunity to study the differences in lability of benzyl ester, and phenolic and aliphatic ethers. The analysis of mass spectra obtained after MW-assisted removal of peptide 8 from Merrifield resin shows that benzyl ester is the first one removed from the peptide; however, the Z group seems to be even more susceptible to TFA under reaction conditions. As expected, the nitro and acetyl groups in peptides 6 and 7 were not affected by the cleavage procedure. Once the product is removed from the resin, the TFA solution can be easily evaporated, and the product either used for characterization and further reactions, or, in the case of partially protected product mixture, subjected to hydrogenation, in order to complete the protecting group removal with no risk of product deterioration.

The partial removal of protecting groups may be inconvenient when the TFA cleavage is used to confirm the product formation, but in our opinion the advantages of short reaction time and the possibility of immediate analysis of the released product by ESI-MS, compensate for the effort spent in calculating the formulas of possible forms of product.

Conclusions

We have demonstrated that peptides could be successfully removed from Merrifield resin using TFA in a microwave-assisted procedure. Although the cleavage is not as efficient as the TFMSA method, most of the product is released from the resin during a 5-min reaction. The fact that the amount of peptide removed from the resin in MW-assisted reaction is higher than that in reaction run at 45 °C (conventional heating) suggests a possibility of nonthermal effects. The quick removal of the product from Merrifield support using TFA makes the procedure developed by us a method of choice in monitoring the reactions carried out on Merrifield resin due to the short reaction time and compatibility with HPLC and ESI-MS conditions.

References

1 Yu H-M, Chen S-T, Wang K-T. Enhanced coupling efficiency in solid-phase peptide synthesis by microwave irradiation. *J. Org. Chem.* 1992; **57**: 4781–4784.
2 Li X-M, Le G-W, Shi Y-H. Microwave-assisted solid-phase oligosaccharides synthesis reaction and scavenging activity of synthetic product to free radical. *Carbohyd. Polym.* 2006; **64**: 274–281.
3 Antonow D, Graebin CS, Eifler-Lima VL. An efficient monitoring technique for solid-phase reactions by KBr pellets/FT-IR using methyl p-aminobenzoate synthesis assisted by microwave radiation on Merrifield resin. *J. Braz. Chem. Soc.* 2004; **15**: 782–785.
4 Park M-S, Oh H-S, Cho H, Lee K-H. Microwave-assisted solid-phase synthesis of pseudopeptides containing reduced amide bond. *Tetrahedron Lett.* 2007; **48**: 1053–1057.
5 Joshi BP, Park J-w, Kim J-m, Lohani CR, Cho H, Lee K-H. Application of microwave method to the solid phase synthesis of pseudopeptides containing ester bond. *Tetrahedron Lett.* 2007; **49**: 98–101.
6 Sureshbabu V, Krishna KGC. Microwave irradiation accelerated rapid, efficient and high yield esterification of Boc-amino acid to Merrifield resin mediated by KF. *Ind. J. Chem.* 2007; **46B**: 1466–1469.
7 Erdélyi M. Solid-phase methods for the microwave-assisted synthesis of heterocycles. *Top. Heterocycl. Chem.* 2006; **1**: 79–128.
8 Matsushita T, Hinou H, Fumoto M, Kurogochi M, Fujitani N, Shimizu H, Nishimura S-I. Construction of highly glycosylated mucin-type glycopeptides based on microwave-assisted solid-phase syntheses and enzymatic modifications. *J. Org. Chem.* 2006; **71**: 3051–3063.
9 Kappe CO. High-speed combinatorial synthesis utilizing microwave irradiation. *Curr. Opin. Chem. Biol.* 2002; **6**: 314–320.
10 Bacsa B, Desai B, Dibo G, Kappe CO. Rapid solid-phase peptide synthesis using thermal and controlled microwave irradiation. *J. Pept. Sci.* 2006; **12**: 633–638.
11 Kappe CO. Microwave dielectric heating in synthetic organic chemistry. *Chem. Soc. Rev.* 2008; **37**: 1127–1139.
12 Rizzolo F, Sabatino G, Chelli M, Rovero P, Papini AM. A convenient microwave-enhanced solid-phase synthesis of difficult peptide sequences: case study of gramicidin A and CSF114(Glc). *Int. J. Pept. Res. Ther.* 2007; **13**: 203–208.
13 Palasek SA, Cox ZJ, Collins JM. Limiting racemization and aspartimide formation in microwave-enhanced Fmoc solid phase peptide synthesis. *J. Pept. Sci.* 2007; **13**: 143–148.

14 Cemazar M, Craik DJ. Microwave-assisted Boc-solid phase peptide synthesis of cyclic cysteine-rich peptides. *J. Pept. Sci.* 2008; **14**: 683–689.
15 Harris PWR, Williams GM, Shepherd P, Brimble MA. The synthesis of phosphopeptides using microwave-assisted solid phase peptide synthesis. *Int. J. Pept. Res. Ther.* 2008; **14**: 387–392.
16 Cini E, Lampariello LR, Rodriquez M, Taddei M. Synthesis and application in SPPS of a stable amino acid isostere of palmitoyl cysteine. *Tetrahedron* 2009; **65**: 844–848.
17 Brandt M, Gammeltoft S, Jensen KJ. Microwave heating for solid-phase peptide synthesis: general evaluation and application to 15-mer phosphopeptides. *Int. J. Pept. Res. Ther.* 2006; **12**: 349–357.
18 Méret M, Bienz S. Efficient and flexible solid-phase synthesis of N-hydroxypolyamine derivatives. *Eur. J. Org. Chem.* 2008; 5518–5525.
19 Bisegger P, Manov N, Bienz S. Solid-phase synthesis of cyclic polyamines. *Tetrahedron* 2008; **64**: 7531–7536.
20 Gao D, Zhai H, Parvez M, Back TG. 1,3-Dipolar cycloadditions of acetylenic sulfones in solution and on solid supports. *J. Org. Chem.* 2008; **73**: 8057–8068.
21 Elliott EL, Ray CR, Kraft S, Atkins JR, Moore JS. Solid-phase synthesis of m-Phenylene ethynylene heterosequence oligomers. *J. Org. Chem.* 2006; **71**: 5282–5290.
22 Patteux C, Foucout L, Bohn P, Dupas G, Leprince J, Tonon M-C, Dehouck B, Marsais F, Papamicaela C, Levacher V. Solid phase synthesis of a redox delivery system with the aim of targeting peptides into the brain. *Org. Biomol. Chem.* 2006; **4**: 817–825.
23 Dorwald FZ. *Organic Synthesis on Solid Phase*. Wiley-VCH: Weinheim, 2002.
24 Stadler A, Kappe CO. High-speed couplings and cleavages in microwave-heated, solid-phase reactions at high temperatures. *Eur. J. Org. Chem.* 2001; 919–925.
25 Lee BS, Krishnanchettiar S, Lateef SS, Lateef NS, Gupta S. Characterization of oligosaccharide moieties of intact glycoproteins by microwave-assisted partial acid hydrolysis and mass spectrometry. *Rapid Commun. Mass Spectrom.* 2005; **19**: 2629–2635.
26 Staszewska A, Stefanowicz P, Szewczuk Z. Direct solid-phase synthesis of quinoxaline-containing peptides. *Tetrahedron Lett.* 2005; **46**: 5525–5528.
27 Koprowska-Ratajska M, Kluczyk A, Stefanowicz P, Bartosz-Bechowski H, Szewczuk Z. Solid phase synthesis of peptides containing novel amino acids, substituted 3-benzimidazolealanines. *Amino Acids* 2009; **36**: 309–315.
28 Kluczyk A, Siemion IZ, Wieczorek Z. The "two-headed" peptide inhibitors of interleukin-1 action. *Peptides* 2000; **21**: 1411–1420.
29 Wieczorek Z, Słoń J, Kluczyk A, Zbozień R, Stefanowicz P, Siemion IZ. The immunomodulatory diversity of the proteins of the transforming factor β (TGFβ) family. *Int. J. Pept. Prot. Res.* 1995; **46**: 113–118.
30 Słoń JJ, Kluczyk A, Stefanowicz P, Zimecki M, Wieczorek Z, Siemion IZ. Immunological and chemical studies in the tuftsin-kentsin group. *Polish J. Chem.* 1994; **68**: 1043–1046.
31 Gisin BF. The preparation of Merrifield-resins through total esterification with cesium salts. *Helv. Chim. Acta* 1973; **56**: 1476–1482.
32 Dressen MHCL, van de Kruijs BHP, Meuldijk J, Vekemans JAJM, Hulshof LA. Vanishing microwave effects: influence of heterogeneity. *Org. Process Res. Dev.* 2007; **11**: 865–869.
33 Perreux L, Loupy A. A tentative rationalization of microwave effects in organic synthesis according to the reaction medium, and mechanistic considerations. *Tetrahedron* 2001; **57**: 9199–9223.
34 Herrero MA, Kremsner JM, Kappe CO. Nonthermal microwave effects revisited: on the importance of internal temperature monitoring and agitation in microwave chemistry. *J. Org. Chem.* 2008; **73**: 36–47.
35 Stuerga D, Gaillard P. Microwave heating as a new way to induce localized enhancements of reaction rate. Non-Isothermal and heterogeneous kinetics. *Tetrahedron* 1996; **52**: 5505–5510.
36 Yan B, Kumaravel G, Anjaria H, Wu A, Petter RC, Charles F, Jewell J, Wareing JR. Infrared spectrum of a single resin bead for real-time monitoring of solid-phase reactions. *J. Org. Chem.* 1995; **60**: 5736–5738.
37 Yan B, Kumaravel G. Probing solid-phase reactions by monitoring the IR bands of compounds on a single "flattened" resin bead. *Tetrahedron* 1996; **52**: 843–848.
38 Mitchell AR, Kent SBH, Engelhard M, Merrifield RB. A new synthetic route to tert-butyloxycarbonylaminoacyl-4-(oxymethyl) phenylacetamidomethylresin, an improved support for solid-phase peptide synthesis. *J. Org. Chem.* 1978; **43**: 2845–2852.
39 Bodansky M, Bodansky A. *The Practice of Peptide Synthesis*. Springer-Verlag: Berlin, 1984.

