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## Solid Phase Synthesis and Biological Activities of [Arg<sup>8</sup>]-Vasopressin Methylenedithioether

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### Abstract:

Solid phase synthesis of [Arg<sup>8</sup>]-vasopressin methylenedithioether, an analog of vasopressin which contains an extra methylene group between the two sulfur atoms of Cys<sup>1</sup> and Cys<sup>6</sup>, is described. Methylene insertion occurred easily when the thiol free peptide on a solid support was treated with tetrabutylammonium fluoride in dichloromethane at room temperature for 3 h. The uterotonic *in vitro*, pressor, and antidiuretic activities of the compound were reduced in comparison to [Arg<sup>8</sup>]-vasopressin by one order of magnitude. © 1999 Elsevier Science Ltd. All rights reserved.

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### Introduction

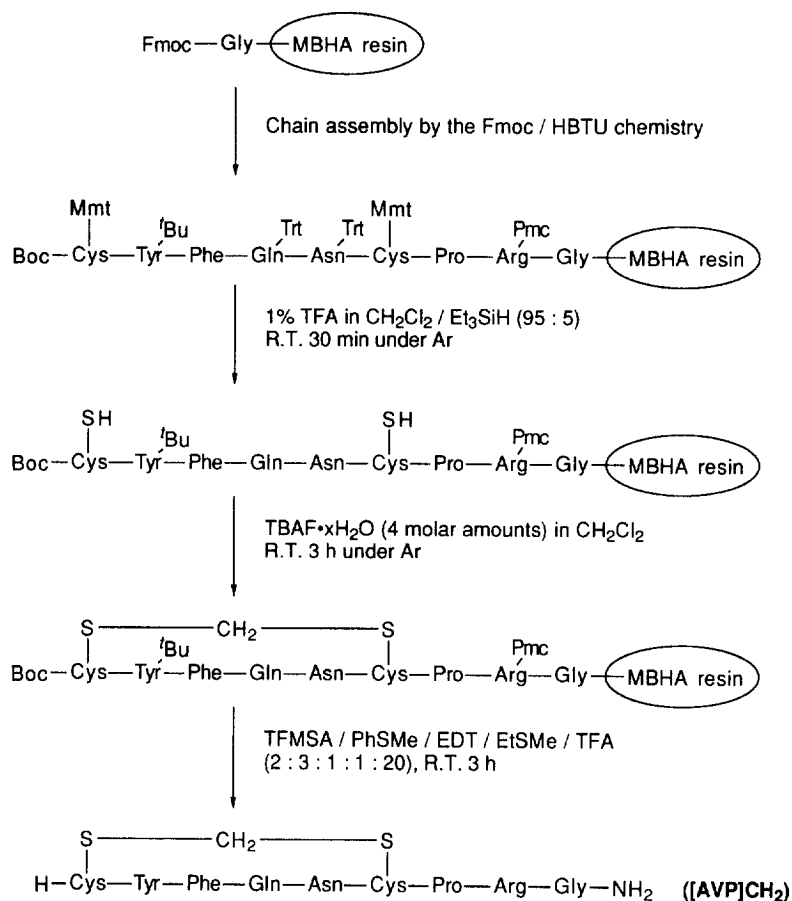
Recently a trisulfide derivative was found in biosynthetic human growth hormone produced in *E. coli* [1]. This finding aroused a strong interest in the activities of trisulfide analogs of biologically active peptides [2,3]. The trisulfide bond is rather stable at acid pH [2,3], however at pH 7 its stability is much lower and at pH 9 it rapidly converts to a disulfide [2]. In the past years we have been establishing a very mild method for inserting a methylene group to form the S-CH<sub>2</sub>-S bond, which corresponds to a mono-carba analog of the trisulfide bond [4, 5, 6, 7]. However, these works have so far been performed in solution. In this study we extended this methodology to perform the highly efficient solid phase cyclization to give an [Arg<sup>8</sup>]-vasopressin methylenedithioether analog.

### Synthesis

The above mentioned S-CH<sub>2</sub>-S bond-forming reaction was found to occur when the thiol-protecting dimethylphosphinothioyl (Mpt) group [8, 9] of Z-Cys(Mpt)-OMe was removed with tetrabutylammonium fluoride hydrate (TBAF·xH<sub>2</sub>O) in CH<sub>2</sub>Cl<sub>2</sub> [4, 7]. The reaction has

already been applied to a highly efficient intramolecular cyclization of Cys-containing peptides prepared by the Boc strategy in solution [5, 6, 7]. However, we have not so far obtained any reproducible result in the solid-phase cyclization of a linear precursor prepared by the Boc strategy. In some cases, many peaks, which did not correspond to the desired compound with S-CH<sub>2</sub>-S bond, were observed by HPLC. Since the thioacetal structure is generally stable under acidic conditions, there must be a number of side reactions occurring in the solid matrix used for the Boc synthesis to prevent the S-CH<sub>2</sub>-S bond formation. In parallel with studies to identify and remove these side reactions, the Fmoc strategy was tried.

The *S*-Mpt group is very labile under basic conditions and its use is limited to the Boc strategy. However, we found that the thiol-free form reacted similarly to give the desired methylene insertion products [6, 7]. This extension has made it possible to synthesize cyclic S-CH<sub>2</sub>-S analogs of peptides containing penicillamine, that did not allow the use of an *S*-Mpt derivative [6]. Through these studies, the optimal conditions for the highly efficient S-CH<sub>2</sub>-S bond forming cyclization in solution were established. Subsequently, in this study, we applied successfully these conditions to solid phase side-chain cyclization.



Scheme 1.

A synthetic route to [Arg<sup>8</sup>]-vasopressin methylenedithioether ([AVP]CH<sub>2</sub>) was summarized in Scheme 1. To accomplish selective deprotection of thiol groups on a solid support, we used 4-methoxytrityl (Mmt) group [10] for the thiol protection and 4-methylbenzhydrylamine (MBHA) resin [11] as a solid support. The linear sequence of [Arg<sup>8</sup>]-vasopressin (AVP) was assembled using the Fmoc/*N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU) chemistry [12] excluding the *N*-terminal Cys residue, which was incorporated in the Boc form. Two *S*-Mmt groups were removed selectively with 1% trifluoroacetic acid (TFA) in CH<sub>2</sub>Cl<sub>2</sub>/triethylsilane (95:5) for 30 min [10] and then the resin was treated with 4 molar amounts (2 molar amounts each for a thiol group) of TBAF·xH<sub>2</sub>O in CH<sub>2</sub>Cl<sub>2</sub> at R.T. for 3 h. Release with trifluoromethanesulfonic acid (TFMSA)/thioanisole (PhSMe)/1,2-ethanedithiol (EDT)/ethyl methyl sulfide (EtSMe)/TFA (2:3:1:1:20) gave a crude peptide in 88% yield based on the content of Gly in the starting resin. The pure material, as shown in Fig. 1 [13], could easily be obtained by preparative HPLC giving a total yield of 73%. FAB-MS, as shown in Fig. 2, and amino acid analysis confirmed the structure of the product [14].

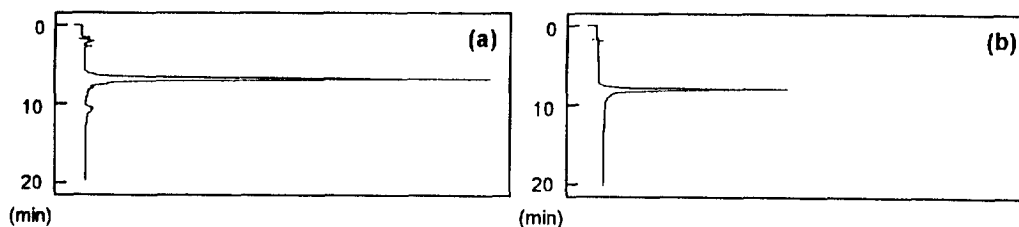


Figure 1. HPLC profiles of crude (a) and purified (b) [AVP]CH<sub>2</sub>.

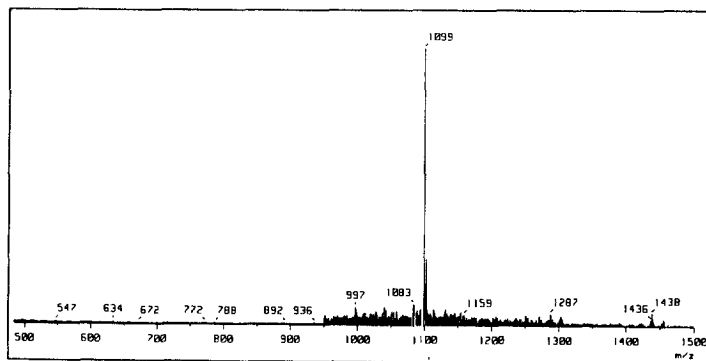


Figure 2. FAB-MS spectrum of [AVP]CH<sub>2</sub>.

### NMR analysis

In order to confirm the product and obtain the structural information in solution, the NMR observation of [AVP]CH<sub>2</sub> was performed. The sample (5.0 mg) was dissolved in 0.5 mL DMSO-d<sub>6</sub>. The DQF-COSY and NOESY spectra were observed on a Bruker AM-500 spectrometer. All the data processing were performed on Felix v-9.50 program on SGI computer. The assignment was performed with these 2D NMR technique and with reference to the previous assignment of AVP [15]. The <sup>1</sup>H chemical shift values are listed in Table 1 together with the assignment. No significant

difference in the chemical shifts was observed between [AVP]CH<sub>2</sub> and AVP taken from the literature [15] in parenthesis in Table 1, except for the NH protons of Tyr<sup>2</sup> and Cys<sup>6</sup>. It should be noted that the two protons of S-CH<sub>2</sub>-S were separately observed ( $J = 13.1$  Hz). Restriction of movement of this methylene group suggests that, in spite of ring expansion, the new S-CH<sub>2</sub>-S bond-forming cyclization would be a useful method to supply conformationally restricted cyclic analogs of peptides. The temperature coefficients of NH protons summarized in Table 2 showed that the hydrogen bond between Asn<sup>5</sup> N and Tyr<sup>2</sup> O was preserved in the CH<sub>2</sub>-expanded ring analog.

Table 1.

<sup>1</sup>H chemical shift values of [Arg<sup>8</sup>]-vasopressin methylenedithioether in (CD<sub>3</sub>)<sub>2</sub>SO at 300K.\*

Residue	Chemical shift of			
	NH	H $\alpha$	H $\beta$	others
Cys 1	8.26(8.32)	4.05(4.05)	3.34/2.95(3.46/3.06)	—
Tyr 2	9.32(8.72)	4.30(4.41)	2.73/2.66(2.83/2.60)	$\delta$ 6.88(6.95), $\epsilon$ 6.59(6.64)
Phe 3	8.26(8.29)	4.43(4.23)	3.25/2.91(3.19/2.93)	$\delta$ 7.31(7.19), $\epsilon$ 7.22(7.28), $\zeta$ 7.22(7.28)
Gln 4	8.58(8.39)	3.93(3.93)	1.94/1.82(1.96/1.89)	$\gamma$ 2.20(2.14), N $\epsilon$ 2 7.29/6.83(7.29/6.84)
Asn 5	8.02(7.95)	4.53(4.54)	2.68/2.52(2.61/2.61)	N $\delta$ 2 7.55/7.02(7.44/6.95)
Cys 6	7.74(8.19)	4.75(4.72)	2.90/2.94(3.00/3.13)	—
-CH <sub>2</sub> -	4.12/3.87			
Pro 7	—	4.26(4.30)	1.94/1.82(2.02/1.83)	$\gamma$ 1.86(1.88), $\delta$ 3.72/3.56(3.63/3.57)
Arg 8	8.10(8.17)	4.18(4.17)	1.80/1.64(1.75/1.56)	$\gamma$ 1.50(1.50), $\delta$ 3.12(3.10), $\epsilon$ 7.56(7.58)
Gly 9	7.91(7.94)	3.68/3.57(3.60/3.53)		NH <sub>2</sub> 7.16/7.08(7.18/7.08)

\* The values in parenthesis are assignment of AVP [15].

Table 2.

Temperature coefficients of backbone amide <sup>1</sup>H-chemical shifts values.

Residue	$\Delta\delta/\Delta T$ (ppb/K)		Residue	$\Delta\delta/\Delta T$ (ppb/K)	
	AVP	[AVP]CH <sub>2</sub>		AVP	[AVP]CH <sub>2</sub>
Cys 1	-1.8	-1.0	Asn 5	-1.7	-1.3
Tyr 2	-2.6	-4.5	Cys 6	-4.8	-2.0
Phe 3	-4.4	-3.4	Arg 8	-4.5	-3.9
Gln 4	-2.6	-5.5	Gly 9	-3.9	-2.6

### Biological activities

Activities of the AVP methylenedithioether analog were determined in the following tests: uterotonic test *in vitro* in the absence of magnesium ions [16, 17], in the vasopressor test using phenoxybenzamine treated male rats [18], and in the antidiuretic test on conscious rats [19, 20]. Wistar rats were used in all experiments. Female rats were estrogenized 24–48 h before conducting the tests. Synthetic oxytocin was used as a standard in the uterotonic test and synthetic AVP in the pressor test. Cumulative dose-response curves or dose-response

curves were constructed. The activity was determined by comparing the threshold doses of the standard and the synthetic analog (IU/mg). The results are summarized in the Table 3.

Direct comparison of the activities of our methylenedithioether AVP analog and the isosteric trisulfide analog of AVP [2] is not possible because in ref. [2] only binding affinities to the receptors are given. The activity of the AVP methylenedithioether analog is reduced in comparison to AVP in all tests by almost the same factor, i.e., one order of magnitude. Dose-response curves for the analog were in all tests parallel to that of AVP. This means that methylenedithioether analogs could be used as chemically and physiologically stable substitutes of biologically active peptides with disulfide bond.

Table 3.  
Biological activities of the [Arg<sup>8</sup>]-vasopressin methylenedithioether

Test	Activity (IU/mg)	
	AVP*	[AVP]CH <sub>2</sub>
Uterotonic <i>in vitro</i>	17	1.4±0.3 (n=4)
Pressor	412	55.3±14.3 (n=4)
Antidiuretic	465	46.5

\* Values according to Ref 21.

## Conclusion

A highly efficient S-CH<sub>2</sub>-S bond-forming cyclization method on the solid support became available. The chemistry used is very simple with no accompanying side reactions. Although the potencies of the methylenedithioether analog of AVP were reduced, the profile of the biological activities was quite similar to that of [Arg<sup>8</sup>]-vasopressin. Considering the general stability of the S-CH<sub>2</sub>-S bond under chemical and physiological conditions, methylenedithioether analogs would have a potential as good substitutes of disulfide-containing peptides. Syntheses of methylenedithioether analogs of other biologically active peptides are now in progress in our laboratory.

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- [13] HPLC conditions: Column:  $\mu$ Bondasphere 5 $\mu$  C18 (3.9 x 150 mm); Gradient: acetonitrile-0.1% TFAaq. 15/85 to 25/75 over 20 min (linear gradient); Flow rate: 1.0 ml/min; Detection: 220 nm.
- [14] FAB-MS: Found: m/z 1099. Calcd for C<sub>47</sub>H<sub>68</sub>N<sub>15</sub>O<sub>12</sub>S<sub>2</sub>: (M+H)<sup>+</sup>, 1099. Amino acid ratios (4% HSCH<sub>2</sub>COOH in 6 M HCl, 120°C, 24 h): Asp 1.08(1), Glu 1.06(1), Gly 1.11(1), Tyr 0.90(1), Phe 1.11(1), Arg 1.07(1), Pro 1.01(1), Djerkolic acid 0.95(1).
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