JOC Article

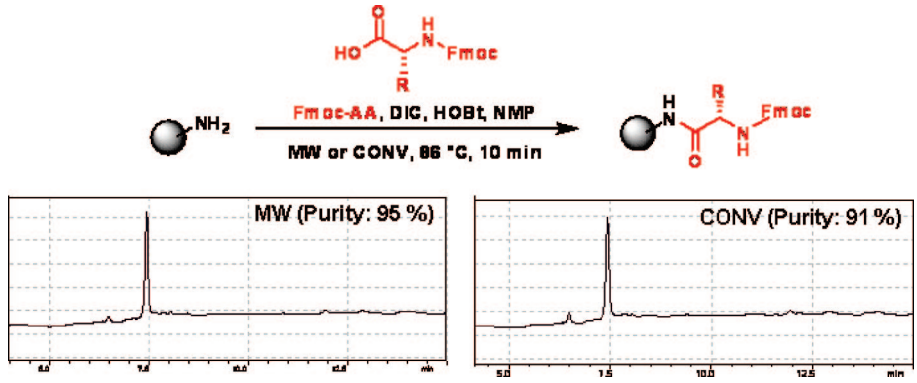
Solid-Phase Synthesis of Difficult Peptide Sequences at Elevated Temperatures: A Critical Comparison of Microwave and Conventional Heating Technologies

Bernadett Bacsa,[†] Kata Horváti,[‡] Szilvia Bõsze,[‡] Fritz Andreae,[§] and C. Oliver Kappe*,[†]

Christian Doppler Laboratory for Microwave Chemistry (CDLMC) and Institute of Chemistry, Karl-Franzens-University Graz, Heinrichstrasse 28, A-8010 Graz, Austria, Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös Loránd University, 1117 Budapest, Hungary, and piCHEM Forschungs und EntwicklungsgmbH, Kahngasse 20, A-8045 Graz, Austria

oliver.kappe@uni-graz.at

Received June 25, 2008



The Fmoc/*t*-Bu solid-phase synthesis of three difficult peptide sequences (a 9-mer, 15-mer, and 24-mer) was performed using *N*,*N*′-diisopropylcarbodiimide/1-hydroxybenzotriazole as coupling reagent on polystyrene, Tentagel, and ChemMatrix resins. In order to obtain an insight into the specific role of the elevated temperature and/or the electromagnetic field for peptide syntheses carried out using microwave irradiation, peptide couplings and Fmoc-deprotection steps were studied under microwave and conventionally heated conditions at the same temperature. While room temperature couplings/deprotections generally produced the difficult peptides in rather poor quality, excellent peptide purities were obtained using microwave heating at a temperature of 86 °C for both the coupling and deprotection steps in only 10 and 2.5 min reaction time, respectively. While for most amino acids no significant racemization was observed, the high coupling temperatures led to considerable levels of racemization for the sensitive amino acids His and Cys. It was demonstrated for all three peptide sequences that when performing the coupling/deprotection steps at the same reaction temperature using conventional heating, nearly identical results in terms of both peptide purity and racemization levels were obtained. It therefore appears that the main effect of microwave irradiation applied to solid-phase peptide synthesis is a purely thermal effect not related to the electromagnetic field.

Introduction

The advent of solid-phase peptide synthesis (SPPS) has led to dramatic developments in peptide chemistry and related fields. Since Merrifield's pioneering work on SPPS in the 1960s, peptide preparation on a small to medium scale has almost exclusively been performed on solid supports.[1,2] Along with changes in protection group strategies and the introduction of different types of solid supports, interest during the past decades has mainly focused on developing more effective coupling procedures/reagents in order to increase peptide yields and to

* To whom correspondence should be addressed. Phone: +43-316-380-5352. Fax: +43-316-380-9840.
[†] Karl-Franzens-University Graz.
[‡] Hungarian Academy of Sciences.
[§] piCHEM.

(1) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154.

10.1021/jo8013897 CCC: $40.75
Published on Web 08/27/2008

minimize undesired side reactions during both the coupling and deprotection steps.[3] The carefully optimized SPPS protocols available today can therefore reliably generate a very wide range of peptides which can be efficiently synthesized in a fully automated and routine fashion using commercially available peptide synthesizers.

A common phenomenon in SPPS, however, is the occurrence of so-called "difficult sequences" which are problematic—if not impossible—to synthesize using standard coupling and deprotection protocols.[4] The difficulties associated with those sequences are mainly related to intra- and/or intermolecular aggregation, secondary structure formation, and steric hindrance of protecting groups which can generate premature termination of the sequence. Problems occurring during the assembly of protected peptides on a solid support can occur as early as from the fifth residue coupled and are thought to be mainly the result of internal aggregation of peptide chains with the peptide−resin matrix. The driving forces for this intrachain and interchain association are most likely related to hydrogen bonding and hydrophobic forces. The tendency for aggregation/folding depends critically on the nature of the peptide chain with sequences containing a high proportion of Ala, Val, Ile, Asn, or Gln residues showing the highest propensity for aggregation effects.[4] Severe steric hindrance commonly results, leading to reduced reagent penetration and significantly reduced reaction rates in both coupling (acylation) and deprotection steps. As a consequence, the desired peptide products are often contaminated by a series of structurally and chemically very similar peptides such as incomplete, mismatch, or deletion sequences. The separation of these undesired byproducts from the target peptide can sometimes be very tedious and often impossible to achieve on a preparative scale.

Attempts to suppress or to reduce these phenomena during the SPPS of difficult sequences have until now involved mainly external factors like changes in the solvent composition,[5] the use of chaotropic salts,[6] the incorporation of solubilizing[7] or reversible amide protection groups,[8] and a variety of other methods.[9] Notably, while all steps in the SPPS cycle are traditionally carried out at room temperature, significant improvements for difficult peptide sequences were obtained in several cases by performing peptide coupling steps at elevated temperatures (30−80 °C).[10−12] In some instances, also the deprotection and washing steps have been successfully carried out using an elevated temperature regime.[10,12] A major concern in this context clearly is the possibility of racemization and the occurrence of other undesired side reactions such as, for example, aspartimide formation when applying higher than ambient reaction temperatures in the coupling and/or deprotection steps.[13]

During the past few years, the use of microwave irradiation to enhance solid-phase peptide synthesis has been growing at a rapid rate.[14] Impressive improvements both in terms of coupling/deprotection speed and in terms of product purity/yield using microwave-assisted SPPS have been reported by a number of research groups.[15−30] Most of the recent efforts have focused on applying microwave irradiation technology to difficult

(2) (a) Chan, W.; White, P. D. *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*; Oxford University Press: Oxford, U.K., 2000. (b) Kates S. A.; Albericio, F. *Solid-Phase Synthesis: A Practical Guide*; CRC Press: Basel, Switzerland, 2000. (c) Benoiton, N. L. *Chemistry of Peptide Synthesis*; CRC Press: Basel, Switzerland, 2006.

(3) (a) Albericio, F.; Bofill, J. M.; El-Faham, A.; Kates, S. A. *J. Org. Chem.* **1998**, *63*, 9678–9683. (b) Carpino, L.; El-Faham, A. *Tetrahedron* **1999**, *55*, 6813–6830. (c) Albericio, F. *Curr. Opin. Chem. Biol.* **2004**, *8*, 211–221. (d) Han, S. Y.; Kim, Y.-A. *Tetrahedron* **2004**, *60*, 2447–2467. (e) Kamiński, Z. J.; Kolesińska, B.; Sabatino, G.; Chelli, M.; Rovero, P.; Blaszczyk, M.; Glŏwka, M. L.; Papini, A. M. *J. Am. Chem. Soc.* **2005**, *127*, 16912–16920. (f) El-Faham, A.; Albericio, F. *J. Org. Chem.* **2008**, *73*, 2731–2737.

(4) (a) Hyde, C.; Owen, J. D.; Quibell, M.; Sheppard, R. C. *Int. J. Peptide Protein Res.* **1994**, *43*, 431–440. (b) Dettin, M.; Pegoraro, S.; Rovero, P.; Bicciato, S.; Bagno, A.; Di Bello, C. *J. Peptide Res.* **1997**, *49*, 103–111. (c) Larsen, B. D.; Holm, A. *J. Peptide Res.* **1998**, *52*, 470–476. (d) Henkel, B.; Bayer, E. *J. Peptide Sci.* **1998**, *4*, 461–470. (e) McNamara, J. F.; Lombardo, H.; Pillai, S. K.; Jensen, I.; Albericio, F.; Kates, S. A. *J. Peptide Sci.* **2000**, *6*, 512–518. (f) Carpino, L. A.; Krause, E.; Sferdean, C. D.; Schümann, M.; Fabian, H.; Bienert, M.; Beyermann, M. *Tetrahedron Lett.* **2004**, *45*, 7519–7523.

(5) (a) MutterM.; Pillai, V. N. R.; Anzinger, Bayer, E.; Toniolo, C. In *Proceedings of the 16th European Peptide Symposium*; Brunfeldt, K., Ed.; Scriptor: Copenhagen, Denmark, 1981; pp 660−665. (b) Hyde, C.; Johnson, T.; Sheppard, R. C. *J. Chem. Soc., Chem. Commun.* **1992**, *21*, 1573–1575.

(6) (a) Thaler, A.; Seebach, D.; Cardinaux, F. *Helv. Chim. Acta* **1991**, *74*, 628. (b) Stewart, J. M.; Klis, W. A. In *Innovation and Perspective in Solid Phase Synthesis: Peptides, Polypeptides and Oligonucleotides*; Epton, R., Ed.; SPCC: Birmingham, U.K., 1991; pp 1−9. (c) Hendrix, J. C.; Halverson, K. J.; Jarett, J. T.; Lansbury, P. T. *J. Org. Chem.* **1990**, *55*, 4517.

(7) (a) Mutter, M.; Oppliger, H.; Zier, A. *Macromol. Chem. Rapid Commun.* **1992**, *13*, 151–157.

(8) (a) Johnson, T.; Quibell, M.; Owen, D.; Sheppard, R. C. *J. Chem. Soc., Chem. Commun.* **1993**, *11*, 369–372. (b) Nicolas, E.; Pujades, M.; Bacardit, J.; Giralt, E.; Albericio, F. *Tetrahedron Lett.* **1997**, *38*, 2317–2320. (c) Zeng, W.; Regamey, P. O.; Rose, K.; Wang, Y.; Bayer, E. *J. Peptide Res.* **1997**, *49*, 273–279.

(9) Pseudoproline derivatives: Whör, T.; Wahl, F.; Nefzi, A.; Sato, T.; Mutter, M. *J. Am. Chem. Soc.* **1996**, *118*, 9218–9227. Sampson, W. R.; Patsiouras, H.; Ede, N. J. *J. Peptide Sci.* **1999**, *5*, 403–409. Cremer, G. A.; Tariq, H.; Delmas, A. F. *J. Peptide Sci.* **2006**, *12*, 437–442. Gacía-Martín, F.; White, P.; Steinauer, R.; Côté, S.; Tulla-Puche, J.; Albericio, F. *Biopolymers* **2006**, *84*, 566–575. (b) Depsipeptide derivatives: Sohma, Y.; Sasaki, M.; Kimura, T.; Kiso, Y. Chem. Commun. 2004, *124*125. Coin, I.; Dölling, R.; Krause, E.; Bienert, M.; Beyermann, M.; Sferdean, C. D.; Carpino, L. A. *J. Org. Chem.* **2006**, *71*, 3171–6177.

(10) Rivier, J. E.; Miranda, M. T. M. In *Synthesis of Peptides and Peptidomimetics*; Goodman, M.; Felix, A.; Moroder, L.; Toniolo, C., Eds.; Georg Thieme: Stuttgart, Germany, 2002; Houben-Weyl, Vol. E22, pp 117−128.

(11) (a) Tam, J. P. *Int. J. Pept. Prot. Res.* **1987**, *29*, 421–431. (b) Barlos, K.; Papaionannou, D.; Patrianakou, S.; Tsegenidis, T. *Liebigs Ann. Chem.* **1986**, 1950–1955. (c) Lloyd, D. H.; Petrie, G. M.; Noble, R. L.; Tam, J. P. In *Peptides: Chemistry, Structure and Biology. Proceedings of the 11th American Peptide Symposium*; Rivier, J. E., Marshall, G. R., Eds.; Escom Science: Leiden, The Netherlands, 1990; pp 909−910. (d) Wang, S.; Foutch, G. L. *Chem. Eng. Sci.* **1991**, *46*, 2373–2376. (e) Tam, J. P.; Lu, Y.-A. *J. Am. Chem. Soc.* **1995**, *117*, 12058–12063. (f) Kaplan, B. E.; Hefta, L. J.; Blake, R. C.; Swiderek, K. M.; Shively, J. E. *J. Peptide Res.* **1998**, *52*, 249–260.

(12) (a) Rabinovich, A. K.; Rivier, J. E. *Am. Biotech. Lab.* **1994**, *12*, 48–51. (b) Varanda, L. M.; Miranda, M. T. M. *J. Peptide Res.* **1997**, *50*, 102–108.

(13) Souza, M. P.; Tavares, M. F. M.; Miranda, M. T. M. *Tetrahedron* **2004**, *60*, 4671–4681.

(14) Reviews: (a) Collins, J. M.; Leadbeater, N. E. *Org. Biomol. Chem.* **2007**, *5*, 1141. (b) Collins, J. M.; Collins, M. J. In *Microwaves in Organic Synthesis*, 2nd ed.; Loupy, A., Ed.; Wiley-VCH: Weinheim, Germany, 2006; pp 898−958.

(15) (a) Yu, H. M.; Chen, S-T.; Wang, K. T. *J. Org. Chem.* **1992**, *57*, 4781–4784. (b) Santagada, V.; Fiorino, F.; Perissutti, E.; Severino, B.; De Filippis, V.; Vivenzio, B.; Caliendo, G. *Tetrahedron Lett.* **2001**, *42*, 5171–5173. (c) Erdelyi, M.; Gogoll, A. *Synthesis* **2002**, *11*, 1592–1596. (d) Finaru, A.; Berthault, A.; Basson, T.; Guillaumet, G.; Berteina-Raboin, S. *Org. Lett.* **2002**, *4*, 2613–2615. (e) Zhang, H. B.; Chi, Y. S.; Huang, W. L.; Ni, S. J. *Chin. Chem. Lett.* **2007**, *18*, 902–904. (f) Wisén, S.; Androsavich, J.; Evans, C. G.; Chang, L.; Gestwicki, J. E. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 60–65. (g) Bando, T.; Fujimoto, J.; Minoshima, M.; Shinohara, K.; Sasaki, S.; Kashiwazaki, G.; Mizumura, M.; Sugiyama, H. *Bioorg. Med. Chem.* **2007**, *15*, 6937–6942. (h) Nagashima, I.; Shimizu, H.; Matsushita, T.; Nishimura, S.-I. *Tetrahedron Lett.* **2008**, *49*, 3413–3418. (i) Katritzky, A. R.; Khashab, N. M.; Yoshioka, M.; Haase, D. N.; Wilson, K. R.; Johnson, J. V.; Chung, A.; Haskell-Luevano, C. *Chem. Biol. Drug Des.* **2007**, *70*, 465–468. (j) Lecaillon, J.; Giles, P.; Subra, G.; Martinez, J.; Amblard, M. *Tetrahedron Lett.* **2008**, *49*, 4674–4676. (k) Chi, Y.; Zhang, H.; Zhou, J.; Huang, W.; Ni, S. *Lett. Org. Chem.* **2008**, *5*, 399–402.

(16) Bacsa, B.; Desai, B.; Dibó, G.; Kappe, C. O. *J. Pept. Sci.* **2006**, *12*, 633–638.

(17) Bacsa, B.; Kappe, C. O. *Nat. Protoc.* **2007**, *2*, 2222–2227.

(18) Fara, M. A.; Díaz-Mochón, H. J.; Bradley, M. *Tetrahedron Lett.* **2006**, *47*, 1011–1014.

(19) Rizzolo, F.; Sabatino, G.; Chelli, M.; Rovero, P.; Papini, A. M. *Int. J. Pept. Res. Ther.* **2007**, *13*, 203–208.

(20) Coantic, S.; Subra, G.; Martinez, J. *Int. J. Pept. Res. Ther.* **2008**, *14*, 143–147.

(21) Abdel Rahman, S.; El-Kafrawy, A.; Hattaba, A.; Anwer, M. F. *Amino Acids* **2007**, *33*, 531–536.

α-peptide sequences using conventional Fmoc/*t*-Bu orthogonal protection strategies.[18−22] Successful applications of this enabling technology have additionally been published for the generation of notoriously difficult to prepare peptide motives such as *β*-peptides,[23−26] glycopeptides,[27] phosphopeptides,[28] and certain types of peptoids/peptidomimetics.[18,29] Apart from solid-phase techniques utilizing conventional resin beads, microwave-assisted SPPS has been used in conjunction with MicroKan technology,[16] SynPhase Lanterns[30] and parallel library synthesis in microtiter plates[25] and for the construction of combinatorial split-and-mix libraries on macrobeads.[24]

In addition to peptide couplings on solid phase, microwave irradiation has also been applied in related fields of peptide chemistry, including 2,5-diketopiperazine formation,[31] macrocyclization reactions,[32] and other transformations of resin-bound peptide structures,[33] for the activation[34] and cleavage[35] of safety-catch linkers, and for the regeneration of resins.[36] Furthermore, the technology has been used successfully to enhance peptide bond formation[37] and other transformations of peptide structures in solution phase.[38]

While the reported improvements using microwave-assisted SPPS in comparison to conventional SPPS in many cases have been impressive,[15−30] little effort has so far been devoted to provide a definitive scientific rationalization for the observed effects. The question must be asked if the experienced enhancements are of purely thermal origin (the result of efficient dielectric heating during the irradiation processes)[39] or if so-called nonthermal microwave effects[40] are implicated that would involve a direct interaction of the electromagnetic field (not related to a macroscopic temperature effect) with, for example, the peptide backbone or other substrates/intermediates in the reaction mixture. It has recently been suggested that due to the very high dipole moment of an amide bond, irradiation of peptides with microwave energy may lead to a deaggregation of the peptide backbone via direct interaction of the peptide chain with the electric field.[14] Microwave effects of this type would not be reproducible by conventional heating at the same measured bulk reaction temperature. Herein a detailed evaluation of microwave-assisted Fmoc solid-phase peptide synthesis involving several difficult sequences under strictly controlled conditions is presented. Using recently developed fast responding internal fiber-optic temperature probes,[41] the reaction temperatures experienced in microwave-assisted peptide couplings/deprotections have been carefully optimized. Adequate control experiments between microwave and conventional heating at the same reaction temperature have been performed in order to distinguish between thermal and nonthermal microwave effects.

Results and Discussion

General Considerations. For all peptide syntheses described herein, peptide chain elongation employing conventional Fmoc/*t*-Bu orthogonal protection strategy was employed.[2] Although a number of innovative and highly efficient peptide-coupling reagents (for example, aminium/uronium and phosphonium salts) have been introduced over the past decade,[3] the standard *N*,*N*′-diisopropylcarbodiimide/1-hydroxybenzotriazole (DIC/HOBt) method was applied for all microwave-assisted and conventional peptide couplings. This choice was made mainly on the basis of the known overall good coupling efficiency of DIC/HOBt and high reagent stability,[42] which appeared to be important in the context of performing high-temperature peptide couplings. Due to the higher thermal stability of NMP compared to the more traditionally used DMF, the former solvent was used for all peptide couplings.[17,28] Control experiments using DMF under identical conditions did generally show slightly lower peptide purities (data not shown). For removal of the Fmoc group 30% piperidine in DMF was used for all deprotections. Solid-phase syntheses of difficult peptide sequences

(22) For an example of microwave-assisted Boc-SPPS, see: Èemaar, M.; Craik, D. J. *J. Peptide Sci.* **2008**, *14*, 661–669.
(23) Murray, J. K.; Gellman, S. H. *Org. Lett.* **2005**, *7*, 1517–1520.
(24) (a) Murray, J. K.; Farooqi, B.; Sadowsky, J. D.; Scalf, M.; Freund, W. A.; Smith, L. M.; Chen, J.; Gellman, S. H. *J. Am. Chem. Soc.* **2005**, *127*, 13271–13280. (b) Murray, J. K.; Sadowsky, J. D.; Scalf, M.; Smith, L. M.; Tomita, Y.; Gellman, S. H. *J. Comb. Chem.* **2008**, *10*, 204–215.
(25) (a) Murray, J. K.; Gellman, S. H. *J. Comb. Chem.* **2006**, *8*, 58–65. (b) Murray, J. K.; Gellman, S. H. *Nat. Protoc.* **2007**, *2*, 624–631.
(26) (a) Petersson, E. J.; Schepartz, A. *J. Am. Chem. Soc.* **2008**, *130*, 821–823. (b) Miyake, R.; Tashiro, S.; Shiro, M.; Tanaka, K.; Shionoya, M. *J. Am. Chem. Soc.* **2008**, *130*, 5646–5647.
(27) (a) Matsushita, T.; Hinou, H.; Kurogochi, M.; Shimizu, H.; Nishimura, S.-I. *Org. Lett.* **2005**, *7*, 877–880. (b) Matsushita, T.; Hinou, H.; Fumoto, M.; Kurogochi, M.; Fujitani, N.; Shimizu, H.; Nishimura, S.-I. *J. Org. Chem.* **2006**, *71*, 3051–3063.
(28) Brandt, M.; Gammeltoft, S.; Jensen, K. J. *Int. J. Pept. Prot. Res.* **2006**, *12*, 349–357.
(29) (a) Olivos, H. J.; Alluri, P. G.; Reddy, M. M.; Salony, D.; Kodadek, T. *Org. Lett.* **2002**, *4*, 4057–4059. (b) Gorske, B. C.; Jewell, S. A.; Guerard, E. J.; Blackwell, H. E. *Org. Lett.* **2005**, *7*, 1521–1524. (c) Joshi, B. P.; Park, J.-W.; Kim, J.-M.; Lohani, C. R.; Cho, H.; Lee, K.-H. *Tetrahedron Lett.* **2008**, *49*, 98–101. (d) Diaz-Mochon, J. J.; Fara, M. A.; Sanchez-Martin, R. M.; Bradley, M. *Tetrahedron Lett.* **2008**, *49*, 923–926. (e) Fowler, S. A.; Stacy, D. M.; Blackwell, H. E. *Org. Lett.* **2008**, *10*, 2329–2332.
(30) Monroc, S.; Feliu, L.; Planas, M.; Bardaji, E. *Synlett* **2006**, 1311–1314.
(31) Tullberg, M.; Luthman, K.; Grtli, M. *J. Comb. Chem.* **2006**, *8*, 915–922.
(32) (a) Grieco, P.; Campiglia, P.; Gomez-Monterrey, I.; Lama, T.; Novellino, E. *Synlett* **2003**, 2216–2218. (b) Grieco, P.; Cai, M.; Liu, L.; Mayorov, A.; Chandler, K.; Trivedi, D.; Lin, G.; Campiglia, P.; Novellino, E.; Hruby, V. J. *J. Med. Chem.* **2008**, *51*, 2701–2707. (c) Byk, G.; Cohen-Ohana, M.; Raichman, D. *Biopolymers* **2006**, *81*, 274–282. (d) Chapman, R. N.; Arora, P. S. *Org. Lett.* **2006**, *8*, 5825–5828. (e) Robinson, A. J.; Elaridi, J.; Van Lierop, B. J.; Mujcinovic, S.; Jackson, W. R. *J. Pept. Sci.* **2007**, *13*, 280–285.
(33) (a) Campiglia, P.; Gomez-Monterrey, I.; Longobardo, L.; Lama, T.; Novellino, E.; Grieco, P. *Tetrahedron Lett.* **2004**, *45*, 1453–1456. (b) Lama, T.; Campiglia, P.; Carotenuto, A.; Auriemma, L.; Gomez-Monterrey, I.; Novellino, E.; Grieco, P. *J. Peptide Res.* **2005**, *66*, 231–235. (c) Park, M.-S.; Oh, H.-S.; Cho, H.; Lee, K.-H. *Tetrahedron Lett.* **2007**, *48*, 1053–1057. (d) Nagaike, F.; Onuma, Y.; Kanazawa, C.; Hojo, H.; Ueki, A.; Nakahara, Y.; Nakahara, Y. *Org. Lett.* **2006**, *8*, 4465–4468. (e) Grieco, P. *Chim. Oggi* **2004**, *22*, 18–20.
(34) Merkx, R.; van Haren, M. J.; Rijkers, D. T. S.; Liskamp, R. M. J. *J. Org. Chem.* **2007**, *72*, 4574–4577.
(35) Isidro-Llobet, A.; Alvarez, M.; Burger, K.; Spengler, J.; Albericio, F. *Org. Lett.* **2007**, *9*, 1429–1432.
(36) García-Martín, F.; Bayó-Puxan, N.; Cruz, L. J.; Bohling, J. C.; Albericio, F. *QSAR Comb. Sci.* **2007**, *26*, 1027–1035.
(37) (a) Paolini, I.; Nuti, F.; de la Cruz Pozo-Carrero, M.; Barbetti, F.; Kolesinska, B.; Kaminski, Z. J.; Chelli, M.; Papini, A. M. *Tetrahedron Lett.* **2007**, *48*, 2901–2904. (b) Santagada, V.; Frecentese, F.; Fiorino, F.; Cirillo, D.; Perisutti, E.; Severino, B.; Terracciano, S.; Caliendo, G. *QSAR Comb. Sci.* **2004**, *23*, 899–901. (c) Katritzky, A. R.; Todadze, E.; Angrish, P.; Draghici, B. *J. Org. Chem.* **2007**, *72*, 5794–5801. (d) Cianci, J.; Baell, J. B.; Harvey, A. J. *Tetrahedron Lett.* **2007**, *48*, 5973–5975. (e) Tantry, S. J.; Ramana Rao, R. V.; Suresh Babu, V. V. *ARKIVOC* **2006**, 21–30. (f) Porcheddu, A.; Ruda, G. F.; Sega, A.; Taddei, M. *Eur. J. Org. Chem.* **2003**, 907–912.
(38) (a) Sureshbabu, V. V.; Patil, B. S.; Venkataramanarao, R. *J. Org. Chem.* **2006**, *71*, 7697–7705. (b) van Dijk, M.; Mustafa, K.; Dechesne, A. C.; van Nostrum, C. F.; Hennink, W. E.; Rijkers, D. T. S.; Liskamp, R. M. J. *Biomacromolecules* **2007**, *8*, 327–330. (c) Rijkers, D. T. S.; van Esse, G. W.; Markx, R.; Brouwer, A. J.; Jacobs, H. J. F.; Pietrs, R. J.; Liskamp, R. M. J. *Chem. Commun.* **2005**, 4581–4583. (d) Campiglia, P.; Aquino, C.; Bertamino, A.; Sala, M.; Gomez-Monterrey, I. M.; Novellino, E.; Grieco, P. *Tetrahedron Lett.* **2008**, *49*, 731–734.
(39) (a) Gabriel, C.; Gabriel, S.; Grant, E. H.; Halstead, B. S.; Mingos, D. M. P. *Chem. Soc. Rev.* **1998**, *27*, 213. (b) Mingos, D. M. P.; Baghurst, D. R. *Chem. Soc. Rev.* **1991**, *20*, 1.
(40) (a) De La Hoz, A.; Diaz-Ortiz, A.; Moreno, A. *Chem. Soc. Rev.* **2005**, *34*, 164. (b) Perreux, L.; Loupy, A. In *Microwaves in Organic Synthesis*, 2nd ed.; Loupy, A., Ed.; Wiley-VCH: Weinheim, Germany, 2006; Chapter 4, pp 134−218.
(41) Herrero, M. A.; Kremsner, J. M.; Kappe, C. O. *J. Org. Chem.* **2008**, *73*, 36.
(42) Hachmann, J.; Lebl, M. *Biopolymers* **2006**, *84*, 340–347.

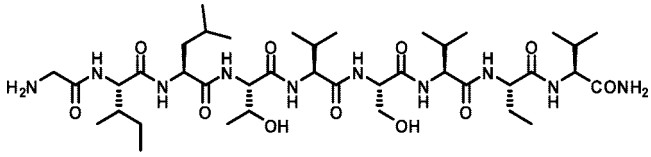


FIGURE 1. Structure of model peptide GILTVSVAV.

were carried out on three different solid supports. Apart from the traditional polystyrene (PS) resin, poly(ethylene glycol) (PEG)-modified PS support (Tentagel, TG)[43] and a new generation of fully PEG-based ChemMatrix resins (CM)[44] were employed. While classical PS supports have certain limitations for the synthesis of complex/difficult peptides due to their high hydrophobicity, purely PEG-based supports such as CM have recently been shown to be very good supports for the generation of difficult sequences by SPPS.[45] In all cases, the resins were modified with Rink Amide linker, which generates peptide amides.[46]

Microwave-assisted SPPS was performed using a dedicated 300 W single-mode manual microwave peptide synthesizer (Discover SPS).[17] The DIC/HOBt couplings and Fmoc-deprotection steps were carried out in a solid-phase reaction vessel under atmospheric conditions, while the reaction temperature was measured continuously with a fiber-optic probe inserted into the reaction vessel. The reaction vessel is a polypropylene tube with a frit attached and designed for solid-phase synthesis allowing for bottom filtration, therefore mimicking the workflow of a conventional peptide synthesizer.[17] Of critical importance for our work, the same vessel and temperature monitoring system used in the microwave synthesizer could also be employed in conjunction with conventional SPPS at room temperature or at elevated temperature, allowing for accurate comparison experiments between microwave-assisted and conventional SPPS (see below). Both the coupling and the deprotection steps under microwave conditions were generally performed applying a pulsed temperature control program (see below) using comparatively small maximum microwave power levels (5−10 W for coupling and 20 W for deprotection) in order to rapidly achieve the desired reaction temperatures (typically 60−75 °C for both coupling and deprotection). Reaction times under microwave conditions represent total irradiation times that include the time required to reach the desired maximum temperature (ramp time). Microwave-assisted deprotection with piperidine/DMF was achieved in two steps: after an initial 30 s of microwave irradiation (60 or 75 °C), the resin was washed and subsequently exposed to a fresh portion of the cleavage cocktail and irradiated for an additional 2.5 min (60 or 75 °C). In general, deprotections were performed at the same temperature as the coupling step for 3 min and were not further optimized.

Reaction Optimization for Model Peptide GILTVSVAV. As a first model peptide the synthetically difficult nonapeptide (H-Gly-Leu-Ile-Thr-Val-Ser-Val-Ala-Val-$CONH_2$) (Figure 1), a predicted CD8+ T-cell epitope of an immunodominant protein of *Mycobacterium tuberculosis*, was chosen.[47] To identify the difficult part of the peptide, the sequence was analyzed using the "Peptide Companion" software package.[48] This prediction program characterizes the coupling difficulty of the amino acids from the fifth residue in the sequence. According to the prediction (see Figure S1, Supporting Information), the sequence is prone to be difficult from the fifth residue onward (GILTV), probably as a result of the high proportion of hydrophobic Ala, Val, and Ile residues.

Microwave-assisted SPPS of nonapeptide H-Gly-Ile-Leu-Thr(*t*-Bu)-Val-Ser(*t*-Bu)-Val-Ala-Val-$CONH_2$ (GILTVSVAV) was first evaluated on Rink Amide MBHA PS resin. For the DIC/HOBt-mediated peptide couplings using 3−10 equiv of Fmoc-amino acids two different nominal reaction temperatures (60 and 75 °C) were selected.[17,18,20] Different combinations of coupling solvents (NMP, DMF, DMSO/NMP)[5] and chaotropic salt additives (0.8 M LiCl/NMP)[6,23] were investigated, but ultimately NMP provided the best results in terms of peptide purity and yield (Table S1, Supporting Information). Using PS resin, the most efficient coupling conditions involved microwave irradiation at 60 °C for 20 min employing 10 equiv of Fmoc-amino acid. The purity of the crude peptide was 85% with an overall isolated yield of 61%. Reducing the excess of coupling reagents gave poor results, probably as a consequence of the hydrophobic nature of the PS polymer support. For comparison purposes, the nonapeptide was also synthesized using standard room-temperature SPPS conditions using the identical reaction vessel and coupling/deprotection protocols. DIC/HOBt coupling applying 10 equiv of Fmoc-amino acid (60 min coupling time) followed by deprotection (2 + 20 min) provided the desired peptide in a moderate 44% purity, confirming the relative difficulty in synthesizing this peptide using conventional conditions. The microwave protocol therefore allowed the generation of a significantly higher purity peptide in a somewhat shorter time frame (4.5 versus ca. 10 h overall reaction time) but ultimately could not prevent the formation of deletion sequences (Table S1 and Figure S2, Supporting Information).

After the initial attempt to synthesize this difficult sequence on PS resin a more detailed optimization study of coupling and deprotection conditions was subsequently performed on RAM-Tentagel resin, in the hope that the less hydrophobic nature of this PEG/PS-derived solid support would facilitate the synthesis of the GILTVSVAV peptide. In a set of experiments involving standard room-temperature SPPS, the influence of coupling time and excess of Fmoc-amino acids was established. The data presented in Table 1 (see also Figures S3 and S4, Supporting Information) clearly indicate that a minimum of 5 equiv of activated amino acid is required to achieve efficiency in the coupling step (compare entries 1 and 2). Reducing the coupling time at room temperature from 60 min to 20 or 10 min, a typical coupling time in a microwave experiment, also led to a dramatic decrease in efficiency (compare entries 2 and 5).

In comparison to standard room-temperature SPPS on RAM-Tentagel, microwave-assisted couplings and deprotections proved to be far more efficient, providing peptides of high purity in considerably shorter processing times (Table 2, Figure 2). When a 10-fold excess of the coupling cocktail at 60 °C nominal

(43) Rapp, W. In *Combinatorial Peptide and Nonpeptide Libraries: A Handbook*; Jung, G., Ed.; VCH: Weinheim, Germany, 1996; pp 425−464.
(44) Camperi, S. A.; Marani, M. M.; Iannucci, N. B.; Côte, S.; Albericio, F.; Cascone, O. *Tetrahedon Lett.* **2005**, *46*, 1561–1564.
(45) (a) Garcia-Martin, F.; Quintanar-Audelo, M.; Garcia-Ramos, Y.; Cruz, L. J.; Gravel, C.; Furic, R.; Côte, S.; Tulla-Puche, J.; Albericio, F. *J. Comb. Chem.* **2006**, *8*, 213–220. (b) Garcia-Martin, F.; White, P.; Steinauer, R.; Côte, S.; Tulla-Puche, J.; Albericio, F. *Biopolymers* **2006**, *84*, 566–575. (c) de la Torre, B. G.; Jakab, A.; Andreu, D. *Int. J. Pept. Res. Ther.* **2007**, *13*, 265–270.
(46) Bernatowicz, M. S.; Daniels, S. B.; Köster, H. *Tetrahedron Lett.* **1989**, *30*, 4645–4648.
(47) Caccamo, N.; Milano, S.; Di Sano, C.; Cigna, D.; Ivanyi, J.; Krensky, A. M.; Dieli, F.; Salerno, A. *J. Infect. Dis.* **2002**, *186*, 991–998.
(48) Lebl, M.; Krchnak, V.; Lebl, G. Peptide Companion; San Diego, CA, 1995; http://www.5z.com/psp/software.shtml.

TABLE 1. Room-Temperature Synthesis of GILTVSVAV on RAM-Tentagel Resin[a]

	Fmoc-amino acid				
entry	equiv	concn (M)	coupling time (min)	deprotection time (min)	purity[b] (%)
1	3	0.11	60	2 + 20	<1
2	5	0.18	60	2 + 20	32
3	10	0.36	60	2 + 20	37
4	5	0.18	10	2 + 20	<1
5	5	0.18	20	2 + 20	5

[a] Peptide synthesis was performed at room temperature (25 °C) on a 0.036 mmol scale using RAM-Tentagel resin (loading 0.24 mmol/g) in a 10 mL solid-phase reaction vessel (ca. 1.1 mL of solvent for the coupling step; 2 mL of 30% piperidine in DMF for the deprotection step), PLS 4 × 6 synthesizer. [b] Purity of crude peptides (analytical RP-HPLC peak area %, UV absorbance at 215 nm). Peaks between 5 and 8 min retention time were used for integration. Chromatograms are reproduced in the Supporting Information (Figures S3 and S4). The identity of the target peptide was established by MALDI-TOF MS.

temperature was applied, the peptide was obtained with similar purity (85%) using the same reaction conditions as employed for Rink Amide PS resin (entry 1). To reduce the high molar excess of amino acid, peptide syntheses were repeated with 5-fold and 3-fold excess of coupling reagents using the same coupling time (20 min). Gratifyingly, coupling using a 5-fold excess (entry 2) did not have a negative influence on the result (83%), while a further reduction employing only 3-fold excess of amino acid (entry 3) led to a significantly lower peptide purity (68%). Shortening the reaction time to 10 min at 60 °C resulted in a further drop in peptide purity (entry 4). Optimum conditions at 60 °C coupling temperature therefore employed a 5-fold excess of coupling reagents for 20 min. The purity of the crude peptide under these conditions was reproducibly ca. 83% with an overall isolated peptide yield of 61% (entry 2). HPLC chromatograms of the crude peptides show that the main impurity in all cases corresponds to a deletion peptide sequence lacking Leu (or Ile) residues as identified by MALDI-TOF MS (Figure S5, Supporting Information). To improve the synthetic protocol, different variations of double coupling and deprotection steps were performed at the critical positions in the sequence, but no significant improvement was observed (entries 5−8).

In an attempt to minimize the occurrence of deletion sequences and to further reduce the required coupling time, the microwave-assisted SPPS of nonapeptide GILTVSVAV was additionally investigated at 75 °C coupling temperature (entries 9−12). Using 10 min of irradiation time at 75 °C (10 W), the peptide was produced in 92% purity (65% isolated yield) by applying a 5-fold molar excess of the coupling reagents (entry 9) (Figure 2c). The use of 5 equiv of Fmoc-amino acid apparently represents the minimum for achieving high purity peptides on Tentagel resin under these conditions. Further reduction to 3 equiv (entry 10) resulted in a notably reduced peptide purity (71%). It should be emphasized that for peptide couplings at 75 °C best results were achieved by choosing 10 W of maximum microwave power. When 10 W microwave output power was applied, the selected reaction temperature of 75 °C was reached within <1 min. Selecting only 5 W microwave power (as for the 60 °C runs) led to a considerably longer ramp time to reach the 75 °C (ca. 2 min) and therefore effectively to a reduced reaction time at the optimized temperature of 75 °C (entry 11). This resulted in a diminished peptide purity of 61%. The reaction time for the coupling step is apparently critical as a 5 min total coupling time also led to noticeable reduced peptide purity (entry 12). It has to be noted that the diminished peptide purities employing 3 equiv of amino acid on Tentagel resin cannot be fully compensated by further increasing the coupling temperature to 85 °C (entry 13).

Figure 2shows a comparison of HPLC chromatograms obtained for the synthesis of GILTVSVAV nonapeptide on Tentagel resin using 5 equiv of Fmoc-amino acids. The dramatic effect on switching from conventional SPPS to microwave-assisted SPPS using 10 min coupling time are clearly evident (compare parts b and c of Figure 2). By extending the coupling times to the traditionally used 60 min at room temperature, significantly higher purities can be obtained as compared to the 10 min experiment (compare parts a and b of Figure 2), but it has to be emphasized that the major product (49%) at room temperature is still the deleted sequence missing Leu (or Ile). The striking influence of the reaction temperature in the range

TABLE 2. Microwave-Assisted Synthesis of GILTVSVAV on RAM-Tentagel Resin[a]

	Fmoc-amino acid		coupling				
entry	equiv	conc (M)	temp[b] (°C)	power[c] (W)	time (min)	deprotection temp[b] (°C)	purity[d] (%)
1	10	0.36	60	5	20	60	85
2	5	0.18	60	5	20	60	83/82[e]
3	3	0.11	60	5	20	60	68
4	3	0.11	60	5	10	60	42
5[f]	5	0.18	60	5	20	60	78
6[g]	5	0.18	60	5	20	60	83
7[h]	5	0.18	60	5	20	60	77
8[i]	5	0.18	60	5	20	60	85
9	5	0.18	75	10	10	75	92/93[j]
10	3	0.11	75	10	10	75	71
11	3	0.11	75	5	10	75	61
12	3	0.11	75	5	5	75	54
13	3	0.11	85	5	10	75	78
14	3	0.11	40	5	10	40	9

[a] Peptide synthesis was performed on a 0.036 mmol scale using RAM-Tentagel resin (loading 0.24 mmol/g) in a 10 mL solid-phase reaction vessel (ca. 1.1 mL of solvent for the coupling step; 2 mL of 30% piperidine in DMF for the deprotection step, 0.5 + 2.5 min deprotection time, 20 W maximum microwave power), CEM Discover SPS. [b] Set temperature monitored by internal fiber-optic probe. [c] Maximum magnetron microwave output power for pulsing. [d] Purity of crude peptides (analytical RP-HPLC peak area %, UV absorbance at 215 nm). Peaks between 5 and 8 min retention time were used for integration. The identity of the target peptide and the deletion sequences was established by MALDI-TOF MS. [e] Isolated yield 61% (crude product). [f] Double coupling of Leu. [g] Double coupling of Ile. [h] Double deprotection of Thr. [i] Double deprotection of Leu. [j] Isolated yield 65% (crude product).

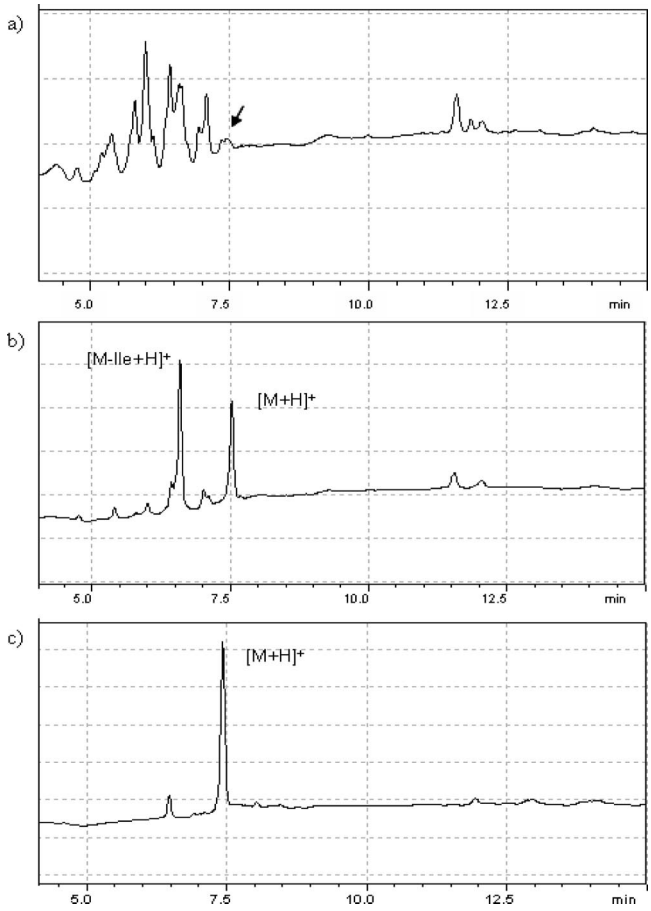


FIGURE 2. Comparison of peptide purities (HPLC chromatograms, UV absorbance at 215 nm) of GILTVSVAV peptide synthesized on Tentagel resin using 5 equiv of Fmoc-amino acids under conventional (25 °C) and microwave (75 °C) conditions. For exact conditions, see Table 1, entries 4 and 2 (for Figures 2a and 2b), and Table 2, entry 9 (for Figure 2c). The target peptide and deletion sequences were identified by MALDI-TOF MS (see Figure S5, Supporting Information). Figure 2b: $[M + Na]^+ = 880.3$, $[M + K]^+ = 896.3$, $[M + H]^+ = 858.3$; $[M - Ile + Na]^+ = 880.3$, $[M - Ile + K]^+ = 896.3$, $[M + H]^+ = 858.3$. Figure 2c: $[M + Na]^+ = 880.2$, $[M + K]^+ = 896.2$, $[M + H]^+ = 858.1$; $M_{cal} = 857.0$.

of 40−85 °C on microwave-assisted couplings involving 3 equiv of Fmoc-amino acid is shown in Figure S6 in the Supporting Information.

The best support for the preparation of the nonapeptide GILTVSVAV proved to be the fully PEG-based ChemMatrix material.[44,45] Using RAM-ChemMatrix resin, the desired peptide could be synthesized in very high purity (ca. 95%) virtually free from any impurities such as deletion sequences experienced with Polystyrene and Tentagel resins. An additional advantage is the fact that here a 3-fold excess of Fmoc-amino acid was sufficient to allow the generation of a high purity peptide at 75 °C applying a 10 min coupling time (Table 3, entry 4). A control experiment at room temperature using 10 equiv of amino acid and 60 min coupling time (22 min for deprotection) furnished the desired peptide in moderate 47% purity.

A comparison between microwave-assisted and conventional room temperature SPPS for the difficult nonapeptide sequence GILTVSVAV demonstrates the apparent advantages of microwave technology. Using microwave irradiation, the desired peptide was synthesized in a much shorter time frame since both the time for the coupling and deprotection steps were significantly reduced as compared to standard room-temperature experiments (10 vs 60 min for coupling, 3 vs 22 min for deprotection). While the overall time required for the synthesis of the nonapeptide at room temperature is on the order of 12−13 h, the same peptide can be synthesized in less than 2 h using microwave-assisted SPPS. Clearly more important than the time-saving aspect is the fact that peptide purity can be dramatically enhanced switching from conventional SPPS (<50%) to a microwave-mediated approach (>90%). Although the performance of the three different resins in these studies can not be directly compared because of the somewhat different loadings, it appears that PEG-based ChemMatrix resins are very well suited for the microwave synthesis of difficult peptide sequences where aggregation phenomena play a critical role.[44,45]

Elevated Temperature Synthesis of GILTVSVAV: Microwave Versus Conventional Heating. The results presented above clearly demonstrate the effectiveness of microwave-assisted solid-phase synthesis for the generation of difficult peptide sequences and therefore confirm previous reports on the general usefulness of microwave-assisted in comparison to conventional SPPS carried out a room temperature.[14−30] However, in order to establish the true value of using microwave technology for peptide synthesis a comparison of microwave-heated with conventionally heated coupling and deprotection reactions *at the exact same temperature* must be conducted. It has to be noted that while in many instances a comparison between results obtained by conventional room-temperature SPPS with data from microwave-assisted SPPS have been reported,[14−30] in only a very few cases have attempts been made to adequately compare the results of microwave-heated and conventionally heated peptide coupling and deprotection chemistry.[16,23,24] In this context, it should be noted that the accurate measurement of reaction temperature in microwave-assisted reactions is a nontrivial affair that cannot be achieved with standard temperature monitoring tools such as external IR sensors.[41,49]

In the Discover microwave peptide synthesizer, the reaction temperature is monitored internally in a continuous fashion employing a fiber-optic probe (Discover FO) that provides feedback to the magnetron so that an appropriate amount of magnetron output power is delivered to achieve and maintain the desired reaction temperature.[17] In order to apply microwave irradiation throughout the full cycle time of the coupling and deprotection steps, a pulsed programming sequence is typically used that provides short bursts of microwave irradiation in order to keep the temperature close to the desired set temperature. When the programmed set temperature of, e.g., 75 °C is reached, the initially used microwave power (10 W) is regulated down to 0 W. When the temperature has fallen to 72 °C ($\Delta T = 3$ °C), the power activates itself to 10 W until the temperature reaches 75 °C again. Note that some overshooting to temperatures above 75 °C (up to 79 °C) typically occurs during this process so that the average temperature over the full cycle corresponds closely to the set temperature of 75 °C. The irradiation cycle is repeated until the end of the preset reaction time (Figure 3).[17] In this way, microwave energy is applied

(49) (a) Hosseini, M.; Stiasni, N.; Barbieri, V.; Kappe, C. O. *J. Org. Chem.* **2007**, *72*, 1417–1424. (b) Kremsner, J. M.; Kappe, C. O. *J. Org. Chem.* **2006**, *71*, 4651–4658. (c) Nüchter, M.; Ondruschka, B.; Bonrath, W.; Gum, A. *Green Chem.* **2004**, *6*, 128–141. (d) Nüchter, M.; Ondruschka, B.; Weiβ, D.; Bonrath, W.; Gum, A. *Chem. Eng. Technol.* **2005**, *28*, 871–881. (e) Leadbeater, N. E.; Pillsbury, S. J.; Shanahan, E.; Williams, V. A. *Tetrahedron* **2005**, *61*, 3565–3585.

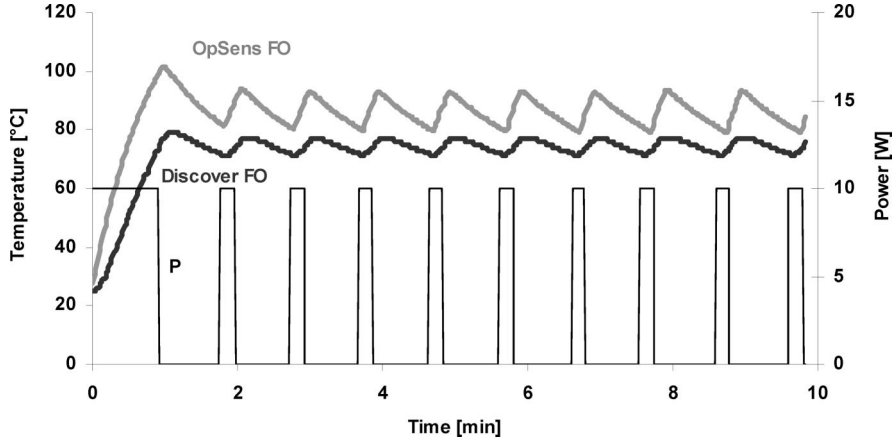


FIGURE 3. Temperature (Discover FO, OpSens FO) and microwave power (P) profiles for a typical microwave-assisted peptide coupling step (3-fold excess of Fmoc-amino acid, DIC and HOBt) in 1 mL of NMP at 75 °C (set temperature) for 10 min irradiation time using the Discover SPS program (maximum power 10 W, $\Delta T = 3$ °C). The average temperature measured by the OpSens FO probe is 86 °C as opposed to 75 °C shown by the Discover FO probe.

throughout the coupling/deprotection steps and therefore potentially allows for a direct interaction of the electromagnetic field with the peptide chain as discussed above (deaggregation via nonthermal microwave effects).[14] Employing a standard temperature-controlled program not using power pulsing sequences, as in traditional microwave chemistry,[50] would lead to a situation where microwave irradiation is only applied in the initial phase of the experiment. Once the set temperature has been reached, little or no additional microwave power will be applied.[16] This is mainly a consequence of the fact that both the coupling and deprotection cocktails are strongly microwave absorbing and therefore even small amounts of microwave power will lead to a rapid rise in temperature, in particular when small volumes are irradiated. Notably, NMP is a solvent that has a considerable dissipation factor (tan δ 0.275),[51] and control experiments have demonstrated that the presence of Fmoc-amino acids, DIC, and HOBt (3, 5, and 10 equiv) has an additional influence on the microwave absorption (Figure S8, Supporting Information).

Recent investigations from our laboratory have demonstrated that fiber-optic probes in combination with their protective immersion wells (including the system used with the Discover SPS) have delay times of several seconds before the correct temperature is displayed by the sensor.[41] A control experiment was therefore performed by measuring the temperature inside the reaction vessel simultaneously with a fast-responding external probe (OpSens FO) attached to the Discover FO sensor. These measurements revealed that the actual reaction temperature during the coupling process applying a set temperature of 75 °C at 10 W maximum power is probably close to 86 °C (Figure 3). For a typical coupling step carried out at 60 °C at 5 W power this value is 67 °C (Figure S9, Supporting Information). It should be emphasized that these discrepancies in temperature monitoring mainly occur using very small reaction volumes (1−2 mL). Here, the rise in temperature applying 10 W microwave power is so fast that the comparatively slow Discover SPS sensor controlling the magnetron power significantly lags behind, leading to considerable overshooting of temperature. For volumes ≥3 mL the two sensors show a much better agreement (Figure S10, Supporting Information). When higher initial power levels (20 W) are employed, the temperature overshooting effect for small scale experiments is even more dramatic leading to temperature differences up to ca. 70 °C between the different fiber-optic probes (Figure S11, Supporting Information). It should be noted that these differences in temperature measurements were also experienced for the deprotection step (Figure S12, Supporting Information).

Based on the results described above, comparison studies applying conventionally heated SPPS were therefore conducted at 67 °C (representing a 60 °C set nominal temperature) and at 86 °C (representing 75 °C set nominal temperature) employing both Tentagel and ChemMatrix resin. For this purpose, the same solid-phase reaction vessel used in the Discover SPS for microwave-assisted couplings and deprotections was applied in a conventional manual solid-phase synthesizer (ACT PLS 4 × 6 platform) keeping all other reaction parameters the same. In order to mimic the two heating modes as closely as possible, two modifications were made, however. Since heating of the strongly microwave absorbing coupling and deprotection cocktails is far more rapid in a microwave reactor as compared to a conventional synthesizer, reaction times for couplings and deprotections under conventional conditions were extended by 1 min (coupling) and 2 min (deprotection, 1 min each for both cycles), respectively. These values were based on measurements of the heating profiles for conventionally heated coupling and deprotection steps using internal fiber-optic probes. As shown in Figure S19 (Supporting Information), it takes ca. 1 min longer for the coupling cocktail to reach the desired set temperature using conductive heat transfer in the solid-phase reaction vessel compared with direct microwave heating (113 versus 40 s). Second, since microwave heating, in contrast to conventional heating by conduction and convection phenomena, provides volumetric heating without temperature gradients on these small scales,[50] the conventionally heated experiments were performed applying a gentle vortexing in order to minimize temperature gradients. Peptide couplings without vortexing did provide

(50) Kappe, C. O. *Angew. Chem., Int. Ed.* **2004**, *43*, 6250–6284.

(51) The ability of a specific solvent to convert microwave energy into heat at a given frequency and temperature is determined by the so-called loss tangent (tan δ), expressed as the quotient tan $\delta = \epsilon''/\epsilon'$. A reaction medium with a high tan δ at the standard operating frequency of a microwave synthesis reactor (2.45 GHz) is required for good absorption and, consequently, for efficient heating. Solvents used for microwave synthesis can be classified as high (tan $\delta > 0.5$), medium (tan δ 0.1−0.5), and low microwave absorbing (tan $\delta < 0.1$). See ref 50 for further details.

(52) de la Torre, B. G.; Jakab, A.; Andreu, D. *Int. J. Pept. Res. Ther.* **2007**, *13*, 265–270.

TABLE 3. Microwave-Assisted Synthesis of GILTVSVAV on RAM ChemMatrix Resin[a]

	Fmoc-amino acid		coupling				
entry	equiv	conc (M)	temp[b] (°C)	power[c] (W)	time (min)	deprotection temp[b] (°C)	purity[d] (%)
1	10	0.75	60	5	20	60	90
2	5	0.38	60	5	20	60	91/89
3	3	0.23	60	5	20	60	77
4	3	0.23	75	10	10	75	95/91[e]

[a] Peptide synthesis was performed on a 0.08 mmol scale applying RAM ChemMatrix resin (loading 0.50 mmol/g) in a 10 mL solid-phase reaction vessel (ca. 1.1 mL solvent for the coupling step; 2 mL 30% piperidine in DMF for the deprotection step, 0.5 + 2.5 min deprotection time, 20 W maximum microwave power), CEM Discover SPS. [b] Set temperature monitored by internal fiber-optic probe. [c] Maximum magnetron microwave output power for pulsing. [d] Purity of crude peptides (analytical RP-HPLC peak area %, UV absorbance at 215 nm). Peaks between 5 and 8 min retention time were used for integration. The identity of the target peptide and the deletion sequence was established by MALDI-TOF MS. Selected chromatograms are reproduced in the Supporting Information (Figure S7). [e] Isolated yield 61% (crude product).

consistently lower peptide purities, although the differences were comparatively small (<5%).

As can be seen from the data presented in Table 4, there is a surprisingly close match in terms of peptide purity between the results obtained using conventional heating and microwave heating at the same coupling and deprotection temperatures. In particular for SPPS on ChemMatrix resin, the peptide purities between microwave and conventional processing are virtually identical (Figure 4), indicating that nonthermal microwave effects are probably not involved. The fact that the purities under microwave conditions are in some instances still somewhat higher as compared to conventional heating (1−5%) may perhaps be rationalized by the fact that the fast-responding OpSens probe has to be employed in combination with a quartz immersion well in order to protect the sensor crystal from the chemically aggressive coupling solution. Therefore, this probe will also show some delay under microwave conditions and thus may not entirely accurately reflect the actual reaction temperature.[41] It can be assumed that the true coupling/deprotection temperatures are in fact a few degrees higher than 67 or 86 °C, respectively. In order to substantiate this hypothesis, the conventionally heated peptide coupling experiment described in Table 4, entry 1 was repeated at 70 °C (as compared to 67 °C) for both the coupling and deprotection steps. Indeed, the purity of the obtained peptide synthesized under these conditions was now 82% (Figure S20, Supporting Information), somewhat higher than the purity obtained using the previously optimized temperature of 67 °C (77%), and now closely matched the purity obtained in the microwave experiment (Table 4, entry 2).

The microwave-assisted synthesis of nonapeptide GILTVSVAV on ChemMatrix resin was additionally performed on a 3-fold scale (0.225 mmol) in a 25 mL reaction vessel, applying a linear scale-up using ca. 3 mL of coupling mixture and 4 mL of deprotection cocktail. Under these conditions, an increased 30 W of maximum power was used for the coupling steps and 40 W for the deprotection steps in order to achieve similar heating profiles as on the smaller scale. Note that under these conditions there is good agreement between the two fiber-optic probes (Figure S10, Supporting Information). The outcome from the 0.225 mmol scale SPPS was comparable with the results of the 0.075 mmol run, leading to a similar peptide purity of 88% (Figure S14, Supporting Information).

Elevated Temperature Synthesis of Cecropin A(1−7)−Mellitin (2−9) Hybride Peptide. In order to test the general effectiveness of elevated temperature SPPS on other difficult and longer peptide sequences, the generation of the 15-mer H-Lys-Trp-Lys-Leu-Phe-Lys-Lys-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-$CONH_2$ was attempted. This peptide sequence is a hybrid consisting of the first seven residues of the antimicrobial peptide cecropin A and residues 2−9 of the bee venom peptide mellitin.[54] This 15-mer peptide was identified as the minimal sequence with strong antimicrobial activity comparable to the full-length cecropins and lacking the hemolytic properties associated with mellitin. Like the native cecropins, KWKLFKKIGAVLKVL has a highly basic N-terminal domain and a relatively hydrophobic C-terminal domain and is known for its synthetic difficulty.[54] The peptide was chosen for its high aggregation potential (see Figure S15, Supporting Information) containing several hydrophobic Ala, Val, Leu, and Ile residues and because of the comparatively large number of Boc-protection groups present on the Lys and Trp amino acid residues. It should be noted that during elevated temperature SPPS, regardless if conventional[10−13] or microwave heating[14−30] is employed, the side-chain protection groups may be exposed to comparatively high temperatures (70−90 °C) for a considerable amount of time. This may be a concern for thermolabile groups such as the Boc protection group, in particular for cases where side-chain protection starts early on in the sequence. The synthesis of the 15-mer H-Lys(Boc)-Trp(Boc)-Lys(Boc)-Leu-Phe-Lys(Boc)-Lys(Boc)-Ile-Gly-Ala-Val-Leu-Lys(Boc)-Val-Leu-$CONH_2$ was performed on ChemMatrix resin on a 0.075 mmol scale applying the microwave-assisted DIC/HOBt coupling and piperidine/DMF deprotection conditions optimized for nonapeptide GILTVSVAV described above (see Table 4). Utilizing 3-fold excess of activated Fmoc-amino acids at 86 °C coupling temperature (10 min reaction time) and 3 min deprotection cycles at 86 °C provided the target peptide in remarkable 91% purity. Similar to the GILTVSVAV sequence described above, the identical experiment using conventional heating at 86 °C for coupling and deprotection (see Table 4) led to a similar peptide purity (87%). No evidence for any byproduct formation resulting from the loss of Boc side-chain protection was obtained. An attempted room temperature SPPS of this peptide, even using extended reaction times (3 equiv of amino acid, 60 min coupling time, 22 min deprotection time), led to a very poor peptide purity (<20%) (see Figure S18 in the Supporting Information for a comparison of HPLC chromatograms). The application of elevated temperature SPPS therefore has allowed the preparation of the 15-mer KWKLFKKTGAVLKVL peptide in a notably shorter time frame (21 versus 3.3 h) and, more importantly, in significantly improved purity.

Racemization Studies Involving a Magainin-II-Amide Model Peptide Derivative. An obvious concern when dealing with peptide couplings and deprotection reactions at elevated temperatures is the racemization of amino acids at the α-carbon atom and/or or in the side chain (for Ile and Thr). The biological properties of proteins and peptides are critically dependent on the configuration of the backbone chiral centers, so maintaining the integrity of these centers is of significant importance in

(53) (a) Hogue Angeletti, R.; Bibbs, L.; Bonewald, L. F.; Fields, G. B.; Kelly, J. W.; McMurray, J. S.; Moore, W. T.; Weintraub, S. T. *Techniques in Protein Chemistry VIII*; Academic Press: San Diego, 1997. (b) Han, Y.; Albericio, F.; Barany, G. *J. Org. Chem.* **1997**, *7*, 4307–4312. (c) Van Den Nest, W.; Yuval, S.; Albericio, F. *J. Pept. Sci.* **2001**, *7*, 115–120.

(54) Palasek, S. A.; Cox, Z. J.; Collins, J. M. *J. Pept. Sci.* **2007**, *13*, 143–148.

TABLE 4. Synthesis of GILTVSVAV Using Microwave and Conventional Heating at the Same Temperature on Tentagel and ChemMatrix Resins[a]

		Fmoc-amino acid		coupling			deprotection			
entry		equiv	conc (M)	temp[b] (°C)	power[c] (W)	time (min)	temp[b] (°C)	power[c] (W)	Time (min)	purity[d] (%)
					Tentagel					
1	CONV	5	0.18	67		21	67		1.5 + 3.5	77
2	MW	5	0.18	67	5	20	67	20	0.5 + 2.5	82/83
3	CONV	5	0.18	86		11	86		1.5 + 3.5	89
4	MW	5	0.18	86	10	10	86	20	0.5 + 2.5	92/93
					ChemMatrix					
5	CONV	5	0.38	67		21	67		1.5 + 3.5	90
6	MW	5	0.38	67	5	20	67	20	0.5 + 2.5	91/89
7	CONV	3	0.23	86		11	86		1.5 + 3.5	91
8	MW	3	0.23	86	10	10	86	20	0.5 + 2.5	95/91

[a] Peptide synthesis was performed on a 0.036 mmol scale using RAM-Tentagel resin (loading 0.24 mmol/g) or 0.075 mmol of RAM-ChemMatrix resin (loading 0.50 mmol/g) in a 10 mL solid-phase reaction vessel (ca. 1.1 mL of solvent for the coupling step; 2 mL of 30% piperidine in DMF for the deprotection step), CEM Discover SPS (MW) or Advanced ChemTech PLS 4 × 6 (CONV). [b] Average temperature monitored by internal fiber-optic probe (OpSens FO, see Figure 3 and S9, Supporting Information). [c] Maximum magnetron microwave output power for pulsing. [d] Purity of crude peptides (analytical RP-HPLC peak area %, UV absorbance at 215 nm). Peaks between 5 and 8 min retention time were used for integration. The chromatograms are reproduced in Figure 4 and the Supporting Information (Figure S13). The identity of the target peptide was established by MALDI-TOF MS.

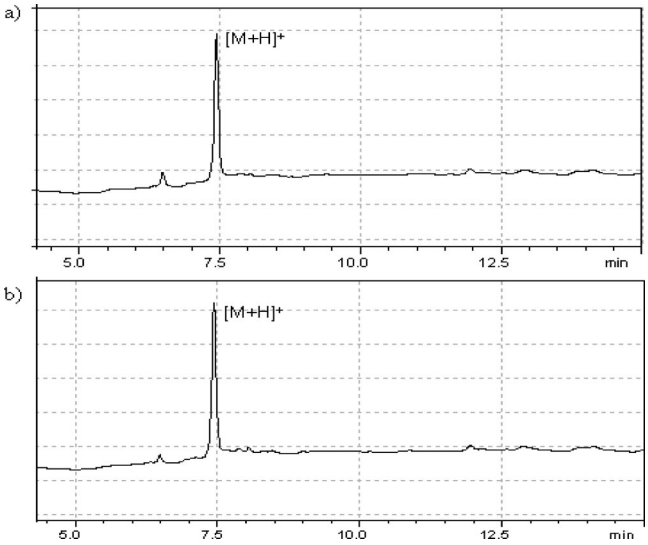


FIGURE 4. Comparison of peptide purities (HPLC chromatograms, UV absorbance at 215 nm) of GILTVSVAV peptide synthesized on ChemMatrix resin using 3 equiv of Fmoc-amino acids under (a) conventional and (b) microwave conditions at 86 °C (coupling and deprotection). For exact conditions, see Table 4, entries 7 and 8). The identity of the target peptide was established by MALDI-TOF MS (see Figure S5, Supporting Information). Figure 4a: $[M + Na]^+ = 880.6$, $[M + K]^+ = 896.6$. Figure 4b: $[M + H]^+ = 858.6$, $[M + Na]^+ = 880.6$, $[M + K]^+ = 896.6$; $M_{cal} = 857.0$.

peptide synthesis. The mechanisms leading to the, often base-induced, racemization of specific amino acids during standard Fmoc/*t*-Bu SPPS are well studied and appropriate measures to prevent or to minimize these undesired effects have been extensively documented.[2,53] Employing higher reaction temperatures for both peptide coupling and Fmoc deprotection it can be expected that racemization effects (and other side reactions like aspartimide formation) will be aggrevated.[13,54]

In order to investigate the effect of temperature on amino acid racemization during the SPPS of model peptide GILTVSVAV, samples of the purified (semipreparative HPLC) peptides resulting from the room-temperature (Table 1, entry 3) and microwave-assisted SPPS at 86 °C (Table 2, entry 9) were analyzed for amino acid racemization following standard protocols. For this purpose, the nonapeptide samples were hydrolyzed using deuterated solvents (6 N D_2O/DCl) and the enantiomeric purity of the amino acids subsequently determined by GC−MS analysis after derivatization with 2-propanol and pentafluoropropionic anhydride.[55] Gratifyingly, for all six different amino acids in the sequence there was no detectable racemization (<0.26%) in both the nonapeptide sample synthesized at 25 °C and the peptide prepared under microwave conditions at 86 °C (Table S2, Supporting Information).

Since the model nonapeptide GILTVSVAV does not contain any amino acids that are known to be prone to racemization like Cys, His, or Ser, a second model peptide was designed that contained all of the racemization-sensitive amino acids in addition to Met, which shows a tendency to oxidation. The basis for the model peptide was the difficult to synthesize 23-mer magainin-II-amide (Figure S17, Supporting Information). Magainin-II-amide is a 23-amino acid antimicrobial peptide identified from the skin of the African clawed frog *Xenopus laevis*. This peptide is water soluble, nonhemolytic at effective antimicrobial concentrations, and potentially amphiphilic. At low concentrations, it inhibits the growth of numerous species of bacteria and fungi and induces osmotic lysis of protozoa.[56,57] Since the original magainin-II-amide sequence does not contain Cys, this sensitive amino acid was incorporated on the N-terminus. When the optimized coupling/deprotection SPPS conditions described above (Table 4) were applied, the 24-mer H-Cys(Trt)-Gly-Ile-Gly-Lys(Boc)-Phe-Leu-His(Trt)-Gly-Ala-Lys(Boc)-Lys(Boc)-Phe-Gly-Lys(Boc)-Ala-Phe-Val-Gly-Glu-(OtBu)-Ile-Met-Asn(Trt)-Ser(tBu)-$CONH_2$ was obtained on ChemMatrix resin in 54% purity using microwave conditions, compared to 48% purity applying conventional heating at the same temperature. This again demonstrates the absence of any significant nonthermal microwave effect, even for longer peptide sequences. Standard room-temperature SPPS was not successful in providing this peptide in a reasonable purity (Figure S18, Supporting Information).

(55) Frank, H.; Nicholson, G. J.; Bayer, E. *J. Chromatogr.* **1978**, *167*, 187–196.
(56) Zasloff, M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5449–5453.
(57) Wenschuh, H.; Beyermann, M.; Krause, E.; Brudel, M.; Winter, R.; Schuemann, M.; Carpino, L.; Bienert, M. *J. Org. Chem.* **1994**, *59*, 3275–3280.

As expected from recently published racemization studies concerning microwave-assisted SPPS at elevated temperatures,[54] a significant amount of racemization was found for both His (ca. 7% D-His) and Cys (ca. 2% D-Cys) in the synthesis of the 24-mer using the DIC/HOBt coupling conditions at 86 °C. Importantly, the racemization levels were very similar comparing peptide samples obtained from microwave and conventionally heating experiments at 86 °C (Table S3, Supporting Information). This again indicates that the mode of heating in SPPS at elevated temperature does not have an effect on peptide purity and racemization and therefore supports the notion that nonthermal microwave effects are not involved. As demonstrated previously,[54] the racemization of sensitive amino acids in microwave-assisted SPPS can be minimized by carrying out problematic coupling steps (mainly for His and Cys) at a lower temperature regime (<50 °C).

Concluding Remarks

In summary, a critical investigation of microwave-assisted Fmoc/*t*-Bu solid-phase peptide synthesis (SPPS) under carefully controlled conditions was performed. A number of reports in the literature have advocated the use of this technology to obtain peptides not only faster but also in higher purity as compared to conventional room temperature SPPS. However, adequate control experiments performing comparison studies involving conventionally heated SPPS *at the same temperature* as in the microwave experiments have not been performed to date. Therefore, speculation on the potential involvement of nonthermal microwave effects have persisted in the literature.

Of critical importance for our evaluation of microwave-assisted SPPS was the introduction of a fast responding fiber-optic probe system as accurate temperature measurement device in *both* the microwave and the conventionally heated solid-phase reactors.[41] An initial optimization of microwave-assisted reaction conditions for the DIC/HOBt coupling and piperidine-based Fmoc deprotection steps in the SPPS of several difficult sequences has provided conditions that allow the preparation of high purity peptides on Tentagel or (preferably) ChemMatrix resin in comparatively short overall time. One of the best sets of conditions utilized 3 equiv of activated Fmoc-amino acid at 86 °C with a 10 min coupling time. For deprotection, a 3 min reaction time at the same temperature has been found to be sufficient. These reaction conditions involving elevated temperatures were found to be far superior to conventional coupling/deprotection at room temperature. Even by increasing the number of Fmoc-amino acid equivalents in the coupling step or by prelongation of the reaction time to 60 min (22 min for deprotection) the purity of the obtained peptides was significantly lower compared to the microwave-heated experiments. In the majority of cases involving the difficult sequences studied herein, room-temperature SPPS was not able to deliver the desired peptides in a synthetically useful state of purity, and deletion sequences could not be avoided.

While this clearly represents a significant practical advantage, it has to be stressed that experiments performed by conventional heating at the same temperature did provide peptides in virtually the same purity as compared to the microwave-heated experiments. For all three peptides (a 9-mer, 15-mer, and 24-mer), the purity using conventional heating was within 5% of the purity obtained by microwave heating. The small differences in performance are probably due to the fact that the temperature in the microwave-heated coupling and deprotection steps is actually a few degrees higher than measured by the fiber-optic probe. This hypothesis was supported by a control experiment performing a conventionally heated SPPS at 70 °C (instead of 67 °C), delivering the desired peptide in exactly the same purity as under microwave conditions (see above). The close match between microwave- and conventional heating in SPPS was not only evident by comparing peptide purities but did also extend to racemization studies. For peptides containing racemization-prone amino acids such as His and Cys the determined racemization levels at 86 °C (microwave or conventional heating) were nearly identical.

It can therefore be concluded that the observed enhancement effects in the microwave-assisted SPPS of the specific peptides investigated in this study are of purely thermal nature and not related to the microwave field. No evidence for a proposed deaggregation of the peptide backbone via direct interaction of the peptide chain with the microwave field could therefore be obtained for the comparatively short peptides studied herein.[14] Finally, it should be emphasized that increasing the reaction temperature from ambient conditions by 60 °C for both coupling and deprotection steps represents an estimated 50-fold increase in the reaction rate for both processes based on the Arrhenius equation. This kinetic effect is probably responsible for the highly efficient coupling and deprotection in microwave-assisted solid-phase peptide synthesis, providing peptides in high speed and purity.

Experimental Section

Microwave Irradiation Experiments. All microwave irradiation experiments described herein were performed using a single-mode Discover SPS reactor from CEM Corp. (Matthews, NC) using standard 10 or 25 mL solid-phase reaction vessels.[17] Experiments were performed in pulsed temperature control mode (SPS mode) where the temperature was controlled with an internal Discover fiber-optic probe. In control experiments, the internal reaction temperature was monitored by an additional fiber-optic probe sensor (GaAs principle, OpSens) as previously reported.[41]

Optimized Microwave-Assisted Solid-Phase Peptide Synthesis Using ChemMatrix Resin. To a 10 mL bottom-filtration reaction vessel was transferred 0.075 mmol (150 mg, loading 0.50 mmol/g) of ChemMatrix resin, which was swollen in 4 mL of DCM/DMF (1:1) for 30 min. After this time, 2 mL of 30% piperidine in DMF was added to the resin. The reaction vessel was placed into the microwave cavity and irradiated for 30 s at 75 °C (SPS mode, maximum power 20 W, $\Delta T = 3$ °C). The resin was subsequently washed with 4 mL of DMF and 2 mL of 30% piperidine in DMF were added to the sample and irradiated for an additional 2.5 min at 75 °C (SPS mode, maximum power 20 W, $\Delta T = 3$ °C). The suspension was then washed with DMF and DCM (5 × 4 mL each) and NMP (2 × 4 mL). In a separate vial the corresponding Fmoc-amino acid (0.225 mmol), DIC (0.225 mmol, 35 μL) and HOBt (0.225 mmol, 36 mg) were combined in 1 mL of NMP. The preactivated coupling cocktail was added to the resin after 2 min and the reaction mixture was irradiated at 75 °C for 10 min using the SPS program (maximum power 10 W, $\Delta T = 3$ °C). After the last deprotection step, the peptidyl resin was dried under reduced pressure.

Solid-Phase Peptide Synthesis Using Conventional Heating (Comparison Experiment). To a 10 mL bottom-filtration reaction vessel was transferred 0.075 mmol (150 mg, loading 0.50 mmol/g) of ChemMatrix resin, which was swollen in 4 mL of DCM/DMF (1:1) for 30 min. After this time, 2 mL of 30% piperidine in DMF was added to the resin. The reaction vessel was placed in an Advanced ChemTech PLS 4 × 6 synthesizer (preheated to 86 °C) and gently agitated at 86 °C for 1.5 min. The resin was then washed

with 4 mL of DMF, and 2 mL of 30% piperidine in DMF was added subsequently added to the sample which was placed in the PLS 4 × 6 synthesizer for an additional 3.5 min at 86 °C. The suspension was then washed with DMF and DCM (5 × 4 mL each) and NMP (2 × 4 mL). In a separate vial, the corresponding Fmoc-amino acid (0.225 mmol), DIC (0.225 mmol, 35 μL), and HOBt (0.225 mmol, 36 mg) were combined in 1 mL of NMP. The preactivated coupling cocktail was added to the resin after 2 min, and the reaction mixture was heated with agitation at 86 °C for 11 min in the PLS 4 × 6 synthesizer. After the last deprotection step, the peptidyl resin was dried under reduced pressure.

Final Cleavage from the Resin. The H-Gly-Ile-Leu-Thr-Val-Ser-Val-Ala-Val-$CONH_2$ peptide was cleaved from the solid support with a cleavage cocktail (5 mL) of TFA/triisopropylsilane/water (95:2.5:2.5 v/v) at ambient temperature for 2 h. For cecropin A(1−7)−mellitin(2−9) hybride peptide and mangainin-II-amide, TFA/ethanedithiol/thioanisole/water (90:4:4:2 v/v) was used as a cleavage cocktail. The resin was filtered and washed with a small amount of cleavage cocktail. The residual product was precipitated with ice-cold diethyl ether and the peptide was collected by filtration, dissolved in deionized water, and lyophilized. Further details of peptide coupling protocols are given in the Supporting Information.

Acknowledgment. This work was supported by a grant from the Christian Doppler Society (CDG). We thank Thorsten Radolf for performing the MS experiments and Dr. Gerald Rechberger for helpful discussions.

Supporting Information Available: Description of general experimental procedures, heating profiles for reactions, chromatograms, and MS spectra of peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

JO8013897