SYNTHESIS OF [1-β-MERCAPTOPROPIONIC ACID, 8-D-ARGININE]-VASOPRESSIN (DDAVP) IN SOLID PHASE. SIMPLE OPTIMALIZATION*

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Received March 16th, 1978

A series of solid-phase syntheses of the protected precursor *II* of DDAVP was carried out. Experimental conditions were developed under which practically pure *II* can reproducibly be obtained in yields better than 60%. The protected precursors of DDAVP obtained by liquid- and solid-phase synthesis and DDAVP samples obtained from these precursors were undistinguishable by conventional analytical or pharmacological assays.

During the past ten years we have employed the method of solid-phase synthesis[1-3] of peptides in many isolated cases. The results of our experiments, judged by the yield and purity of the products obtained, and thus also the efficiency of the synthetic procedure, widely varied. This is not a drawback in isolated cases, at least not an essential one. If, however, our aim is to provide a routine base for solid-phase synthesis – and this is the case of syntheses of a numerous of vasopressin and oxytocin analogs – we are faced with the problem of optimalization of the procedure.

A great number of various solid-phase syntheses of oxytocin, vasopressin, and their analogs have been reported. Since the early applications of Merrifield's method[4-9] in this field the reaction cycle has undergone numerous important changes and methodical alterations decreasing the time of operation, increasing the purity of reaction products, and improving the analytical purity of the process. It has been stated in the early days[5] that the preparation of oxytocin by solid-phase synthesis is much faster than by liquid-phase synthesis. During the past few years considerable attention has been focused on the optimalization of the yields of the process[10-13]. The synthesis of oxytocin on the benzylhydrylamine and the Merrifield resins was recently examined from this aspect[12]. The majority of syntheses of oxytocin and vasopressin peptides are effected at present by a "mixed-type synthesis", *i.e.* the condensation is carried out by the carbodiimide method (with the addition of N-hydroxybenzotria-

* Part CLI in the series Amino Acids and Peptides; Part CL: This Journal *44*, 275 (1979). The symbols and abbreviations common in the chemistry of amino acids and peptides are used in this paper. Unless stated otherwise the amino acids are of L-configuration.

zole if necessary[14]), asparagine and glutamine are incorporated *via* activated esters (mostly *p*-nitrophenyl esters, the reaction being catalyzed by hydroxybenzotriazole[9,15]). Syntheses of the "single type" have been described in the oxytocin series[16–19]. The vasopressins, including DDAVP, have so far been prepared by the "mixed-type procedure". The "single-type" synthesis of arginine-vasopressin on the benzylhydrylamine resin was described not long ago[13].

This study was intended to follow the possibly simplest synthetic scheme ("single-type" synthesis) which would be reliable and would give good yields. The classical Merrifield resin was used as a carrier. The synthetized peptide was split off the resin by ammonolysis[5,20]. This approach permits the purity of the peptide to be checked or, if necessary, the protected peptide to be purified or further modified. We carried out several experiments designed to examine the relation between several basic indices (coupling period, repeating of coupling step, concentration, deblocking time) and the yield and purity of the reaction product. We used the usual combination of protecting groups (Boc – mobile; Tos – stable; Bzl – thiol groups; we did not protect the hydroxyl group of tyrosine). The condensation was effected by dicyclohexylcarbodiimide, the removal of the Boc residue by trifluoroacetic acid. The purity of the protected peptide was checked by the determination of melting point, optical activity, amino acid composition, and by thin-layer chromatography. To save the relatively expensive D-arginine, the initial experiments were carried out with L-arginine.

It has been shown[4–7,21] that oxytocin and vasopressin prepared by liquid- and solid-phase synthesis have the same chemical, pharmacological, and biological properties. However, even to date we meet with an opposite opinion according to which the peptides prepared by solid-phase synthesis are inferior and unsuitable for pharmacological and particularly clinical studies. The question of equivalence of peptides prepared by liquid- and solid-phase synthesis was of essential importance for our future work. Therefore, simultaneously with studies on the methodical angle of the problem, we also compared the products prepared by liquid- and solid-phase synthesis. For this reason we chose DDAVP for our experiments whose properties are well known and can be analyzed by reliable and dependable tests.

The quality of the products prepared under different conditions, as judged by the melting point, was essentially the same, naturally with the exception of the product prepared in the first experiment where the unreacted amino groups were not blocked after the first condensation. (The key experiments are listed in Table I. Each value is a mean of two experiments.) In all cases we were able to obtain by one single crystallization from aqueous acetic acid a product of practical purity, comparable with the purity of products obtained by liquid-phase synthesis and used for the preparation of DDAVP. The optical activity of once crystallized samples was by about 2° lower than the activity of comparable products prepared by liquid-phase synthesis. After additional crystallization from the same solvent system their optical activity

increased to a value corresponding to that of a pure product prepared by liquid-phase synthesis. The yields varied widely according to the reaction conditions. From this viewpoint repeated condensation was more favorable than single-run condensation or repeated single-run condensation carried out for a prolonged reaction period. Repeated short-time deblocking in 30% solution of trifluoroacetic acid in dichloromethane was superior than long-time deblocking in 80% solution. Very satisfactory yields were obtained with a combination of repeated, short-time deblocking in 30% trifluoroacetic acid, even when a 1·5-fold excess of Boc-amino acids was used. The increase of the concentration of the reaction components resulted in a small yet marked increase of the yield. If the reaction cycle is viewed from the point of time consumption, simplicity, and yields, then the most convenient one appears the procedure employed in experiments 5 and 7: either simple, relatively long coupling (4 h) combined with repeated deblocking in 30% trifluoroacetic acid (exp. 5) or repeated, short-time coupling with a 1·5-fold excess of Boc-amino acids, carried out in a more concentrated solution and with repeated deblocking in 30% trifluoroacetic acid. Other possibilities of optimalization of the process are obvious.

The liquid-phase synthesis of *II* is intended as a fragment condensation and designed so that D-Arg is involved in the possibly smallest number of reaction steps[22]. The yields of *II* prepared by routine syntheses are 20–35% (calculated in terms of Gly

TABLE I
Solid-phase Synthesis of DDAVP

Experiment	Condensation			Deblocking			Yield crude %	M.p. °C	Yield cryst. %	M.p. °C
	Boc-amino acid, equiv.	time, min		concentration of CF_3COOH/CH_2Cl_2 [%]	time, min					
		1.	2.		1.	2.				
1[a]	3	120	—	80	60	—	61·6	65–180	24	196–198
2[b]	3	120	120	80	60	—	40	175–185	23	195–197
3	3	120	120	80	60	—	72·5	183–195	50	197–199
4	3	240	—	80	60	—	50·5	184–191	35	195–198
5	3	240	—	30	5	15	88·5	187–194	63	195–198
6	1·5	20	30	30	5	15	79·6	180–189	45	195–198
7[c]	1·5	20	30	30	5	15	82·5	185–194	54	195–198

[a] No blocking after condensation of Boc-Arg(Tos); [b] the condensation of Boc-Gln and Boc-Tyr only was repeated. Deblocking repeated only with Boc-Gln; [c] reaction mixture whose concentration was 5 times higher.

or D-Arg, respectively; the number of reaction steps involving D-Arg is 4 and the average yields are 67·5 – 77·5%). The solid-phase process represents stepwise synthesis from the carboxyl terminus. The best yield obtained in this study is 63% (calculated in terms of the first amino acid attached to the resin; 8 condensation steps, the average yield of individual step 94·5%). The yield of the solid-phase process is 1·8 – 3·15 times higher than the yield of the liquid-phase synthesis. The disadvantage of the solid-phase synthesis which requires an approximately three-fold excess of the Boc-amino acids is thus practically eliminated (we disregard the possibility of regeneration of Boc-amino acids). The mass balance of the synthesis of *II* in solid and liquid phase is therefore practically the same. The solid-phase version of the synthesis of *II* is considerably more simple from the point of operation and substantially shorter. From the point of effectiveness of synthetic work solid-phase synthesis is less laborious and less time-consuming.

Product *II* obtained by solid-phase synthesis was converted into *I* by conventional procedures[23]. The product could not be distinguished by the conventional tests used as purity checks of *I* (TLC, electrophoresis, amino acid analysis, determination of nitrogen, optical activity and UV-spectrum, polarography) from the product which had been prepared from the protected peptide obtained by liquid-phase synthesis. The composition of crude *I* was the same regardless of the procedure of preparation of *II*. Hence, the by-products found in the crude product are formed while the protecting groups are split off in liquid ammonia and during the subsequent operations. They are not formed during the synthesis of *II* and they are not characteristic of the procedure of preparation of *I*.

The biological activity of *I* prepared from *II* obtained by solid-phase synthesis was examined in the usual manner[24] with two samples from two independent runs. The antidiuretic effect was 83% and 106% of a standard sample in the first and second case, respectively. Therefore *I* prepared from *II* obtained by solid-phase synthesis has full biological activity.

EXPERIMENTAL

The instruments and analytical procedures used in this study are the same as those described elsewhere[23]. The chromatographic purity of the protected peptides was carried out in the system tert-butanol–n-butanol–acetic acid–water (2 : 2 : 1 : 1). The syntheses were performed in an instrument operated manually. Chloromethylated polystyrene cross-linked by 2% divinylbenzene served as a carrier (Calbiochem, Los Angeles, U.S.A.; chlorine content 5·9%). The preparation of the resin for the synthesis and its esterification were carried out in the usual manner. The glycine content of the resin was 0·88 mmol/g. Dried and freshly redistilled solvents were used.

Syntheses of β-Benzylthiopropionyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl--S-benzylcysteinyl-prolyl-N^G-tosyl-D-arginyl-glycine Amide (*II*)

The reactions were carried out with 1·0 g of esterified resin in a 45 ml reaction vessel. The volume of the reaction mixture (in all steps) was c. 20 ml. The Boc-groups were removed by a solution of trifluoroacetic acid in dichloromethane. When two-step deblocking was used the washing of the resin between the steps was omitted. At the end of the deblocking the resin was washed three times with dichloromethane, neutralized by triple washing with 10% solution of triethylamine in dichloromethane, and finally washed 5 times with dichloromethane. The condensation reactions were effected mostly in dichloromethane, the condensation of Boc-Asn and Boc-Tyr in a mixture of dichloromethane and dimethylformamide (1 : 1). When repeated condensation was used the resin was washed with dimethylformamide (3 times) and dichloromethane (3 times) between the two condensations. The initial concentrations of the solutions were chosen so that the reaction mixture be 1 molar with respect to the Boc-amino acid. After the first condensation the unreacted amino groups were blocked by a solution of acetic anhydride and triethylamine in dichloromethane (2 ml of acetic anhydride, 0·7 ml of triethylamine, and 17 ml of dichloromethane) (except for the first experiment). The condensations were effected by dicyclohexylcarbodiimide. When the peptide chain was extended by Boc-Asn and other Boc-amino acids, an equivalent quantity of hydroxybenzotriazole was added to the reaction mixture. When the Boc-group was removed from tyrosine, anisol (2 ml) was added to the reaction mixture. At the end of the synthesis the washed and dried resin was suspended in methanol (40 ml of methanol per 1 g of resin) in a pressure vessel, the suspension was saturated with ammonia at 0°C, the vessel was sealed off, and the mixture was stirred 24 h at room temperature. The resin was filtered off and extracted with hot (100°C) dimethylformamide (3 times with 20 ml). The pooled filtrates were taken to dryness at reduced pressure, the dry residue was triturated with ether, filtered off, dried, and weighed. It was subsequently recrystallized from aqueous acetic acid, filtered off, washed on the filter with water, dried, and weighed.

[1-β-Mercaptopropionic Acid, 8-D-arginine]vasopressin *I*

Product *I* was prepared from the protected peptide according to[23]. In two independent experiments 504 mg (227 mg) of the 1st lyophilisate and 202 mg (105 mg) of the 2nd lyophilisate were obtained from 870 mg (420 mg) of the protected peptide. For $C_{46}H_{64}N_{14}O_{12}S_2$ (1069·2) calculated: 18·34% N; found: 14·90% N (15·45% N). The samples contained therefore 81% (84%) of peptide, according to polarographic determination 75% (78%). $[\alpha]_D^{20}$ −60·3° (−62·1°) (*c* 0·5, 1M-CH_3COOH). Amino acid analysis: Tyr 0·98 (0·97), Phe 1·03 (1·03), Glu 0·97 (1·00), Asp 0·98 (1·01), Pro 1·03 (1·05), Arg 1·00 (1·00), Gly 1·00 (0·95).

* See Table I for complementary data.

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Translated by V. Kostka.