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Communications

Enhanced Coupling Efficiency in Solid-Phase Peptide Synthesis by Microwave Irradiation

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Summary: Procedures have been developed for increasing coupling efficiency in solid-phase peptide synthesis by microwave irradiation using a kitchen microwave oven.

Recently there has been growing interest in applying microwave heating to rapid thermal digestion prior to element and chemical analysis of inorganic and biological samples.[1] The rapid heating capability of the microwave oven leads to a considerable saving in dissolution time, which may eventually replace some of the conventional flame and hot-plate heating procotols.[2,3] We describe here a novel application of microwave technology to enhance coupling efficiency in solid-phase peptide synthesis. A significant improvement of the coupling efficiency (a rate increase of at least 2–4-fold), especially in side-chain-hindered amino acids, was obtained in the study.

Figure 1 shows the reaction apparatus and vessels.[4] The custom-made solid-phase reaction vessel was placed in the middle of the microwave oven, and a Teflon tube from the side arm of the reaction vessel was connected to a nitrogen source to introduce a stream of nitrogen. The Fmoc-protected amino acids with two different coupling methods, symmetric anhydride and pre-formed *N*-hydroxybenzotriazole active ester (HOBt), were used in the synthesis.[5,6]

In a preliminary test, two symmetric anhydride derivatives of Fmoc-Ile and Fmoc-Val were coupled with Gly-HMP-resin,[7] respectively. The reaction was conducted via microwave irradiation for 2–6 min and stopped by filtering off the reaction solution via the side arm. For measuring the concentration of unreacted amino acid residue on the resin, the resulting resin (containing Fmoc-peptide-resin and unreacted Gly-Resin) was further coupled with three equiv of Fmoc-Ala. The peptides were cleaved from the resin using trifluoroacetic acid. The yield of product was measured by HPLC analysis, and the peak identification was compared with the authentic sample. Figure 2 shows the time course for the dipeptide formation. The coupling reaction of these two refractory and sterically hindered β-branched amino acid derivatives to form protected dipeptide on resin usually takes a longer time than do most other amino acid derivatives.[8] The reaction was slow when

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(1) (a) Nadkarni, R. *Anal. Chem.* **1984**, *56*, 2233. (b) Fischer, L. B. *Anal. Chem.* **1986**, *58*, 261. (c) Fernando, L. A.; Heavner, W. D.; Gabrielli, C. C. *Anal. Chem.* **1986**, *58*, 511. (d) Kingston, H. M.; Jassie, L. B. *Anal. Chem.* **1986**, *58*, 2534.

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(4) The microwave oven used was a commercially available cooking apparatus without any modification (Tatung microwave oven TMO-110, Tatung Co., Taipei, Taiwan). The total power of the microwave was 650 W with a 9 power setting from 72 W.

(5) (a) Atherton, E.; Sheppard, R. C.; Wade, J. D. *J. Chem. Soc., Chem. Commun.* **1983**, 1060. Fields, G. B.; Noble, R. L. *Int. J. Peptide Protein Res.* **1990**, *35*, 161. (b) Atherton, E.; Cameron, L.; Meldal, M.; Sheppard, R. C. *J. Chem. Soc., Chem. Commun.* **1986**, 1763. (c) Paquet, A. *Can. J. Chem.* **1982**, *60*, 976.

(6) The symmetric anhydride was commercially available, and the pre-formed N-protected amino acid active ester was prepared by using 1 equivalent (equiv) of *N*-Fmoc amino acid, 1.2 equiv of HOBt, and 1.0 equiv of DCC.

(7) (a) Wang, S. S. *J. Am. Chem. Soc.* **1973**, *95*, 1328. (b) Lu, G.; Mojsov, S.; Tam, J. P.; Merrifield, R. B. *J. Org. Chem.* **1981**, *46*, 3433. (c) Sieber, P. *Tetrahedron Lett.* **1987**, *28*, 6147. (d) Bernatowicz, M. S.; Kearney, T.; Neves, R. S.; Koster, H. *Ibid.* **1989**, *30*, 4341.

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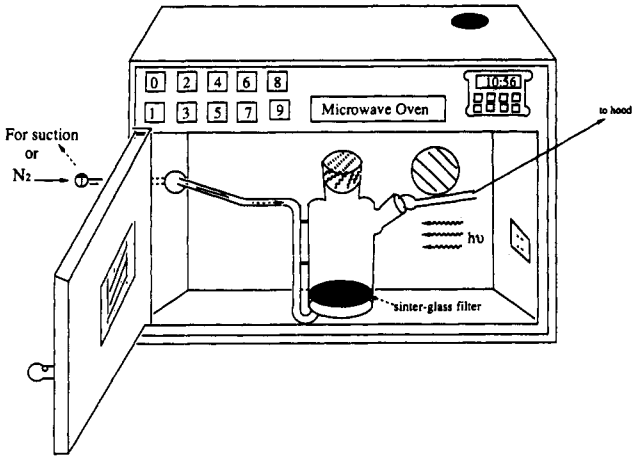

Figure 1. Microwave oven and the custom-made solid-phase reaction vessel. The vessel was left in the middle of the microwave oven, and a Teflon tube from the side arm of the reaction vessel was connected to a nitrogen source. During microwave irradiation, a stream of nitrogen was blown into the reaction vessel, and the nitrogen gas bubbles served as a stirrer. After irradiation was stopped, the reaction solution was filtered off via the side arm by suction.

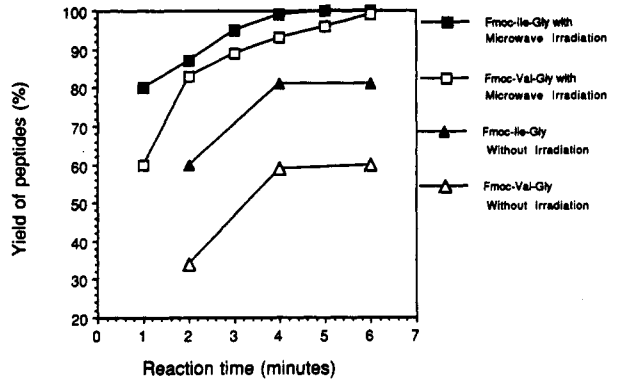

Figure 2. Time courses for the solid-phase peptide synthesis of Fmoc-peptides with and without microwave irradiation in DMF solution: -■-, Fmoc-Ile-Gly-resin with microwave irradiation; -▲-, Fmoc-Ile-Gly-resin without microwave irradiation; -□-, Fmoc-Val-Gly-resin with microwave irradiation; -△-, Fmoc-Val-Gly-resin without microwave irradiation.

the coupling was conducted without microwave irradiation. In the case of microwave irradiation using the lowest power (10% of full power), the reaction rate increased at least 2–3-fold under comparable conditions, and the coupling reaction of both dipeptides could be completed in 6 min whereas without irradiation both reactions only reached a level of 60% and of 79%, respectively. Under these reaction conditions, the temperature of the reaction solution was near 55 °C.[2b,c]

Significantly improved coupling efficiency with microwave irradiation was observed in all the tested amino acid derivatives.[9] In a further study, two peptide fragments, Fmoc-Val-Ile-OH and Fmoc-Ala-Val-Ile-OH, were coupled with Gly-HMP-resin. Surprisingly, all the coupling reactions were completed within 2 min. The coupling yield of each step was determined by quantitative ninhydrin assay[10] and by calculating the relative concentration of the

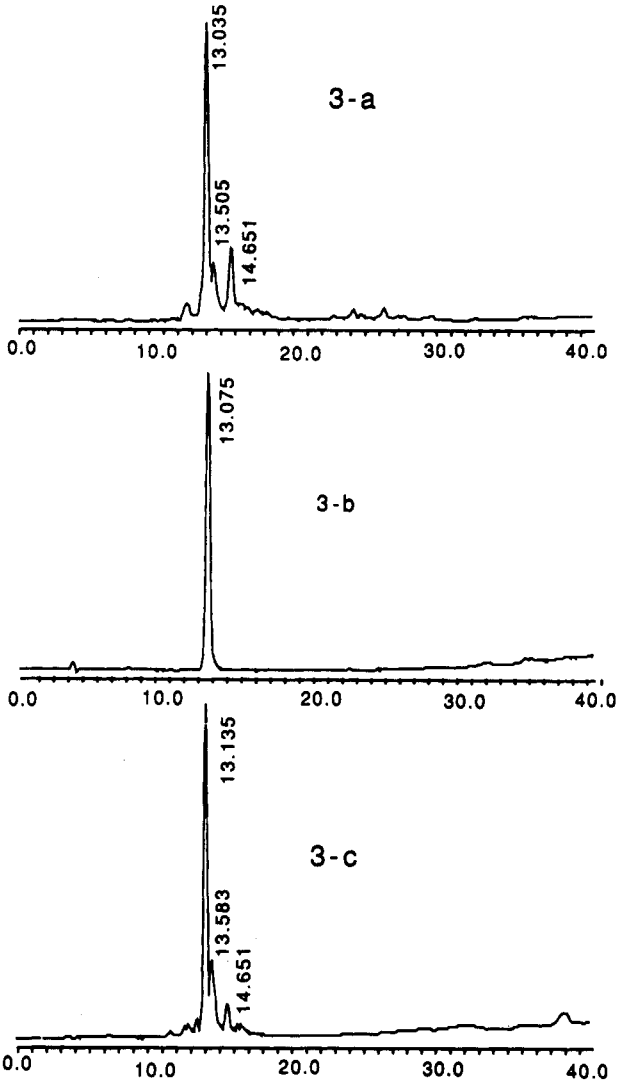

Figure 3. Hplc analysis of three $^{65-74}$Acp's. 3-a is a crude $^{65-74}$Acp synthesized using microwave irradiation. 3-b is a pure $^{65-74}$Acp purified by HPLC from 3-a. 3-c is a crude $^{65-74}$Acp synthesized by an autosynthesizer method. The HPLC was performed on a Gilson instrument, which consisted of a Model 302 pump, a Model 305 pump, a Model 115 UV detecter, and a Model 811b dynamic mixer. The signal of analysis was collected and plotted by a Rainin Dynamax which runs on a Macintosh LC. The conditions for the analysis are as follows: eluent A, 0.1% TFA in water/acetonitrile (9:1), eluent B, 0.1% TFA in water/acetonitrile (1:9), gradient, B%, 0 → 100%, 40 min, UV, 214 nm, flow rate, 1 mL/min, column, RP-18, 4.6 × 250 mm.

product and the unreacted peptide using HPLC analysis.[11]

For a representive test, three peptides, $^{65-74}$fragment of acyl carrier protein ($^{65-74}$Acp, sequence see Scheme I), Gly-Val-Gly-Phe-Val-Ile-Gly, and Gly-Phe-Gly-Val-Ala-

(8) (a) Kent, S.; Clark-Lewis, I. In *Synthetic Peptides in Biology and Medicine*; Alitalo, K., Partanen, P., Vaheri, A., Eds.; Elsevier: Amersterdam, 1985; p 29. (b) Van Woerkom, W. J., Van Nispen, J. W. *Int. J. Peptide Protein Res.* **1991**, *38*, 103–113.

(9) Enhancement of coupling efficiency of following amino acid derivatives with prelode-amino acid resin was observed also: Phe+Val; Ile+Val; Leu+Val; Val+Ile; Gly+Val; Ala+Gly–Val; Leu+Ala–Gly–Val.

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(11) In a typical reaction, to amino acid on HMP-resin (0.5 g, ca. 0.25 mequiv of amino acid) suspended in dimethylformamide (10 mL) was added 3.0 equiv (0.75 mmol) of symmetric anhydride or of the pre-formed N-protected amino acid active ester. Nitrogen gas was bubbled through the side arm into the reaction vessel and served as a stirrer. The reaction was conducted via microwave irradiation for 2–6 min and stopped by filtering off the reaction solution via the side arm. Small amounts of the sample with resin were taken for ninhydrine test. Then, Fmoc-Ala (3 equiv), DCC (3 equiv) was added and reacted at room temperature for 6 h. The product was cleaved from the resin using trifluoroacetic acid (cleavage conditions followed the procedure of the *Howard Florey Fmoc Workshop Manual* (1985) by J. Wade & G. Tregear or see Applied Biosystem User's Manual, pp 2–21). The purity of product was measured by HPLC analysis, and the peak identification was compared with the authentic sample.

Scheme I[a]

$^{65-74}$Acp: H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-Resin

with microwave irradiation:	99.9	100	100	99.8	99.5	99.9	99.9	99.7	99.8
without microwave irradiation:	80.4	83.1	87.9	94.8	93.1	76.7	70.5	79.9	83.4
ABI 431-A Synthesizer:	99.4	99.9	100	99.8	98.9	99.9	98.9	99.7	99.5

peptide-1: H-Gly-Phe-Gly-Val-Ala-Val-Ile-Gly-Resin

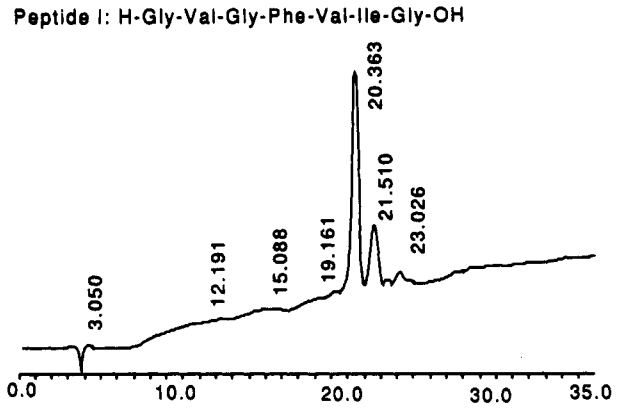
with microwave irradiation:	100	99.9	99.8
without microwave irradiation:	76.4	71.2	46.8

peptide-2: H-Gly-Val-Gly-Phe-Val-Ile-Gly-Resin

with microwave irradiation:	99.8	99.7	100
without microwave irradiation:	58.5	73.7	47.9

[a] Based on a quantitative ninhydrine test in which the average coupling yield in $^{65-74}$Acp is 99.14%; for the other two peptides the positions of the peptide bond formation are denoted by an array, and the yields of each coupling step are shown on the array.

Val-Ile-Gly, were synthesized using pre-formed active ester in DMF, and each coupling step included irradiation for 4 min. The $^{65-74}$Acp was synthesized by stepwise coupling of amino acid derivatives, and the other two peptides were synthesized by fragment coupling. After the elongation finished, the weight increase by the peptide coupled to the resin was measured and calculated to have an average coupling yield of 99.65%. The coupling efficiency (by ninhydrine assay) in each step is shown on Scheme I. With microwave irradiation, peptide bond formation was completed within 4 min. Compared with the reaction without microwave irradiation at room temperature for 30 min, the latter was relative slow, especially in the case of fragment coupling. Figure 3 shows the Hplc analysis results of crude $^{65-74}$Acp, pure $^{65-74}$Acp (obtained by collecting the desired peak in crude Hplc analysis), and a crude $^{65-74}$Acp synthesized by an autosynthesizer method (ABI-431A). The peak of crude $^{65-74}$Acp synthesized by the microwave irradiation method had a yield of 79.0% (3-a), whereas the peak of $^{65-74}$Acp from the autosynthesizer had a yield of 69.4% (3-c). Figure 4 shows the HPLC analysis of the two crude products that were made by fragment coupling of Fmoc-Val-Ile-OH and Fmoc-Ala-Val-Ile-OH. The purity of each major peak was >85%. The product was characterized by amino acid analysis and FABMS.[12] The D-amino acid content of each synthetic peptide was measured by a GC analysis of the TFA derivatives of the peptide hydrolysates using a chiral column.[13] No detectable racemization was observed. This results coincided with a recent report which showed that when a pre-formed active ester of Fmoc-Val or of Fmoc-Me-Leu coupled with



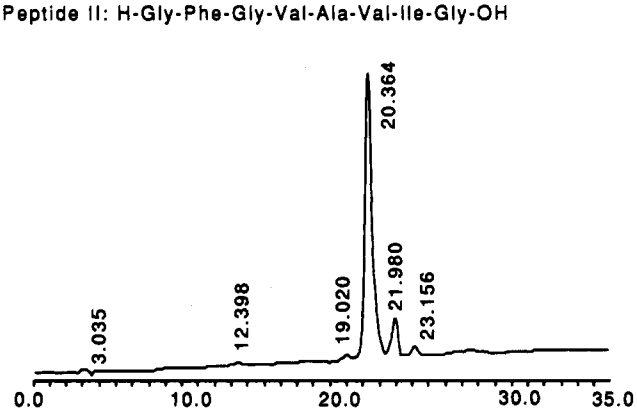


Figure 4. HPLC analysis of peptides H-Gly-Phe-Gly-Val-Ala-Val-Ile-Gly-OH (4-a) and H-Gly-Val-Gly-Phe-Val-Ile-Val-Ile-Gly-OH (4-b) using the same HPLC. The conditions for analysis of both samples are as follows: eluent A, 0.1% TFA in water/acetonitrile (95:5), eluent B, 0.1% TFA in water/acetonitrile (25:75), gradient B%, 0 → 100%, 0 → 35 min, UV, 214 nm, flow rate, 1 mL/min, column, RP-18, 4.6 × 250 mm.

an MBHA-resin or with an Me-Leu-BHA-resin in DCM/DMF (1:1) solution, respectively, no racemization (<0.1%) was found in HPLC.[14] We reasoned that the reaction step that can easily cause racemization is during DCC activated to form peptide bond in the presence of tertiary amine[15] and not in the formation of a symmetric anhydride or an active ester in the absence of amino component.

Using microwave irradiation to hydrolyze peptides and proteins will result in substantially less racemization.[16] We have studied microwave-accelerated coupling reactions in different solvents and at different reaction temperatures by control of energy input in microwave power.[17] It has been found that no significant racemization side reactions have occurred in the dipeptide products from the coupling reaction between different amino acid derivatives. Increased coupling efficiency in solid-phase peptide synthesis using elevated temperature has been shown,[18] but the use of microwave irradiation has not been investigated before. In conclusion, we have presented here a new reaction

(12) The data for all compounds are listed as follows: peptide (mw), MS, amino acid analysis, calcd, found. (a) Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly (1063.3), 1063, Asp–Glu–Gly–Ala–Val–Ile–Tyr, calcd 2:1:1:2:1:2:1, found 2.0:1.0:1.0:2.0:1.0:1.8:0.9. (b) Gly-Phe-Gly-Val-Ala-Val-Ile-Gly (719.0), 719.5, Gly–Ala–Val–Ile–Phe, calcd 3:1:2:1:1:1, found 3.0:1.0:1.9:0.9:1.0. (c) Gly-Val-Gly-Phe-Val-Ile-Gly (647.9), 648.6, Gly–Val–Ile–Phe, calcd 3:2:1:1, found 3.0:1.9:0.8:1.0.

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protocol by using a common microwave oven in solid-phase peptide synthesis. The procedure not only reduced the needed reaction time of 2–3 h reacting at room temperature, or of 30 min by reacting at 60 °C, to less than 6 min via the microwave irradiation method but also accomplished the complete coupling of difficult sequence peptides. The reaction apparatus is simple and can potentially be designed for an autosynthesizer. Under microwave irradiation conditions, the peptide fragments have higher reactivity than do the amino acid derivatives, which is very useful for the synthesis of big peptides. That using a dipeptide or a tripeptide instead of amino acid derivatives in the same synthesis steps will make a peptide which has longer amino acid residue. Also, in the synthesis of same peptide, the fragment coupling will only need half or one-third of the coupling steps especially for sterectically hindered amino acid. The development of a convenient method for preparation of peptide fragment will be very useful for the synthesis of big peptides.[19]

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Amination of Nitroarenes with Sulfenamides via Vicarious Nucleophilic Substitution of Hydrogen[1]

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Summary: Nitroarenes react with sulfenamides $RSNH_2$ in the presence of strong bases to give *p*- and *o*-nitroanilines.

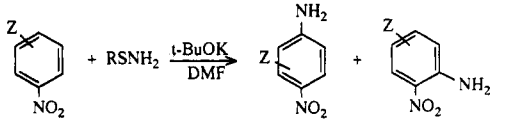
The vicarious nucleophilic substitution of hydrogen (VNS) is presently a well-established methodology for introduction of carbon substituents into electrophilic aromatic rings.[2] Recently, a similar reaction was discovered between nitroarenes and the anions of alkyl hydroperoxides to produce nitrophenols.[3]

Although direct nucleophilic amination of electrophilic arenes, particularly heterocycles, has been thoroughly investigated,[4] and important methods such as the Chichibabin reaction or oxidative amination[5] are known, no simple and general method for direct amination of mononitroarenes is available. An old method of amination with hydroxylamine is applicable only to nitro derivatives of bicyclic arenes or dinitroarenes.[6] A much more general method of amination with 4-amino-1,2,4-triazoles, described recently by Katrizky, is of somewhat limited use because of moderate availability of the triazoles and some limitations of its scope.[7]

Our experience in the VNS reaction with carbanions suggested that X in an aminating agent of general structure $X-NH_2$ should be able to stabilize the negative charge on the neighboring atom and to be eliminated from the intermediate σ-adduct in the form of HX. These requirements should be best fulfilled by groups X = RS which are known to stabilize carbanions[8] and are widely used in the VNS reactions.[9] We expected therefore that sulfenamides of general structure $RSNH_2$ should be able to aminate nitroarenes in the presence of strong bases, similarly as $RSCH_2CN$[10] or $RSCH_2SR$[11] effect corresponding alkylation.

The possibility of using sulfenamides for nucleophilic amination of nitroarenes was, however, not obvious to us, because very little is known about anions of sulfenamides and existing data indicate that they are of low stability.[12] Only sulfenamides which contain additional electron-withdrawing substituents at the nitrogen atom form stable anions,[13] but the latter are insufficiently nucleophilic for

Table I



Z	$RSNH_2$	position of NH_2	yield (%)
H	1	2	14
		4	71
	2	2	34
		4	35
2-MeO	1	4	39
2-CF_3	1	4	71
3-Cl	1	4	86
3-CF_3	1	4	91[a]
4-tBu	2	2	63
4-Cl	2	2	60

[a] The reaction was carried out using KOH in liquid NH_3.

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