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The Solid Phase Synthesis of Peptides Containing an Arginine Residue with an Unprotected Guanidine Group

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Abstract—A new variant of the solid phase synthesis of arginine-containing peptides was proposed. The conditions for the attachment to the Wang polymer of N^{α}-Fmoc-arginine containing a protonated guanidine group were found. We demonstrated that this attachment is accompanied by neither racemization nor the attachment of the second Arg residue. Side reactions involving the guanidine group of arginine were studied, and methods for their prevention were proposed. The comparison of the carbodiimide method with a 1-hydroxybenzotriazole additive and a modified method with the use of Kastro's reagent for the introduction of N^{α}-Fmoc-Arg residue with the unprotected guanidine group into the growing peptide chain demonstrated the advantages of the second method. Bradykinin and a peptide corresponding to the 584-591 sequence of the transmembrane gp41 from HIV-1 were synthesized by the method proposed here.

Key words: arginine, guanidine group; peptides, solid phase synthesis; Kastro reagent; ^1H NMR spectroscopy; bradykinin

INTRODUCTION

The solid phase synthesis of peptides containing one or several Arg residues in their chains inevitably confronts problems associated with the protection of the guanidine group (N^{G}), because this group can be acylated during the coupling process and the removal of the N^{G}-protecting groups might also cause some complications.[2] It has been found that the protection of N^{α}-Fmoc-derivatives of arginine by the N^{G}-protecting urethane-type groups (Boc or Adoc) does not prevent the acylation of the guanidine function and the formation of ornithine [1, 2]. On the other hand, the deprotection of a peptide containing several Mtr-protected Arg residues requires rigorous conditions and prolonged treatment that can result in the formation of some by-products [3, 4].

We faced exactly this problem while synthesizing the 584-618 fragment of the transmembrane HIV gp41 and using the Mtr protecting group [4]. After the deprotection and cleavage of the peptide from the polymer, we isolated and characterized a by-product formed by the interaction of the tryptophan indole ring with TFA and 1,2-ethanedithiol. We prevented the formation of this by-product by using an N^{ω}-Pmc protecting group, whose removal required a less prolonged treatment. However, the Pmc derivative of arginine is an expensive compound whose synthesis consists of multiple stages.

The use of arginine protected by protonation in the SPPS has been described several times. An antagonist of Luteinizing Hormone Releasing Hormone was synthesized thus using Boc-arginine hydrochloride [5]. The successful synthesis of the 4–15 fragment of λ-crorepressor with the use of the Fmoc-strategy of SPPS and with the protection of the guanidine group of arginine by protonation has also been reported [6]; however, no experimental details were given. A quite inadequate attempt to synthesize decapeptide containing one Arg residue using protonation for arginine protection was described in [7]: the resulting peptide contained a 25% admixture of the peptide with no Arg residue.

RESULTS AND DISCUSSION

Taking all these facts into account along with our own experience in the conventional syntheses of peptides containing Arg residues with a protonated guanidine group in solution [8, 9], we decided to study the application of this type of arginine protection in SPPS with the Fmoc strategy. We had to solve two problems: the attachment of the *C*-terminal Fmoc-derivative of protonated arginine to the polymer and the introduction

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[2] Abbreviations: Adoc, adamantyloxycarbonyl; BOP, 1-benzotriazoleyloxy-tris(dimethylamino)phosphonium hexafluorophosphate (Kastro's reagent); DCC, *N,N'*-dicyclohexylcarbodiimide; DIPCDI, *N,N'*-diisopropylcarbodiimide; DMAP, 4-dimethylaminopyridine; HOBt, 1-hydroxybenzotriazole; EDT, 1,2-ethanedithiol; ESI MS, the electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethyloxycabonyl; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; ONp, *p*-nitrophenyl; *P*, the Wang polymer; Pmc, 2,2,5,7,8-pentamethylchromane-6-sulfonyl; SPPS, solid phase peptide synthesis; and TFA, trifluoroacetic acid.

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Table 1. Attachment of Fmoc-Arg(H+)-OH to the Wang polymer with a content of hydroxymethyl groups of 0.45 mmol/g by the DIPCDI method*

Catalyst	Amount of the catalyst, mol/mol of amino acid	Reaction time, h	Substitution degree	
			mmol/g	%
DMAP	0.2	1	0.056	12
		24	0.160	36
Imidazole	1	24	0.037	8
	2	24	0.040	9
Imidazole-HOBt	0.7	1	0.320	71
1-Methylimidazole	0.7	2	0.420	93
		24	0.410	91

* The amino acid excess was 5 equiv in relation to the hydroxymethyl groups in all cases.

of Fmoc-Arg into the growing peptide chain without any additional protection of its guanidine function.

We used Wang polymer [10] (copolymer of styrene and divinylbenzene with 4-hydroxymethylphenyloxymethyl anchoring group) with the 0.45 mmol/g content of hydroxymethyl groups as the SPPS support.

Fmoc amino acids are usually attached to the polymer carrier using a carbodiimide (DCC or DIPCDI) in the presence of DMAP catalyzing the alcoholysis [11, 12, 25]. We also used this method at first but failed to completely attach Fmoc-Arg(H+)-OH to the polymer. The amino acid content in the polymer was only 0.05–0.16 mmol/g, which corresponded to a substitution degree of 10–35%. Note that Fmoc-Arg(Pmc)-OH was attached to the same carrier under the same conditions without any complication, and the substitution degree was 82% (0.37 mmol/g).

Pyridine [13] and imidazole [14] are also well-known catalysts for the attachment of N^{α}-protected amino acids to the hydroxyl groups of the polymer. Bodanszky and Fagan have studied the kinetics and mechanism of the acetylation of hydroxyl compounds catalyzed by pyridine, imidazole, and 1-methylimidazole [15]. 1-Methylimidazole was shown to be the most effective catalyst in this process, since it provides a reaction rate two to three orders of magnitude higher than pyridine or imidazole.

We also found that 1-methylimidazole is a better catalyst than the other bases for the attachment of Fmoc-Arg(H+)-OH to the Wang resin by the carbodiimide method (Table 1), and we established that the conditions providing for the 0.42 mmol/g (93%) content of the starting amino acid in the resin should be the following: a fivefold excess (in relation to the content of hydroxyl groups in the polymer) of equimolar amounts of Fmoc-Arg-OH, pyridine hydrobromide, and DIPCDI, a 0.7 equiv of 1-methylimidazole in relation to the amino acid, and a reaction time of 2 h at room temperature.

We used the resulting Fmoc-Arg(H^+)-O**P** with an amino acid content of 0.42 mmol/g for the synthesis of the first model peptide, bradykinin (H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH), whose conventional synthesis in solution had been well studied.

The presence of a base (e.g., DMAP) at the attachment of Fmoc-arginine (like any other amino acid) to the polymer can induce side reactions such as racemization and the formation of an Arg-Arg dipeptide substituent [11, 12, 16]. 1-Methylimidazole is a weaker base (pK_a 7.0) [13] than DMAP (pK_a 9.7) [17] and is not expected to facilitate these reactions. However, we needed to examine the possibility of its use.

We synthesized the model peptides H-Phe-Arg-OH, H-Phe-*D*-Arg-OH, and H-Phe-Arg-Arg-OH and characterized them by ^{1}H NMR and ESI MS in order to determine the degree of Arg racemization and to reveal the possible formation of the dipeptide substituent during arginine attachment to the polymer.

When preparing H-Phe-Arg-OH, we found that the raw product obtained after the cleavage from the polymer by 95% TFA consisted of several compounds according to HPLC but contained neither a significant amount of the *D*-Arg containing isomer nor a considerable quantity of H-Phe-Arg-Arg-OH (Figs. 1a and 1b). We thus demonstrated that the attachment of Fmoc-Arg(H^+)-OH to the resin was accompanied neither by racemization of Arg nor by formation of the Arg-Arg substituent.

Since the content of the target dipeptide H-Phe-Arg-OH in the raw product turned out to be only 60%, we separated it by HPLC and isolated a number of by-products. According to their ^{1}H NMR spectra, these were products of the acylation of the arginine guanidine

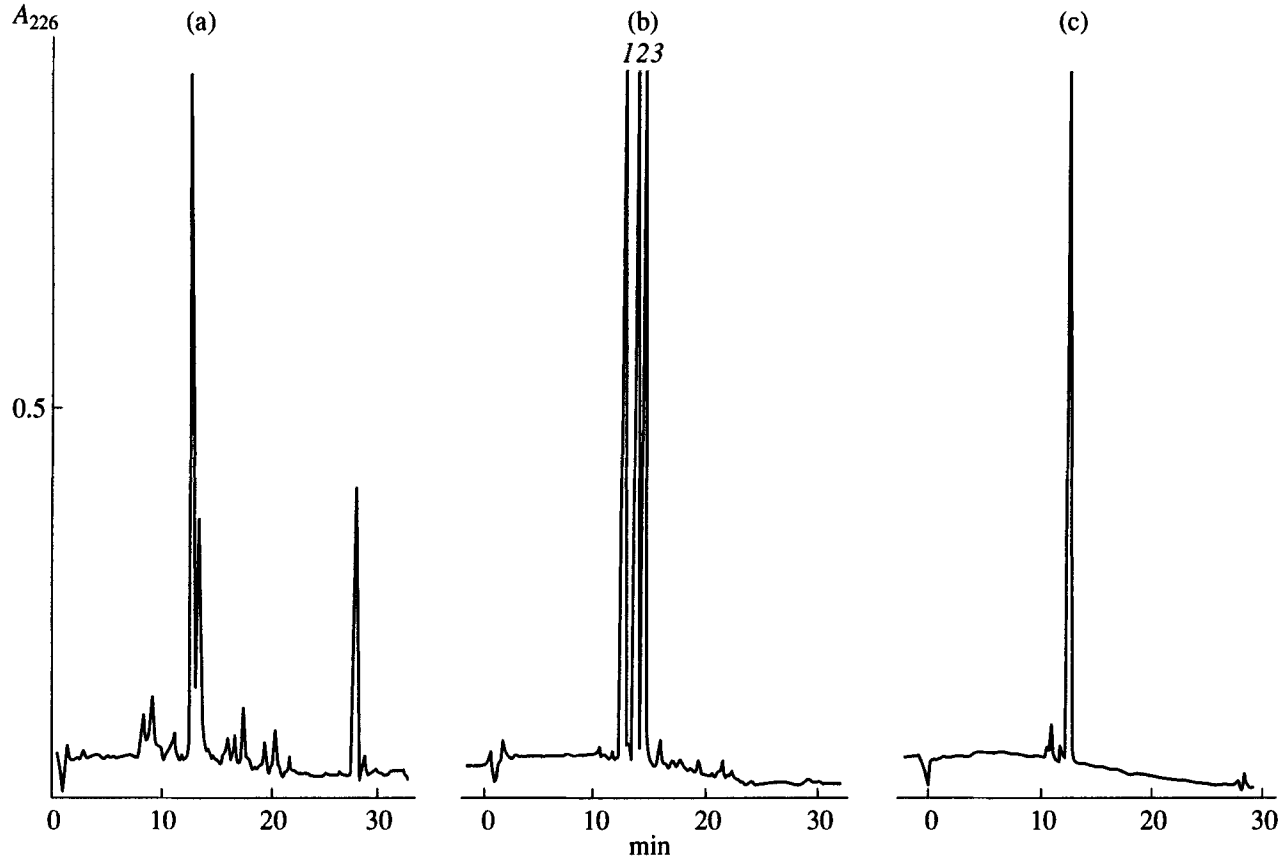
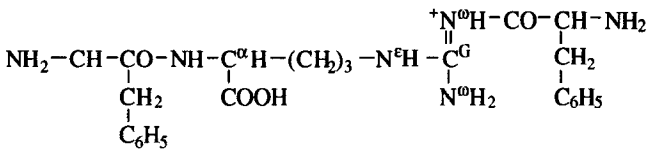


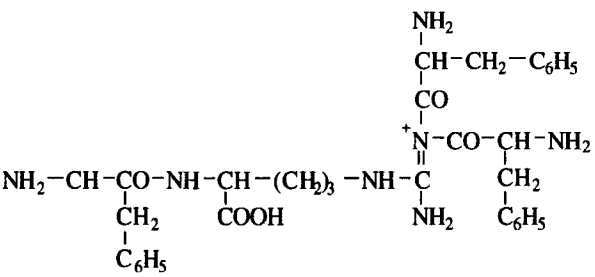
Fig. 1. Analytical HPLC of (a) raw H-Phe-Arg-OH; (b) artificial mixture of (*1*) H-Phe-Arg-OH, (*2*) H-Phe-*D*-Arg-OH, and (*3*) H-Phe-Arg-Arg-OH; and (c) raw H-Phe-Arg-OH prepared by synthesis with an additional protonation step for the guanidine group. Conditions: a Nucleosil C-18 column (5 μm, 4.6 × 150 mm), elution with gradient 1 (see the Experimental section for details).

group by phenylalanine: H-Phe-Arg(Phe)-OH and H-Phe-Arg(Phe-)$_2$-OH.

H-Phe-Arg(Phe-)-OH



H-Phe-Arg(Phe-)$_2$-OH



The assignment of resonances in their ^{1}H NMR spectra and the determination of their structures were performed by the methods of double resonance and the nuclear Overhauser effect and on the basis of comparison of the chemical shifts of these peptides.

The singlet low-field resonance at 10.1 ppm, additional resonances from $C^{\alpha}H$ and $C^{\beta}H$ at 4.71 ppm and 3.09 ppm, and the resonances from the aromatic ring protons of Phe at 7.30 and 7.20 ppm confirmed the formation of a bond between one of the ω-amino groups of the Arg guanidine group and the carboxyl group of phenylalanine (Fig. 2c).

It should be noted that, at 27°C, three (rather than one) singlet low-field resonances from $N^{\omega}H$ at 10.1, 10.01, and 9.65 ppm, three resonances from $C^{\alpha}H$ of arginine at 4.26, 4.21, and 4.14 ppm, the triplet resonances from $N^{\varepsilon}H$ of the guanidine group of arginine at 9.54, 9.43, and 8.73 ppm, and at least two resonances from $C^{\alpha}H$ of Phe residues bound to the guanidine group at 4.71 and 4.64 ppm are observed. Three different sets of resonances instead of one from each proton in the spectrum suggest the presence of conformers in the NMR time scale. They result from the restricted rotation around the C^{G}–N^{ε} and C^{G}–N^{ω} bonds of the guanidine group in H-Phe-Arg(Phe)-OH. This rotation is accelerated upon heating, which results in a broadening of the resonance signals and in changes in their chemical shifts. When saturating the proton resonance at 10.10 ppm, the intensity of the resonance from the sim-

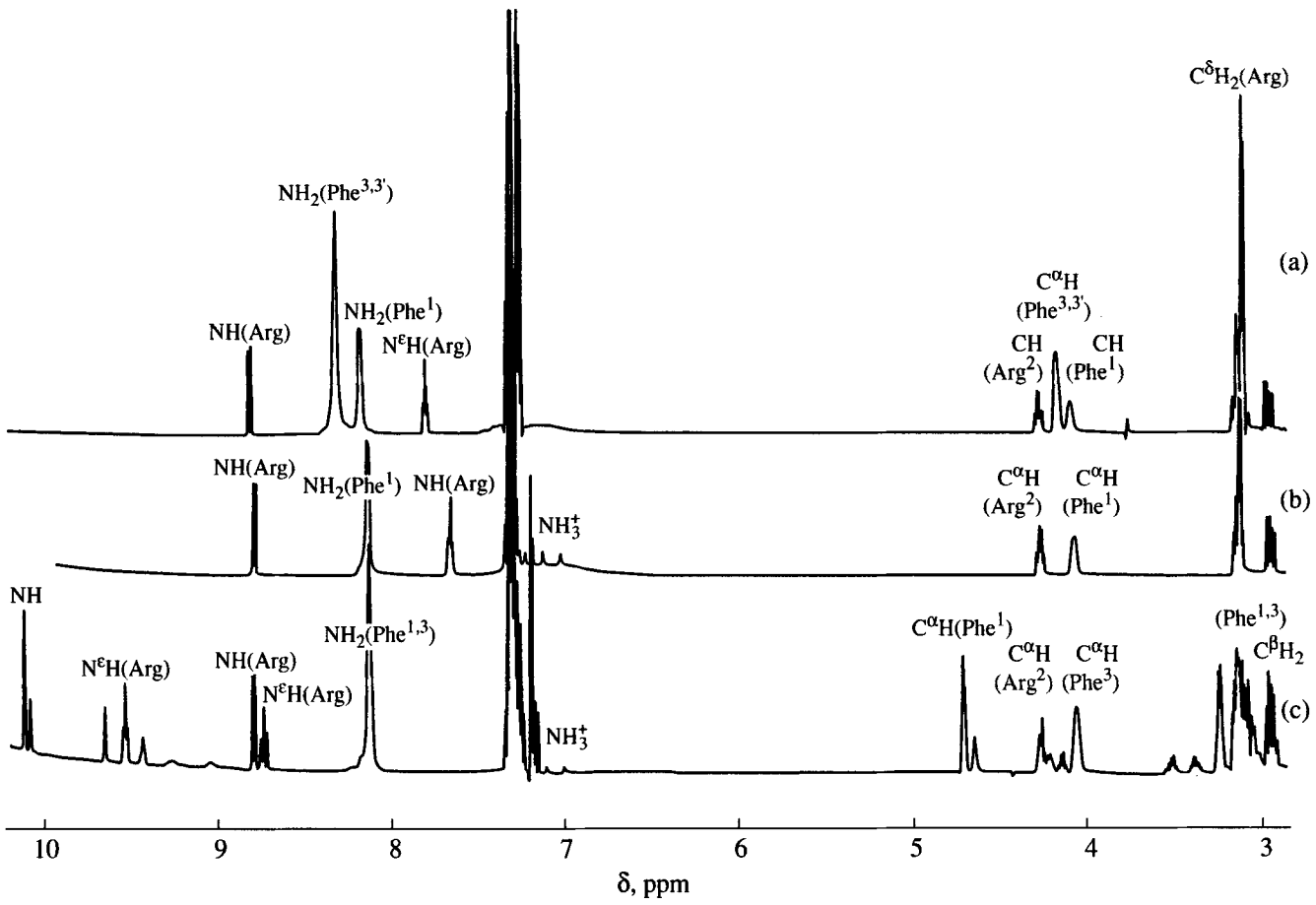


Fig. 2. ^{1}H NMR spectra of (a) H-Phe-Arg(Phe)$_2$-OH, (b) H-Phe-Arg-OH, and (c) H-Phe-Arg(Phe)-OH in DMSO-d_6 in the presence of TFA at 27°C

ilar proton at 9.65 ppm is observed in the ^{1}H NMR spectrum.

One can easily see from the spectrum of H-Phe-Arg(Phe-)$_2$-OH in Fig. 2a that two Phe residues are attached to the same N^{ω} atom of the Arg guanidine group. The absence of resonances from the $N^{\omega}H$ protons of the guanidine group (cf. Figs. 2a and 2c) confirms this. At the same time, the resonances from $C^{\alpha}H$ (4.71 ppm), $C^{\beta}H$ (3.09 ppm), and $N^{\alpha}H_2$ (8.31 ppm) from Phe3 and Phe3' completely coincide, suggesting a symmetric structure for the chimerical tetrapeptide. The molecular masses of the isolated peptides determined by ESI MS additionally confirmed the structures of these peptides.

These by-products are probably formed at the stage of deprotection of α-amino group as a result of the partial deprotonation of the guanidine group during treatment with piperidine (pK_a 11.12) and subsequent acylation in the presence of a large excess of the acylating agent. Actually, the arginine guanidine group is known to be readily acylated [1, 2]. This side reaction can be completely prevented by an additional stage of guanidine group protonation by a washing of the peptidylpolymer with a solution of the excess pyridine hydrobromide in dimethylformamide after deprotection of the amino group and before its subsequent coupling. After cleavage of the peptidylpolymer with 95% aqueous TFA, the resulting H-Phe-Arg-OH had a 97% purity according to HPLC (Fig. 1c). Its structure was confirmed by ^{1}H NMR spectroscopy (Fig. 2b).

We should stress that the *C*-terminal Arg residue with the protonated guanidine group must be attached to the polymer with the maximal degree of substitution of the polymer hydroxymethyl groups, because the standard treatment of the polymer with acetic anhydride for the blockage of unreacted hydroxyl groups is in this case impossible.

We used this treatment in the synthesis of a *C*-terminal dipeptide fragment of bradykinin (H-Phe-Arg-OH) starting from Fmoc-Arg(H^+)-**OP** having a 49% substitution degree (0.22 mmol/g) and isolated a by-product identified as H-Phe-Arg(Ac)-OH by ^{1}H NMR.

On the basis of these results, we carried out further synthesis of bradykinin involving in the protocol additional protonation steps after every deprotection of an amino group. The manual variant of SPPS was used with stepwise elongation of the peptide chain by the

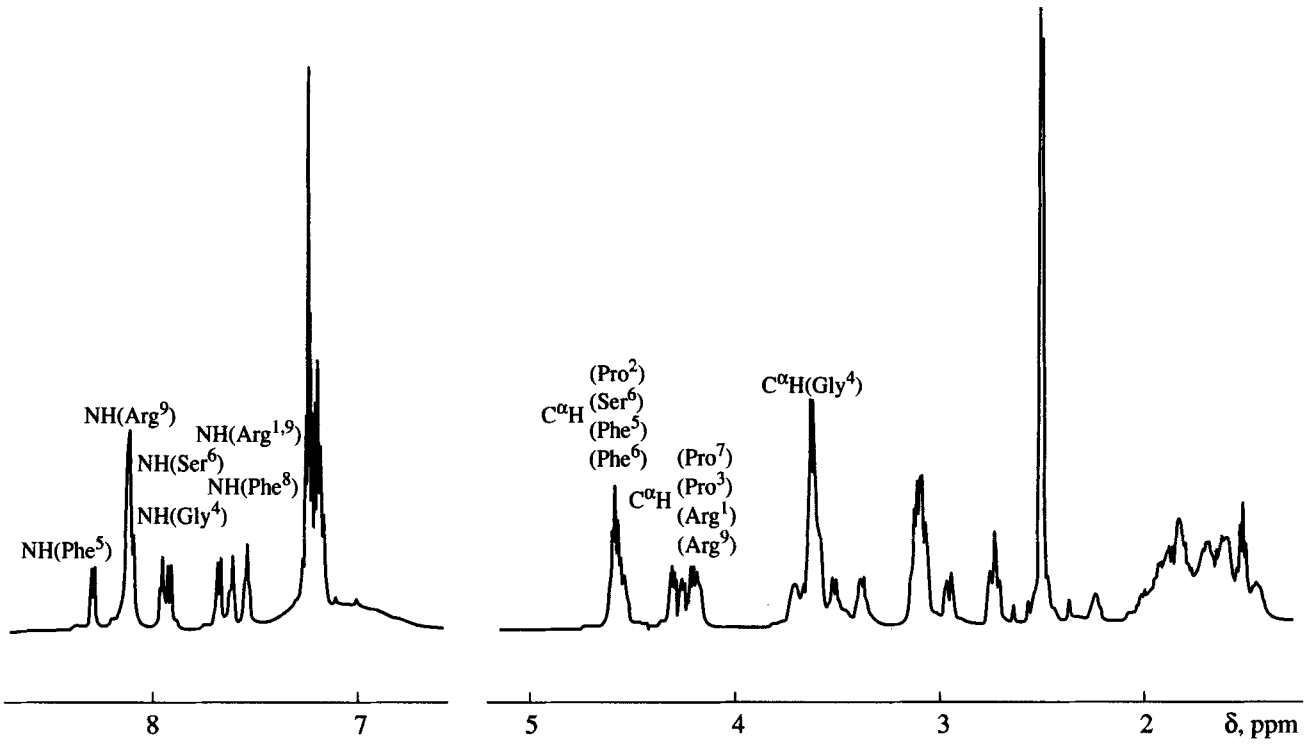


Fig. 3. ^{1}H NMR spectrum of bradykinin in DMSO-d_6 at 27°C.

DIPCDI/HOBt method. A 5-equiv excess of activated Fmoc-amino acid relative to the peptidylpolymer was used. The coupling reactions were monitored by the ninhydrin test [18] and were repeated when necessary. The control cleavage of the peptide from the polymer was carried out after every two steps, and the content of peptide was measured by analytical HPLC. Before the last condensation, the content of des-Arg^1-bradykinin in the sample was 96%.

The *N*-terminal arginine was coupled using twofold condensation by the BOP-method. As follows from the literature data, the use of BOP-reagent helps significantly accelerate the condensation reaction [19]. When condensation is performed using the Kastro reagent, several equivalents of base are added to the reaction mixture [19–21]. In the case of Fmoc-Arg with a free guanidine group, as expected, this group can serve as the organic base (pK_a 12.5) necessary for the activation. The excess base in the reaction mixture is known to promote racemization, and the absence of an additional organic base during the synthesis of peptide with the free guanidine group is advantageous in this case.

The content of bradykinin in the raw product after cleavage from the polymer and deprotection with 95% aqueous TFA was 92% according to analytical HPLC, and its yield was 65% relative to the starting amino acid. The resulting bradykinin was purified by preparative reversed-phase HPLC (for the conditions, see the Experimental section). Its structure was confirmed by ^{1}H NMR (Fig. 3) and ESI MS.

Simultaneously, we developed a method for the introduction of arginine into the growing peptide chain while synthesizing octapeptide H-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-OH corresponding to the 584-591 sequence of the transmembrane gp41 of HIV-1.

We used the Fmoc-strategy and an Applied Biosystems Model 431A synthesizer. The Fmoc-aminoacylpolymer on the basis of the Wang resin, Fmoc-Tyr(Bu^t)-OP, with an amino acid content of 0.65 mmol/g was used as a support. The Fmoc-group was removed by treatment with a 20% solution of piperidine in dimethylformamide. The side functions of the amino acids, except for the guanidine function, were protected by *tert*-butyl groups. A 10-equiv excess (relative to the peptidylpolymer) of the Fmoc-amino acid was taken.

At first, we synthesized the octapeptide with the PMC-protection of the guanidine groups of both Arg residues by the DIPCDI/HOBt method. The target peptide was cleaved from the support and deprotected by treatment with a mixture of TFA (82.5%), phenol (5%), water (5%), thioanisole (5%), and ethanedithiol (2.5%) for 1.5 h [22]. The content of the target peptide was 85% according to HPLC (Fig. 4a). The yield of the peptide after preparative reversed-phase HPLC was 59%, and the peptide had the correct amino acid composition. Its structure was confirmed by ESI MS.

We next compared two reagents, DIPCDI/HOBt and BOP, for the introduction of arginine with a free guanidine group into the growing peptide chain. In the

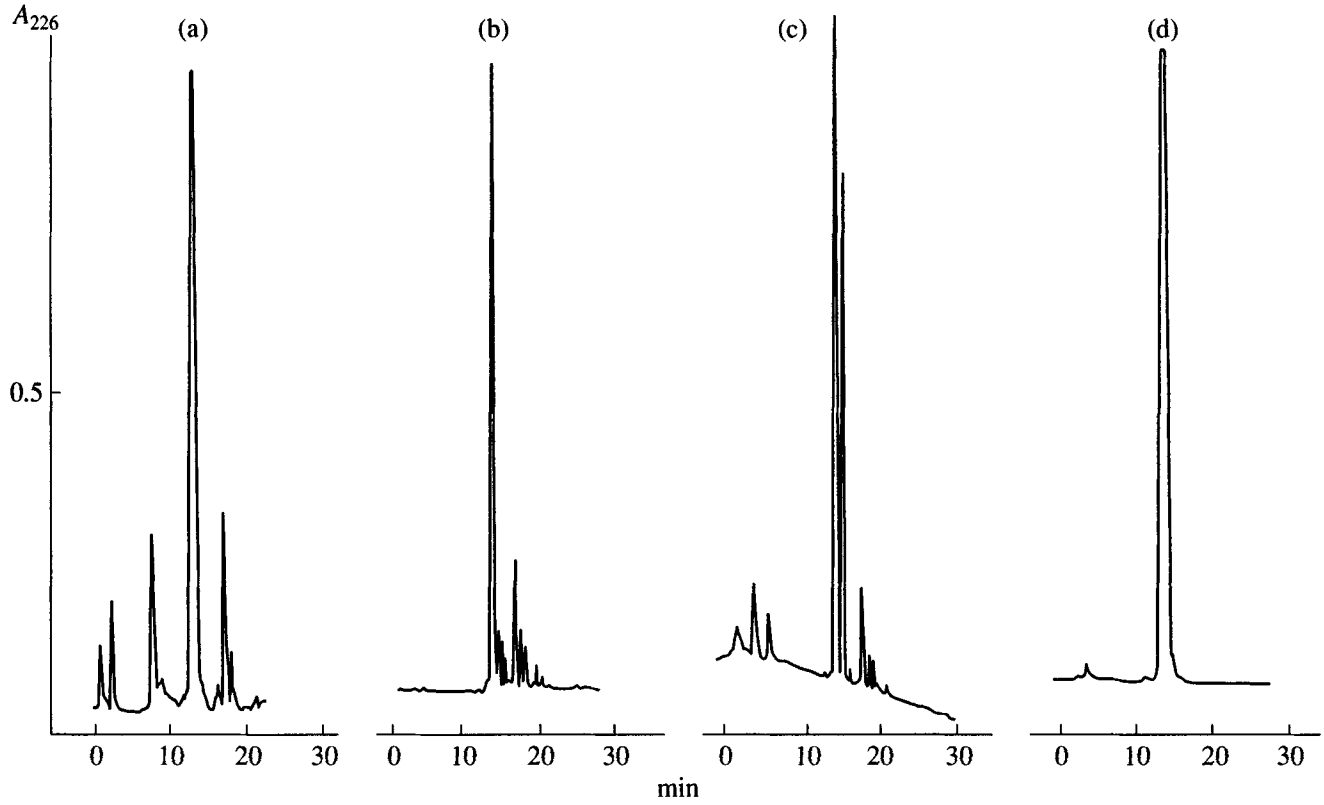


Fig. 4. Analytical HPLC of (a) raw H-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-OH prepared by the DIPCDI/HOBt method with the use of Fmoc-Arg(Pmc)-OH; (b) raw octapeptide prepared by synthesis using BOP without protection of the guanidine group; (c) artificial mixture of the *L*-octapeptide and its *D*-Arg[1]-analogue; and (d) *L*-octapeptide after preparative HPLC on a Ultrasphere ODS column (5 μm, 4.6 × 250 mm) eluted with gradient 2 (see the Experimental section for details).

first case, the guanidine group was protonated by the preliminary treatment of Fmoc-arginine with one equiv of pyridine hydrobromide, and the condensation was performed by the standard DIPCDI/HOBt method. With the second reagent, we used equivalent quantities of BOP and Fmoc-arginine without any additional protonation of the guanidine group and without any additional base in the reaction mixture.

We varied the method and the number of repetitions at both steps of the Fmoc-arginine coupling. All the other Fmoc-amino acids were attached by single DIPCDI/HOBt condensation. The peptides were cleaved from the polymer and deprotected by treatment with 95% aqueous TFA. The content of the target product was determined by analytical HPLC at every step. The impurities isolated after purification by preparative HPLC were characterized by ESI MS and quantitative amino acid analysis. These were peptides with Arg deletions in positions 1 and/or 7. It should be noted that, unlike in the bradykinin synthesis, we used no additional protonation in this synthesis. The results of the syntheses are presented in Table 2.

One can see from these data that the best results were obtained with the use of the BOP-reagent and twofold coupling at both steps of the introduction of arginine. The introduction of the Arg residue in position 1 proceeded worse than that in position 7. This can be explained by the influence of the preceding sterically hindered amino acid sequence Ile-Leu.

We synthesized the H-*D*-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-OH analogue in order to measure the possible racemization degree as the syntheses described above were performed. It was synthesized using the Fmoc-strategy on an Applied Biosystems Model 431A synthesizer by the standard DIPCDI/HOBt method. The *N*-terminal *D*-arginine was introduced by twofold coupling using the BOP reagent. The resulting peptide was purified by preparative HPLC, and its structure was confirmed by ^{1}H NMR spectroscopy. Analytical HPLC (Figs. 4b and 4c) demonstrated the complete absence of the *D*-isomer in the target peptides.

CONCLUSION

We thus demonstrated that Arg can successfully be introduced into a peptide chain using the Fmoc-strategy of SPPS without any additional protection of the guanidine group.

We recommend the use of carbodiimide in the presence of 1-methylimidazole for the attachment of Fmoc-

Table 2. A comparison of the results of the syntheses of H-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-OH under various condensation conditions at the stages of introduction of Arg1 and Arg7

Introduction stage				Content of the target product according to HPLC, %
Arg1		Arg7		
condensation method*	number of repetitions	condensation method*	number of repetitions	
DIPCDI/HOBt	2	DIPCDI/HOBt	2	55
DIPCDI/HOBt	3	DIPCDI/HOBt	2	64
BOP	1	DIPCDI/HOBt	2	58
BOP	1	BOP	2	65
–	–	DIPCDI/HOBt	2	67**
–	–	BOP	1	70**
BOP	2	BOP	1	67
BOP	2	BOP	2	80

* Other amino acid residues were introduced by the DIPCDI/HOBt method.

** The content of H-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-OH was determined by HPLC.

Arg(H^+)-OH to the Wang polymer. In this case, the maximal attachment degree of the amino acid to the polymer should be achieved and merely varying the content of hydroxymethyl groups in the starting polymer should regulate the content of the starting amino acid in the polymer. This method for the attachment of Fmoc-Arg(H^+)-OH to the resin does not result in detectable racemization or in the formation of the Arg-Arg dipeptide substituent.

The BOP method without the addition of a base in the reaction mixture is optimal for the introduction of Fmoc-Arg-OH into the growing peptide chain without the protection of its guanidine group. For example, the yields of H-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-OH obtained without guanidine group protection and with the use of Fmoc-Arg(Pmc)-OH were comparable: according to analytical HPLC, they were 80% and 85%.

In order to prevent possible acylation of the guanidine group of arginine in the peptidylpolymer during SPPS by the DIPCDI/HOBt method, we recommend including the additional protonation step after each deprotection of the Fmoc-group.

A combination of all these methods and techniques in the synthesis of bradykinin allowed us to prepare the target peptide with a high level of purity. The content of bradykinin in the raw product was 92% according to analytical HPLC.

EXPERIMENTAL

The N^{α}-Fmoc-derivatives of *L*-amino acids and *D*-arginine were from Bachem (Switzerland), Fluka (Switzerland), and Reanal (Hungary); DIPCDI, BOP, 1-methylimidazole, DMAP, HOBt, and EDT were from Fluka (Switzerland); and the Wang resin (copolymer of styrene and 1% divinylbenzene) with 4-hydroxymethylphenyloxymethyl anchoring group) and Fmoc-Tyr(Bu^t) attached the Wang resin were from Bachem (Switzerland).

DMF was successively distilled over CaO and, then, in a vacuum over ninhydrin and dried over 4 Å molecular sieves. Piperidine was distilled over NaOH. Methylene chloride was washed with concentrated sulfuric acid and water, dried, and distilled over $CaCl_2$.

Pyridine hydrobromide was prepared by bubbling gaseous hydrogen bromide through a cooled solution of pyridine in hexane. The precipitate was filtered, washed with hexane, and dried in a vacuum-desiccator.

TLC was carried out on precoated Kieselgel 60 plates (Merck, Germany) in a 60 : 45 : 20 chloroform-methanol-32% acetic acid mixture. The substances were detected by treatment with Cl_2 and benzidine [23].

Analytical HPLC was carried out on a Gilson chromatograph (France) equipped with Nucleosil C-18 (4.6 × 150 mm, 5 μm, Alltech, United States) and Ultrasphere ODS (4.6 × 250 mm, 5 μm, Beckman, United States) columns. The eluents were buffer A, 0.1% TFA in water, and buffer B, 80% acetonitrile in buffer A. The columns were eluted with gradient 1 (from 0 to 30% of buffer B in buffer A within 30 min) and gradient 2 (from 10 to 70% of buffer B in buffer A within 30 min). The flow rate was 1 ml/min, and the detection was at 226 nm. For the preparative HPLC, a Beckman chromatograph (United States) equipped with a Diasorb C-16 column (24 × 250 mm, 10 μm) was used. The buffers were the same as for the analytical

HPLC; the flow rate was 10 ml/min, and the detection was at 226 nm. Acetonitrile for HPLC was purchased from Technopharm (Russia).

The peptides were hydrolyzed by 6 N HCl containing 2% phenol at 110°C for 24 h and subjected to amino acid analysis on an automatic Biotronic LC 5001 analyzer (Germany).

A Finnigan MAT TSQ 700 quadrupole spectrometer (Germany) equipped with a Finnigan MAT API electrospray ionic source was used for ESI MS.

The ^{1}H NMR spectra were recorded on a WM-500 spectrometer (Bruker, Germany, 500 MHz) at 27°C in DMSO-d_6. The chemical shifts were measured relative to tetramethylsilane.

The attachment of Fmoc-Arg(H$^+$)-OH to the Wang polymer. A mixture of Fmoc-Arg-OH (0.892 g, 2.25 mmol) and pyridine hydrobromide (0.405 g, 2.25 mmol) was dissolved in DMF (5 ml), cooled to 0°C, and treated with DIPCDI (0.348 ml, 2.25 mmol). The reaction mixture was kept for 5 min, added to the Wang resin (1.00 g, content of hydroxymethyl groups was 0.45 mmol/g), treated with 1-methylimidazole (0.125 ml, 1.57 mmol), and stirred for 2 h at 20°C. The resin was filtered, washed with DMF (3 × 10 ml), methanol (3 × 10 ml), and methylene chloride (3 × 10 ml), and dried in a vacuum-desiccator. The content of Fmoc-Arg in the polymer determined according to the technique described below was 0.42 mmol/g (the substitution degree was 93%).

Attachment with the use of other catalysts was carried out according to the same procedure with a fivefold excess Fmoc-Arg(H$^+$)-OH and DIPCDI. The catalyst quantity and the reaction time were varied (Table 1).

The substitution degree of the resin was determined by spectrophotometry. A 20% solution of piperidine in DMF (5 ml) was added to the Fmoc-Arg-polymer (20 mg), the reaction mixture was stirred for 20 min at 20°C, and the resin was filtered off. An aliquot was taken from the filtrate, and the absorption of the resulting solution of *N*-(9-fluorenylmethyl)piperidine was measured at 301 nm. The substitution degree was calculated using the formula $A_{301} \times V(\text{ml})/7800 \times W(\text{g})$ [11].

H-Phe-*D*-Arg-OH. A solution of Boc-Phe-ONp (463 mg, 1.2 mmol) in DMF was added to a cooled suspension of H-*D*-Arg-OH (174 mg, 1 mmol) in DMF. The reaction mixture was stirred for 24 h at 20°C, evaporated, and the residue was dissolved in chloroform and chromatographed on a silica gel column. The column was eluted by a stepwise gradient of ethanol in chloroform (from 0 to 100% ethanol) with the percent of ethanol being increased at 10% intervals. The eluent volume at every step was equal to three free volumes of the column. Boc-Phe-*D*-Arg-OH was obtained in a 90% yield (380 mg); R_f 0.74.

Boc-Phe-*D*-Arg-OH (380 mg) was dissolved in 95% aqueous TFA and stirred for 1 h at 20°C. TFA was evaporated, and the residue was triturated with ether, filtered, and washed with ether. The yield of H-Phe-*D*-Arg-OH was 279 mg (87%); R_f 0.46; RT 15.38 min [Nucleosil C-18 column 5 μm (4.6 × 150 mm) eluted with gradient 1].

H-Phe-Arg-OH was prepared according to the procedure described for H-Phe-*D*-Arg-OH. The yield of H-Phe-Arg-OH was 255 mg (79%); R_f 0.46; RT 14.30 min on the Nucleosil C-18 column (5 μm, 4.6 × 150 mm) eluted with gradient 1.

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH (bradykinin). The manual SPPS was carried out starting from Fmoc-Arg-**P** (1.0 g) with an amino acid content of 0.42 mmol/g by stepwise elongation of the peptide chain by the DIPCDI/HOBt method. The synthetic cycle involved (1) a 20-min activation of the Fmoc-amino acid (5 equiv) to be coupled by the DIPCDI (5 equiv) with the addition of HOBt (5 equiv) in DMF (5 ml); (2) deprotection of α-amino groups by treatment with a 20% solution of piperidine in DMF (2 × 5 ml) for 20 min; (3) washing of the peptidylpolymer with DMF (4 × 10 ml for 5 min); (4) an additional washing of the peptidylpolymer with a solution of pyridine hydrobromide (0.42 mmol) in DMF (5 ml) for 10 min; (5) condensation of the activated Fmoc-amino acid (5 equiv) with peptidylpolymer for 2 h; and (6) a series of washings of the peptidylpolymer with DMF (4 × 10 ml for 5 min) and methylene chloride (2 × 10 ml for 5 min); (7) the monitoring of the substitution of the amino groups at each coupling step was carried out by the ninhydrin test [18]. If the test was positive, the coupling step was repeated. Pro, Gly, and Ser residues were introduced into positions 2, 4, and 6, respectively, by twofold condensation.

The *N*-terminal Arg1 was attached by twofold condensation using the BOP-reagent in DMF for 1 h (each time) with 2–3 min preactivation; Fmoc-Arg-OH and BOP were taken in equimolar amounts.

After completion of the synthesis and deprotection of the α-amino group, the peptidylpolymer was washed with DMF and methylene chloride and dried in a vacuum-desiccator. The peptide was cleaved from the resin and deprotected by treatment with 95% aqueous TFA (10 ml) for 1.5 h. The polymer was filtered, and the filtrate was evaporated in a vacuum at 40°C. The residue was triturated with cool anhydrous ether, filtered, washed with ether, and dried in a vacuum-desiccator. The content of bradykinin in the resulting raw product (389 mg) was 92% according to analytical HPLC; R_t was 15.43 min on a Ultrasphere ODS column (5 μm, 4.6 × 250 mm) eluted with gradient 2.

The peptide was purified by preparative HPLC. The gradient of buffer B was from 0 to 10% for 5 min and, then, from 10 to 50% for 80 min. The yield of bradykinin was 307 mg (69% relative to the starting amino acid). The structure of the resulting peptide was confirmed by ^{1}H NMR (Fig. 3). The molecular mass of this compound was determined to be 1060 according to ESI

MS, which corresponded to the calculated molecular mass.

H-Phe-Arg-Arg-OH. The manual SPPS was started from Fmoc-Arg-polymer (0.24 g, 0.10 mmol; content of the amino acid was 0.42 mmol/g). Fmoc-Arg-OH (5 equiv) was attached by twofold condensation with the BOP-reagent according to the procedure described for bradykinin. Fmoc-Phe-OH was coupled by a single condensation by the DIPCDI/HOBt method. After cleavage from the polymer by treatment with 95% aqueous TFA for 1.5 h and isolation by the standard technique, the raw product contained 87% H-Phe-Arg-Arg-OH according to analytical HPLC; R_t 16.1 min on a Nucleosil C-18 column (5 μm, 4.6 × 150 mm) eluted with gradient 1. The peptide was purified by preparative HPLC in a gradient of buffer B from 0 to 40% for 80 min. The yield of H-Phe-Arg-Arg-OH was 35 mg (73%); R_f 0.09.

H-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-OH. SPPS was performed with the use of the Fmoc-strategy on an Applied Biosystems Model 431A synthesizer. The Wang polymer (0.1 mmol for each synthesis) with attached Fmoc-Tyr(Bu^t) was used, with the content of the amino acid being 0.65 mmol/g. The synthesis was carried out according to the standard protocol with the use of a tenfold excess of the activated Fmoc-amino acid and a single condensation at each cycle. Exceptions were made for Ile2, Arg1, and Arg7, which were introduced by twofold condensations. The condensation methods for the introduction of these Arg residues were varied as described above. The synthetic cycle involved (1) a 20-min activation of the Fmoc-amino acid to be coupled (1 mmol) with equivalent amounts of DIPCDI and HOBt in DMF; (2) deprotection of α-amino groups by treatment with a 20% solution of piperidine in DMF for 17 min; (3) condensation of the activated Fmoc-amino acid (1 mmol) with the peptidylpolymer for 37 min; and (4) all necessary intermediate washings of the peptidylpolymer. After completion of the synthesis and deprotection of the α-amino group, the peptidylpolymer was washed on a filter with methylene chloride and dried in a vacuum-desiccator. The peptide was cleaved from the polymer and deprotected by treatment with 10 ml of a mixture of TFA (82.5%), phenol (5%), water (5%), thioanisole (5%), and ethanedithiol (2.5%) for 1.5 h. The polymer was filtered and the filtrate was evaporated in a vacuum at 40°C. The residue was triturated with cool anhydrous ether, filtered, washed with ether, and dried in a vacuum-desiccator.

The content of the target peptide in the raw product was 85% according to analytical HPLC; R_t 13.60 min on a Ultrasphere ODS column (5 μm, 4.6 × 250 mm) eluted with gradient 2.

The peptide was purified by the preparative reversed-phase HPLC using a gradient of buffer B from 0 to 20% for 10 min and from 20 to 50% for 60 min. The yield of H-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-OH was 60 mg (59% relative to the starting amino acid); the amino acid analysis: Arg 2.03 (2), Ile 0.79 (1), Leu 0.87 (1), Ala 1.0 (1), Val 1.02 (1), Glu 1.10 (1), and Tyr 0.98 (1); ESI MS: 1019 [M].

H-*D*-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-OH was synthesized similarly to its *L*-analogue starting from 0.1 mmol of the starting amino acid. Arg7 was introduced by twofold condensation using the DIPCDI/HOBt method with arginine protection by protonation. *D*-Arg1 was introduced by twofold condensation using BOP. After cleavage from the polymer by treatment with 95% aqueous TFA for 1.5 h, the content of the target peptide in the raw product was 76% according to analytical HPLC; R_t 14.38 min on an Ultrasphere ODS column (5 μm, 4.6 × 250 mm) eluted with gradient 2. After preparative HPLC, the yield of the peptide was 51 mg (50% from the starting amino acid). The structure of the peptide was confirmed by ^{1}H NMR spectroscopy.

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