Amino Acid Sequence of the *Anthopleura xanthogrammica* Heart Stimulant, Anthopleurin A†

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ABSTRACT: A highly potent heart stimulant, anthopleurin A, from *Anthopleura xanthogrammica* was shown to exist as a single polypeptide chain consisting of 49 amino acid residues. The sequence of the peptide was shown to be: Gly-Val-Ser-Cys-Leu-Cys-Asp-Ser-Asp-Gly-Pro-Ser-Val-Arg-Gly-Asn-Thr-Leu-Ser-Gly-Thr-Leu-Trp-Leu-Tyr-Pro-Ser-Gly-Cys-Pro-Ser-Gly-Trp-His-Asn-Cys-Lys-Ala-His-Gly-Pro-Thr-Ile-Gly-Trp-Cys-Cys-Lys-Gln as judged by Edman degradation of the carboxymethylcysteine derivative and the tryptic peptides obtained from the derivative. Although six carboxymethylcysteine residues were present in the polypeptide, no cysteine residues were detectable in the native protein, indicating that there are three cystine residues in anthopleurin A.

In a series of earlier publications (Shibata et al., 1974; Quinn et al., 1974), it was reported that extracts of sea anemones caused a marked positive inotropic effect on isolated rat atria. The 50% effective dosage was reported to be 4.4×10^{-9} M for the purified peptide on isolated guinea pig atria (Shibata et al., 1975). The heart stimulant has been named anthopleurin A (AP-A)[1] and was found to have inotropic but no chronotropic effects on mammalian heart preparations as well as on the in situ heart. AP-A appeared to be superior to ouabain, especially in its lack of side effects, and, therefore, if clinical trials confirm this finding, it may be useful as a human heart stimulant. Anthopleurin A has been shown to have no effect on the Na^+/K^+ ATPase, monoamine oxidase, nor on adenosine 3′,5-phosphodiesterase (Shibata et al., 1975). At the present time, its mode of action on the heart is unknown.

In order to study its mechanism of action and for structure–function studies, purified preparations were needed and a procedure for purifying the heart stimulant from the sea anemone, *Anthopleura xanthogrammica,* was developed (Norton and Kashiwagi, 1976). Anthopleurin A, the major component isolated, was shown to be a polypeptide consisting of about 53 amino acid residues. In the present investigation, the amino acid sequence of anthopleurin A has been determined and the details of the sequence determination are presented in this publication.

Experimental Procedures

Isolation of Anthopleurin A. The AP-A was isolated from the sea anemone, *Anthopleura xanthogrammica,* from Bodega Bay, Calif., as previously described (Norton and Kashiwagi, 1976). In a typical experiment, about 5 mg of native AP-A was further purified on a column (1.0 × 10 cm) of SE-Sephadex C-25 equilibrated with 0.05 M phosphate (pH 6.0). The sample was eluted by linear gradient elution obtained by mixing 50 ml of the equilibrating buffer with 50 ml of the same buffer containing 0.25 M NaCl. The flow rate was 66 ml/hr and volume of each fraction was 2.2 ml. The fractions were detected by measuring absorbance at 280 nm.

Preparation of the Carboxymethylcysteine Derivative of AP-A. The procedure described by Crestfield et al. (1963b) was used to convert the AP-A to the carboxymethylcysteine derivative. The carboxymethylated reaction mixture was immediately put onto Sephadex G-50 gel and eluted with 0.1 M ammonia.

Amino Acid Analyses. The acid hydrolysates of the AP-A and peptides were analyzed in the Beckman Model 120 C automatic amino acid analyzer as described by Spackman et al. (1958).

End Group Analyses. The NH_2-terminal amino acid analysis was carried out as described by Edman and Sjoquist (1956). COOH-terminal analyses used were the carboxypeptidase A–B procedure described by Ambler (1967) and the hydrazinolysis procedure of Bradbury (1958).

Sequence Determinations. The NH_2-terminal sequences of the AP-A were determined on both the carboxymethyl-AP-A and the 4-sulfophenylthiocarbamoyl derivative of the carboxymethyl-AP-A. The reaction of carboxymethyl-AP-A with 4-sulfophenyl isothiocyanate was carried out according to Braunitzer et al. (170). The NH_2-terminal sequences of all the other peptides were determined by the usual manual Edman degradation procedure (Edman and Sjoquist, 1956). All of the reagents used for sequence analyses were of sequanal quality and were purchased from the Pierce Chemical Co. The amino acid phenylthiohydantoins were identified by gas chromatography as described by Pisano and Bronzert (1969), by thin-layer chromatography as described by Edman and Begg (1967), and by amino acid analyses of acid hydrolysates of the amino acid phenylthiohydantoins as described by Van Orden and Carpenter (1964).

Tryptic Digestion of AP-A and Purification of the Tryptic Peptides. About 0.8 μmol of the carboxymethyl-AP-A was digested with 5% trypsin for 24 h at 28 °C. Prior to the use, trypsin was treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (Wang and Carpenter, 1965). The tryptic digest was fractionated on a Dowex 1-X2 column (0.7 × 10 cm). For the elution of the peptides, linear gradient elution was first used in which 50 ml of 0.1 M pyridine was added to the mixing chamber and 50 ml of 0.5 M acetic acid was added to the reservoir with the mixing chamber open. After the gradient elution step was finished, the peptides were then eluted with

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[1] Abbreviations used: AP-A, anthopleurin A; Pth, phenylthiohydantoin; CM, carboxymethyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

TABLE I: Amino Acid Composition Data of Anthopleurin A.

Amino Acid	Residues per Mole: Previous Data[a]	From Present Sample[b]	From the Sequence
Lys	2	1.98 (2)	2
His	2	1.94 (2)	2
Arg	2	0.96 (1)	1
Asp	5	3.92 (4)	4[e]
Thr	3	2.93 (3)	3
Ser	5	5.75 (6)	6
Glu	3	1.06 (1)	1[f]
Pro	5	3.85 (4)	4
Gly	8	7.90 (8)	8
Ala	2	1.00 (1)	1
½-Cys	6	6.03[c] (6)	6
Val	2	1.94 (2)	2
Met	0	(0)	0
Ile	1	1.02 (1)	1
Leu	4	4.00 (4)	4
Tyr	2	1.02 (1)	1
Phe	1	(0)	0
Trp	0	3.06[d] (3)	3
Total residues	53	49	49
Mol wt	5500		5133

[a] Shibata et al. (1975). [b] Acid hydrolyses were performed on carboxymethylanthopleurin A for 24 and 48 h at 110 °C with 5.7 N HCl. The amino acid residues were calculated on the basis of an alanine content of 1.00 mol/mol of pure protein. Extrapolations to zero time were made for threonine and serine. Values in parentheses indicate values rounded off to nearest whole number. [c] Determined as *S*-β-carboxymethylcysteine. [d] Determined spectrophotometrically as described by Goodwin and Morton (1946). [e] Sum of 2 aspartic acid and 2 asparagine residues. [f] Exists as glutamine in the COOH terminus of the protein.

20 ml of 6.0 M acetic acid. Fractions of 2.5 ml were collected and an aliquot of each fraction was assayed by a ninhydrin procedure following alkaline hydrolysis (Crestfield et al., 1963a). Each fraction with a positive ninhydrin reaction was pooled and further purified by paper chromatography using the solvent system of 1-butanol–pyridine–acetic acid–water (60:40:12:48, v/v/v/v).

Peptide Nomenclature. Peptides obtained from the tryptic hydrolysis of the carboxymethyl-AP-A are designated by the symbol T.

Results

Purity of Anthopleurin A. Anthopleurin A, when tested for purity, showed a single band by sodium dodecyl sulfate disc electrophoresis or isoelectric focusing and showed a single peak when chromatographed on Sephadex G-50. The amino acid composition of material at this stage of purification is shown in Table I. However, when the sample was applied to a SE-Sephadex column, a slight impurity was separated from the main peak as shown in Figure 1. The main peak fractions were pooled and subjected to desalting by Sephadex G-50 column. The eluting solvent was 0.1 M acetic acid. The sample thus obtained was finally purified by paper chromatography using the solvent system of 1-butanol–pyridine–acetic acid–water (60:40:12:48, v/v/v/v). The R_f value of AP-A was 0.52 and, after spraying with ninhydrin, the color of the peptide changed from yellow to violet. The amino acid composition of the sample thus obtained is shown in column 2 of Table I. The

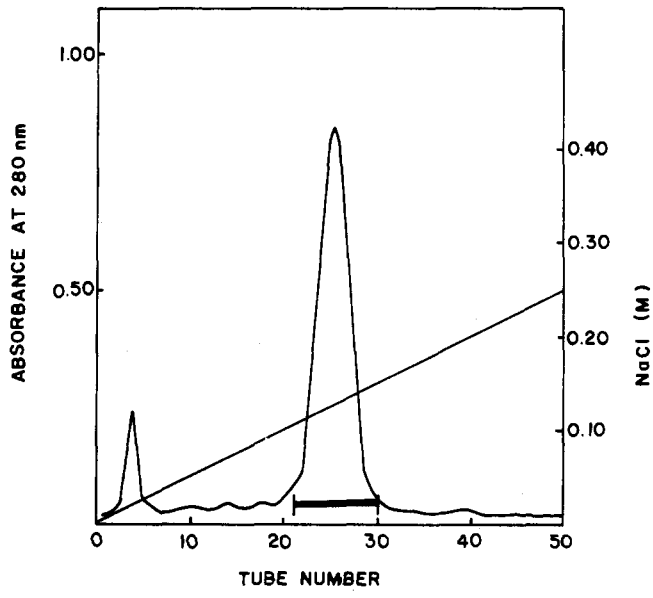

FIGURE 1: Cation exchange column chromatography of native *Anthopleura xanthogrammica* anthopleurin A (about 5 mg) on SE-Sephadex C-25. See Experimental Procedures for experimental details. Fractions under a peak which were pooled are shown by a solid bar.

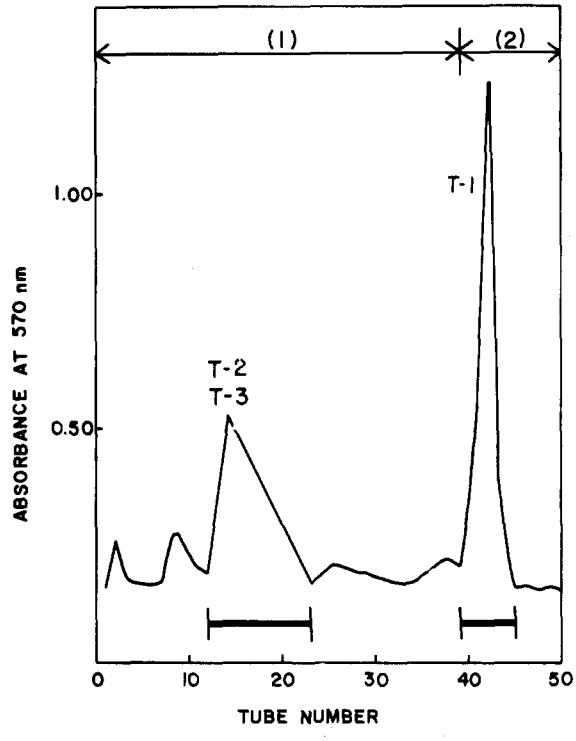

FIGURE 2: Anion exchange column chromatography of the tryptic peptides of carboxymethylanthopleurin A on Dowex 1-X2. See Experimental Procedures for details of experimental conditions. Fractions under each peak which were pooled are indicated by solid bars.

amino acid composition was now in complete agreement with the sequence found for anthopleurin A.

Tryptic Digestion of the Carboxymethylcysteinylanthopleurin A. The tryptic digest of carboxymethyl-AP-A was applied to a Dowex 1-X2 column and the chromatogram obtained is shown in Figure 2. Two main peaks were observed. The first peak contained two peptides, T-2 and T-3. The second peak contained peptide T-1. The separation of peptides, T-2 and T-3, was accomplished by band separation of the mixture

TABLE II: Amino Acid Composition[a] and Properties of the Tryptic Peptides from Carboxymethylanthopleurin A.

Amino Acid	T-1	T-2	T-3	Total Residues
Lys		1.01 (1)	1.00 (1)	2
His		0.94 (1)	0.93 (1)	2
Arg	0.95 (1)			1
CM-Cys	1.84 (2)	1.88 (2)	1.90 (2)	6
Asp	2.05 (2)	1.98 (2)		4
Thr		2.09 (2)	1.04 (1)	3
Ser	2.80 (3)	2.86 (3)		6
Glu			1.03 (1)	1
Pro	0.90 (1)	1.98 (2)	1.02 (1)	4
Gly	1.99 (2)	3.97 (4)	2.02 (2)	8
Ala			1.00 (1)	1
Val	1.96 (2)			2
Ile			0.99 (1)	1
Leu	1.00 (1)	2.96 (3)		4
Tyr		1.03 (1)		1
Trp[b]		2.02 (2)	1.04 (1)	3
Total residues	14	23	12	49
Yield (%)	81	60	60	
R_f in BPAW[c]	0.25	0.60	0.38	
Color reaction with ninhydrin	Yellow to violet	Yellow to violet	Violet	
Ehrlich reaction		Blue	Blue	
Pauly reaction		Pink	Pink	
Purification method	BPAW[c]	BPAW[c]	BPAW[c]	

[a] Results from 22-h 6 N HCl hydrolysates. The numbers in parentheses refer to the assumed stoichiometric number of residues per molecule of pure peptide. Amino acids present in amounts equivalent to <0.1 residue are not shown. [b] Determined by the method of Goodwin and Morton (1946). [c] Paper chromatography in the solvent system, 1-butanol–pyridine–acetic acid–water (60:40:12:48, v/v/v/v) was used.

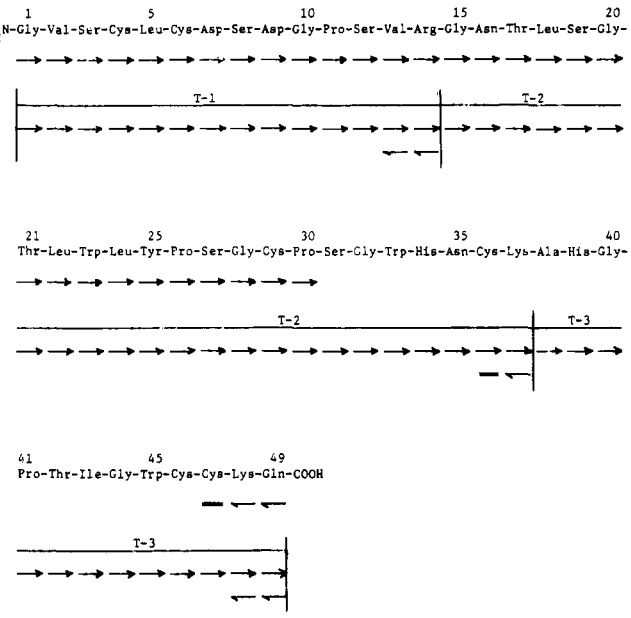

FIGURE 3: Reconstruction of the amino acid sequence of *Anthopleura xanthogrammica* anthopleurin A from the sequence data derived with the intact protein and the tryptic peptides. Full arrows pointing to the right indicate sequences determined by manual Edman degradation. Half arrows pointing to the left indicate sequences determined by the action of carboxypeptidase A or B, while ═══ indicates sequences determined by hydrazinolysis.

on Whatman No. 1 paper. The solvent used was 1-butanol–pyridine–acetic acid–water (60:40:12:48, v/v/v/v). The R_f of peptides, T-2 and T-3, were 0.60 and 0.38, respectively. The peptides were eluted from the paper and freeze-dried.

The amino acid compositions and some properties of peptides, T-1, T-2, and T-3, were determined and the results of these analyses are summarized in Table II.

NH_2- and COOH-Terminal Sequence Analyses of the Intact Carboxymethylcysteinylanthopleurin A. Manual Edman degradation was carried out on both the carboxymethylcysteinylanthopleurin A and the 4-sulfophenylthiocarbamoyl derivative of carboxymethylcysteinylanthopleurin A. It was possible to identify the first 30 amino acid residues from the NH_2-terminal end of the polypeptide. The yields of the various Pth-amino acids and the methods used to identify the various amino acids are summarized in Table III.

Action of carboxypeptidase A on the intact carboxymethylcysteinylanthopleurin A gave glutamine in 72.6% yield after 2 h and in 99.0% yield after 4 h. After the reaction time of 4 h, the digestion mixture was immediately chromatographed on a Sephadex G-50 column to obtain a peptide (by measuring the absorbance at 233 nm) and an amino acid fraction which was detected by the ninhydrin procedure. The amino acid fraction contained glutamine which was identified by combination of direct amino acid analysis and amino acid analysis after 6 N HCl hydrolysis. The peptide peak fractions were pooled, lyophilized, and again digested with carboxypeptidase B and A. Action of carboxypeptidase B (1 h) on the residual peptide liberated lysine in 100% yield. Subsequent carboxypeptidase A action on the digest with carboxypeptidase B did not liberate any additional amino acids. However, hydrazinolysis of the digest after carboxypeptidase B–A digestion yielded *S*-β-carboxymethylcysteine in 96% yield. No amino acids were liberated on the intact carboxymethylcysteinylanthopleurin A by hydrazinolysis. Thus, COOH-terminal sequence of intact anthopleurin A was determined to be -Cys-Lys-Gln-COOH.

TABLE III: Manual Edman Degradation Results of Carboxymethylanthopleurin A.[a]

Step No.	Pth-Amino Acid	% Yield	Methods of Analysis[b]		
1	Gly	100	GC	HYD	TLC
2	Val	92	GC	HYD	TLC
3	Ser	84	GC	TLC	
4	CM-Cys	83	GC	TLC	
5	Leu	79	GC	HYD	TLC
6	CM-Cys	74	GC	TLC	
7	Asp	68	HYD	TLC	
8	Ser	63	GC	TLC	
9	Asp	62	HYD	TLC	
10	Gly	60	GC	HYD	TLC
11	Pro	54	GC	HYD	TLC
12	Ser	50	GC	TLC	
13	Val	49	GC	HYD	TLC
14	Arg	10	HYD		
15	Gly	44	GC	HYD	TLC
16	Asn	41	HYD	TLC	
17	Thr	37	GC	TLC	
18	Leu	36	GC	HYD	TLC
19	Ser	33	GC	TLC	
20	Gly	32	GC	HYD	TLC
21	Thr	29	GC	TLC	
22	Leu	28	GC	HYD	TLC
23	Trp	+	TLC		
24	Leu	24	GC	HYD	TLC
25	Tyr	21	GC	HYD	TLC
26	Pro	20	GC	HYD	TLC
27	Ser	18	GC	TLC	
28	Gly	18	GC	HYD	TLC
29	CM-Cys	16	GC	TLC	
30	Pro	15	GC	HYD	

[a] Manual Edman degradation was carried out on the 4-sulfophenylthiocarbamoyl derivative of the carboxymethylanthopleurin A. See Experimental Procedures for experimental details. [b] The abbreviations used are: GC, gas chromatography of Pth-amino acid; TLC, thin-layer chromatography of Pth-amino acid; and HYD, 6 N HCl hydrolysis of the Pth-amino acid and subsequent amino acid analysis.

Sequence Determinations of Peptides, T-1, T-2, and T-3. Manual Edman degradation and carboxypeptidase B–A procedure on peptide T-1 confirmed the sequence determined on the intact protein and, therefore, the results will not be mentioned further.

Manual Edman degradation of peptide T-2 yielded the results shown in Table IV. After the 22nd step of Edman degradation, direct amino acid analysis of the sample without acid hydrolysis yielded ϵ-phenylthiocarbamoyllysine in 8% yield and free lysine after 6 N HCl hydrolysis of the sample. Carboxypeptidase B digestion (1 h) of peptide T-2 yielded lysine in 100% yield. Subsequent hydrazinolysis of the carboxypeptidase B digest yielded *S*-β-carboxymethylcysteine in 60% yield.

Manual Edman degradation results of peptide T-3 are shown in Table V. Action of carboxypeptidase A on peptide T-3 yielded glutamine after 4 h in 100% yield. The following carboxypeptidase B action (1 h) on the digestion mixture with carboxypeptidase A yielded lysine in 82% yield in addition to glutamine (100% yield).

Reconstruction of the Complete Sequence. Manual Edman degradation of the intact protein derivative showed that peptide T-1 was the NH_2-terminal peptide and that peptide T-2 followed it. Only peptide T-3 was left as the COOH-terminal peptide. The COOH-terminal analysis of the intact protein also

TABLE IV: Manual Edman Degradation Results of Peptide T-2.

Step No.	Pth-Amino Acid	% Yield	Methods of Analysis[a]	
1	Gly	100	GC	HYD
2	Asn	90	HYD	TLC
3	Thr	84	GC	TLC
4	Leu	82	GC	HYD
5	Ser	72	GC	TLC
6	Gly	65	GC	HYD
7	Thr	60	GC	TLC
8	Leu	57	GC	HYD
9	Trp	40	GC	TLC
10	Leu	48	GC	HYD
11	Tyr	42	GC	HYD
12	Pro	40	GC	HYD
13	Ser	35	GC	TLC
14	Gly	33	GC	HYD
15	CM-Cys	31	GC	TLC
16	Pro	27	GC	HYD
17	Ser	24	GC	TLC
18	Gly	23	GC	HYD
19	Trp	+	TLC	
20	His	10	HYD	
21	Asn	16	HYD	TLC
22	CM-Cys	12	GC	TLC
23	Lys	8	HYD	

[a] See footnote *b* in Table III.

TABLE V: Manual Edman Degradation Results of Peptide T-3.

Step No.	Pth-Amino Acid	% Yield	Methods of Analysis[a]	
1	Ala	100	GC	HYD
2	His	40	HYD	
3	Gly	84	GC	HYD
4	Pro	70	GC	HYD
5	Thr	66	GC	TLC
6	Ile	64	GC	HYD
7	Gly	55	GC	HYD
8	Trp	42	GC	TLC
9	CM-Cys	40	GC	TLC
10	CM-Cys	36	GC	TLC
11	Lys	28	HYD	
12	Gln	25	HYD	TLC

[a] See footnote *b* in Table III.

showed that peptide T-3 was the COOH-terminal peptide since both contained Gln as the COOH-terminal amino acid. Thus, the complete sequence of anthopleurin A could be reconstructed readily from the sequences of the tryptic peptides. The sequence results necessary to establish the total sequence (which have been covered in the previous section) and also additional sequence results which were determined but are not described are summarized in Figure 3.

Discussion

The heart stimulant, anthopleurin A, proved to contain 49 amino acid residues rather than the 53 amino acid residues previously reported. Part of this difficulty arose from the presence of peptide impurities containing arginine, glutamate, alanine, tyrosine, and phenylalanine and which were tightly bound to the polypeptide. The impurities could not be removed by chromatography on Sephadex G-50 or by cation-exchange chromatography on CM-Sephadex C-25. However, they could be removed by chromatography on SE-Sephadex C-25. The

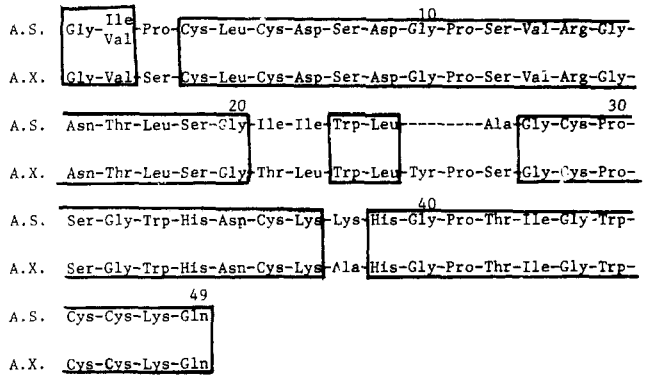

FIGURE 4: Comparison of the amino acid sequences of the *Anemonia sulcata* toxin II (A.S.) and the *Anthopleura xanthogrammica* heart stimulant, anthopleurin A (A.X.). The identical residues are squared off.

amino acid composition of the AP-A, purified in this manner and with an additional step of paper chromatography, agreed with the composition determined from the amino acid sequence of the protein. Some of the interesting aspects of the amino acid composition is the lack of methionine and the high contents of cystine, proline, and tryptophan. The AP-A is a basic protein and the IEP has been reported to be 8.2 (Norton and Kashiwagi, 1976).

As for the sequence studies of AP-A, the Lys-48-Gln-49 linkage in carboxymethyl-AP-A was not hydrolyzed with trypsin in the present investigation, while Wunderer et al. (1976) have reported a cleavage of Lys-46-Gln-47 bond in *Anemonia sulcata* toxin II by trypsin.

The sequence of AP-A shows great resemblance to the toxin II isolated from the sea anemone, *Anemonia sulcata,* as shown in Figure 4. However, the pharmacological actions of AP-A and toxin II appear to differ greatly. Toxin II is not as effective as AP-A in stimulating the heart and appears to be more toxic (Shibata and Norton, unpublished results). However, further studies with *Anemonia sulcata* toxin II are necessary to settle this point.

Determination of the cysteine content of AP-A by the DTNB method of Ellman (1959) disclosed the lack of such residues indicating that 3 cystine residues were present in the native AP-A.

Attempts are underway in our laboratory to etermine the pairing of the cysteine residues. Knowledge of the sequence and cysteine pairing of AP-A will make it possible to synthesize the heart stimulant. From the considerable studies on animal heart preparations and the in situ heart experiments, AP-A appears to be superior to the cardiac glycosides and isoproterinol, the current heart stimulants used clinically.

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