Russian Journal of Bioorganic Chemistry, Vol. 30, No. 2, 2004, pp. 101–110. Translated from Bioorganicheskaya Khimiya, Vol. 30, No. 2, 2004, pp. 115–125.

The Use of Hydrogen Peroxide for Closing Disulfide Bridges in Peptides

M. V. Sidorova*,[1] A. S. Molokoedov*, A. A. Az'muko*, E. V. Kudryavtseva*, E. Krause, M. V. Ovchinnikov*, and Zh. D. Bespalova***

**Cardiological Research and Production Association, Russian Ministry of Health, ul. Tret'ya Cherepkovskaya 15a, Moscow, 121552 Russia*

***Institute of Molecular Pharmacology, Berlin, Germany*

Received October 2, 2002; in final form, November 29, 2002

Abstract—The use of hydrogen peroxide for the formation of disulfide bridges was studied in 15 peptides of various lengths and structures. The oxidation of peptide thiols by hydrogen peroxide was shown to proceed under mild conditions without noticeable side reactions of Trp, Tyr, and Met residues. Yields of the corresponding cyclic disulfides were high and mostly exceeded those obtained with other oxidative agents, in particular, iodine. It was established that the use of hydrogen peroxide in organic medium also provided sufficiently high yields when large-scale syntheses of oxytocin and octreotide (up to 10 g) were carried out.

Key words: chemical synthesis, disulfide bond, hydrogen peroxide, natural peptides and their analogues

INTRODUCTION

The development of peptide chemistry currently allows the preparation of practically any peptides, their analogues, and even small proteins; however, the synthesis of peptides with internal disulfide bridges is still a real challenge [1, 2].[2] Low yields at the stage of formation of SS bonds often reduce to zero all the efforts made for the preparation of linear precursors. The preparation of cyclic peptide disulfides from the corresponding SH-precursors (variant A) and the direct conversion of the cysteine-protected derivatives into cyclic products (deprotection of the SH-groups with simultaneous cyclization, variant B) are most widely used among the diversity of methods known for the synthesis of disulfide-containing peptides. As a rule, in both cases, the cyclization is carried out in very dilute solutions, with the peptide concentration being of 10^{-4}–10^{-5} M in order to avoid an intermolecular aggregation and side reactions [1]. The directed formation of S–S bonds in the highly diluted solutions significantly depends on structural peculiarities of a peptide, in particular, on the nature and the number of amino acid residues between Cys residues [3, 4]. The cyclization of free thiols (variant A) by the air oxygen usually leads to low yields of target products (9–15%) [2]. The application of potassium ferricyanide or dimethyl sulfoxide usually result in homogeneous reaction mixtures and the yields of cyclic products are considerably higher (from 20 to 60%, and, in some cases, up to 80%, which depends on the peptide structure [2, 5]). However, a multistage purification of product is necessary for the removal of excess of these oxidative agents [1]. A very attractive one-step formation of the disulfide bridges by the action of iodine is often accompanied by side reactions [1, 2, 6–8] and has only a limited use in the case of Trp- and Met-containing peptides.

Our synthesis of peptides with intramolecular disulfide bridges on a scale far exceeding usual laboratory needs prompted us to search for such oxidation method for SH groups in various peptides that could provide sufficiently homogeneous reaction mixtures and a simple (preferably one-step) isolation of highly pure product. Hydrogen peroxide attracted our attention, because we found separate references [9] to its application for cyclization of peptide thiols. We compared the efficiency of various oxidative agents—O_2, I_2, $K_3[Fe(CN)_6]$, and H_2O_2—and demonstrated the advantages of hydrogen peroxide application to the disulfide bond formation in the fragments of HIV gp41 glycoprotein from various strains (Z3, ELI, BRU, and ROD) [6, 10, 11]. Our conclusions about the efficiency of hydrogen peroxide are in a good agreement with the results in [12], also devoted to a comparison of different cyclization methods.

[1] Corresponding author; phone: +7 (095) 414-6716; fax: +7 (095) 414-6786; e-mail: peptide@cardio.ru

[2] The abbreviations recommended by the IUPAC–IUB commission in *Eur. J. Biochem.*, 1984, vol. 183, pp. 9–37, are used along with the following ones: AcOH, acetic acid; Acm, acetamidomethyl; DIC, *N*,*N*'-diisopropylcarbodiimide; *d*F, *D*-Phe; *d*W, *D*-Trp; ESI MS, electrospray ionization mass spectrometry; ET, endothelin; Fmoc, 9-fluorenylmethyloxycarbonyl; MALDI MS, matrix-assisted laser desorption and ionization mass spectrometry; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; β-Mpa, β-mercaptopropionic acid; NMP, *N*-methylpyrrolidone; and u-PAR1, urokinase receptor.

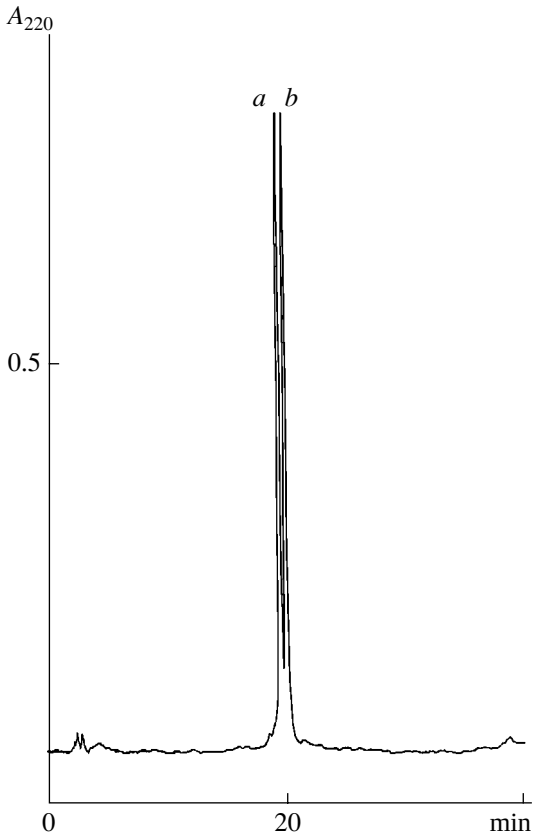

Fig. 1. Analytical HPLC of the mixture of (*a*) oxidized (SS form) and (*b*) reduced (SH form) of rat atriopeptin II (Table 1) on an Ultrasphere ODS column (4.6 × 250 mm, 5 μm) eluted with a gradient of 70% acetonitrile in 0.05 M phosphate buffer (pH 3) from 10 to 60% for 40 min.

RESULTS AND DISCUSSION

This paper generalizes the results of our studies of the hydrogen peroxide use for the formation of disulfide bonds in peptides of various lengths and structures. Amino acid sequences of the compounds synthesized are given in Table 1. Potentials and limitations of this approach to the cyclization were studied in the case of peptides with one or two disulfide bridges and with various cycle sizes (from 6 to 17 amino acid residues) that involved Met, Tyr, and Trp residues sensitive to oxidation. We also studied the reproducibility of cyclization results at the increased synthesis scale.

The syntheses of antigenic determinants of gp41 (peptides P1–P3 and P5) [6, 10, 11], ET 1 and 3 [7], and the 1–16 fragment of u-PAR1 [13] were previously described. The P4 peptide (an antigenic determinant of gp41 glycoprotein of HIV-2), rat atriopeptins II and III, the rat 1–28 atrial natriuretic factor, and (1–14)-somatostatin fragment were prepared by the solid phase method of peptide synthesis on the Wang polymer. The following protecting groups were used for the side functions of trifunctional amino acids: Bu^t for Ser, Thr, Tyr, Asp, and Glu; Boc for Lys, Trt for His, Asn, and Gln, and Pmc for Arg. The Fmoc group was chosen for the temporary protection of the N$^\alpha$-amino function. Cys residues were blocked by the Acm group for all the peptides, except for the u-PAR1-(1–16) fragment (Table 1). In this fragment, Cys3 was blocked by the Acm group, whereas two remaining Cys residues (Cys6 and Cys12), which are connected by the disulfide bridge in the protein, were protected by the Trt group. The coupling was achieved by the DIC/HOBt method. The peptides were cleaved from the support by the treatment with the reagent K [14]. The linear Acm-precursors of peptides and the linear SH-precursor of the (1–16)-u-PAR1 fragment were purified by preparative HPLC to the purity of 95%. The linear precursors of oxytocin, octreotid, and [βMpa^1,Arg^8]-vasopressin were prepared by ZAO Sintez peptidov (Peptide synthesis) using the conventional methods of peptide synthesis in solution.

Mercury acetate (2 equiv per SH function) in acidic aqueous medium (30–50% AcOH or 30% formic acid) was used to cleave Acm groups from all the peptides, and hydrogen sulfide to subsequently remove mercuric ions.

We conditionally divided all the synthesized peptides into the following groups: peptides with one disulfide bridge containing no Met (P1–P5, APII$_r$, APIII$_r$, α-ANF$_r$, the (1–14)-somatostatin, oxytocin, octreotide, and a vasopressin analogue); Met-containing peptides [ET-1 and u-PAR1-(1–16)]; and peptides with two disulfide bridges (ET-1 and ET-3).

Disulfide bridges were closed in aqueous or aqueous–organic (methanol–water, DMF–water, and dioxane–water) solutions [1] under the condition of high dilution at the peptide concentrations of 0.5–1 mg/ml at pH 6.5–8.5. The peptides without Met residues (Table 1) were cyclized with the use of 5–15-fold excesses of hydrogen peroxide, whereas 1.5–2-fold excesses were applied to Met-containing peptides. The processes were monitored by HPLC. The conditions of analytical HPLC at which the corresponding SH- and SS-forms of the peptides were well resolved were found for all of the studied peptides (Fig. 1). Oxidation was completed within 15–40 min for all the peptides studied. After the completion of oxidation, the reaction mixture was acidified with acetic acid to pH 4, and the target peptides were isolated by the preparative HPLC on Diasorb 130T (C16). The resulting cyclic disulfides of the peptides were characterized by amino acid analysis, mass spectrometry, and analytical HPLC (Table 3 in the Experimental section). Their yields are listed in Table 1. The yields given in Table 1 and in the Results and Discussion section are not corrected to the content of peptides after lyophilization for the sake of convenience at comparing with literature data.

The closing of disulfide bridges in the peptides with a relatively small cycle (P1–P5 peptides, oxytocin, and octreotide) proceeded without a considerable formation of side products. The content of cyclic peptides in the

Table 1. A comparison of yields of the peptide cyclic disulfides prepared by the treatment with iodine or with hydrogen peroxide under a high dilution

Designation	Peptide	Amino acid sequence	Yield[a], %		Content in lyophilizate[d], %
			I_2	H_2O_2	
P1	HIV-1 gp41-(598–609) (Z3) fragment	H-LGLWGCSGKLIC-NHEt	51[11]	75–85[11]	82
P2	HIV-1 gp41-(598–609) (ELI) fragment	H-LGIWGCSGKHIC-NHEt	56[11]	85–90[11]	85
P3	HIV-2 gp41-(593–603) (ROD) fragment	H-NSWGCAFRQVC-NHEt	66[11]	80–87[11]	89
P4	HIV-2 gp-41-(586–607) (ROD) fragment	H-LQDQARLNSWGCAFRQVCHTTV-OH	<5	82	90
P5	HIV-1 gp41-(601–617) (BRU) fragment	H-WGCSGKLICTTAVPWNA-OH	25[11]	85–90[11]	92
	Oxytocin	H-CYIQNCPLG-NH_2	52	75	94
	Octreotide	H-*d*FCF*d*WKTC-NHCH[CH(OH)CH_3]CH_2OH	–	80	87
	Deamino-[Arg^8]-vasopressin	βMpa-YFQNCPRG-NH_2	–	81	88
$APII_r$	Rat atriopeptin II	H-SSCFGGRIDRIGAQSGLGCNSFR-OH	23[19, 20]	83	86
$APIII_r$	Rat atriopeptin III	H-SSCFGGRIDRIGAQSGLGCNSFRY-OH	26[19, 20]	50	82
	Somatostatin-(1-14)	H-AGCKNFFWKTFTSC-OH	–	51	91
α-ANF_r-(1–28)	Rat atrial natriuretic factor-(1–28)	H-SLRRSSCFGGRIDRIGAQSGLGCNSFRY-OH	20[19, 20]	62	82
u-PAR1-(1–16)	Urokinase receptor-(1–16) fragment	H-LRC(Acm)MQCKTDGDCRVEE-OH	–	30[b][13]	84
ET-1	Endothelin-1	H-CSCSSLMDKECVYFCHLDIIW-OH	–	26[c][7]	91
ET-3	Endothelin-3	H-CTCFTYLDKECVYYCHLDIIW-OH	–	20[c][7]	80

[a] The yields from the $[Cys(Acm)]_2$-derivatives of the corresponding linear peptides are given. The disulfide bridges were formed starting from 100–500 mg of the corresponding linear precursors of the peptides at their concentrations of 0.5–1.0 mg/ml. No less than three cyclization experiments were carried out for each of the listed compounds; yields varied within 10%. To simplify the comparison of our results with literature data, the yields were not corrected for the content of peptides in the corresponding lyophilyzates.

[b] The yields from the $[Cys(SH)]_2$-derivatives of the corresponding peptides are given.

[c] The yields from the $[Cys(Acm)]_4$-derivatives of the corresponding linear peptides are given.

[d] The results for the compounds synthesized using hydrogen peroxide are obtained by the quantitative amino acid analysis.

Table 2. The results of model experiments on the disulfide bond formation in oxytocin and octreotide using hydrogen peroxide in aqueous and organic media at different concentrations of the starting dithiols

Peptide	Solvent	Concentration, mg/ml	Content of target product in reaction mixture, % according to HPLC
Oxytocin	Water	1	82
		5	58
		10	50
	DMF–CH_3OH, 1 : 5	1	87
		5	71
		10	72
		20	58
Octreotide	CH_3OH	1	98
		10	98
		20	96
		50	94
		100	93

reaction mixtures after the oxidation was sufficiently high (85–95%). The yields of cyclic products (see Table 1) from the corresponding Acm-protected precursors were also sufficiently high (from 69 to 90%).

The cyclization of deamino analogues of some natural peptides is known to proceed under rather rigorous conditions. For example, the SS bond in oxytocin is easily closed even on the exposure to air oxygen, whereas the closing of SS bridge in deaminooxytocin requires the treatment with $K_3[Fe(CN)_6]$ [15]. In the case of a vasopressin analogue containing a residue of unnatural β-mercaptopropionic acid, the formation of SS bond by the action of hydrogen peroxide (see Table 1) proceeded as quickly and easily as in the natural peptides. The yield of [βMpa^1,Arg^8]vasopressin was 81%.

The formation of SS bonds in somatostatin (10 aa between two Cys residues) and in atrial peptides (15 aa between two Cys residues) was accompanied by a formation of side products (possibly disulfide oligomers [12]), which decreased the content of target cyclic disulfides in the reaction mixtures to 70–75% (according to HPLC). In this case, the yields of the corresponding products dropped to 50–60% (see Table 1). The formation of SS bridge in oxytocin was the most difficult. Its yield was practically independent of the concentration of starting compound (within the range from 0.2 to 1.0 mg/ml), but it significantly changed upon the transition from aqueous to aqueous–organic solutions in the reaction medium: 26% in 0.1 M ammonium acetate (pH 6.8), 35% in methanol–water (pH 6.8), and 51% in dioxane–water (pH 6.8). The side product isolated from the reaction mixture after oxidation was converted into a thiol after the treatment with dithiothreitol and, presumably, was a dimer of somatostatin; the formation of such dimers is characteristic of somatostatin [16].

Note that the yield of all the natural peptides with one disulfide bridge we prepared by the hydrogen peroxide oxidation were significantly higher than those obtained at the iodine treatment (Table 1).

The application of hydrogen peroxide to the cyclization of Met-containing peptides is regarded as problematic due to a very easy oxidation of methionine into the corresponding sulfoxide [12]. Previously, we published the results of using hydrogen peroxide for the formation of SS bridges in Met-containing peptides in detail [7, 13] and here only briefly describe the cyclization conditions. We had already found that the process of formation of SS bridge itself by the action of hydrogen peroxide is not accompanied by any noticeable oxidation of methionine into the corresponding sulfoxide. This side reaction proceeds after the completion of cyclization as a result of acidification of the reaction mixture before isolation of the target disulfide. Therefore, an increase in pH of the reaction mixture to 8–9, the use of a small excess of hydrogen peroxide (no more than two equiv), and bubbling of an inert gas through the reaction mixture using a glass capillary for 30 min before the acidification allow a successful use of hydrogen peroxide for Met-containing peptides ET-1 and u-PAR1-(1–16) too.

These conditions were used for a simultaneous formation of two SS bridges in ET 1 and 3 [7]. The isomers with the natural positions of SS bridges were predominantly formed after the treatment with hydrogen

Table 3. Characteristics of the cyclic peptides prepared with the use of H_2O_2

Peptide	Empirical formula	Purity according to HPLC	Molecular mass		Amino acid analysis*
			calculated	found by MS	
gp41-(586–607) (P4)	$C_{108}H_{167}N_{35}O_{32}S_2$	96.0	2531.9	2531.4[a]	D/N 1.9(2), T 1.7(2), S 0.8(1), E/Q 3.1(3), G 1.0(1), A 1.7(2), V 1.8(2), L 2.0(2), F 1.1(1), H 1.0(1), W 0.6(1), R 2.2(2)
Oxytocin	$C_{43}H_{66}N_{12}O_{12}S_2$	97.0	1007.2	1007.7[b]	D/N 1.0(1), E/N 1.1(1), G 1.0(1), I 0.9(1), L 0.9(1),Y 0.8(1)
Octreotide	$C_{49}H_{66}N_{10}O_{10}S_2$	98.2	1019.3	1020.3[b]	T 0.9(1), F 2.0(2), W 0.7(1), K 1.0(1)
[βMpa[1], Arg[8]]-vasopressin	$C_{46}H_{64}N_{14}O_{12}S_2$	98.0	1069.2	1068.0[b]	D/N 1.0(1), E/Q 1.1(1), G 1.0(1), Y 0.9(1), F 1.0(1), R 1.0(1)
$APII_r$	$C_{98}H_{156}N_{34}O_{32}S_2$	98.1	2386.6	2386.0[a]	D/N 2.2(2), S 3.6(4), E/Q 1.2(1), G 5.2(5), A 1.0(1), I 1.9(2), L 1.0(1), F 2.1(2), R 3.0(3)
$APIII_r$	$C_{107}H_{165}N_{35}O_{34}S_2$	98.3	2549.8	2549.5[a]	D/N 2.1(2), S 3.6(4), E/Q 1.2(1), G 5.2(5), A 1.0(1), I 1.9(2), L 1.0(1),Y 0.9(1), F 2.1(2), R 3.1(3)
Somatostatin-(1–14)	$C_{76}H_{104}N_{18}O_{19}S_2$	97.5	1637.9	1640.5[b]	D/N 1.1(1), T 1.8(2), S 0.9(1), G 1.0(1), A 1.0(1), F 2.9(3), W 0.7(1), K 2.1(2)
α-ANF_r	$C_{128}H_{205}N_{45}O_{39}S_2$	98.0	3062.4	3062.4[a]	D/N 2.1(2), S 4.3(5), E/Q 1.2(1), G 5.2(5), A 1.0(1), I 1.8(2), L 2.0(2), Y 0.9(1), F 2.0(2), R 4.9(5)

*The contents of proline and cysteine in the hydrolyzates of the corresponding peptides were not determined.

[a] Molecular mass was determined by ESI MS.

[b] Molecular mass was determined by MALDI-MS, the $[M + H]^+$ values are given.

peroxide as was the case with the air oxygen [17]. However, the ratio of the natural isomer and two other possible isomers of ET 1 was 3 : 1 : 0 with the use of air oxygen and 10 : 1 : 0 with the use of hydrogen peroxide. In the case of ET 3, this ratio was 4 : 1 : 2 [7]. Moreover, the oxidation was completed already after 40 min, whereas the oxidation by the air oxygen required several hours.

Thus, we demonstrated a doubtless advantage of using hydrogen peroxide for the conversion of various peptide thiols into the corresponding disulfides. The next stage of our investigation was the study of the potential use of hydrogen peroxide for the synthesis of large quantities of oxytocin and octreotide (both peptides exhibit a hormone activity). Octreotide (sandostatin) is a synthetic analogue of somatostatin proposed by Sandoz [18]. It has the following structure:

H-*D*-Phe-Cys-Phe-*D*-Trp-Lys-Thr-Cys-NHCH[CH(OH)CH_3]CH_2OH.

Both peptides are therapeutic agents certified in Russia, and the necessity of large-scale synthetic schemes for these peptides (dozens and hundreds grams), including the cyclization step, is evident. As follows from Table 1, the use of hydrogen peroxide to the SS bridge formation in oxytocin and octreotide is promising under the conditions of high dilution (the peptide concentration was approximately 1 mg/ml or $\approx 10^{-3}$ M). Under these conditions, the yields of oxytocin and octreotide from their linear [Cys(Acm)]$_2$-precursors were 75 and 80%, respectively. However, technologically, high dilutions are not good for large-scale syntheses, because volumes of reaction mixtures dramatically increase. For example, even the cyclization of 20 g of the linear precursor of the corresponding peptide requires a 20-l volume of the reaction mixture. Therefore, we studied the effect of increasing concentrations of the linear precursors of the peptides on the

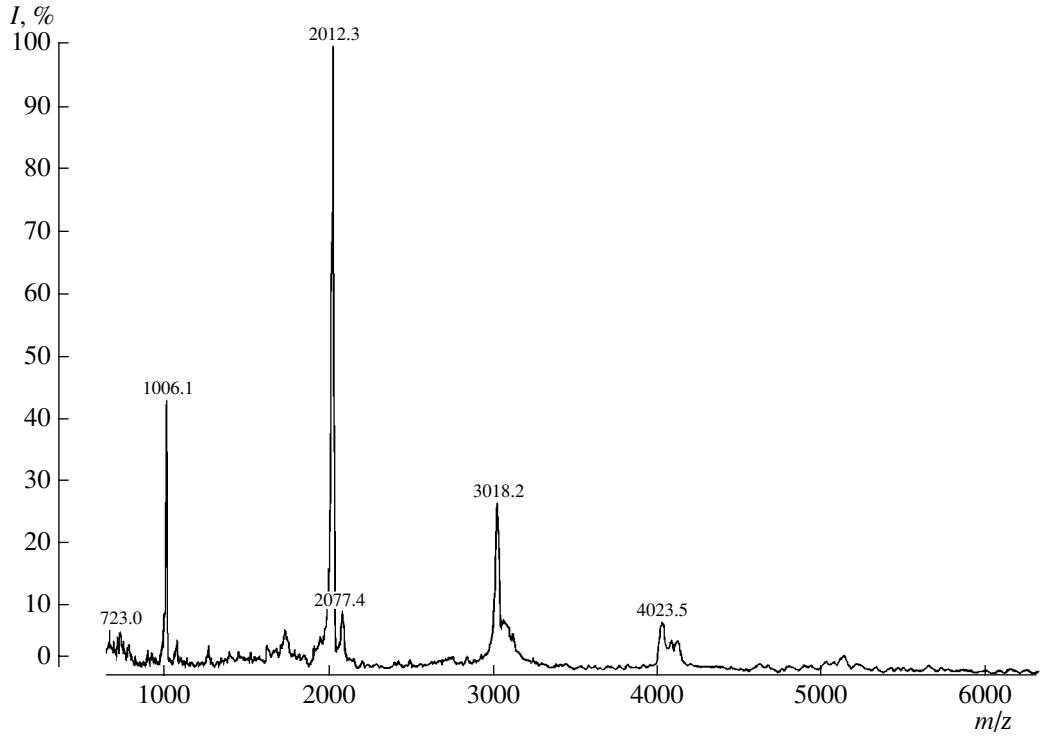

Fig. 2. Mass spectrum of the reaction mixture of the oxytocin preparation at 10-mg/ml concentration of its [$Cys(SH)_2$]-precursor in aqueous medium at pH 7.2. The calculated molecular mass of oxytocin is 1007.7. The values of $[M + H]^+$ are given on the X-axis.

cyclization process. The results of this study are given in Table 2.

The percent content of oxytocin dramatically (almost two times) decreased in the reaction mixture along with increase in the concentration of its linear precursor (from 81.1% at the peptide concentration of 1 mg/ml to 49.8% at the concentration of 10 mg/ml) when the SS bonds of oxytocin were formed with the use of hydrogen peroxide in aqueous medium at pH 7.2 (see Table 2). In this case, the area of the peak corresponding to oxytocin approximately 3.5 times decreases in the reaction samples brought to equal concentrations. The most probable reason for this decrease is the formation of the corresponding oxytocin oligomers along with its dimeric forms. These oligomers could be irreversibly sorbed on chromatographic sorbents or, under the HPLC conditions, gave no clear peaks due to exclusion, which did not allow us to register the oligomers. A mass-spectrometric analysis of the reaction mixture of oxytocin preparation at the concentration of 10 mg/ml confirmed the formation of not only oxytocin and its dimer but also oxytocin trimers and tetramers as well (see Fig. 2). It should be noted that, in this case, we could not observe any correlation between the ratios of the target product and by-products in the HPLC chromatograms (see Table 2) and in mass spectra (Fig. 2).

There are some reports in literature that the introduction of organic solvents into reaction mixtures during the SS bridge formation facilitates the formation of intramolecular disulfides [4]. Our results on the synthesis of the 1–14 fragment of somatostatin also confirm these publications. Therefore, we tried to cyclize oxytocin in an organic medium the more so, as the solubility of its linear precursor considerably decreased in aqueous solution at pH 6.5–8.5. We failed to use methanol as a solvent for this reaction, because the linear peptide has a low solubility in it under the oxidation conditions. Satisfactory results were obtained, when the cyclization was carried out in a 1 : 5 DMF–methanol mixture. One can see from Table 2 that, in this case, the quality of reaction mixture does not decrease so substantially with the increase in the concentration of linear peptide to 10 mg/ml (approximately 10^{-2} M). The oxytocin content was 71.6%, and no sharp decrease in the area of peak corresponding to oxytocin was observed.

The solubility of the linear dithiol precursor of octreotide is limited in aqueous media with pH 6.5–8.0 even at the concentration of 1 mg/ml, and no comparison of the results of its cyclization in aqueous and organic solvents is possible. Bauer and Pless [18] formed SS bridge in octreotide in highly dilute solutions (0.25 mg/ml) using a dioxane–water mixture. Surprisingly, the octreotide linear precursor was easily cyclized in methanol without noticeable formation of oligomeric by-products in the wide range of concentrations (from 1 to 100 mg/ml) (Fig. 3). The content of tar-

get product in the reaction mixture insignificantly changed from 98 to 93% (Table 2) and no considerable decrease in the area of peak corresponding to octreotide (no more than 5–10%) was also observed in the samples of the oxidation reaction mixtures brought to the equal concentrations (HPLC). We cannot now explain this interesting phenomenon and can only presume that the absence of intermolecular interactions at such high concentrations of the peptide is associated with formation of a compact conformation of the octreotide molecule in methanol that favors the predominant formation of the intermolecular SS bridge. A mass-spectrometric analysis of the reaction mixture (Fig. 4) demonstrated that, under these conditions, the main oxidation product was the target cyclic peptide, and the corresponding oligomers were present in insignificant amounts.

We chose 10 mg/ml as the working concentration and used it for the preparation of oxytocin and octreotide in organic solvent media starting from 20 g of the linear dithiols (see the Experimental section). Note that, in the model experiments, the corresponding linear precursors were purified to >95% HPLC-purity (the results are given in Table 2). However, when carrying out the large-scale experiments, we used the corresponding Acm- and SH-derivatives without any preliminary purification, and the content of the main substance in the starting SH-peptides was 74% for oxytocin and 82% for octreotide (according to HPLC). The yields of oxytocin and octreotide in the preparative experiments were 44 and 64%, respectively.

Thus, we demonstrated the advantages of hydrogen peroxide for the formation of disulfide bonds in natural peptides of various structures. A well-reproducible technology of the oxidation of peptide thiols at high concentrations into the corresponding disulfides by the treatment with hydrogen peroxide in organic solvents was developed by the examples of large-scale experiments with oxytocin and octreotide.

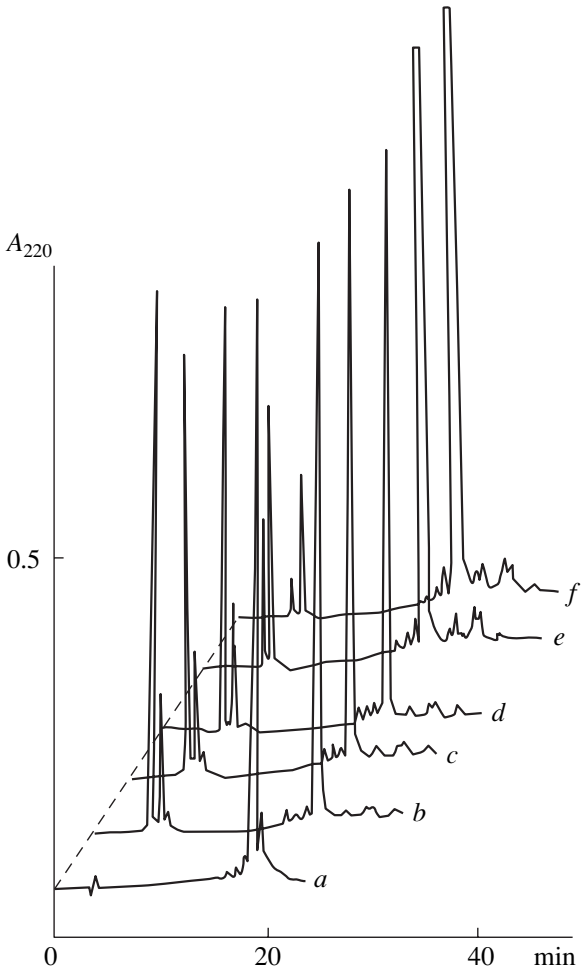

Fig. 3. Analytical HPLC of (*a*) [Cys(SH)$_2$]-precursor of octreotide and the reaction mixtures of its oxidation by the treatment with hydrogen peroxide in methanol at the peptide concentrations of (*b*) 1, (*c*) 10, (*d*) 20, (*e*) 50, and (*f*) 100 mg/ml on an Ultrasphere ODS column (4.6 × 250 mm, 5 μm) eluted with a gradient of 70% acetonitrile in 0.05 M phosphate buffer (pH 3) from 10 to 70% for 30 min.

EXPERIMENTAL

Derivatives of *L*-amino acids (Bachem, Switzerland), phenol (Merck, Germany), mercury acetate, anisole, *p*-cresol, thiophenol, EDT, DIC, βMpa, *D*-phenylalanine, *D*-tryptophan (Fluka, Switzerland), and 50% hydrogen peroxide (Solvay Interox, Belgium) were used in this study. Before the use, hydrogen peroxide was diluted with deionized water to the desired concentration and titrated with 1 N solution of $KMnO_4$. Dichloromethane, *N*-methylpyrrolidone, piperidine, methanol, and trifluoroacetic acid (Applied Biosystems GmbH, Germany) were used in the solid phase peptide syntheses. For analytical HPLC, a Gilson chromatograph (France) equipped with Ultrasphere ODS (4.6 × 250 mm, 5 μm) column (Beckman, United States) and Vydac C18 (4.6 × 250 mm, 5 μm, pore size of 300 Å) column (Sigma, United States) was used. The first column was eluted with 70% acetonitrile in 0.05 M KH_2PO_4 (pH 3, buffer A) and the second, with 80% acetonitrile in 0.1% trifluoroacetic acid (buffer B); the flow rate was 1 ml/min and the detection was at 220 nm. The preparative HPLC was carried out on a Beckman Prep 350 chromatograph (United States) on a Diasorb-130-C16T column (10 μm, ZAO Biokhimmak CT, Russia) with detection at 226 nm. The cyclic peptide disulfides were eluted with a gradient of 80% acetonitrile in 0.01 M ammonium acetate (pH 4.5). Acetonitrile for HPLC was from Technopharm (Russia).

Peptides were hydrolyzed with 6 N HCl containing 2% of thioglycolic acid at 110°C for 24 and 48 h and subjected to amino acid analysis after the hydrolysis on a Biotronik 5001 automatic analyzer (Germany). Mass spectra were registered on a Finnigan MAT TSQ 700 quadrupole mass spectrometer (Germany) with electrospray ion source (API) (Finnigan MAT, United States)

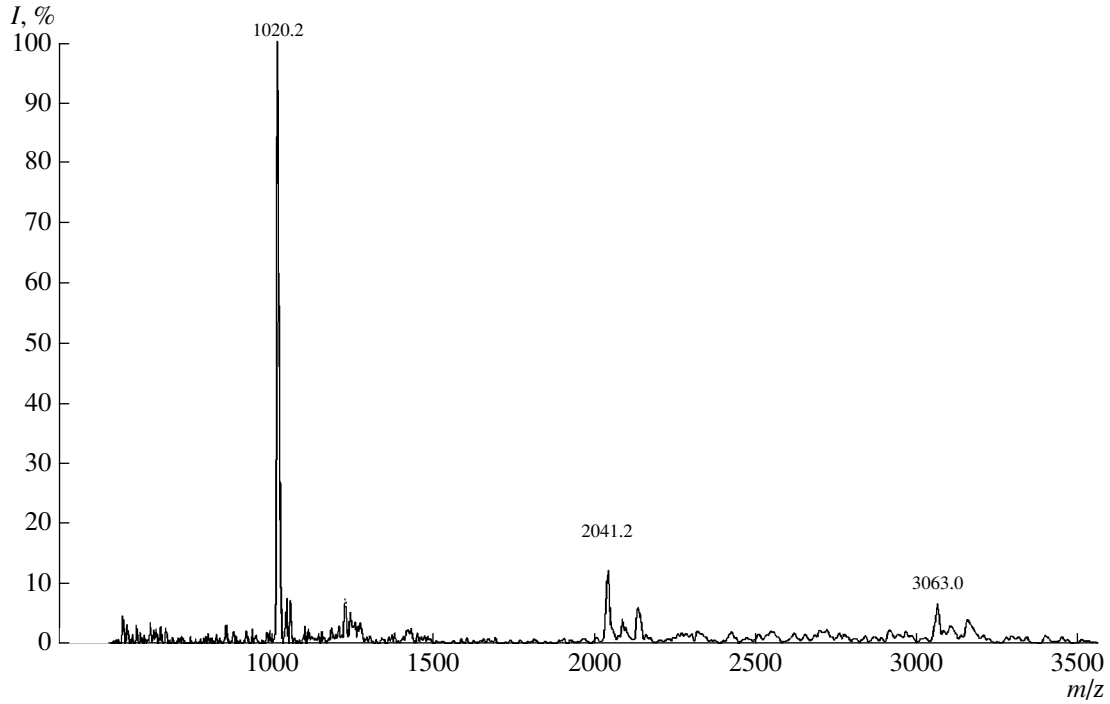

Fig. 4. Mass spectrum of the reaction mixture of the octreotide preparation at the concentration of 10 mg/ml of its [Cys(SH)$_2$]-precursor in methanol. The calculated molecular mass of octreotide is 1019.3. The values of $[M + H]^+$ are given on the X-axis.

(ESI-MS) and on an Analytical Compact MALDI 4 mass spectrometer (Kratos, UK) (MALDI-MS). Nitrogen in cylinders of o.s.ch. (special purity) grade (Rusia) was used in this study.

Solid phase peptide synthesis. We synthesized peptides on a 431A automatic peptide synthesizer (Applied Biosystems). The linear Acm-precursors of P4, APII$_r$, APIII$_r$, α-ANF$_r$, and somatostatin-(1–14) were prepared according to the Fmoc-strategy starting every time from 0.25 mmol of the corresponding Fmoc-aminoacyl polymer (Bachem, Switzerland). The content of starting amino acid in the Fmoc-aminoacyl polymers was 0.5–0.7 mmol/g. A copolymer of styrene and 1% divinylbenzene with 4-hydroxymethylphenoxymethyl anchoring group (the Wang polymer) was used as a support. The amino acid chains were elongated according to the standard programs for the one-step coupling of Fmoc-amino acids. The synthetic cycle involved a 20-min activation of the attached amino acid (1 mmol) in the presence of equimolar amounts of DIC and HOBt in NMP, deprotection of the α-amino groups by the treatment with 20% solution of piperidine in NMP for 20 min, coupling with the fourfold excess of acylating agent (1 mmol) in NMP for 90 min, and all the necessary intermediate washings of the peptidyl polymer.

When the solid phase synthesis was completed, the N$^\alpha$-deprotected peptidyl polymers were suspended in a mixture (10 ml) containing 82.5% TFA, 5% phenol, 5% water, 5% thioanisole, and 2.5% EDT and stirred for 2 h at 25°C. The polymer was filtered off and washed with TFA (3 × 1 ml). The combined filtrate was evaporated to the volume of about 1 ml and mixed with anhydrous ether. The precipitated product was filtered, washed with ether and ethyl acetate, and dried. The resulting [Cys(Acm)]$_2$ derivatives of the P4, APII$_r$, APIII$_r$, α-ANF$_r$ peptides, and the somatostatin-(1–14) were dissolved in 10% acetic acid and fractionated on a column with Diasorb (25 × 250 mm) in portions of 0.15–0.20 g. The peptides were eluted with a gradient of 80% acetonitrile in 0.1% TFA (0.5%/min) from 10 to 50% at a flow rate of 12 ml/min. The fractions containing the target products were combined, evaporated, dissolved in water, and lyophilized.

Our procedures of direct conversion of the [Cys(Acm)]$_2$-precursors into the cyclic disulfides by iodine [6, 10, 11, 19, 20] and deprotection of Cys residues and the subsequent formation of the disulfide bonds by hydrogen peroxide [6, 10, 11] were previously described and used for the preparation of P4 peptides, atrial peptides, and somatostatin-(1–14) (Table 1) and in the procedure of cyclization of a vasopressin analogue (see below). The yields and characteristics of the cyclic peptides we prepared using hydrogen peroxide for the first time are given in Tables 1 and 3. The content of peptide material in the lyophilized cyclic products was determined by the quantitative amino acid analysis; it was from 82 to 90%.

βMpa-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2 ([βMpa^1,Arg^8]vasopressin). βMpa(Acm)-Tyr-Phe-Gln-Asn-Cys(Acm)-Pro-Arg-Gly-NH_2 (0.2 g, 0.16 mmol) was dissolved in a mixture of acetic acid (15 ml) and methanol (5 ml), mercuric acetate (0.2 g, 0.64 mmol) was added to the solution, the reaction mixture was stirred for 1.5 h at 20°C, and hydrogen sulfide was bubbled through it for 30 min. The precipitate was filtered off and washed with a 2 : 1 mixture of acetic acid and methanol (15 ml). The filtrate was evaporated to the volume of approximately 2 ml, and ethyl acetate (20 ml) was added. The precipitated solid was filtered, washed with ethyl acetate and hexane, and dried. The resulting dithiol was dissolved in water (150 ml), adjusted with 25% ammonia to pH 8.0, and treated with a dilute solution of hydrogen peroxide with exact concentration (3 ml, 1.6 mmol). The reaction mixture was stirred for 30 min with the HPLC monitoring of the SS bridge formation. Acetic acid (2 ml) was added, and the reaction mixture was evaporated to the volume of 50 ml and fractionated on a column (25 × 250 mm) with Diasorb. Its elution with a gradient of 80% acetonitrile in 0.01 M ammonium acetate (pH 4.5) (12 ml/min, 1%/min, from 20 to 60%), and the fractions containing the target product were combined and evaporated. The residue was dissolved in water and lyophilized, to give the cyclic peptide; yield 0.13 g (81.0%); mass spectrum (MALDI-MS): found *m*/*z* 1068.0 [$M + H^+$], calculated 1069.24 for $C_{46}H_{64}N_{14}O_{12}S_2$. Amino acid analysis: Asx 1.01(1), Glx 1.10 (1), Gly 1.00 (1), Tyr 0.88 (1), Phe 1.01 (1). The content of peptide in the lyophilized material was 88%. The purity of the peptide was 98.0% according to HPLC.

H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 (oxytocin). A crude trifluoroacetate of H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 (20 g, contains 74% of the main product according to HPLC) was dissolved in a 1 : 5 DMF–methanol mixture (2 ml), adjusted with 25% ammonia to pH 7.5–8 (6 ml), and a solution of hydrogen peroxide (6 ml, 53.0 mmol) was added to the peptide solution. The reaction mixture was kept for 1 h at 25°C with the HPLC monitoring, acidified to pH 4 with acetic acid, and evaporated to the volume of 200 ml at 30°C. The residue was mixed with ether. The precipitate was filtered, washed with ether, dried, dissolved in 3% acetic acid (1 l) and fractionated on a Diasorb column (50 × 250 mm). The target peptide was eluted with a gradient of 80% acetonitrile in 0.01 M ammonium acetate (pH 4.5) (0.3%/min, from 10 to 50%) at a flow rate of 50 ml/min. The yield of oxytocin was 6.2 g (47%); mass spectrum (MALDI-MS): found *m*/*z* 1007.7 [$M + H]^+$, calculated 1007.2 for $C_{43}H_{66}N_{12}O_{12}S_2$. Amino acid analysis: Asx 0.98 (1), Glx 1.08 (1), Gly 1.00 (1), Ile 0.90 (1), Leu 0.80 (1), Tyr 0.84 (1). Content of the peptide in the lyophilized material was 94%, and purity of the peptide was 97.0% according to HPLC.

H-*D*-Phe-Cys-Phe-*D*-Trp-Lys-Thr-Cys-NH-CH-[CH(OH)CH_3]-CH_2OH (octreotide). Crude trifluoroacetate of H-*D*-Phe-Cys-Phe-*D*-Trp-Lys-Thr-Cys-threoninol (20 g of a raw synthetic product containing 82% of the target product according to HPLC) was dissolved in methanol (2 l), adjusted with 25% ammonia to pH 7.5–8 (6 ml), and mixed with a solution of hydrogen peroxide (6 ml, 53.0 mmol). The reaction mixture was kept for 1 h at 25°C with the HPLC monitoring.[3] The reaction mixture was acidified with acetic acid to pH 4, evaporated to the volume of 1 l at 30°C, and diluted with water (1 l). Methanol was evaporated, and the remaining solution was fractionated on a Diasorb column (50 × 250 mm). The target octreotide was eluted with a gradient of 80% acetonitrile in 0.01 M ammonium acetate (pH 4.5) (0.3%/min, from 20 to 60%) at a flow rate of 50 ml/min; yield 8.6 g (64%); mass spectrum (MALDI-MS): found *m*/*z* 1020.3 [$M + H]^+$, calculated 1019.3 for $C_{49}H_{66}N_{10}O_{10}S_2$. Amino acid analysis: Thr 0.87 (1), Phe 2.01 (2), Trp 0.69 (1), Lys 1.03 (1). The content of peptide in the lyophilized material was 87%, and the purity of the peptide was 98.2% according to HPLC.

REFERENCES

1. Andreu, D., Albericio, F., Sole, N.A., Munson, M.C., Ferrer, M., and Barany, G., *Peptide Synthesis Protocols*, Pennington, W. and Dunn, M.B., Eds., Totowa, NJ: Humana, 1994, pp. 91–169.
2. Sidorova, M.V., Kudryavtseva, E.V., and Evstigneeva, R.P., *Usp. Khim.*, 1998, vol. 67, pp. 611–630.
3. Hardy, P.M., Ridge, B., Rydon, H.N., Dos, S.P., and Serräo, F.O., *J. Chem. Soc. (C)*, 1971, pp. 1722–1731.
4. Moroder, L., Besse, D., Musiol, H.-J., Rudolph-Bohner, S., and Siedler, F., *Biopolymers*, 1996, vol. 40, pp. 207–234.
5. Misicka, A. and Hruby, V.J., *Pol. J. Chem.*, 1994, vol. 68, pp. 893–899.
6. Kudryavtseva, E.V., Sidorova, M.V., Ovchinnikov, M.V., Bespalova, Zh.D., and Bushuev, V.N., *J. Peptide Res.*, 1997, vol. 49, pp. 52–58.
7. Kudryavtseva, E.V., Sidorova, M.V., Molokoedov, A.S., Ovchinnikov, M.V., and Bespalova, Zh.D., *Bioorg. Khim.*, 1999, vol. 25, pp. 107–116.
8. Kudryavtseva, E.V., Sidorova, M.V., Ovchinnikov, M.V., Bespalova, Zh.D., Bushuev, V.N., and Evstigneeva, R.P., *Bioorg. Khim.*, 1996, vol. 22, pp. 370–375.
9. Papsuevich, O.S., Chipens, G.I., and Mikhailova, S.V., *Neirogipofizarnye gormony* (Neurohypophysial Hormones), Riga: Zinatne, 1986.
10. Sidorova, M.V., Kudryavtseva, E.V., Molokoedov, A.S., Ovchinnikov, M.V., and Bespalova, Zh.D., *Bioorg. Khim.*, 1995, vol. 21, pp. 675–683.

[3] The octreotide content in various reaction mixtures was from 70 to 75%.

11. Sidorova, M.V., Kudryavtseva, E.V., Antopol'skii, M.L., Pal'keeva, M.E., Ovchinnikov, M.V., and Bespalova, Zh.D., *Bioorg. Khim.*, 1996, vol. 22, pp. 273–279.
12. Eichler, J. and Hougthen, R.A., *Protein Peptide Lett.*, 1997, vol. 4, pp. 157–164.
13. Kudryavtseva, E.V., Sidorova, M.V., Ovchinnikov, M.V., and Bespalova, Zh.D., *J. Peptide Sci.*, 2000, vol. 6, pp. 208–216.
14. King, D.S., Fields, C.G., and Fields, G.B., *Int. J. Peptide Protein Res.*, 1990, vol. 36, pp. 255–266.
15. Hope, D.V., Murti, V.V.S., and du Vigneaud, V., *J. Biol. Chem.*, 1962, vol. 237, pp. 1563–1566.
16. McCurdy, S.N., *User Bulletin Peptide Synthesizer Applied Biosystems*, 1988, no. 25.
17. Kumagaye, S.-I., Kuroda, H., Nakajima, K., Watanabe, T.X., Kimura, T., Masaki, T., and Sakakibara, S., *Int. J. Peptide Protein Res.*, 1988, vol. 32, pp. 519–526.
18. Bauer, W. and Pless, J., Eur. Pat. Appl. 29 579 (1981); US Patent 4395403 (1983).
19. Ovchinnikov, M.V., Bespalova, Zh.D., Molokoedov, A.S., Titov, M.V., Revenko, I.V., Vinogradov, V.A., Korobov, N.V., and Zhukovskii, S.V., *Bioorg. Khim.*, 1988, vol. 14, pp. 777–782.
20. Ovchinnikov, M.V., Bespalova, Z.D., Molokoedov, A.S., Titov, M.I., Revenko, I.V., Vinogradov, V.A., Korobov, N.V., and Zhukovskii, S.V., *Collect. Czech. Chem. Commun.*, 1989, vol. 54, pp. 796–802.