The structure of trifolitoxin.
A bacteriocin from *Rhizobium leguminosarum* biovar *trifolii* strain T24.

by

Benjamin James Lethbridge
B.Ag.Sc. (Hons.) Adelaide

Department of Agricultural Biochemistry
Waite Agricultural Research Institiute
University of Adelaide
South Australia

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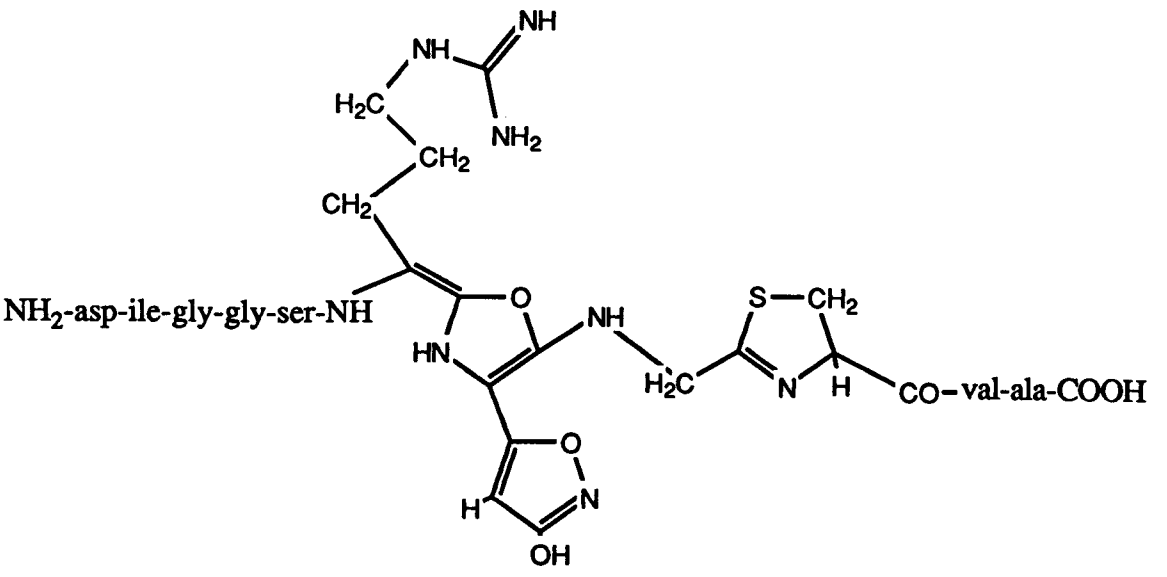

Current model of trifolitoxin.
(10/6/89)

Structure of Trifolitoxin - a bacteriocin from *Rhizobium leguminosarum* biovar *trifolii* Strain T24.

SUMMARY

This study investigates the biochemical and chemical characteristics of a bacteriocin produced by *Rhizobium leguminosarum* biovar *trifolii* Strain T24 (trifolitoxin).

A stable biologically active derivative of trifolitoxin has been purified by reverse phase chromatography, gel filtration and high voltage paper electrophoresis.

Utilizing ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR), fast atom bombardment mass spectroscopy (FAB MS), high voltage paper electrophoresis (HVPE), enzymatic, Edman sequencing, amino acid analysis and hydrolyses techniques, trifolitoxin has been shown to consist of a linear peptide (MW=1037 (FAB MS)) (asp-ile-gly-gly-ser-(arg-X-gly)-cys-val-ala (Edman/partial hydrolysis)). It contains two UV absorbing chromophores. One is an acid-labile thiazoline, the other chromophore,X, (pKa=5.1, λ^{max} pH 7.0, 239 nm, 302nm, $\varepsilon^{239\ nm} = 9700$, $\varepsilon^{302nm} = 5000$) has been characterized but its structure has not been determined. This blue fluorescent chromophore is linked to glycine and a modified arginine, which yields a D-L mixture of arginine after complete acid hydrolysis.

Hydrolysis of the thiazoline ring system reduces toxicity (98%) and leads to a multiplicity of active forms due to the oxidation of the free thiol.

Analysis of proteolytic fragments or acetylated derivatives of trifolitoxin and trifolitoxin sulphonic acid indicate that an N terminal amino group is required for toxicity. Cleavage of C terminal amino acids or reduction of the blue fluorescent chromophore completely eliminates activity.

The significance of the biological activity is discussed in the light of the current structure which is shown opposite.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and that , to the best of the candidates knowledge and belief , the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis .

The author consents to the thesis being made available for photocopying and loan if accepted for the award of the degree.

B. Lethbridge

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ABBREVIATIONS AND SYMBOLS.

α	alpha
Abs.	Absorbance
Ala	Alanine
Asp	Aspartic acid
Arg	Arginine
β	beta
Cys	Cysteine
$CysO_3H$	Cysteic acid
C.P.M.	Counts per minute
d.	Daltons
δ	delta
△	delta (change in)
ε	epsilon or extinction coefficient
DEAE	Diethylaminoethyl
HVPE	High Voltage Paper Electrophoresis
IR	Infrared
Ile	Isoleucine
γ	gamma
Gly	Glycine
Glu	Glutamic acid
λ	lamba (Wavelength)
M.W.	Molecular Weight
N.M.R.	Nuclear Magnetic Resonance
O.D.	Optical Density (Absorbance of a 1ml. solution)
PC	Paper chromatography
PITC	Phenyl Isothiocyanate
PTH	Phenyl thiohydantoin
ppm	parts per million
R_f	chromatographic mobility relative to the solvent front
RM_{OG}	Relative Mobility to Orange G (followed by pH)
RM_{2da}	Relative mobility to 2deoxy adenosine
Ser	Serine
T.L.C	Thin Layer Chromatography
T24	Trifolitoxin
T24SH	Trifolitoxin thiol
T24SST24	Trifolitoxin disulfide
$T24SO_3H$	Trifolitoxin sulphonic acid
UV	Ultraviolet
Val	Valine

CHAPTER 1
GENERAL INTRODUCTION

DEFINITION OF A BACTERIOCIN

A bacteriocin can be defined as a specific non self propagating agent produced by one strain of bacterium and antagonistic toward other similar bacteria (Hirsch 1979, Dowling and Broughton 1986).

AIM OF THIS STUDY

The aim of this project was to isolate and characterize a bacteriocin produced by *Rhizobium leguminosarum* biovar *trifolii* strain T24 (trifolitoxin) utilizing chemical and biochemical techniques.

NODULATION COMPETITION AND BACTERIOCINS

Rhizobial inoculants often do not nodulate the plant due to competition from highly competitive indigenous rhizobia. These rhizobia may nodulate the host plant but fix little or no nitrogen thus reducing the capacity of the nitrogen fixing process. (Hodgson and Stacey 1986, Date and Brockwell 1978, Vance 1983). It is therefore just as important to improve the competitiveness of rhizobium strains, as it is to increase the overall efficiency of nitrogen fixation.(Atkins 1985) From an economic stand point one of the most important aspects of ecology of Rhizobium is interstrain competition (Bushby 1982) The potential of rhizobium antagonism in competition has been recognized for some time (Bergersen and Brockwell *et al.* 1971, Schwinghamer and Belkengren 1968) but the detailed molecular basis for successful competition in rhizobia has so far received limited attention .

Reviews on nodulation competition (Dowling and Broughton 1986), rhizobium improvement (Hodgson and Stacey 1986), strain competition (Date and Brockwell 1978) and biology of rhizobium (Trinick 1982) point to a possible role of bacteriocin production in nodulation competition as one technique for strain improvement. The reviews generally organize the rhizobial populations into different spatial environments, which progressively come closer to the goal , the formation and maintenance of a nodule, starting with the soil, rhizosphere, rhizoplane, root hair attachment and finally nodule initiation. The rate at which the rhizobia survive and pass through these zones during the infectable plant stage, will determine the ultimate nodulation success of each strain and is a complex function of the rhizobial strains properties Factors which have been examined include motility (Ames and Bergman 1981),

adsorption to soil particles (Bushby 1982) , bacteriocin production and sensitivity, interaction with other soil microorganisms (Schwinghamer and Belkengren 1968, Trinick 1982) together with the host plant properties ie. plant variety (Vincent and Waters 1953), specific chemo-attractants (Caento-Annoles *et al.*, 1988), nod inducers and repressors (Redmond *et al.*, 1987, Firmin *et al.*, 1987), attachment phenomena (Dazzo, Truchet *et al.*, 1982), interactions with environmental pH, soil N, salinity, temperature, phage and predators (see Dowling and Broughton 1986).

Bergersen, Brockwell *et al.* (1971) recognized four important features of rhizobium for inoculant improvement. (1) They should have symbiotic effectiveness over the range of environments and host varieties used. (2) An ability to forms nodules promptly. (3) Ability to compete with other rhizobia. (4) An ability to persist in soils concerned.

Rhizobial bacteriocin production can specifically modify the competitive ability between strains for nodulation in laboratory tests (Schwinghamer and Belkengren 1968, Triplett and Barta 1987, Hodgson and Stacey 1985) and hence would provide a rational approach to the improvement of inoculation technology .

THE POTENTIAL ROLE OF BACTERIOCINS IN RHIZOBIAL ECOLOGY, BIOLOGY AND NODULATION COMPETITION

"Understanding the mechanism of biocontrol is imperative if we are to develop rational models for the exploitation of antagonists in agro-ecosystems". (Fravel 1988).

Are bacteriocins important ?

Triplett and Barta (1987) convincingly demonstrated by TN5 mutagenesis that bacteriocin production was the responsible agent for the competitiveness of *Rhizobium leguminosarum* biovar *trifolii* strain T24 in mixed inoculations of producing and sensitive strains in nodulation competition experiments. Earlier Hodgson, Roberts *et al.* (1985) using other strains of *Rhizobium leguminosarum* biovar *trifolii* demonstrated that when a bacteriocin producing non nodulating strain (Strain CB782) was added to a mixed inoculum of a bacteriocin sensitive and bacteriocin resistant nodulating strains, the representation in the nodules of the bacteriocin resistant strain was increased.

Such evidence would indicate that bacteriocin production in the same strain or a related resistant strain could act as an effective agent in controlling nodule proportions under defined conditions.

Is Numerical predominance important?

In the presence of a mixture of bacteriocin-producing, non-nodulating strain, a bacteriocin resistant nodulating strain and a bacteriocin sensitive nodulating strain, the bacteriocin resistant strain has been demonstrated to have a competitive nodulation advantage over the sensitive strain. In the absence of the bacteriocin producer , no such advantage exists. (Triplett and Barta 1987 with strain T24 or Hodgson, Roberts *et al.*, 1985 with strain CB782) . However when bacteriocin producing non nodulating strain is coinoculated with only the bacteriocin sensitive nodulating strain ,nodulation of the bacteriocin sensitive nodulating strain is not suppressed. (Triplett and Barta 1987, Schwinghamer and Belkengren 1968).

Dowling and Broughton's (1986) summation of the effects of bacteriocins was that they affect competition by altering the population of sensitive rhizobia in the rhizosphere . Data on the relative rhizosphere colonization by strains does not always reflect the nodule population (Brewin , Wood *et al.*, 1983, Labadera and Vincent 1975, Vincent and Waters 1953, Marques-Pinto *et al.*, 1974). In an extensive study by Labadera and Vincent (1975) with subclover (*Trifolium subterranean*) and white clover (*Trifolium repens*) the competitive superiority in nodule formation of *Rhizobium leguminosarum* biovar *trifolii* strain TA1 against an ineffective Uruguayan competitor *Rhizobium leguminosarum* biovar *trifolii* strain U73 was overwhelming. Ninety-three to one hundred percent of nodules were occupied by strain TA1 and this was independent of the relative representation on the root surface (Labadera and Vincent 1975). Strain TA1 produces a low molecular weight bacteriocin with a narrow range of antibiosis to *Rhizobium leguminosarum*.(Schwinghamer 1971) but antagonistic effects between the two strains in culture were not tested. On *Trifolium polymorphium* an indigenous Uruguayan legume, strain TA1 was still dominant and was more dependant on root surface representation, both strains nodulated this legume more slowly than either of the subclover or white clover species. Strain TA1 forms slightly earlier nodules than strain U73 in competitive studies (Labadera and Vincent 1975), this should be distinguished from nodulation speed of strains inoculated separately (Marques-Pinto et al 1974). In the latter case no significant difference was observed between strains which would provide an explanation for nodulation superiority . These data suggests that strain-strain interaction and strain-host interaction are important determinants for nodulation superiority and numerical predominance is of lesser importance.

Are Nodulation Rates Important?

Schwinghamer and Belkengren (1968) have demonstrated that in mixed strain inocula the *Rhizobium leguminosarum* biovar *trifolii* strain T24 (a bacteriocin producer) has a competitive advantage against bacteriocin sensitive strain T1 in the nodulation process. Ninety

six percent of nodules were produced by the bacteriocin producing strain . However if the inoculation of strain T24 was delayed 72 hours, then strain T1 was the major nodule occupier (ninety two percent) indicating a time dependant response.

Sargeant, Huang *et al.* (1987) demonstrated with a clover split root assay system, that the rate of nodulation of trifolii strains controlled the relative proportions of nodules. With either nodulation impaired mutants derived from *Rhizobium leguminosarum* biovar *trifolii* strain ANU843, or a delayed inoculation of greater than twenty four hours on one root system, more nodules were formed on one side relative to the other. From these data it was concluded that either early or faster nodule initiations produced a systemic effect impairing the nodulation on the other root system (feedback regulation of nodulation). The nodulation superiority of the wild type was also apparent in mixed strain inocula, but not apparent with the less sensitive analysis of the kinetics of nodule formation of strains inoculated in isolation. Broughton, Samrey *et al.* (1982) experiments on nodulation blocking of strain TOM by strain PF2 on Afghanistan pea could only be attributed to a faster rate of accumulation of strain PF2 on the root surface.No bacteriocin production by strain PF2 against strain TOM could be detected by plate assay and the involvement of a plant component was invoked.

Rhizobia are motile and hence can move towards chemo attractants or away from irritants (antibiotics, bacteriocins, plant antimicrobial compounds) (Dowling and Broughton 1986, Chet and Mitchell 1976). Indeed strain specific positive and negative chemotaxis to root exudates has already been noted for some *Rhizobium meliloti* strains (Currier and Strobel 1986). A nodulating strain specific chemotaxis towards a hydroxylated flavone nodulation inducer (luteolin) in *Rhizobium meliloti* has been reported by Caento-Anolles *et al.* (1988) and suggests a role for specific positive chemotaxis in nodulation. Non motile strains (flagellated and non flagellated) of *Rhizobium leguminosarum* biovar *trifolii* strain TA1 appeared to be less nodulation competitive relative to the wild type. Inoculation ratios of 1:1 consistently gave nodule occupation of 5:1 in favour of the motile strain, although both types formed approximately equal nodules when inoculated in isolation. No difference in growth or rhizosphere abundance was detectable (Mellor *et al.* 1987) indicating that the selective pressure for nodulation is not due numerical predominance at the site of action. The disproportionate nodulation of non motile mutants is similar to that found by Ames and Bergman (1981) in *Rhizobium meliloti* on lucerne. Caento-Anolles *et al.* (1988) was able to demonstrate with *Rhizobium meliloti* strain L530 that the rate of bacteria firmly adsorbed to the host root surface was 5 to 20 fold greater for the parents than either motile or chemotactic mutants, which were less efficient competitors on root adsorption assays and it was also noted that the nodules produced were significantly delayed in their development. The wild type was 10 to 30 times more competitive for nodulation than either mutant. The rate of nodulation of the competing strains would therefore appear to be an important component of nodulation competition.

Nodulation competition and bacteriocin production

Although the possibility of bacteriocin producing strains being numerically predominant in the rhizosphere has not been ruled out, the published data (above) suggests rhizosphere numerical predominance may only be a minor contributing factor to nodulation dominance, but could well be important in saprophytic competence of a strain in the absence of the legume host. The Date and Brockwell (1978) summary of interstrain competition, suggests that the host plant is an important factor in strain selection, but how this operates is unknown. Bacteriocins produced by rhizobia at sublethal levels, may minimize the migration of competing rhizosphere rhizobia towards nodulation sites through negative or retarded chemotaxis or motility and an implied reduction in nodulation rates. Retardation of nodulation rates will significantly affect the rhizobium nodule population. Environmental factors affecting production of bacteriocin, bacteriocin sensitivity or bacteriocin toxicity may also modify a bacteriocin producing strains response to nodulation competition. A knowledge of the structure and properties of bacteriocins will allow rational experiments to be developed to test such environmental variables.

Bacteriocinogenic plasmids

Plasmid coded bacteriocins (Hirsch 1979, Zelana-Kowalska 1979, Van Brussel *et al* 1985, van Veen, Okker *et al.*, 1986) could act as a selective agent for the transfer of genetic material between rhizobia. Hirsch (1979), Brewin, Beringer *et al.* (1980b), Van Brussel *et al.* (1985) have demonstrated the presence of highly transmissible plasmids coding for 'medium' bacteriocin production .'Medium' and 'small' refer to classes of bacteriocin based on molecular size, (Hirsch 1979). Selection of 'small' bacteriocin resistant colonies, from a 'medium' bacteriocin producing strain in some cases results in the elimination of a nodulation plasmid encoding 'medium' bacteriocin production and 'small' bacteriocin sensitivity (Priem 1984). Transfer of this nodulation plasmid encoding 'medium' bacteriocin production to 'small' bacteriocin producing strains represses small bacteriocin production (Hirsch 1979, Wijfelman, Pees *et al.*, 1983) These results indicates a role for bacteriocins in plasmid transfer and maintenance in rhizobium. Modifying plasmid composition has been shown to affect nodulation competitive ability of certain strains (Brewin, Wood *et al* 1983, Wang, Beringer *et al.*, 1986). It is clear that plasmid coded bacteriocin production and immunity could supply a selective pressure and be a component of the natural processes that induce strain diversity amongst the rhizobia bacteria (Young 1985).

AGROBACTERIA / RHIZOBIA

Agrobacteria and rhizobia are both part of the rhizobiacaea family .Physiological and DNA homology studies reveal a relationship between fast growing rhizobia and agrobacteria (White 1972, Dixon 1969).

The respective pathological association of agrobacteria and symbiotic association of rhizobia (although the latter can degenerate into a purely parasitic interaction) have many features in common. Processes of infection of the plant leading to redirected morphogenesis (hypertrophy) have both been shown to be the expression of functions of the Ti (tumour inducing) and Nod (nodulation) plasmids respectively (Vance 1983) Both processes are known to be triggered by exuded plant phenolic compounds (Stachel *et al.*, 1985, Redmond *et al.*, 1987). Indeed it is possible to transfer such plasmid coded functions between agrobacteria and rhizobia and obtain expression of these functions (Hooykaas, Van Brussel *et al.*, 1981, Martinez, Palacios *et al.*, 1986) . Introduction of a root inducing plasmid (the Ri plasmid) from *Agrobacterium rhizogenes* to *Rhizobium meliloti* strain causes an increase in the level of nodulation.(Strobel *et al.*, 1985)

Opines have been defined in the agrobacterium context as plant products produced by transformed tissue and specifically utilized by the inducing strain as a source of nutrients. (ie nopaline, octopine (Tempe *et al.*, 1982), agrocinopines (Ellis *et al.*, 1981) . This broadly based definition of an opine lead Murphy *et al.* (1987) and Scott *et al.* (1987) to compounds produced in nodules induced by Rhizobium strains which had biological characteristics similiar to the above definition of an opine . These compounds were produced in nodules of the inciting strain and were only utilized by the same free living strain.

There are obvious parallels between agrobacteria and rhizobia, it would therefore seem reasonable to hypothesize, as have several workers (Gross and Vidaver *et al* 1983, Hirsch 1979 Van Brussel *et al.* 1985 Triplett and Barta 1987), that bacteriocins produced by each species would show some similar biological and/or chemical properties.

AGROBACTERIAL BACTERIOCINS (AGROCINS)

Agrocins are low molecular weight molecules produced by agrobacteria which act against other strains of Agrobacteria. The agrocins 84,108,d286,434 have all been shown to consist of nucleoside cores .Agrocin 84 (Roberts, Tate *et al* 1977) and D286 (Hendson *et al* 1983) have adenosine cores where as 108 (Kerr and Tate 1984) and 434 have cytidine cores (Donner S., personal communication)

The most thoroughly studied of these is agrocin 84. Agrocin 84 produced by a *Agrobacterium radiobacter* strain 84 is a N6, 5' disubstituted adenine nucleotide .The nucleotide portion is equivalent to a dideoxy nucleotide (a common DNA synthesis terminator). The 5'

phosphoramidate residue is also required for demonstrated toxicity. Specific uptake of this agrocin by sensitive agrobacteria and hence selectivity is determined by the N6 phosphoramidate moiety, this residue shows strong structural homology with the agrocinopines (Ryder, Tate *et al.* 1984) which are the natural substrate for the agrocin uptake mechanism.The uptake and catabolism systems of agrocinopines are coded for by genes present on the tumour inducing plasmid. This results in the selective inhibition of only pathogenic agrocinopine strains.

The non pathogenic strain 84 possesses a plasmid which codes for agrocin synthesis and immunity as well as nopaline catabolism genes. Inoculation of uninfected seedling plants with strain 84, forms the basis of the biological control of crown gall.(Kerr and Tate, 1984).

RHIZOBIUM BACTERIOCINS

It is reasonable to assume that the study of rhizobial bacteriocins in terms of selectivity and toxicity in a similar manner to the agrocins could provide a 'window of enlightenment' for our understanding of rhizobial ecology. It is also conceivable, that bacteriocins may also be utilized in improving the nodulation competitive ability of resistant strains in the rhizosphere of homologous plants. Even though bacteriocin production by rhizobia is common,(Roslycky 1967) structural details of rhizobial bacteriocins are scarce.

In an initial study on rhizobium antagonism, Schwinghamer (1971) demonstrated that bacteriocin production in *Rhizobium trifolii* Schwinghamer (1975) further analysed bacteriocins from six *trifolii* strains (CB782, Ro611, Ro11, Tr4, Tr11, R52) and classified them into two groups on the basis of sensitivity to strains TA1 and CC276 and chemical properties. All were determined to be proteinaceous by there sensitivity to proteolytic enzymes and buoyant density in CsCl gradients. The molecular weight of these bacteriocins were estimated between 180,000 and 200,000 by membrane filterability and glycerol gradient centrifugation.

Skorupska *et al.* (1984), Zelana Kowalska (1979) recognized proteinase sensitive bacteriocins in *Rhizobium leguminosarum* biovar *trifolii* . By contrast Gross and Vidaver (1983) partially characterized bacteriocins from *Rhizobium japonicum* and cowpea rhizobia as low molecular weight (<6000) non proteinaceous compounds because of there insensitive to proteolytic enzymes. Joseph and Desai (1983) found a bacteriocin-like compound in *Rhizobium trifolii* and termed it BLS, and it was characterized as a low molecular weight (dialysable) protein (sensitive to trypsin)

Hirsch (1979) described two classes of bacteriocin in *Rhizobium leguminosarum* defined by the molecular size (dialysability) as 'small' and 'medium' .The 'small' produced by many strains had a similar narrow range of antibiosis (suggesting similar compounds) whereas the 'medium' bacteriocins produced by a few strains differed in the range of antibiosis, suggesting different bacteriocins. None of the bacteriocins found were sensitive to pronase,

however many peptide antibiotics of bacterial origin, are known to have non enzymologically hydrolysable peptide linkages (Chapter 4.1, Introduction).

Van Brussel, Zatt *et al* (1985) were able to demonstrate that the 'small' bacteriocin reported by Hirsch (1979) was chloroform soluble and had an estimated molecular weight between 700 and 1500 by membrane diffusion and gel filtration chromatography, however no reference chloroform soluble compounds were tested under similar conditions, nor was the possibility of micelle formation considered. The chloroform soluble bacteriocin was more heat stable at pH 5.5 than at pH 7.0. The proteolytic insensitivity of 'small' bacteriocin was confirmed and this toxic compound was also found to be insensitive to pectinase and lysozyme.

Bacteriocins from rhizobia would not appear to belong to any general class of compounds. A few groupings do occur, the high molecular weight bacteriocins appear to be proteinaceous in origin perhaps analagous to the the colicins of *Escherichia Coli* (Reeves 1965). The low molecular weight compounds (dialysable) would appear to show a diversity of compounds similar to the antibiotics of bacterial origin (Corcoran 1974)

The following chapters provide a detailed account of the purification, structural determination and biological properties of trifolitoxin, a bacteriocin from *Rhizobium leguminosarum* biovar *trifolii* strain T24 which was first recognized by Schwinghamer and Belkengren in 1968.

CHAPTER 2
MATERIALS AND METHODS

STRAINS USED IN THIS STUDY

Strain	Source	Properties
T24* (K780**)	A.Kerr	Produces trifolitoxin
309*** (K312**)	A.Kerr	Produces small , sensitive to trifolitoxin$^+$
248*** (K313**)	A.Kerr	Produces medium , sensitive to trifolitoxin$^+$

* Schwinghamer strain number (Schwinghamer and Belkengren 1968)
**Kerr strain number (Personal communication)
*** Beringer strain number (Hirsch 1979)
$^+$ This thesis

BIOASSAY FOR TRIFOLITOXIN

Bacteriocin production and sensitivity was monitored by the method of Schwinghamer (1968) where MMG2 media was used as the base agar and overlayed with soft buffered agar (appendix 2) containing approximately 10^8 sensitive bacteria (K312) estimated photospectrometrically at 640 nm. vs a standard curve (appendix 3). To bioassay paper electrophoretograms or small volumes of bacteriocin (<20 microlitres), 1 cm square sections of paper (corners hooked to anchor the segment) are placed on bioassay plates. The plates are sterilized with chloroform vapour for 30 mins and overlaid with the sensitive strain (K312) in soft buffered agar.

MAINTAINENCE AND GROWTH OF STRAINS

All strains were maintained on tryptone/yeast agar (appendix 1) slopes, grown 24 hr. at 28 degrees and stored at 4 degrees until required.

All rhizobial cultures for the production of bacteriocin were grown on MMG2 media (appendix 1).

T24 BACTERIOCIN PRODUCTION

200 ml. cultures of MMG2 cultures were inoculated with a fresh slope of T24 and grown for 24 hrs. at 28 degrees C. by swirl culture.

LARGE SCALE ISOLATION

A 200 ml swirl culture grown for 24 hrs was inoculated into a 4 litre forced air culture and incubated a further 24 hrs . 500 mls of this culture were inoculated into a 8 litre forced air culture for 24 hrs

CULTURE FLUID ISOLATION

For small scale isolation steps (<2 litres) centrifugation at 10K for 20 mins (GSA rotor) was used. For large scale isolation (40 litres) hollow fibre ultrafiltration was utilized (Amicon cartridge type H10P10 cut off limit 10,000).

HIGH VOLTAGE PAPER ELECTROPHORESIS (HVPE)

HVPE was according to the method of Tate (1981). pH mobility profiles were also constructed by the method of Tate (1981) except the buffers used differed from those outlined and are indicated with the relevant text. (appendix 2).

PAPER CHROMATOGRAPHY

Paper chromatography was carried out by the descending method in tanks equilibrated with the appropriate solvent or as indicated in the two dimensional method.

TWO DIMENSIONAL CHROMATOGRAPHY / ELECTROPHORESIS

This method represent a modification of the method used by Smith (1969) to suit the equipment designed by Tate (1981). A 570 mm.by 160 mm. piece of Whatmann Chr1 chromatography paper was run by HVPE in the formic acetic system (pH 1.7) for 15 minutes with the unknown material run 20 mm from and parallel with the bottom edge of the paper . The paper was thoroughly dried and an 180 mm. section was cut where it was anticipated the material of interest would run to yield a 160 mm by 180 mm strip of paper. The paper was suspended in a TLC tank equilibrated with isobutanol: acetic acid: water 12:3:5 so that the solvent level is 10 mm above the level of the bottom of the paper and ascending perpendicularly to the direction of the electrophoresis. The paper is then dried and stained as usual.

ELUTION OF COMPOUNDS FROM PAPER

The position of the compound is first detected (by staining, viewing under shortwave or long wavelength UV light or bioassay) and sections cut out and placed in centrifuge tube with a small hole cut in the bottom ,suspended above another tube .The paper is minimally saturated with water and centrifuged in a bench centrifuge. The water saturation and centrifugation steps are repeated three times.

ULTRAVIOLET SPECTROSCOPY

All ultraviolet spectra were recorded on a Perkin Elmer λ 5 spectrophotometer. The conditions of the sample will be displayed with the appropriate spectra.

INFRARED SPECTROSCOPY

All spectra were run in the solid state as pottasium chloride embedded slotted discs in a Perkin Elmer 983G infrared spectrophotometer .

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Proton n.m.r .and ^{13}C n.m.r. were run on various field strength spectrometers in D_20 or D_20, H_20 mixtures according to standard techniques (Williams and Fleming 1987).

FAST ATOM BOMBARDMENT MASS SPECTROSCOPY

Samples in a glycerol matrix were subjected to fast atom bombardment (argon or xenon) and mass spectra recorded utilizing a ZAB FAB or Finnigan mass spectrometers.

AMINO ACID DETERMINATION

Amino acids were identified by two dimensional HVPE/PC and quantified by HPLC of the phenylthiohydantoins (the picotag method,Waters, U.S.A). To a lyophilized sample of amino acids is added 30 microlitres of derivatization reagent (7:1:1:1 ethanol: triethylamine: phenylisothiocyanate: water) allowed to stand for 20 minutes at 25°C and then lyophilized.The phenylthiohydantoins were then separated by HPLC with Novapak C18 column using a gradient system solvent A per litre water , 19 g sodium acetate, 0.5 ml TEA ,pH 6.4. Solvent B 600 ml acetonitrile 400 ml. water, monitored by UV absorbance at 254 nm.

COMPLETE ACID HYDROLYSIS

Samples were suspended in 5 N HCl solutions in freeze drying tubes, sparged with nitrogen and heat sealed. The tubes were heated at 110°C for 3 hrs the tubes were cooled, opened and the samples lyophilized. These conditions gave reproducible recoveries of all amino acids .

STAINING OF PAPER ELECTROPHORETOGRAMS OR CHROMATOGRAMS

NINHYDRIN for amines (amino acids)

Papers were dipped in 0.25% ninhydrin in acetone, dried and heated at 110°C until coloured spots developed. Compounds having amine groups gave coloured compounds.

PHENANTHRENE QUINONE for guanidines (arginine)

Papers were dipped in 90% (0.2 M phenanthrenequinone in ethanol), 10% (5N NaOH), dried and viewed under a short wavelength UV light where positive guanidine components fluoresced yellow.Yamada (1965)

MATERIALS

Octadecyl reverse phase silica was obtained from Sigma
Biogel P2 came from Biorad Laboratories California USA
DEAE G25, G15, G10 Sephadex were obtained from Pharmacia, Sweden
1Chr, Chr20 paper for HVPE or PC were purchased from Whatmann
Ninhydrin from Ajax Chemicals, Sydney ,Australia
Phenanthrene quinone from Aldrich Chemicals U.K
^{35}S sulphate was from Amersham Australia
All other chemicals or solvents were of reagent grade.

CHAPTER 3
THE NATURE OF TRIFOLITOXIN

INTRODUCTION

PROPERTIES OF TRIFOLITOXIN.

It is now over two decades since *Rhizobium leguminosarum* biovar *trifolii*, strain T24, was first reported by Schwinghamer and Belkengren in 1968. Strain T24, was reported to have been isolated from *Trifolium dubium* nodules and formed ineffective nodules on red or white clover. In this key paper Schwinghamer and Belkengren (1968) showed that this strain of rhizobia was extremely competitive in nodulation-competition experiments on clover. This competitiveness was correlated with the production of a bacteriocin which showed broad activity against fast growing rhizobia.

Schwinghamer and Belkengren (1968) Bergersen, Brockwell *et al.* (1971) Trinick *et al.* (1982) Triplett and Barta (1987) all recognized the potential of this particular strains ability to produce antibiosis in culture and nodulation competitiveness as beneficial to inoculation technology. Triplett and Barta (1987) termed this bacteriocin trifolitoxin and demonstrated conclusively that trifolitoxin production was responsible for this nodulation superiority of this strain. The bacteriocin showed broad but specific activity to the fast growing *Rhizobium leguminosarum* strains.(Schwinghamer and Bergersen 1968, Bergersen, Brockwell *et al* 1971, Triplett and Barta 1987). Agrobacterium strains were insensitive.(Triplett and Barta 1987)

Schwinghamer and Belkengren's (1968) preliminary analysis of the partially purified extracts indicates that the molecular weight of this toxic compound was between 1000 and 1500, determined by dialysis and gel filtration with reference to polar peptide antibiotics. This bacteriocin was not soluble in those organic solvents which were immiscible with water indicating the polar nature of this molecule and distinguishes it from the small bacteriocin identified by Hirsch (1979). The sensitivity of biological activity to papain, a proteolytic enzyme, indicated that trifolitoxin had at least one essential peptide bond. Schwinghamer and Belkengren (1968) also observed that trifolitoxin was found to be partially sensitive to protease and insensitive to trypsin. The partial sensitivity of protease is surprising, this enzyme being the least selective of all the peptidases used. Amino acid analysis of the active component gave recovery of unidentified amino acids. Bacteriocin activity was shown to be sensitive to preincubation at acidic (<4.5) and basic (>9.0) pH. A coincidence of a 280 nm./260 nm. UV absorbance ratio of 0.81. with biological activity was also noted.

The data presented by Schwinghamer *et al.* (1968) would be most consistent with a peptide structure. The 280 nm./ 260 nm. ratio has been widely used as a protein/nucleic acid estimator, the possibility that trifolitoxin had a nucleoside core (260 nm. >280 nm) as has been

found for the agrocins of agrobacteria (Kerr and Tate 1984) was the starting point for our investigations. This chapter is involved with the purification and preliminary structural analysis of a stable biologically active derivative of trifolitoxin . It is demonstrated that trifolitoxin consists of a linear peptide chain with a modified cysteine and an unusual blue fluorescent chromophore linked into the chain as a fraudulent amino acid.

CHAPTER 3
PURIFICATION AND PROPERTIES OF TRIFOLITOXIN

INITIAL PURIFICATION

Culture filtrates or centrifugates were adsorbed to activated charcoal (0.5 g/l). Although trifolitoxin activity was adsorbed onto charcoal this process became irreversible with time and hence was unsuitable for large cultures (approximately 30% recovery could be obtained on small scale cultures provided trifolitoxin was desorbed immediately with 70% propanol).

Four litre centrifuged cultures of Strain T24 were rotary evaporated at 40°C and resuspended in 80% propanol (400 mls) in which trifolitoxin was soluble but the majority of the media components were insoluble (80% recovery of trifolitoxin activity). The sample was further purified by ion exchange chromatography on DEAE Sephadex at pH 8.5.(Figure 3.1) This gave two active fractions. The major fraction was further purified by Sephadex G10 in water. Trifolitoxin elutes in the void volume coincident with blue dextran and absorbance at 300 nm. This was followed by HVPE pH 9.2 (RM_{OG} pH 9.2 = 0.6), and detected by blue fluorescence under UV light (254 nm.) as well as biological activity. Residual salts were removed by repeating the Sephadex G10 step. This gave a sample suitable for amino acid analysis which appears in figures 3.9, 3.10.

FINAL PURIFICATION METHOD ADOPTED

The purification of a stable derivative of trifolitoxin

The ultimately favoured purification procedure depended upon two important observations. First the initial purification demonstrated that biological activity was invariably associated with the presence of a blue fluorescent chromophore which absorbs at 300 nm. Secondly ^{35}S labeling unequivocally confirmed that biological activity was always correlated with the presence of labeled material.(Figure 3.2). This latter observation confirmed the presence of cysteine in the initially purified trifolitoxin acid hydrolysates and more importantly suggested the simple conversion of the multiplicity of observed trifolitoxin species and possibly other uncharacterized oxidation products to a single stable, biologically active sulphonic acid derivative, which will be subsequently be referred to as T24SO3H.

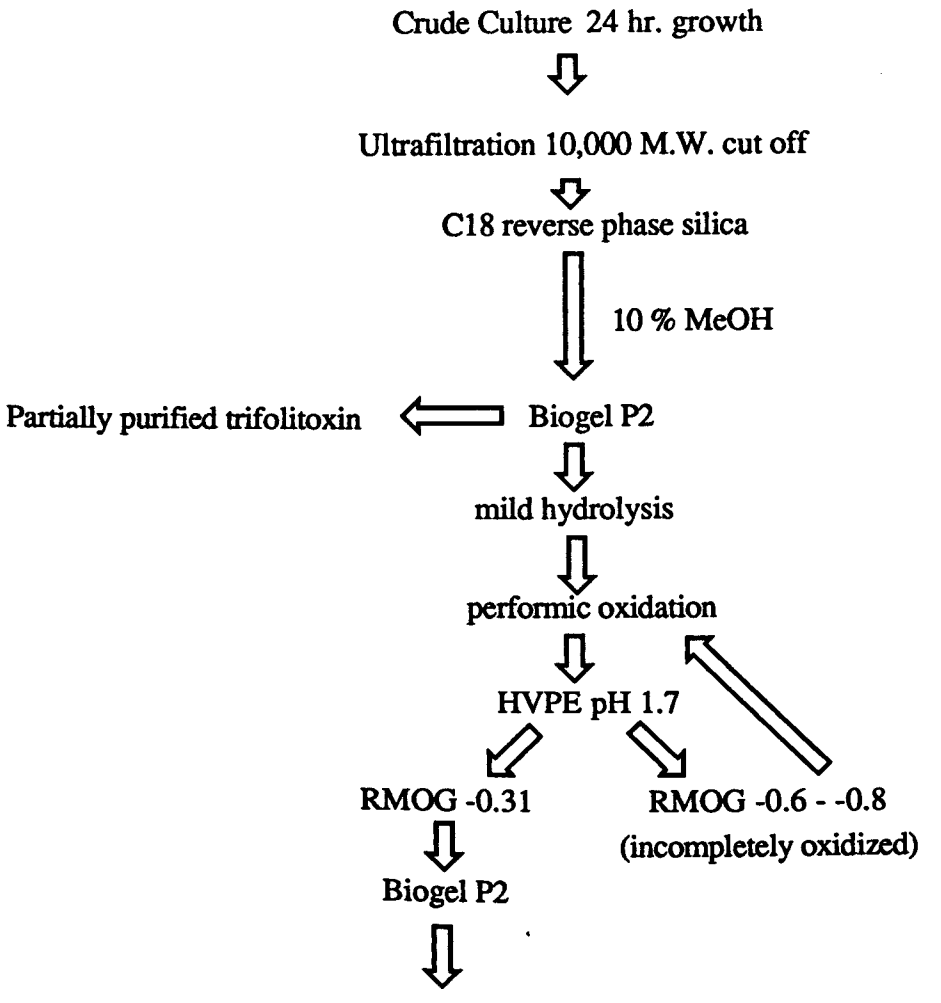
Bacteria were removed from 40 litre cultures by hollow fibre ultrafiltration with an Amicon ultrafiltration unit type H10P10 (10,000 molecular weight cut off). The filtrate is then loaded onto a C18 silica reverse phase column (15 litres per 100 grams), washed with water and eluted with 7 % methanol. 10 ml fractions were collected and monitored in the UV for peaks (or shoulders) at 300 nm. indicating the presence of bacteriocin (Figure 3.3) Positive fractions were rotary evaporated (45°C) to approximately two mls. and loaded onto a biogel P2

column in water (column size 75 cm.by 1.8 cm diameter column) fractions were monitored in the UV for peaks at 300 nm.(figure 3.4). These fractions were pooled and rotary evaporated (45°C) to dryness. For the purpose of structural analysis of trifolitoxin it was oxidized to the sulphonic acid derivative. The partially purified preparation of trifolitoxin was resuspended in 0.75 M formic acid/1M acetic acid buffer (pH 1.7) for one hour at room temperature, rotary evaporated to dryness at 45°C and oxidized with performic acid (9:1 formic acid: 30% H_2O_2) for 20 mins. The preparation is rotary evaporated to dryness and run by preparative HVPE at pH 1.7 for 15 mins at 3500 V. (see figures 3.5 and 3.6). The blue fluorescent products (UV light) with RM_{OG} of -0.30 were eluted from electrophoretograms and concentrated by lyophilization and reloaded onto the biogel column (Figure 3.4). Fractions showing peaks at 300 nm. were pooled. This material was homogeneous as determined by UV, ninhydrin and phenanthrene quinone staining of electrophoretograms at pH 1.7 and 9.2. This material was used for the structural determination of trifolitoxin.

Purification in this laboratory of larger samples of crude trifolitoxin were made possible by the supply and preparation in the Wisconsin laboratories of crude ultrafiltrates of trifolitoxin produced with the recombinant over producing strain of strain T24 (E.Triplett personal communication)

Table 3.1

Purification Flow Diagram



RESULTS

Anion exchange chromatography

Figure 3.1 shows the elution profile of trifolitoxin from a DEAE Sephadex column at pH 8.5. The activity eluting as two peaks.

Reverse phase chromatography

Figure 3.3 indicates the elution of trifolitoxin from C18 reverse phase column. The elution of trifolitoxin at only 7% MeOH is consistent with its hydrophillicity. Note that activity is coincident with absorbance at 302 nm.

FIGURE 3.1

Anion exchange chromatography of trifolitoxin

4 litres of a strain T24 culture was centrifuged. The supernatant was rotary evaporated to dryness (45° C),resuspended in 80% propanol (400 mls). The propanol fraction was redried resuspended in a minimum volume of 5 mM NH_4HCO_3 (adjusted to pH 8.5 with triethylamine) and applied to a column of DEAE Sephadex ($C0_3$ form) (25 cm by 1.5 cm.) Trifolitoxin was eluted with a 5 mM to 200 mM gradient (200 mls) of NH_4HCO_3 adjusted to pH 8.5 with triethylamine. 5 ml. fractions were collected. 0.5 microlitres of each fraction was bioassayed

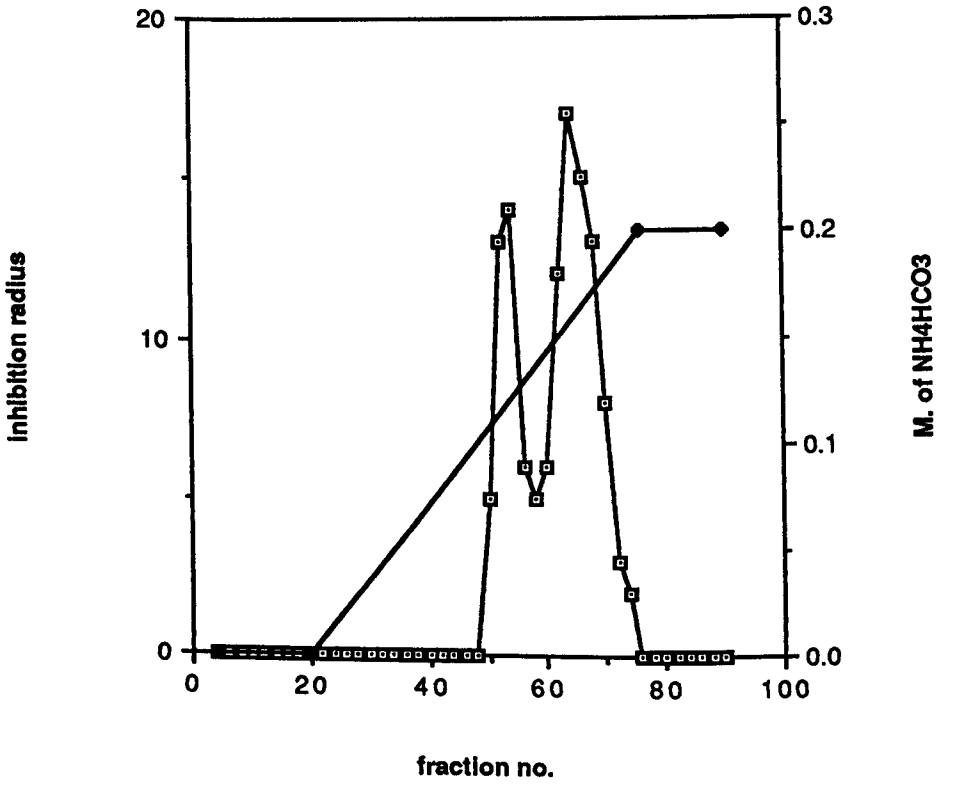

20
10
0
0.3
0.2
0.1
0.0
0
20
40
60
80
100
Inhibition radius
M. of NH4HCO3
fraction no.

FIGURE 3.2

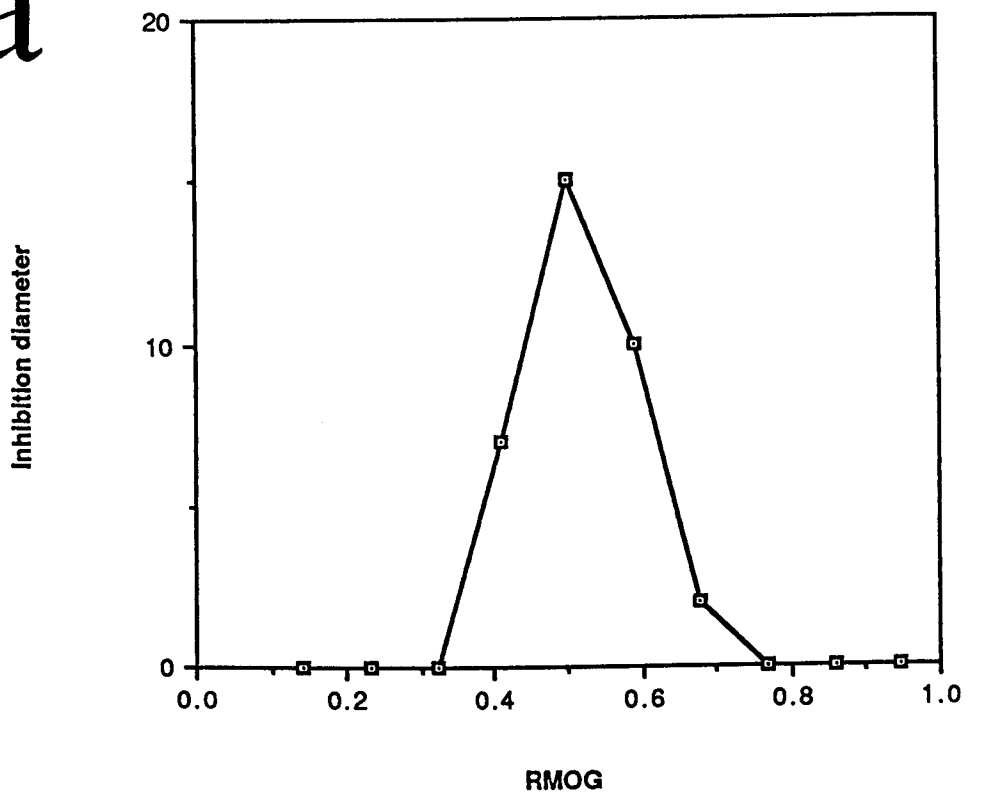
^{35}S labelling of trifolitoxin

A sulphur deficient MMG media (50 ml) (appendix 1) supplemented with 0.1 g/l $MgSO_4$ is inoculated with a fresh slope of strain T24 and grown overnight by swirl culture at 28 degrees C. 20 microlitres of this culture is inoculated into a 2 ml. culture of sulphur deficient MMG media plus 10 microlitres of 2% $MgCl_2$ plus 37 MBq /ml $^{35}SO_4$ (specific activity 39200 MBq/M) and grown overnight by swirl culture at 28 degrees C. Bacteria were removed by centrifugation and BDH activated charcoal (0.5 mg/ml.) added (30 mins).The charcoal was recovered by centrifugation , washed twice with H_2O (centrifuging after each wash) and resuspended in 70% aqueous isopropanol (200 microlitres /1 mg. charcoal) for 30 mins and centrifuged. The supernatant was lyophilized and and run HVPE pH 5.0 (citrate / NaOH)(RM_{OG}= 0.2) the biologically active section was eluted and rerun by HVPE pH 6.0 (citrate/NaOH) 0.5 cm sections were cut, bioassayed and scintillation counted in Beckman ready-solv EP fluor.

a : C.P.M. of 0.5 cm sections of electrophoretogram (HVPE pH 6.0) .

b: Bioassay inhibition diameters of corresponding 0.5 cm sections from electrophoretograms (HVPE pH 6.0).

a


20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
Inhibition diameter
RMOG

b

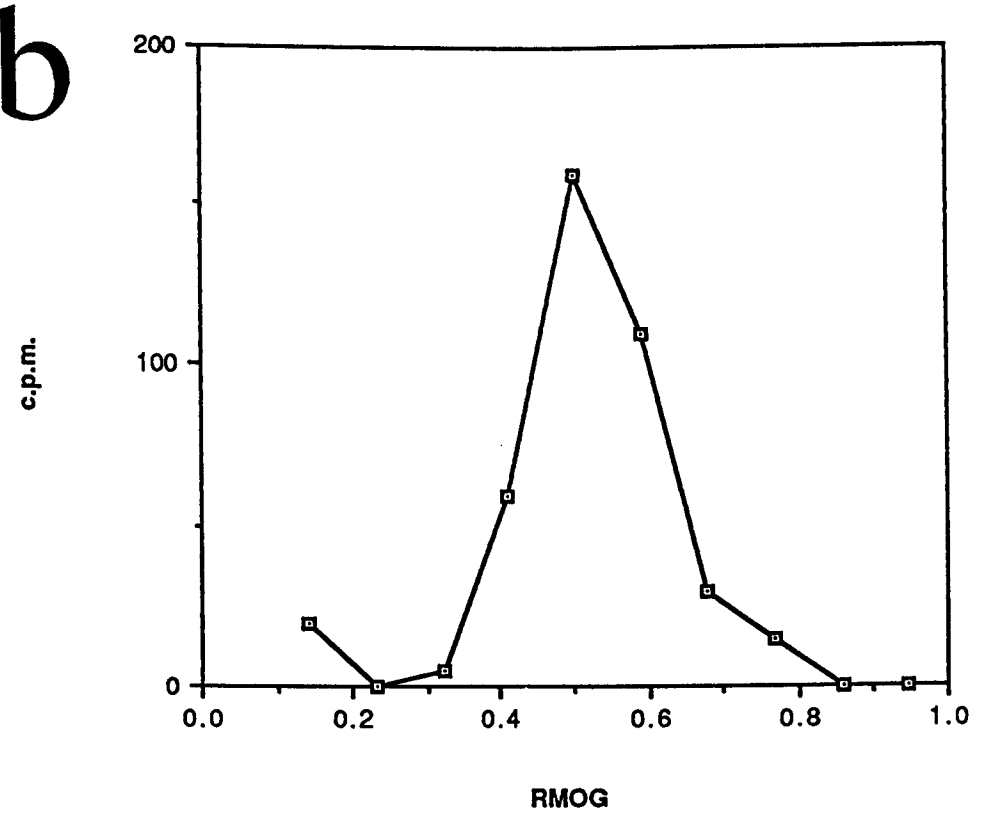

200
100
0
0.0
0.2
0.4
0.6
0.8
1.0
c.p.m.
RMOG

FIGURE 3.3

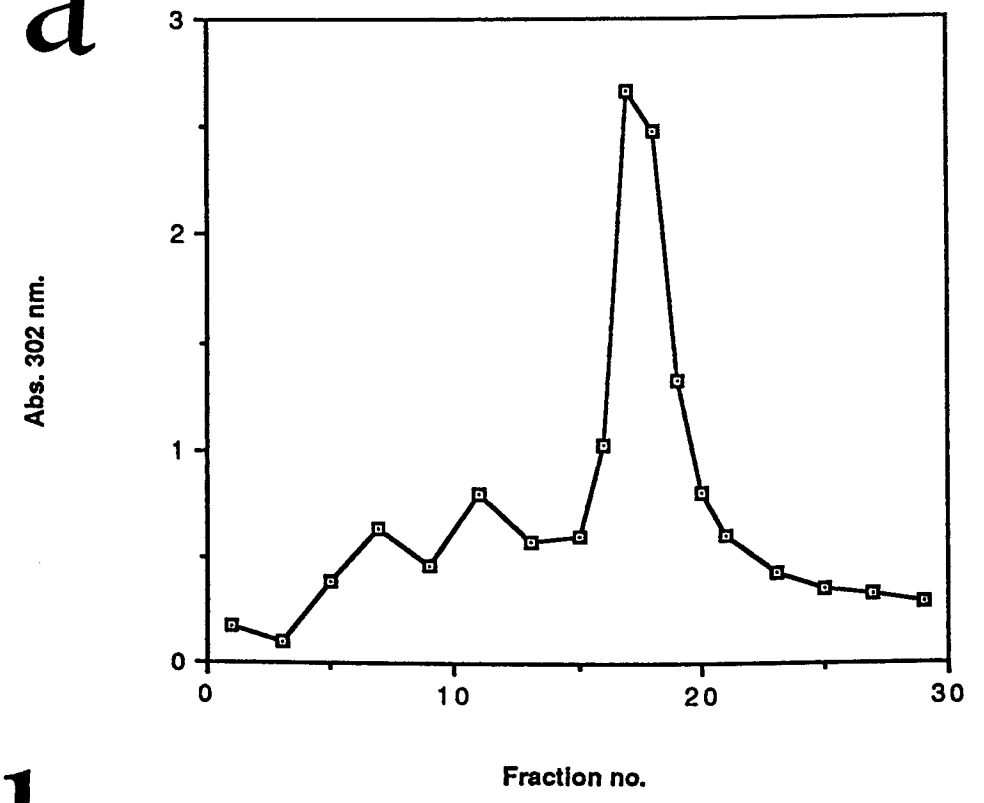
C18 silica reverse phase chromatograpy of trifolitoxin.

15 litres of of ultrafiltered culture was loaded onto a 100 gram column (dry weight) of reverse phase silica., washed with water and eluted with 7% methanol (elution start fraction 11) 10 ml fractions were collected and monitored at 302 nm. and 5 microlitres bioassayed .

a: Absorbance at 302 nm. vs fraction no.

b: Inhibition diameter (—◆—) vs fraction no.
Relative toxicity (—□—) calculated from the data of Figure 3.7

a


Abs. 302 nm.
3
2
1
0
0
10
20
30
Fraction no.

b

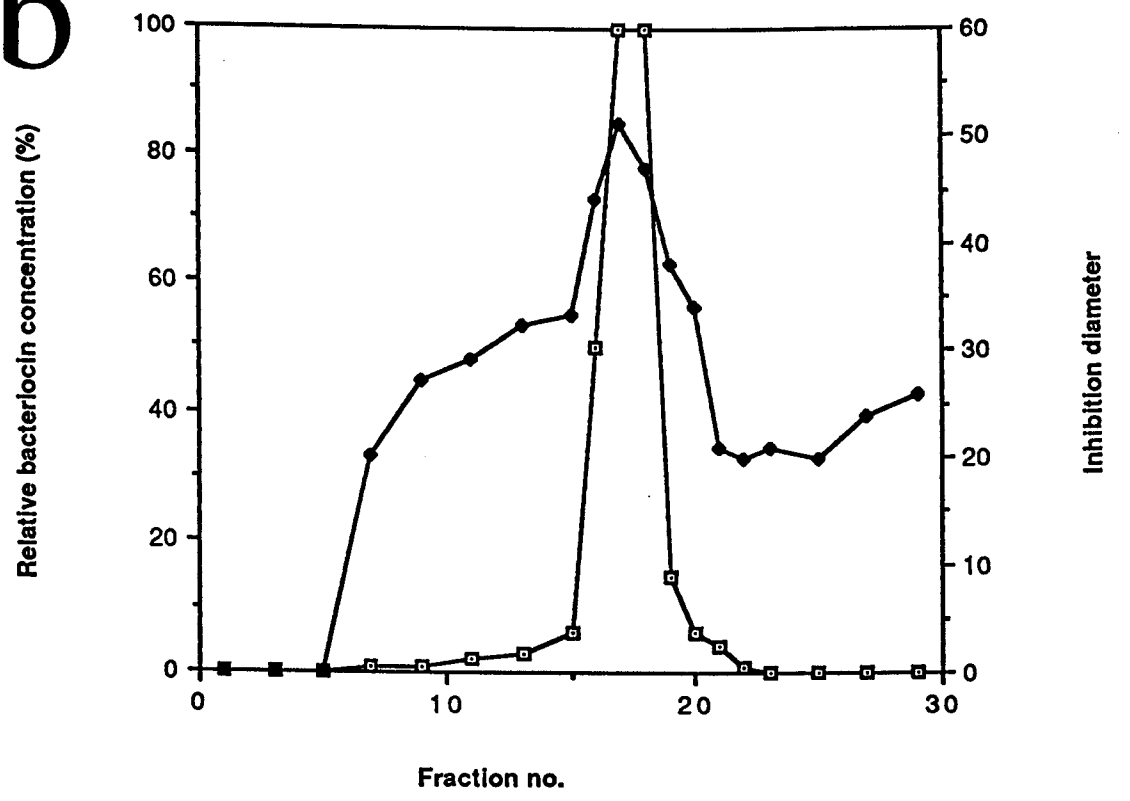

Relative bacteriocin concentration (%)
100
80
60
40
20
0
60
50
40
30
20
10
0
Inhibition diameter
0
10
20
30
Fraction no.

FIGURE 3.4

Biogel P2 gel filtration chromatography of trifolitoxin and trifolitoxin sulphonic acid

Samples of trifolitoxin were run on a Biogel P2 column (25 cm. by 1.7 cm.) in water (1 ml. /min) ,2 ml. fractions were collected and monitored by absorbance at 302 nm.

Trifolitoxin —⊡—

Trifolitoxin sulphonic acid —◆—

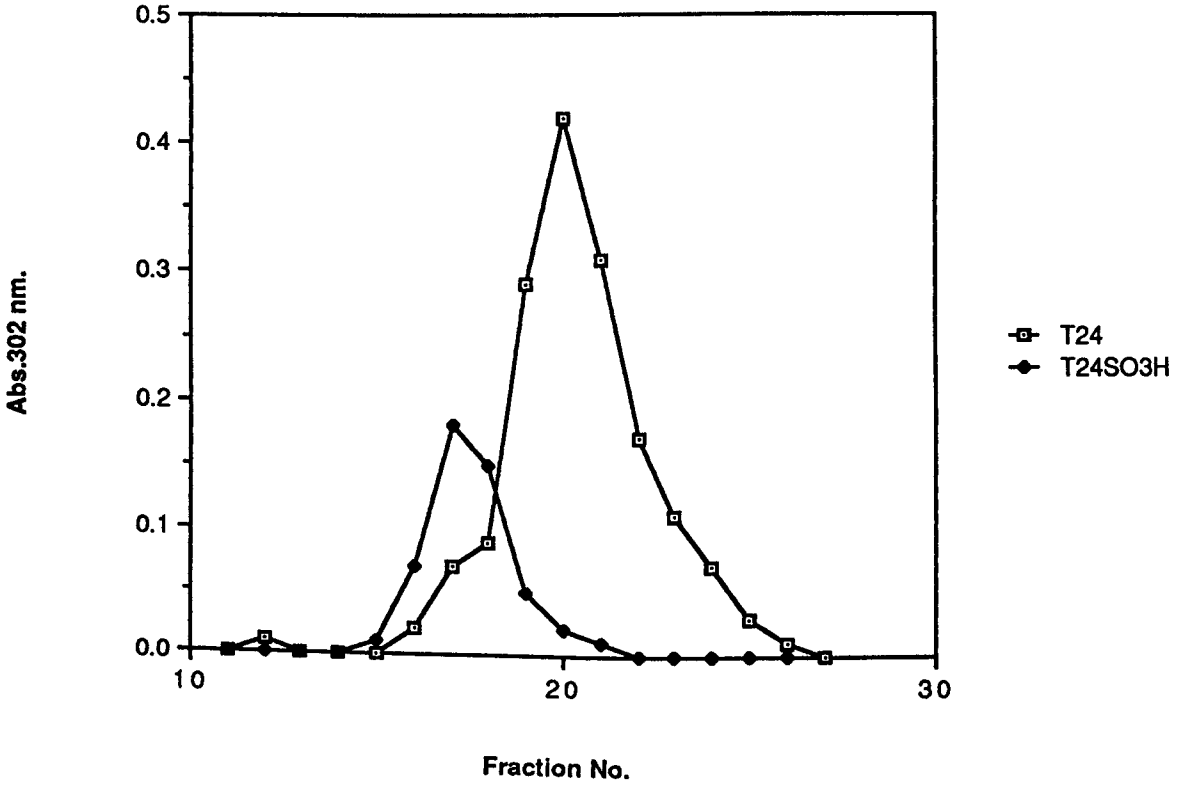

0.5
0.4
0.3
0.2
0.1
0.0
Abs.302 nm.
10
20
30
Fraction No.
T24
T24SO3H

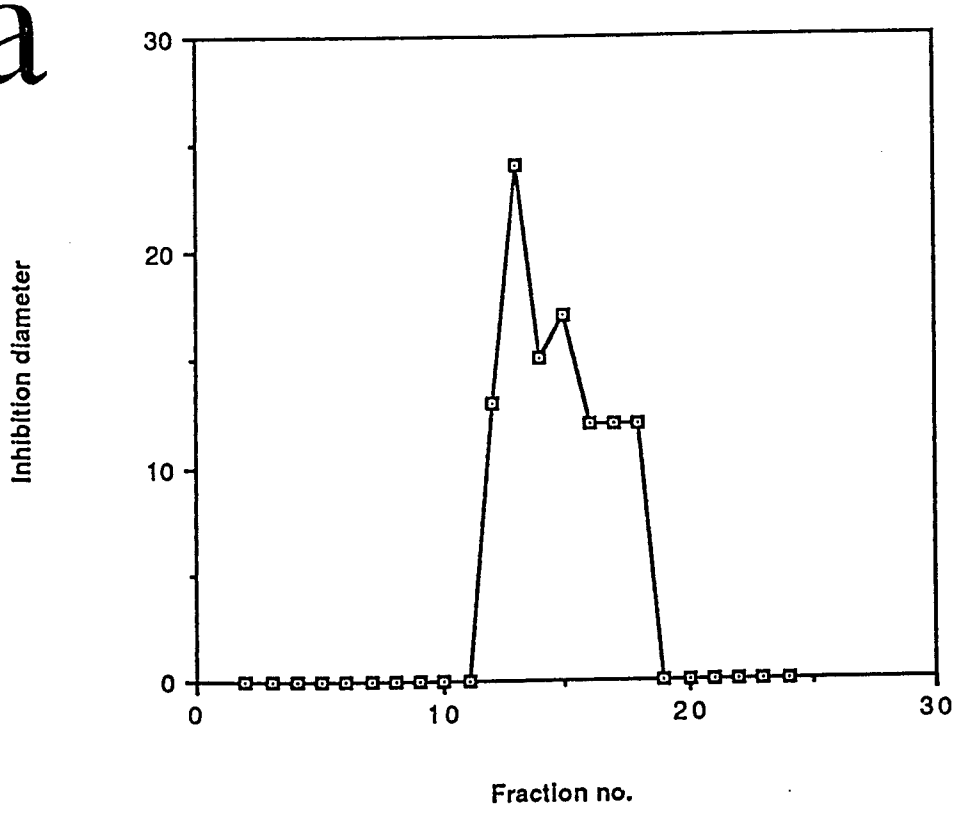
FIGURE 3.5

HVPE pH 1.7 of trifolitoxin

0.5 ODs 302 nm. of trifolitoxin was lyophilized and electrophoresed by HVPE pH 1.7 at 3500 V for 15 mins 0.5 cm sections were eluted ,monitored at 302 nm. and bioassayed.

a: inhibition diameter vs RM_{OG}
b: Absorbance 302 nm. vs RM_{OG}

a


30
20
10
0
0
10
20
30
Inhibition diameter
Fraction no.

b

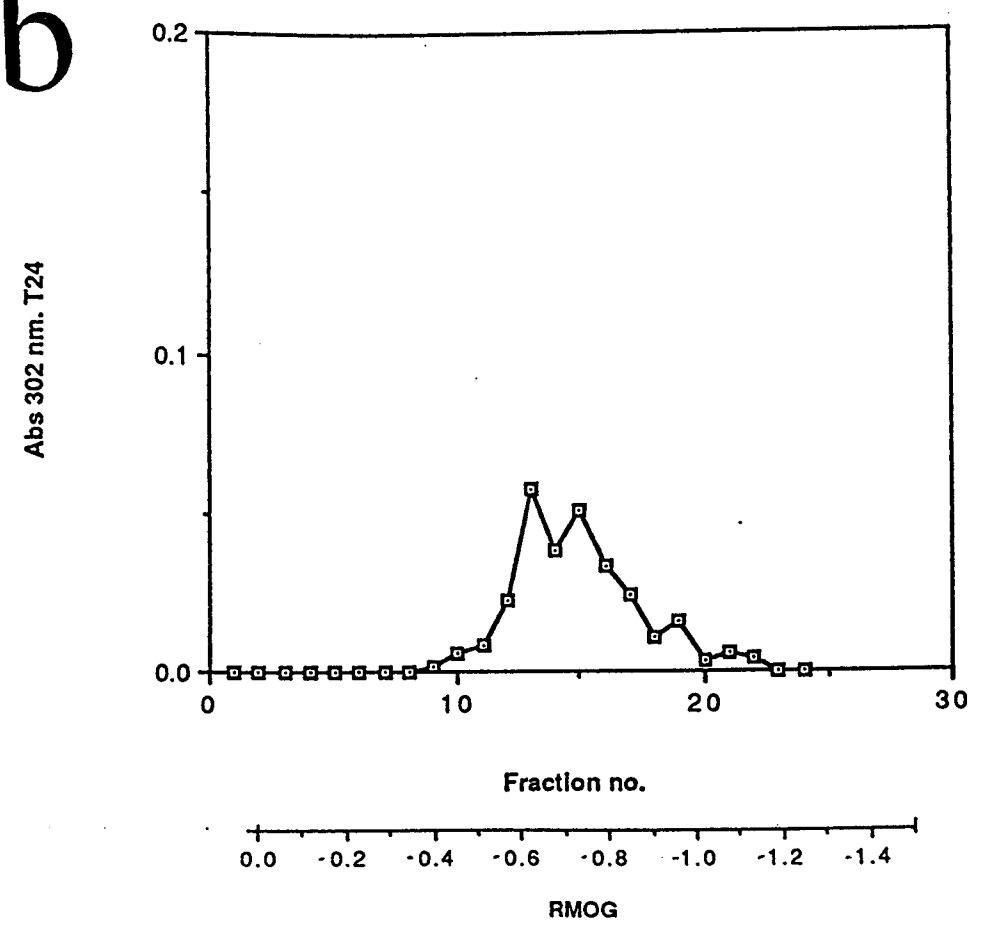

0.2
0.1
0.0
0
10
20
30
Abs 302 nm. T24
Fraction no.
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
RMOG

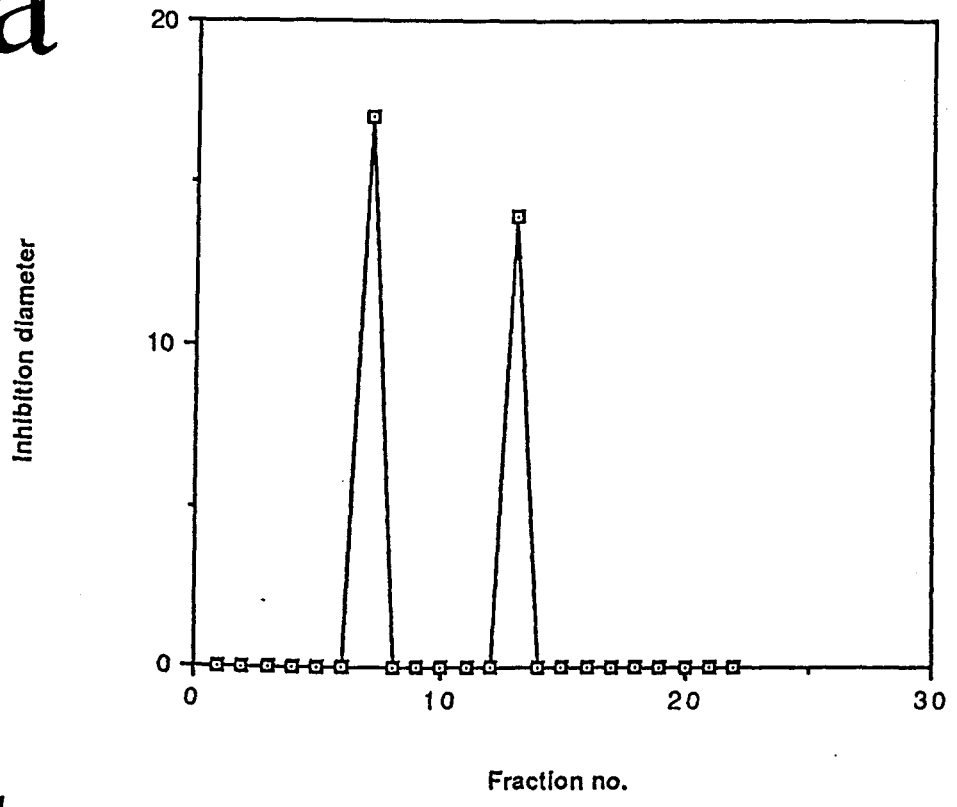
FIGURE 3.6

HVPE pH 1.7 of trifolitoxin treated with performic acid.

Trifolitoxin (0.5 ODs 302 nm.) was oxidized with performic acid for 20 mins, lyophilized and electrophoresed by HVPE at pH 1.7 at 3500 V for 15 mins.). 0.5 cm sections were cut ,eluted ,monitored at 302 nm..and bioassayed.

a: Inhibition diameter vs RM_{OG}
b: Absorbance 302 nm. vs RM_{OG}

a


20
10
0
0
10
20
30
Inhibition diameter
Fraction no.

b

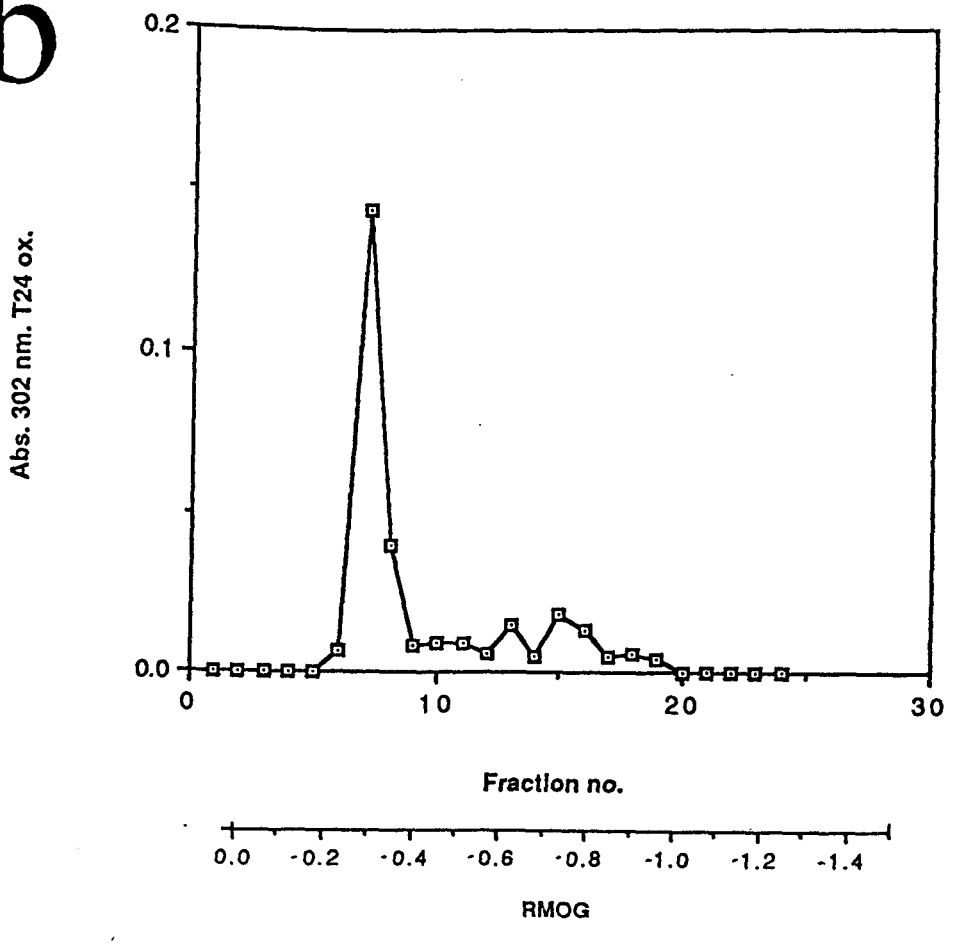
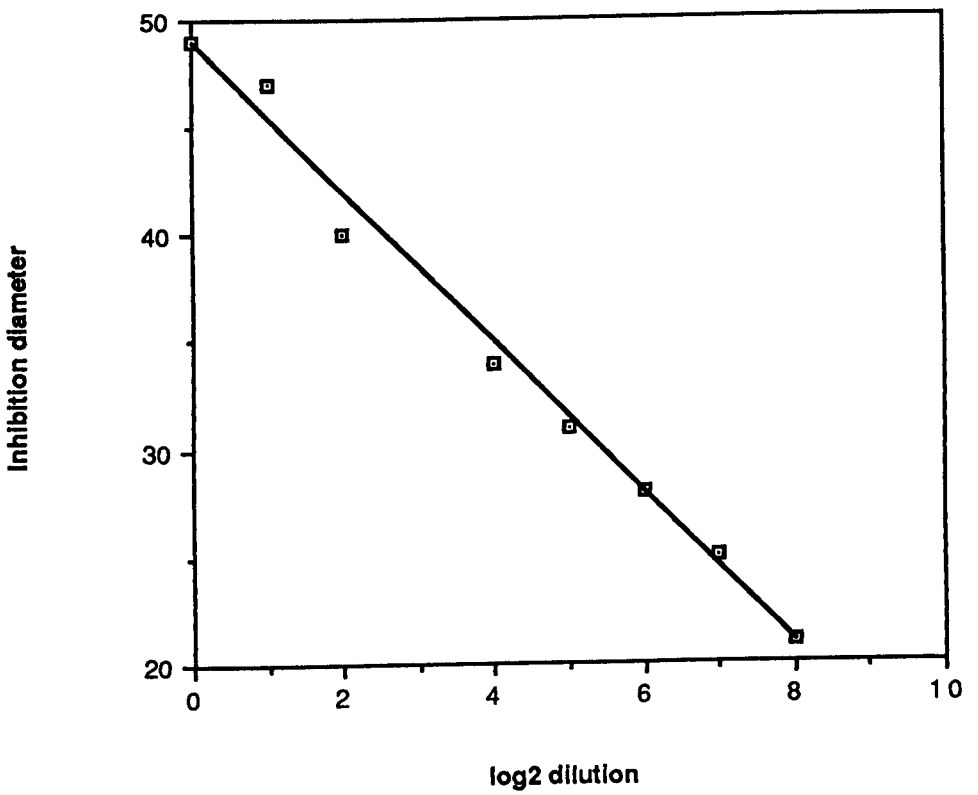

0.2
0.1
0.0
0
10
20
30
Abs. 302 nm. T24 ox.
Fraction no.
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
RMOG

FIGURE 3.7

Dilution series vs Inhibition diameter for trifolitoxin

0.028 ODs 302 nm. of trifolitoxin was resuspended in 10 microlitres of H_2O, 5 microlitres was bioassayed on a 1 cm^2 paper , the remaining 5 microlitres of trifolitoxin was made to 10 microlitres and the process repeated. After 48 hrs inhibition zone diameters were measured.


50
40
30
20
0
2
4
6
8
10
Inhibition diameter
log2 dilution

Biogel P2 gel filtration chromatography

Figure 3.4 shows the biogel P2 chromatography of trifolitoxin and trifolitoxin sulphonic acid. Trifolitoxin is partially included in or absorbed to the gel matrix. Fraction 14 represents the void volume of the column, equivalent to approximately MW1800. Trifolitoxin sulphonic acid elutes slightly earlier than trifolitoxin.

Conversion of trifolitoxin to trifolitoxin sulphonic acid

HVPE of trifolitoxin at pH 1.7 indicated multiple active peaks a consequence of the acid labile group present in trifolitoxin which are coincident with absorbance at 302 nm.(Figure 3.5) Incomplete oxidation of trifolitoxin with performic acid demonstrates that the multiple forms of trifolitoxin can be converted to a single biologically active derivative of trifolitoxin (Figure 3.6)

Inhibition diameter vs.concentration.

The inhibition diameter is linearly related to the log of the concentration (Figure 3.7) in this case base 2 was used. The slope of the line is -0.284. (Correlation 99%). From this value an equation can be formulated relating relative concentration (as a percent) to inhibition diameter. The equation is

$$\text{Relative toxicity \%} = \frac{100}{2^{0.284(I-F)}}$$

Where I is the initial inhibition diameter, F is the final inhibition diameter and relative toxicity % refers to the relative amount of the initial sample required to give the final inhibition diameter.

THE NATURE OF TRIFOLITOXIN : RESULTS

INFRARED Spectra

Infrared analysis of trifolitoxin (Figure 3.8a) showed that trifolitoxin contained the major amide 1 and 2 peaks (Williams and Fleming 1987) consistent with the peptide nature of trifolitoxin and is very comparable to the reference peptide antibiotic bacitracin (figure 3.8 b). A major distinguishing feature between trifolitoxin and bacitracin is a peak at 1600 cm^{-1}.

AMINO ACID ANALYSIS

Amino acid analysis of complete acid hydrolysates of trifolitoxin was quantified by phenylthiohydantoin formation followed by HPLC (Figure 3.9) and qualitatively determined by two dimensional electrophoresis and chromatography of the amino acids (Figure 3.10). Three residues of glycine and one residue each of aspartic acid, isoleucine, serine, arginine, cysteine (as half Cystine), valine and alanine were present.

^{35}S RADIOLABELLING OF TRIFOLITOXIN

The presence of cystine in trifolitoxin hydrolysates was confirmed by utilizing an independent purification technique and ^{35}S labeling (Figure 3. 2) of trifolitoxin. The radiolabel coincides with biological activity. After complete acid hydrolysis of this purified material followed by HVPE pH 1.7 it was found that 83 % of the radiolabel was coincident with cysteine or its oxidation products.

ULTRAVIOLET SPECTRA

UV analysis of partially purified trifolitoxin or material isolated by the ion exchange method, contained a UV chromophore inconsistent with amino acids detected in acid hydrolysates. This UV chromophore fluoresced blue under shortwave (254 nm.) UV light Figure 3.11a b c d shows the pH dependant shifts of the UV chromophore of partially purified trifolitoxin. The maxima are presented in table 3.2. The calculated extinction coefficient for the 302 nm. peak at pH 7.0 in water equals 5000.

Table 3.2

pH	UV maxima (nm.)
-1.0	270
1.0	228,304
7.0	239,302
13.0	239,296

pK of the 228 nm. to 239 nm shift of the blue fluorescent chromophore

Figure 3.12 is a plot of the ratio of 228 nm.to 239 nm.vs pH.of trifolitoxin The spectra are presented in figure 3.11 The shift has a calculated pK of 5.1.

FIGURE 3.8

Infrared spectra of trifolitoxin and bacitracin

a: trifolitoxin (0.5 ODs, 302 nm.)

b: bacitracin

b
a
4000
3000
2000
1600
1200
800
cm-1

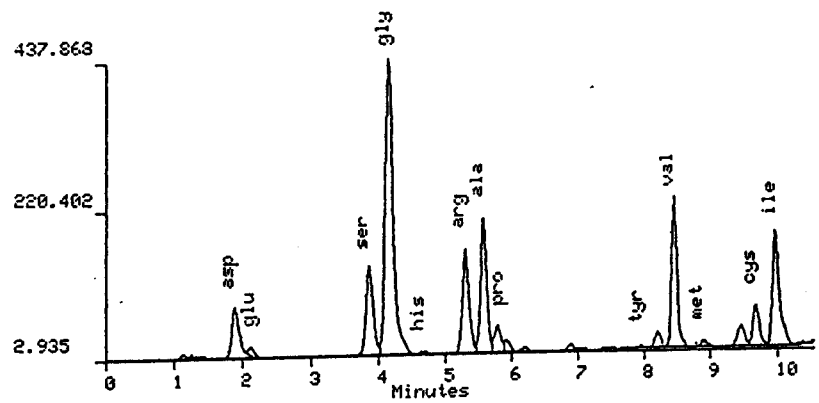
FIGURE 3.9

HPLC of PTH amino acids derived from complete acid hydrolysis of trifolitoxin.

a: PTH amino acids derived from complete acid hydrolysis of trifolitoxin.

b: Standard mixture of PTH amino acids.

a


437.868
220.402
2.935
asp
glu
ser
gly
his
arg
ala
pro
tyr
val
met
cys
ile
0
1
2
3
4
5
6
7
8
9
10
Minutes

b

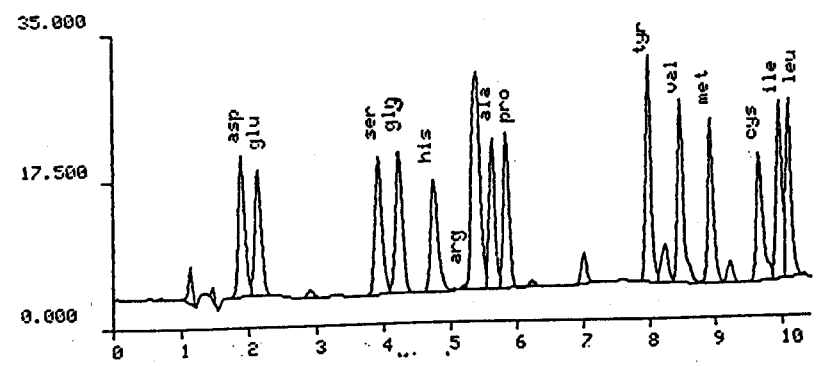

35.000
17.500
0.000
asp
glu
ser
gly
his
arg
ala
pro
tyr
val
met
cys
ile
leu
0
1
2
3
4
5
6
7
8
9
10

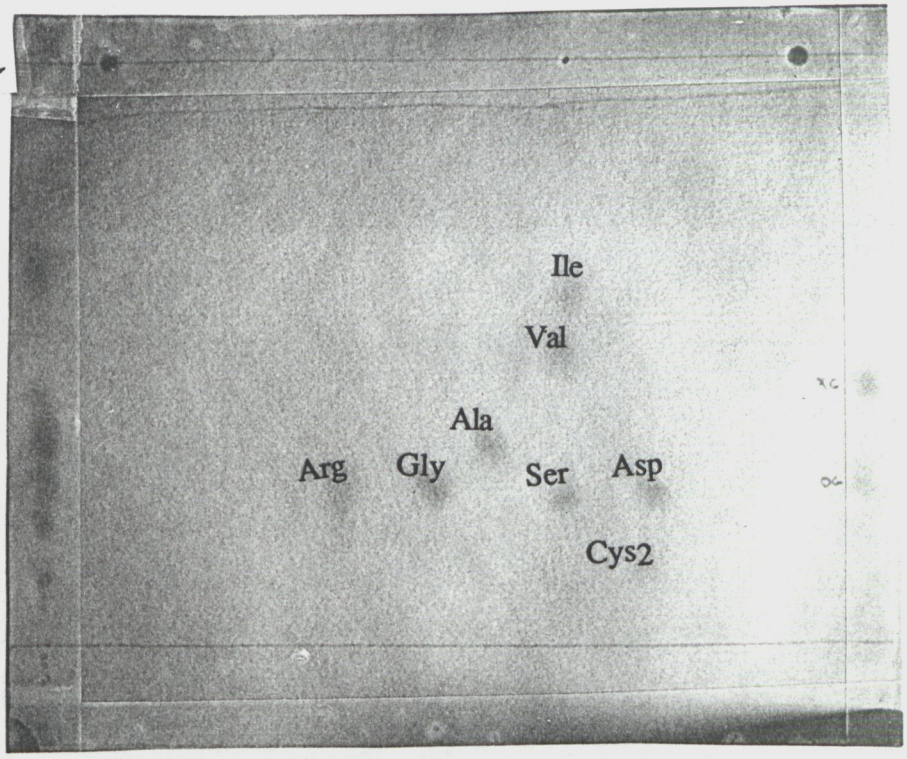
FIGURE 3.10

2D HVPE/PC of amino acids derived from a complete acid hydrolysis of trifolitoxin.

a: trifolitoxin

b: amino acid mixture of amino acids found in trifolitoxin based on the results of figure 3.9

a


Ile
Val
Ala
Arg
Gly
Ser
Asp
Cys2

b

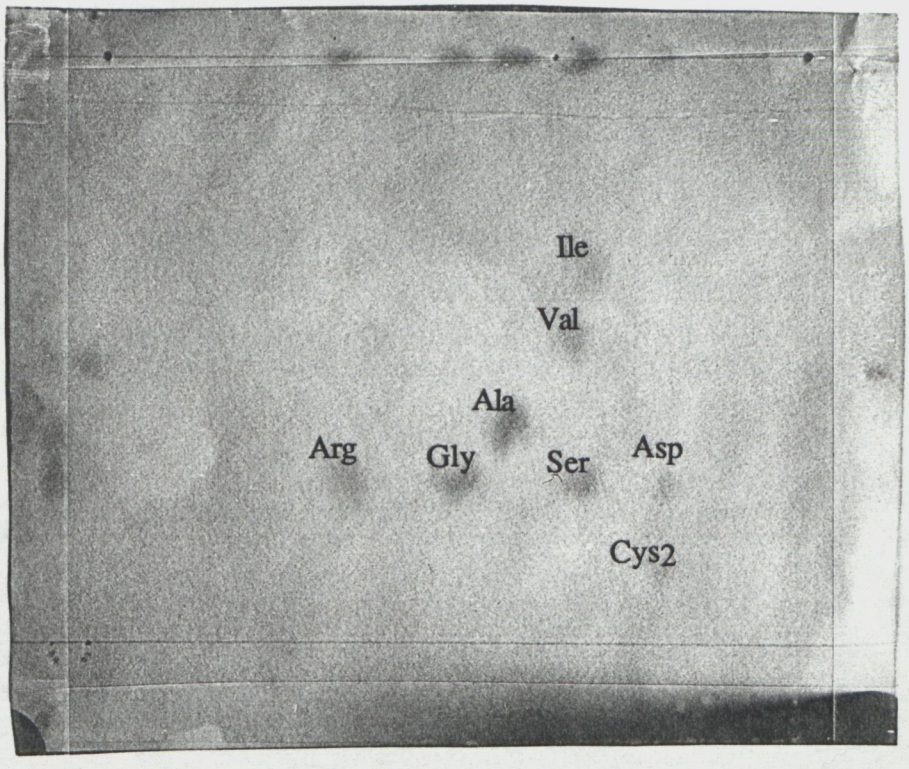

Ile
Val
Ala
Arg
Gly
Ser
Asp
Cys2

FIGURE 3.11

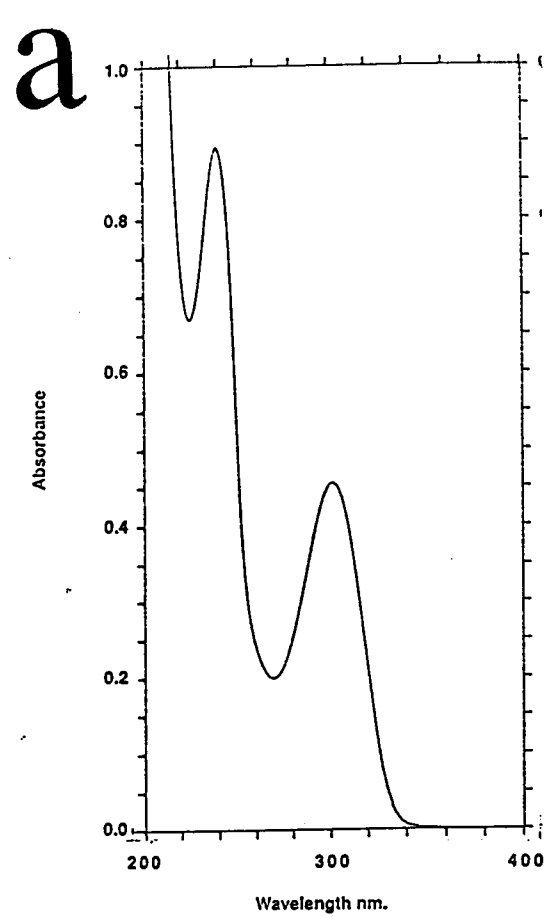
UV spectra of trifolitoxin vs pH

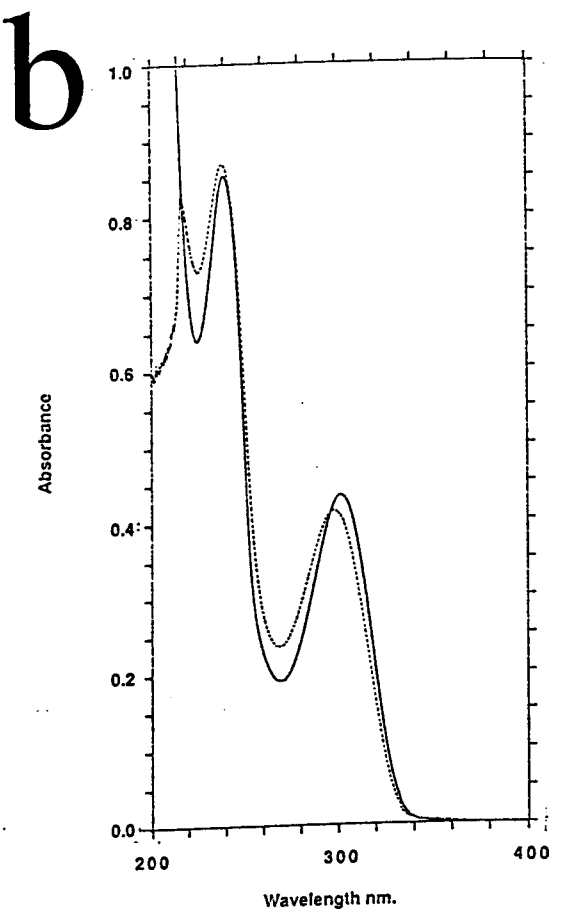
a: trifolitoxin pH 7.0 in H20 (max 239 nm. ,302 nm.)

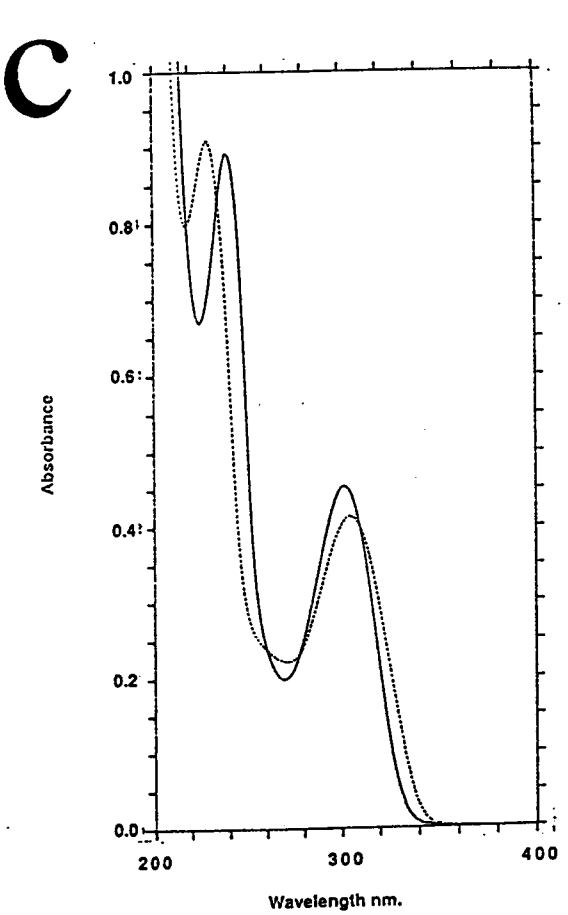
b: _____ a
------ trifolitoxin in 0.1 N NaOH (pH 13.0)

c:_____ a
------ trifolitoxin in 0.1 N HCl (pH 1.0)

d:_____ trifolitoxin in 0.1 N HCl (pH 1.0)
-------trifolitoxin in 10 N HCl (pH -1.0)


a
Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
200
300
400
Wavelength nm.


b
Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
200
300
400
Wavelength nm.


c
Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
200
300
400
Wavelength nm.

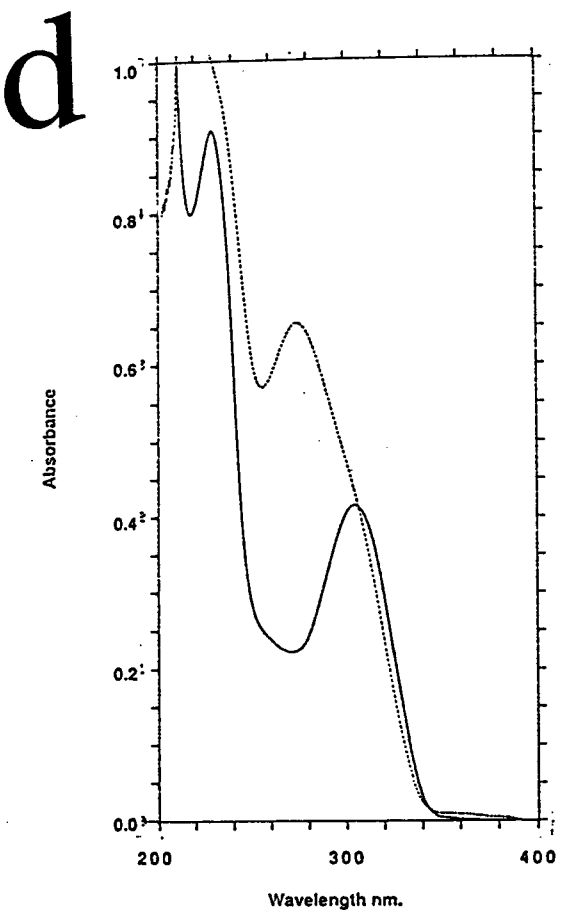

d
Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
200
300
400
Wavelength nm.

FIGURE 3.12

Trifolitoxin UV chromophore vs pH
Ratio of 239 nm. : 228 nm. vs pH

Addition of NaOH solution to a 5 mM solution of citric acid plus trifolitoxin (0.25 ODs 302 nm.)
The pH of a standard 5 mM solution of citric acid plus aliquots of a NaOH solution had been predetermined with a pH Meter. The ratio of 239 nm. to 228 nm. is plotted to account for any dilution effects.

The calculated pK (Albert et al 1971) for the shift equals 5.1

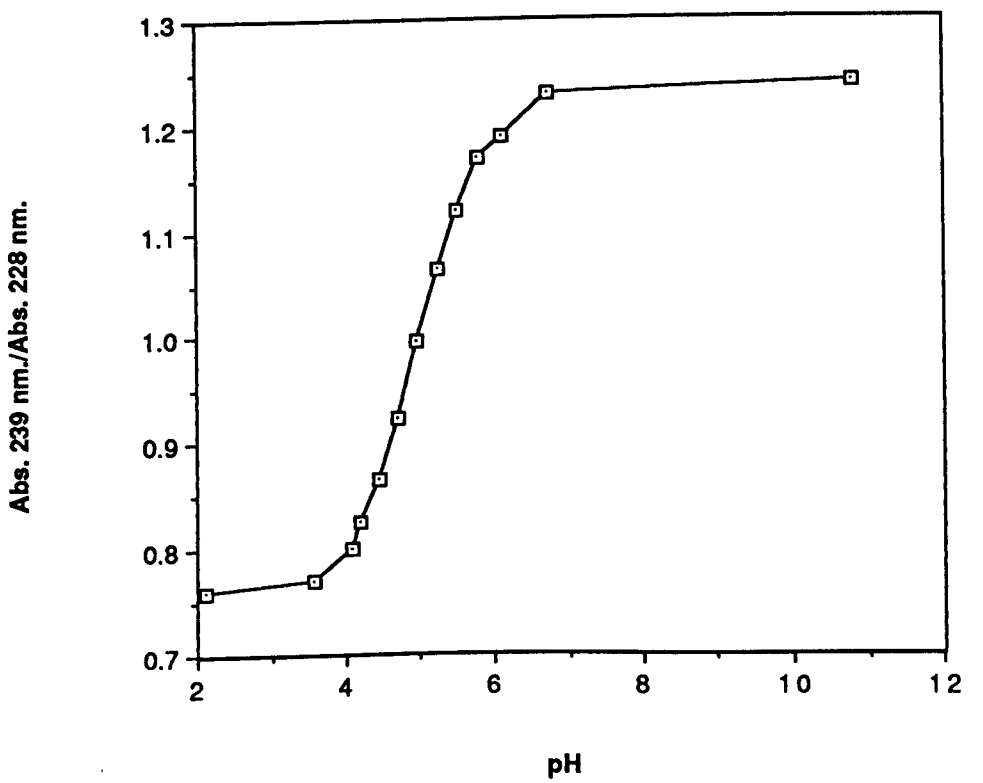
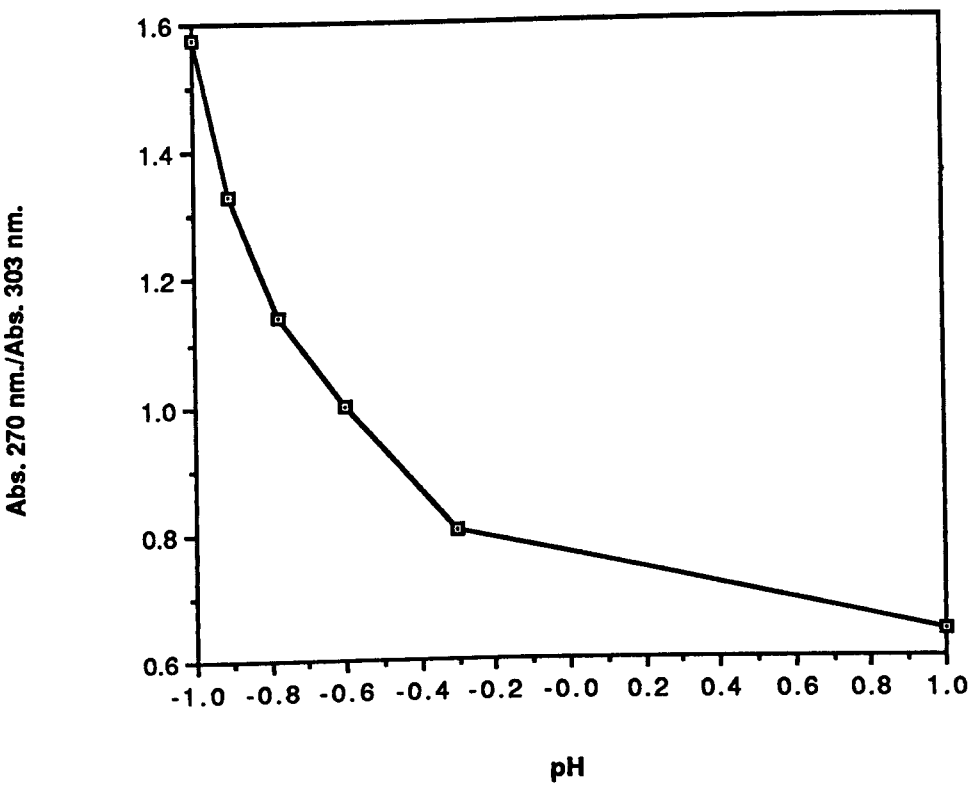

Abs. 239 nm./Abs. 228 nm.
1.3
1.2
1.1
1.0
0.9
0.8
0.7
2
4
6
8
10
12
pH

FIGURE 3.13

A plot of the ratio of 270 nm.UV absorbance to 304 nm UV absorbance as a function of pH (increasing concentrations of HCl) for trifolitoxin.


Abs. 270 nm./Abs. 303 nm.
1.6
1.4
1.2
1.0
0.8
0.6
-1.0 -0.8 -0.6 -0.4 -0.2 -0.0 0.2 0.4 0.6 0.8 1.0
pH

pK of the 270 nm. chromophore

Figure 3.13 is a plot of the ratio of 270 nm. to 304 nm. vs pH (varying concentrations of HCl). The pK for the shift is less than zero. The spectrum is presented in Figure 3.11.

NUCLEAR MAGNETIC RESONANCE SPECTRA

Proton N.M.R.

Proton n.m.r. (Figure 3.14) showed all the protons expected for the amino acids (Jardetsky and Roberts 1987) found in trifolitoxin sulphonic acid (Table 3.3) except for arginine and one glycine residue and an extra proton consistent with an ethylenic proton probably derived from the chromophore. One of the glycine residues shows an A-B pattern distinguishable from the two other glycine resonances and indicates that this glycine residue is in an unusual environment. The apparent absence of an alpha proton for arginine and the shift of the beta protons upfield 0.2 ppm from that of literature values indicates a modified arginine or an unusual environment for these protons. The alpha protons of aspartic acid and alanine are also shifted from that expected from an intrapeptide linkage and can be rationalized on their N-terminal and C terminal sequence locations as discussed in the analysis of the peptide sequence (Chapter 4.1). Two dimensional COSY proton n.m.r. (Figure 3.15) confirmed the assignments of the one dimensional spectra. It must be noted that the ethylenic proton detected in the one dimensional spectra is absent in the 2D spectra indicating the absence of coupling to this proton .

Carbon 13 N.M.R.

The assignments of the ^{13}C spectra in figure 3.16 are presented in Table 3.4. The precise assignment of some signals proved difficult because of overlap especially in the carbonyl region. The ^{13}C n.m.r. indicates the presence of at least two extra signals which cannot be assigned to the amino acids found in trifolitoxin sulphonic acid. (112 ppm and 81 ppm) and have been assigned to the chromophore. The guanidino carbon at 159 ppm is as expected from literature values (Jardetsky and Roberts 1981) indicating that the guanidino group is not modified. This result is relevant to the placement of the chromophore in the peptide chain.

FIGURE 3.14

Proton N.M.R. of trifolitoxin sulphonic acid

400 MHz spectrum of 5 mg. sample of trifolitoxin sulphonic acid in 600 microlitres of D_2O

Proton assignments are based on literature values of Jardetzky and Roberts (1981) where appropriate.

Ethylenic CH
Cys03H α CH
Ser α CH
Gly3 α CH
Asp α CH
Ile α CH
Val α CH
Gly1 Gly2 CH2
Ser β CH2
Arg δ CH2
Cys03H β CH2
Asp β CH2
Arg β CH2
Val β CH
Ile β CH
Arg γ CH2
Ile γ CH
Ala β CH3
Ile γ CH
Ile CH3
Val CH3
Ile CH3
Val CH3
PPM

FIGURE 3.15

Two dimensional proton COSY N.M.R. of trifolitoxin sulphonic acid

Sample prepartion the same as for one dimensional spectra (Figure 3.14)

Horizontal and vertical lines connecting off-diagonals indicate J-connectivities of vicinal or geminal protons as suggested by the assignments.

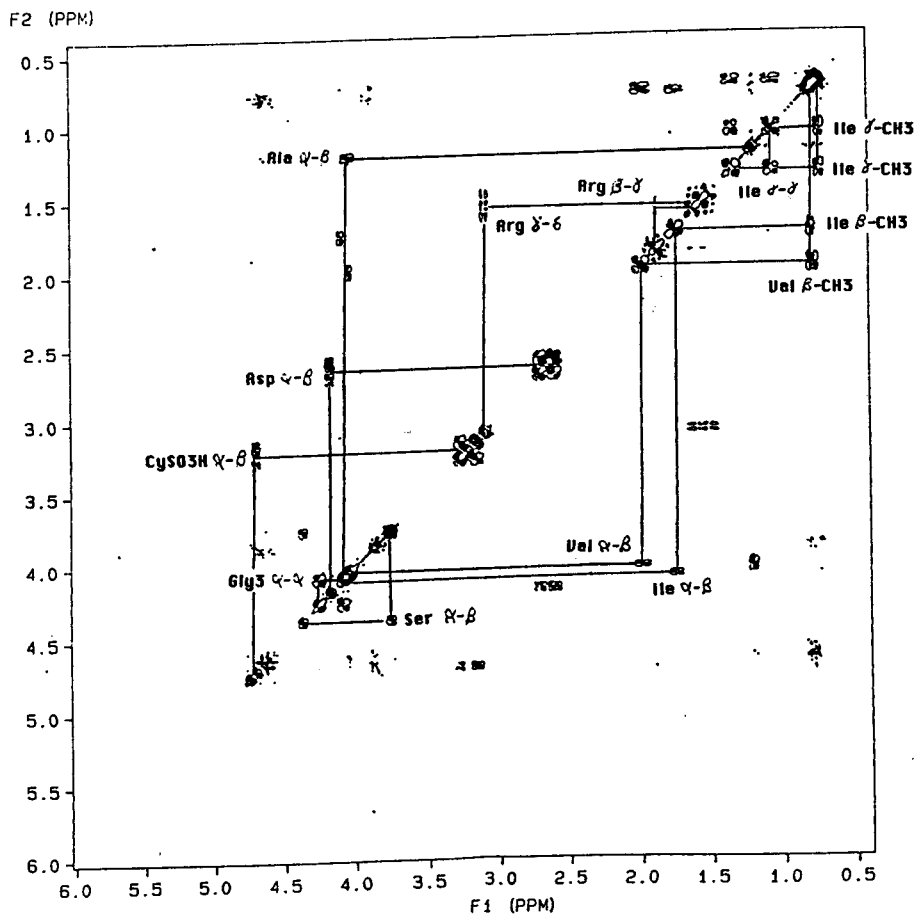

F2 (PPM)
F1 (PPM)
Ile γ-CH3
Ile δ-CH3
Ile δ-δ
Ile β-CH3
Val β-CH3
Arg β-γ
Arg γ-δ
Asp α-β
CysO3H α-β
Val α-β
Gly3 α-α
Ile α-β
Ser α-β

TABLE 3.3

Assignments of proton signals of trifolitoxin sulphonic acid from Figure 3.14
Literature values are from Jardetzky and Roberts (1981)

amino acid	CαH	CβH	CγH	CδH
asp	4.77	2.75,2.84		
asp T24SO3H	4.30	2.74,2.86		
ile	4.22	1.89	1.19,1.48	0.89
ile T24SO3H	4.24	1.87	1.20,1.48	0.90
gly	3.97			
gly T24SO3H	4.00			
gly	3.97			
gly T24SO3H	4.00			
ser	4.50	3.89		
ser T24SO3H	4.50	3.89		
arg	4.40	1.80,1.92	1.72	3.31
arg T24SO3H	?	2.01	1.70	3.23
gly	3.97			
gly T24SO3H	4.39,4.24			
cysO3H	4.7	3.2,3.3		
cysO3H T24SO3H	4.7	3.2,3.3		
val	4.18	2.13	0.94,0.97	
val T24SO3H	4.17	2.13	0.94,0.97	
ala	4.35	1.40		
ala T24SO3H	4.20	1.35		

Unassigned protons

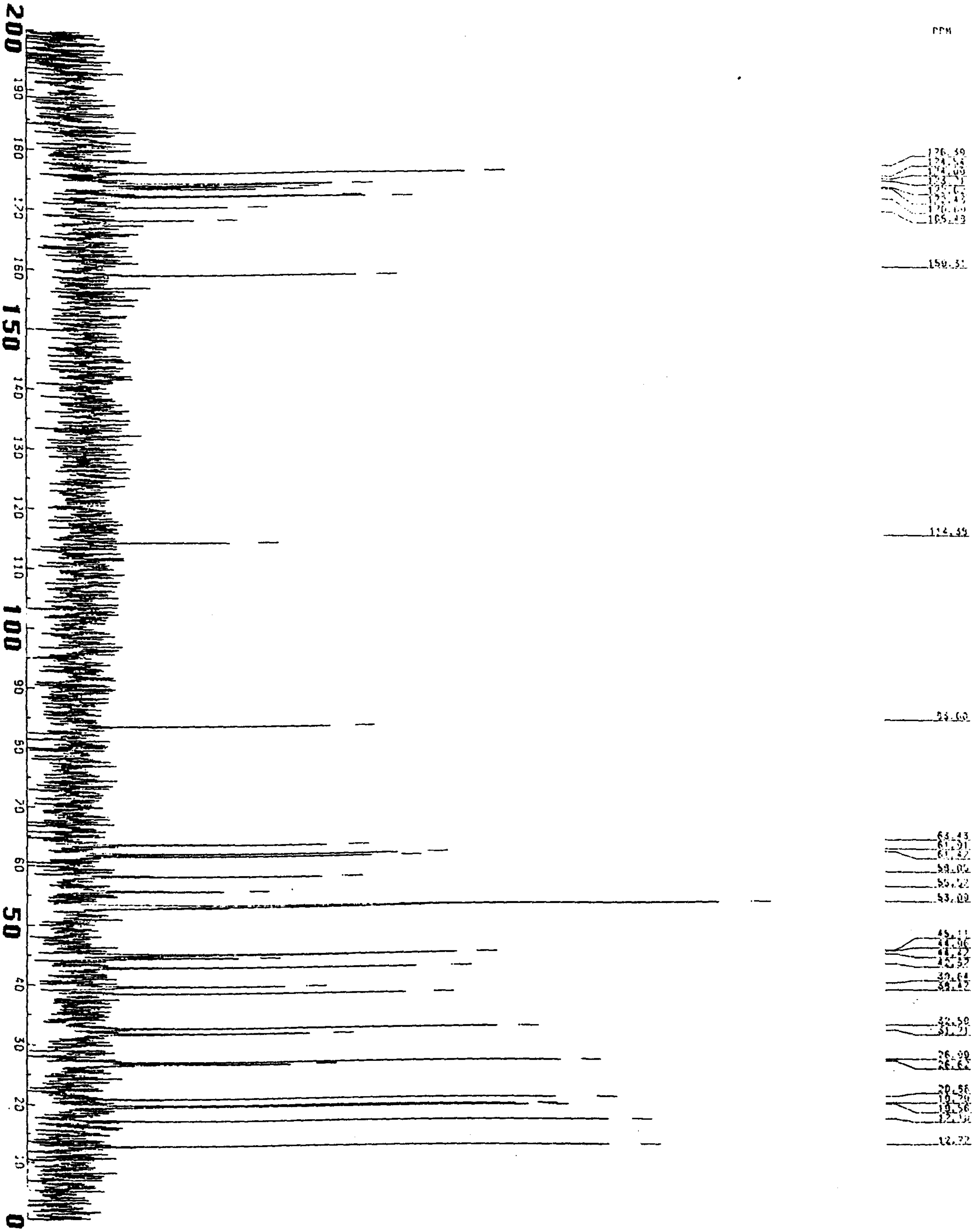
5.8 (ethylenic)

FIGURE 3.16

Carbon 13 N.M.R. spectrum of trifolitoxin sulphonic acid

300 MHz ^{13}C N.M.R. of trifolitoxin sulphonic acid in 90% H_2O / 10% D_2O

Assignments are presented in table 3.4



200
150
100
50
0

TABLE 3.4

^{13}C N.M.R. assignments for trifolitoxin sulphonic acid
Literature values from Jardetzky and Roberts (1981)

Carbon 13 N.M.R.

amino acid	Cα	Cβ	Cγ	Cδ	Cε
asp	52.4	39.4			
asp T24SO3H	51.46	38.01			
ile	59.4	37.0	25.2,15.5	11.0	
ile T24SO3H	59.84	37.01	25.4,15.5	11.14	
gly	43.5				
gly T24SO3H	42.845				
gly	43.5				
gly T24SO3H	43.33				
ser	56.4 62.0				
ser T24SO3H	56.42 61.8				
arg	54.4	29.0	25.2	41.5	157.5
arg T24SO3H	?	29.96	24.99	41.03	157.5#
gly	43.5				
gly T24SO3H	43.48				
cysO3H	52.7	52.0			
cysO3H T24SO3H	?	?			
val	60.05	30.9	19.25,18.75		
val T24SO3H	60.2	30.08	18.16,19.23		
ala	50.65 17.4				
ala T24SO3H	51.46 17.93				

Values (ppm) calculated using the guanidine carbon of arginine # as an internal standard (actual measurement 159 ppm). Carbonyls or carboxyls of amino acids were not assigned because of the large overlap of signals. (170-176 ppm) Cysteic acid values are non literature values.

Unassigned carbons

81.97 112.76 164.85 169.06

FIGURE 3.17

FAB MS spectra of trifolitoxin

a: trifolitoxin

b: bacitracin

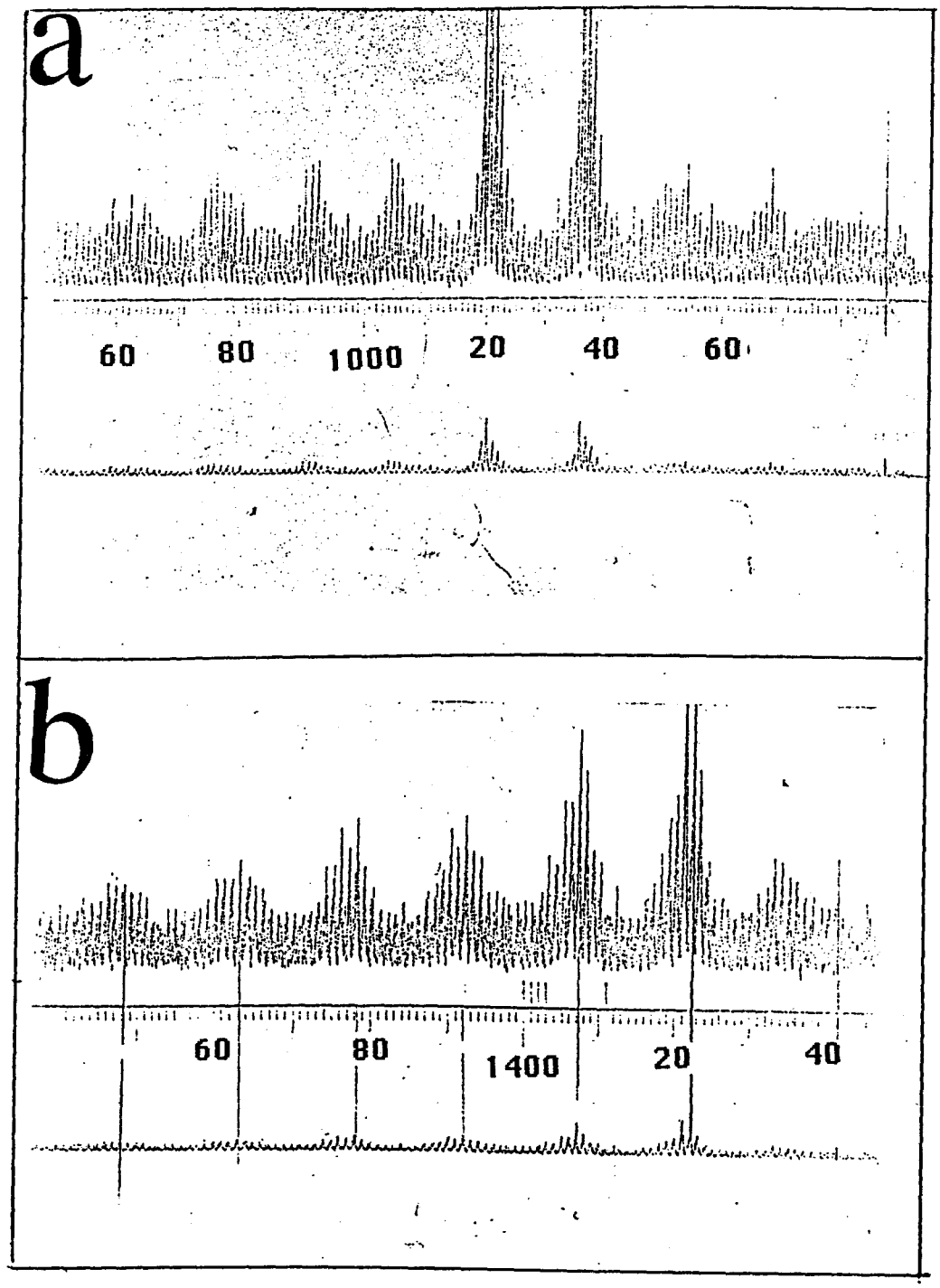

a
60
80
1000
20
40
60
b
60
80
1400
20
40

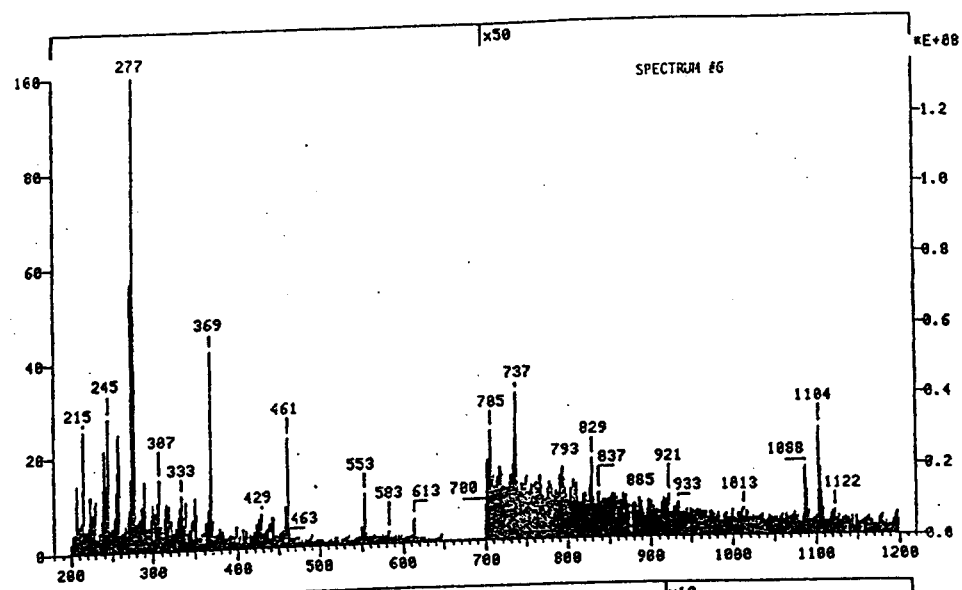
FIGURE 3.18

Fast atom bombardment mass sprectrum of trifolitoxin sulphonic acid. Finnigan Fast atom bombardment mass spectrometer.

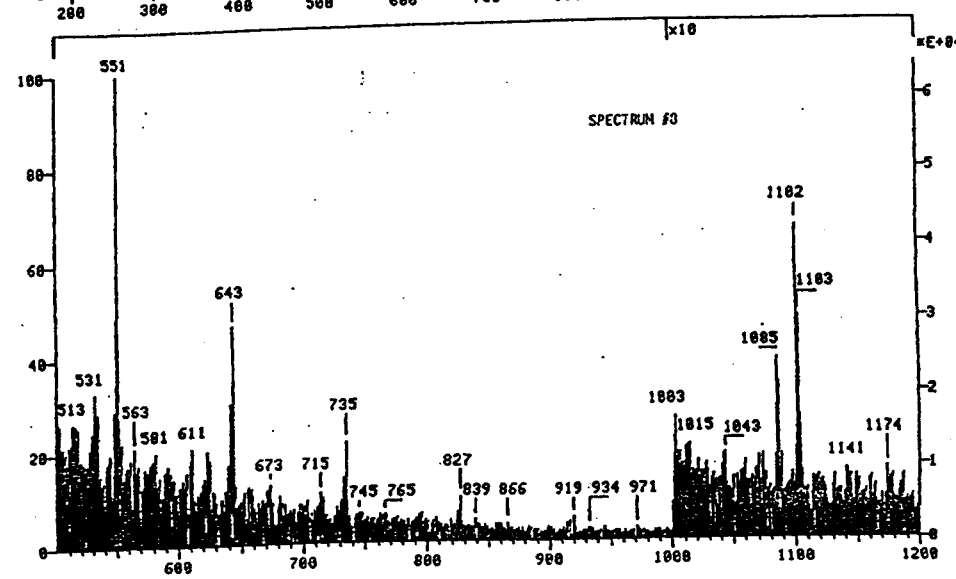
A: positive ion spectra M+H=1104

B: negative ion spectra M-H=1102

a


x50
xE+08
SPECTRUM #6
277
369
245
215
307
333
461
429
463
553
583
613
700
737
785
793
829
837
885
921
933
1013
1088
1104
1122

b


x10
xE+04
SPECTRUM #3
551
643
531
513
563
581
611
673
715
735
745
765
827
839
866
919
934
971
1003
1015
1043
1085
1102
1103
1141
1174

FAST ATOM BOMBARDMENT MASS SPECTROSCOPY

Fast atom bombardment mass spectroscopy of trifolitoxin and trifolitoxin sulphonic acid (Figure 3.17 and 3.18) indicated molecular M+H ions at 1038 and 1104 respectively. Although fragment ions are present the peaks are difficult to interpret. Figure 3.18 also contains a FAB MS of bacitracin (Molecular weight of 1421) This data is currently under clarification with a Finnigan TSQ FAB MS.

DISCUSSION

Trifolitoxin is a polar, anionic at neutral pH, low molecular peptide, its properties are in general agreement with the previously suggested oligo-peptide nature (Schwinghamer and Belkengren 1968) except that purification to homogeneity has permitted characterization of the chromophore as the 300 nm. species shown in Figure 3.11.

The peptide component

It is evident from the infrared spectra that trifolitoxin is very comparable to the infrared spectra of bacitracin, a known peptide. The major features are the large amide 1 and 2 peaks consistent with a substantial number of amide linkages relative to other structural features of peptides. The peak at 1600 cm^{-1} could represent the carbonyl stretch C terminal carboxylate anion. Bacitracin lacks a C terminal carboxyl group.

Acid hydrolysis of trifolitoxin reveals the presence of ten amino acids, eight of which are indistinguishable by proton N.M.R. from intrapeptide residues of amino acids (underlined values represent a divergence of greater than 0.2 ppm). The alpha protons of one glycine residue and the alpha and beta protons of arginine are shifted from literature values indicating a potential linkages for the blue fluorescent chromophore. Most of the carbon signals from amino acids in the ^{13}C N.M.R. can be defined and do not differ from literature values. In particular the chemical shift of the guanidine carbon is the same as published literature value (Jardetsky and Roberts 1987) and this strongly argues against the involvement of the guanidine moiety in a direct linkage to the chromophore. Further ^{13}C assignments will come with the analysis of the protease fragments, currently in progess.

Fast atom bombardment mass spectroscopy of trifolitoxin revealed a molecular weight of trifolitoxin at 1037 which requires to be clarified. Finnigan positive ion spectra of the sulphonic acid revealed a molecular ion at 1104 and a negative ion at 1102 identifying the molecular weight of trifolitoxin sulphonic acid as 1103. Simple analysis of the molecular weights of these two species shows a difference 66 daltons, 18 daltons higher than that expected for a simple oxidation of a thiol to sulphonic acid. The oxidation of trifolitoxin adds

an extra molecule of water to the structure. The finding that native trifolitoxin fails to react with thiol reagents the FABMS results and the 270 nm chromophore in 10 N HCl of partially purified trifolitoxin are consistent with the thiazoline structure for cysteine in the peptide chain of trifolitoxin which is amplified in Chapter 4.2.

Addition of the molecule weights of the amino acids minus 9 water molecules for the peptide bonds, assuming linearity, gives a molecular weight of 915.4, 122 mass units short of that measured, this extra mass has been ascribed to the presence of an unusual chromophore.

The chromophore component

The ultraviolet chromophore (maxima 239 nm 302 nm pH 7.0) associated with trifolitoxin activity does not correspond to any known proteinaceous aromatic amino acids and contradicts Schwinghamers data (1968). The blue fluorescent chromophore is a major distinguishing feature of trifolitoxin from other known peptide antibiotics and has been extremely useful in simplifying purification procedures. Initial analysis of the UV spectrum of trifolitoxin at neutral pH shows a calculated extinction coefficient of 5000 at 302 nm. and UV maxima with peaks at 302 nm and 239 nm and blue fluorescence would be consistent with a compound containing three or four conjugated units (Williams and Fleming 1987). Epsilon values of the order of 1000 to 10000 generally indicate the presence of an aromatic system. Salicylates (Doub and Vandenbelt 1955), 3-hydroxy anthranilic acid (Nyc and Mitchell 1948), or the analogue of anthranilic acid, ortho sulphanilamide represent model compounds with similar UV maxima and epsilon values. On the recognition of the pK at 5.1, potential pyridine compounds were also included, 3-hydroxy or 3-amino pyridines being good examples.(Stack and Ewing 1948) All of the model chromophores show blue fluorescence. It is of interest to note that 3-hydroxy picolinic acid the pyridine equivalent of salicylic acid is linked to the N terminal residue of etamycin a peptide antibiotic compound.(Maeda 1957).

Nevertheless none of the degradative or other spectroscopic data obtained for trifolitoxin support the presence a benzene or pyridine nucleus, for instance no aromatic protons are detectable in the proton N.M.R., all the above compounds are stable to acid and base hydrolysis, a property of benzene and pyridine compounds in general. It is a matter of considerable regret that the trifolitoxin chromophore is not stable to these hydrolytic conditions. Benzene and pyridine derivatives can therefore be excluded in the search for a model chromophore.

The ^{13}C N.M.R. show unassignable peaks at 81 and 112 ppm. ^{13}C DEPT indicate that that these peaks represent CH and quaternary carbons respectively (J Sims personal communication). Comparison to literature values (Williams and Fleming 1987) indicate that the quaternary carbon is probably an alkene and the methine proton could be the olefinic proton detected in the proton N.M.R. Because of the complexity of the amide carbonyl region of the

^{13}C spectra, other potential chromophore carbonyls cannot be ruled out. The fast atom bombardment mass spectra of trifolitoxin or trifolitoxin sulphonic acid indicate that the chromophore has a residue molecular weight of 122. The absence of adequate carbons in the ^{13}C N.M.R. spectra to explain this molecular weight would suggest an even number of nitrogens are present in the chromophore. The perturbed arginine and glycine peaks in the proton N.M.R. spectra suggest a potential linkage point for this unusual chromophore to these amino acids. Proton N.M.R. indicates a single uncoupled olefinic proton at 5.8 ppm, attributed to the chromophore, some conjugated system is required to explain the UV absorption and fluorescence characteristics of trifolitoxin. Potential protons may also be hidden under the water peak and cannot be discounted. Clearly more independent data is required before a clear picture of the make up of the chromophore can be obtained.

The crude structure

From the data currently presented it is apparent that trifolitoxin is a peptide bacteriocin containing a modified cysteine and somewhere in the peptide component probably at a location close to an arginine and glycine residue is an unusual low molecular weight UV fluorescing chromophore with an ionizable group with pK 5.1.

The data so far presented would be consistent with Schwinghamer and Belkengrens (1968) original oligo-peptide postulate. The blue fluorescent chromophore however is not consistent with their data and probably reflects the purity of compound that they were able to obtain.

The following chapter attempts to answer the question of the nature of the peptide (Chapter 4.1), the nature of the cysteine modification (Chapter 4.2), the chromophore linkage (Chapter 4.1 and 4.3) and the characterization of the chromophore.(Chapter 4.3).

CHAPTER 4
THE STRUCTURE OF TRIFOLITOXIN

INTRODUCTION

Structural variation among peptide antibiotics appears to be vast and the diversity of constituent amino acids seems almost unlimited, for example dehydroamino acids, alpha hydroxy acids and D amino acids are common.(Waley 1966). Nevertheless the structural types are more restricted and include linear, cyclic, branched cyclic peptides and depsipeptides.(Kleinhauf 1987). Quite a few of these peptide backbones contain other than alpha amino and alpha carboxyl peptide linkages, such as gamma glutamyl, (glutathione as an example) epsilon (Aspartyl) lysine (bacitracin), esters (etamycin). Ring systems are also not uncommon. As for example thiazoline (bacitracin),thiazole (bottromycin), 1-4 dithiane (Echinomycin) and dioxopiperazine. (Echinulin), lactone and lactam rings. Amino group acylations and formylations (gramicidin D) and oxidative coupling of phenolic rings (Vancomycin) also occurs (See Waley 1966)

Although this list is by no means exhaustive, it does indicates some of the diversity of structures possible in antibiotic peptides and demonstrates that structural determinations of antibiotic peptides is seldom a matter of amino acid composition and sequence data. The following section gives some published examples of peptides with features which distinguish them from simple linear peptides of proteinaceous origin and discusses various aspects which were relevant to the structural determination of trifolitoxin.

(a). BACITRACIN (Johnson *et al.*, 1945, Konisberg and Craig 1961, Lockhart and Abraham 1955, Newton and Abraham 1953a,b, Stoffel *et al.*, 1961). Bacitracin is a branched cyclic peptide produced by *Bacillus licheniformis*

(1) The linkage of the alpha carboxyl of asparagine to the epsilon amino group of lysine forms the branched cyclic structure as shown.

(2) The presence of alternating D and L amino acids, which is thought to inhibit protease action.

(3) Ornithine a non proteinaceous amino acid has been shown to occur.

(4) The thiazoline ring is a consequence of the dehydration of cysteine.

(b). ETAMYCIN (Maeda 1957, Arnold and Johnson 1958, Eastwood and Snell 1958) Etamycin is a monocyclic peptide lactone produced by *Streptomyces* species.

(1) the would be C terminal carboxyl of N methyl phenylalanine is lactonized to the hydroxyl of threonine.

(2) another unusual feature is the the masking of the N terminal amino group by amide bond formation with 3 hydroxy picolinic acid which UV maxima at 300 nm. is close to that of the blue fluorescent chromophore of trifolitoxin.

(3) The majority of peptide bonds have been N methylated, such structures are thought to stabilize peptide bonds against proteolytic cleavage.

(4) Etamycin also contains some unusual non proteinaceous amino acids.

(c). GRAMICIDIN D (Sarges and Witkop 1964) Gramicidin D is linear peptide produced by *Bacillus brevis*

(1) Gramicidin D contains only proteinaceous amino acids but with alternating D and L isomers

(2) The C terminal is blocked by ethanolamine and the N terminal is formylated.

(d).GRAMICIDIN S (Erlanger and Goode 1954) Gramicidin S is a cyclic peptide produced by *Bacillus brevis*

(1) Gramicidin S contains the non proteinaceous amino acid ornithine and D or L amino acids .

(2) Gramicidin S shows a cyclic structure.

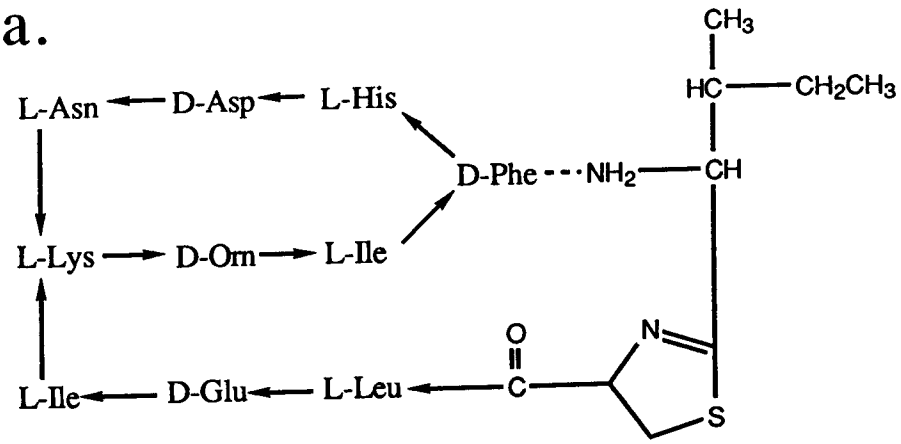
(e). ENNIATIN A (Quitt *et al.* 1963)Enniatin A is a cyclic depsipeptide produced by a *Fusarium* species

(1) Enniatin A contains alpha hydroxy acids as well as alpha amino acids

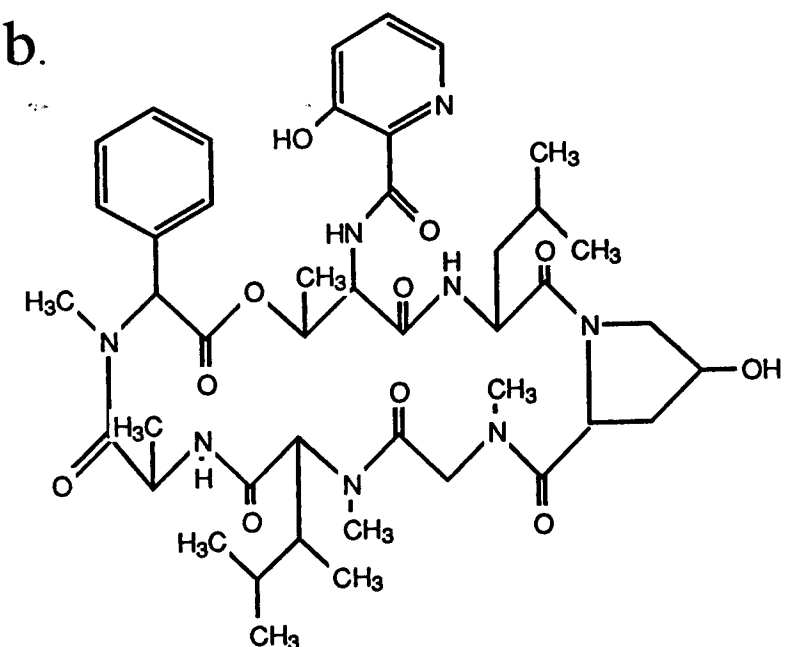
(2) the hydroxy acids are linked through ester rather than amide bonds to form a linear sequence of linkages.

The following sections explores the nature of the peptide trifolitoxin in detail and compares the similarity and differences to the above examples with what has been observed with the selectively toxic trifolitoxin molecule.

a.



b.

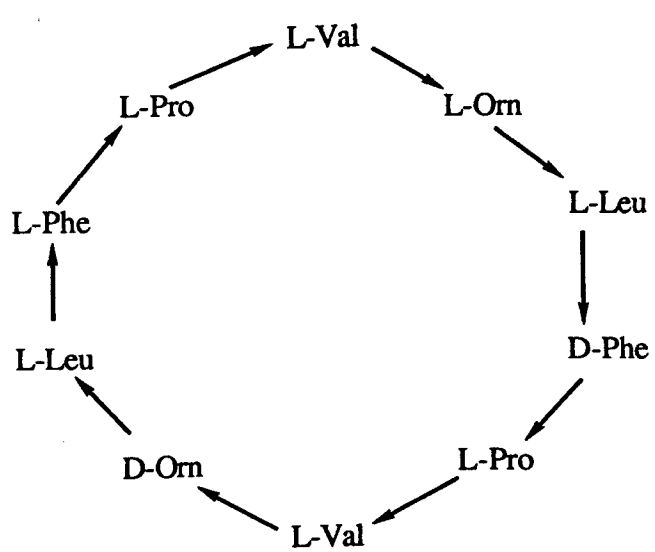


c.

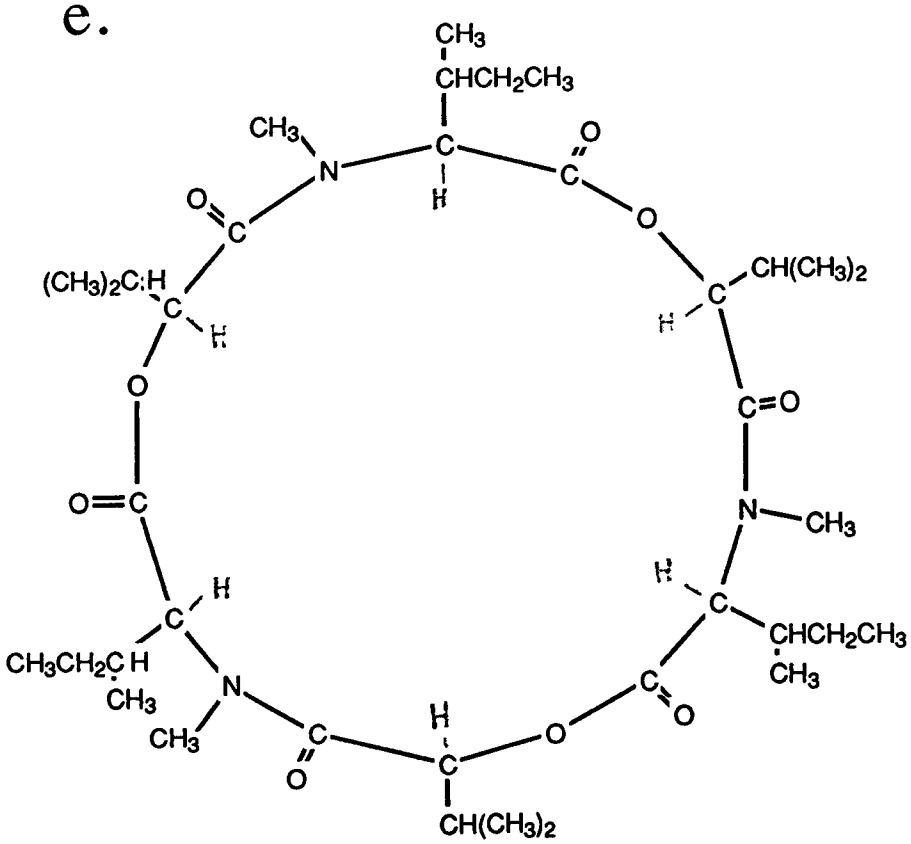
HCO-Val-Gly-Ala-Leu-Ala-Val-Val-Val-Trp-Leu-Trp-Leu-Trp-Leu-Trp-$NHCH_2CH_2OH$

L L D L D L D L D L D L D L

d.



e.


CH3
CHCH2CH3
CH3
N
C
H
O
C
O
O
C
(CH3)2CH
C
H
CH(CH3)2
C
H
O
C=O
O=C
N
CH3
H
C
C
H
CHCH2CH3
CH3CH2CH
CH3
N
CH3
C
O
H
C
O
O
C
O
CH(CH3)2

MATERIALS AND METHODS

AMINO ACID SEQUENCING.

Sequencing was either performed automatically by the Edman method (BRESA Adelaide) or manual Edman degradation according to a modified method of Klemm (1981) and subtractive method of Konisberg (1967).

1. The peptide was lyophilized in a shortened freeze drying tube and resuspended in 25 microlitres of water.
2. 25 microlitres of 5% phenylisothiocyanate in pyridine was added, the tube sealed with a subaseal, flushed with nitrogen for 10 seconds, and incubated for 20 minutes at 50°C.
3. The solution was extracted once with 200 microlitres of hexane: ethyl acetate (10:1) and once with hexane: ethyl acetate (1:2). The organic layer (top) is discarded after centrifugation .(lab top centrifuge) and dried under vacuum
4. For the cleavage step 50 microlitres of trifluoroacetic acid was added and the tube left open in 45°C desiccator (evacuated for 3 seconds) for ten minutes. The sample was dried under vacuum and resuspended in water , a sample taken for amino acid analysis, and the remainder of peptide taken for the next degradation cycle.
5. The amino acid composition of the fragment peptides were monitored by complete acid hydrolysis, HVPE pH 1.7 and ninhydrin staining.

THIOL DETERMINATION (Cysteine)

Thiols were determined with Ellman's reagent, 5'5'dithio bis nitro benzoic acid according to the method of Habeeb (1972)

THIOL OXIDATION (Cysteine)

Thiols of cysteine derivatives were oxidized with performic acid (Formic acid: hydrogen peroxide (100 volume) solution 9:1) according to the method of Sanger (1965) or Hirs (1967)

CATALYTIC HYDROGENATION

Stirred samples of peptides or amino acids were hydrogenated with platinum oxide as catalyst in a 30% aqueous propanol solution at room temperature under a positive pressure atmosphere of hydrogen for the times indicated.

ENZYMIC DIGESTION

Proteolytic fragments of trifolitoxin were generated by incubating the sample in a preparation of insolubilized protease (Sigma) (1 mg of insolubilized enzyme preparation per 0.2 ODs 302 nm. of trifolitoxin) in 50 mM phosphate pH 7.0 for 24 hrs at 28°C. The insolubilized protease was removed by centrifugation. The fragments were isolated by HVPE as indicated in the text, all other enzymic digestions were prepared according to the methods of Smith (1969) or Smyth (1967).

ACETYLATION

Samples were dried and resuspended in 1:1 mixture of acetic anhydride: acetic acid for the times indicated at 25°C. Control incubations in acetic acid were used.(Riordan 1967)

METHYLATION

Lyophilized samples were methylated with Methanol / HCl (0.1M) at 110°C in sealed tubes under N_2 for the times indicated.(Willcox 1967).

HYDRAZINOLYSIS

Trifolitoxin derivatives were treated with anhydrous hydrazine under nitrogen for 48 hrs. at 80°C (Frankael-Conrat 1967). The sample was lyophilized and the amino acid, hydrazide preparation resolved by 2D HVPE/PC.

MATERIALS

Phenyl isothiocyanate and trifluoroacetic acid, insolubilized protease and trypsin, aminopeptidase, hydrazine ,5'5' dithio bis nitro benzoic acid (Ellman's reagent) were purchased through Sigma St Louis U.S.A.

Trypsin and Acetic anhydride were purchased from BDH Poole, England

Carboxypeptidase came from Worthington biochemical corporation Freehold New Jersey

Platinum oxide was from Matthey chemicals limited, London, England

Hydrogen peroxide was from Ajax chemicals Sydney Australia

All other solvents or chemicals not specifically mentioned above were of reagent grade

CHAPTER 4.1
PEPTIDE SEQUENCE RESULTS

RESULTS

Enzymic proteolysis of trifolitoxin.

Schwinghamer and Belkengren (1968) indicated that trifolitoxin was sensitive to papain; Table 4.1.1 shows confirmation of this result. The results also demonstrate that trifolitoxin activity was sensitive to carboxypeptidase, indicating a free C terminal amino acid. Trifolitoxin was found to be insensitive to leucine aminopeptidase and although trifolitoxin contains arginine, trypsin was unable to eliminate biological activity, independent confirmation of Schwinghamer and Belkengren (1968) observation.

Exhaustive protease digestion of trifolitoxin sulphonic acid released aspartic acid, isoleucine, glycine, valine and alanine and small quantities of serine and several fluorescent fragments, (Table 4.1.2) (Figure 4.1.1) the most cationic of these fragments yielded the amino acids arginine, glycine and cysteic acid and aspartic acid after acid hydrolysis (Figure 4.1.2). It is probable that the proximity of the chromophore to and the nature of bonding which is present confers the observed resistance to protease cleavage. Proton N.M.R. of the major blue fluorescent fragment generated by protease digestion of T24SO3H (Fragment 3) (Figure 4.1.3.) shows all the expected signals for a fragment containing serine, arginine, glycine and cysteic acid plus the ethylenic proton at 5.8 ppm attributable to the chromophore but no protons directly attributable to aspartic acid. None of the UV spectra of the blue fluorescent protease fragments differed from that of the original trifolitoxin sulphonic acid indicating that no bond directly linked to the chromophore was susceptible to cleavage with protease.

Table 4.1.1

Sensitivity of trifolitoxin to proteolytic enzymes

	Inhibition radius	
Enzyme	-enzyme	+enzyme
papain	11	0
protease	13	0
Carboxypeptidase	12	0
Aminopeptidase	13	12
Trypsin	13	13

Table 4.1.2

Protease fluorescent fragments.

Fragment No.	RM_{OG} 1.7	RM_{OG} 9.2	Ninhydrin	Inhibition
Control	-0.31	0.76	purple	+
0	0.00	n.d.	-	n.d.
1	-0.32	n.d.	purple	-
2	-0.43	0.92	yellow	-
3	-0.46	0.82	yellow	-
4	-0.51	0.77	pink	n.d.

THE C-TERMINUS

Trifolitoxin toxicity was shown to be sensitive to carboxypeptidase (Table 4.1.1). Hydrazinolysis revealed that alanine was the C terminal amino acid. (Figure 4.1.4). This was the only amino acid which retained its ninhydrin staining properties and position by 2D chromatography and electrophoresis, the remaining amino acids present as the hydazides.

THE N-TERMINUS

The native trifolitoxin reacts only poorly with ninhydrin and is apparently insensitive to leucine aminopeptidase.(Table 4.1.1). However acetylation of trifolitoxin with acetic anhydride caused a nett increase in anionic charge at pH 5.0 (Figure 4.1.5a) consistent with the acetylation of an N terminal amino group, resulting in the loss of biological activity.(Figure 4.1.5b). Protease treatment of the acetylated derivative revealed all the amino acids found in underivatized sample except aspartic acid (Figure 4.1.6) as revealed by ninhydrin staining indicating an N-terminal aspartic acid.

Figure 4.1.7 shows that the removal of the N-terminal aspartic acid of trifolitoxin sulphonic by Edman degradation does not detectably modify the biological activity.

THE AMINO ACID SEQUENCE

Sequential Edman degradation of trifolitoxin sulphonic acid gives the N-terminal sequence asp-ile-gly-gly (Figure 4.1.8) before the reaction sequence faulters. Partial acid hydrolysis (Figure 4.1.9 and Table 4.1.3) reveals the peptides gly-ser (partial acid hydrolysis fragment 3) indistinguishable from a commercial sample by amino acid content, (Figure 4.1.10 Figure 4.1.11, Table 4.3) ninhydrin staining properties (yellow to purple), and position in the

2D chromatography electrophoresis system. Other peptides identified were cysO$_3$H-val-ala (partial acid hydrolysis fragment 1) (zero mobility HVPE pH 1.7, pink to purple with ninhydrin) and minor product gly-cysO$_3$H-val-ala (zero mobility HVPE pH 1.7, yellow to purple ninhydrin stain) which copurified with cysO$_3$H-val-ala.by HVPE pH 1.7. Subtractive Edman degradation of this mixture indicated that no glycine and small amounts of cysteic acid are present after the first round of Edman degradation, (Figure 4.8a) alanine has been determined as C-terminal by hydrazinolysis, therefore it can be deduced that the sequence is gly-cysO$_3$H-val-ala.. A blue fluorescent peptide was also isolated from partial acid hydrolysates (partial acid hydrolysis fragment 4). This peptide contained the amino acids serine, arginine and glycine and small amounts of aspartic acid (Figure 4.1.10, Figure 4.1.11 and Table 4.1.3).

Table 4.1.3

	Partial acid hydrolysis peptides	
Fragment	RM_{OG} pH 1.7	R_f
1	0.00	0.36
2	-0.97	0.37
3	-1.39	0.15
4	-1.54	0.05

Properties of the blue fluorescent partial acid hydrolysis fragment No. 4

A pH mobility profile of a ninhydrin positive, blue fluorescent product containing the amino acids serine, arginine and glycine and aspartic acid obtained from partial acid hydrolysates of trifolitoxin sulphonic contained one N-terminal amino group and one C-terminal carboxyl group (Figure 4.1.12). This conclusion is based on the expected pKa's for a C terminal carboxyl (2-3) and N terminal amino group (8-9) of peptides (Kortum *et al.*, 1961, Perrin 1965) and their products after derivatization. (methylation and acetylation respectively). The difference in RM_{OG} between this fragment and an acetylated fragment at pH 1.7 equals 0.5 RM_{OG} units, on the assumption that this change in mobility is due to the acetylation of the N terminal group then a change in mobility equivalent to 0.5 RM_{OG} units represents one ionization. It can be deduced that the nett charge at pH 9.2 equals minus one. At pH 9.2 summation of the cationic guanidine group, the anionic C-terminal group and the fractionally protonated ($<< 0.5$) N-terminal group indicates that the observed anionic behaviour is consistent with an acidic blue fluorescent chromophore with a pK near 5 consistent with that determined by pH dependence of UV absorbance (pK=5.1)

FIGURE 4.1.1

Trifolitoxin sulphonic acid (0.2 ODs 302 nm.) treated with insolubilized protease. The incubation mixture is run by 2D HVPE/PC and viewed under shortwave UV light and stained with ninhydrin.

Those marked with f are fluorescent fragments

Cysteic acid was not released by protease treatment ,therefore only the cationic portion of the electrophoetogram is shown.

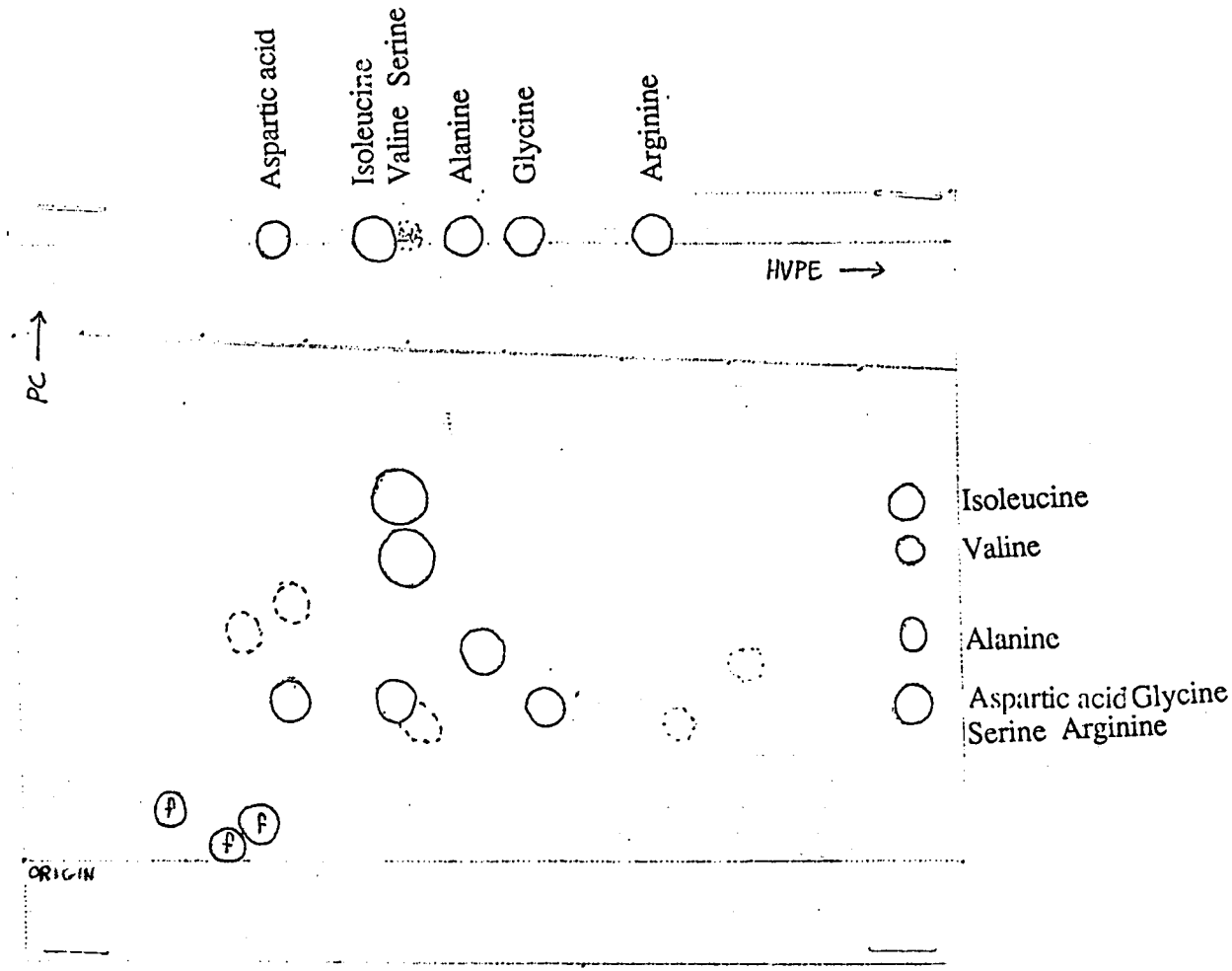
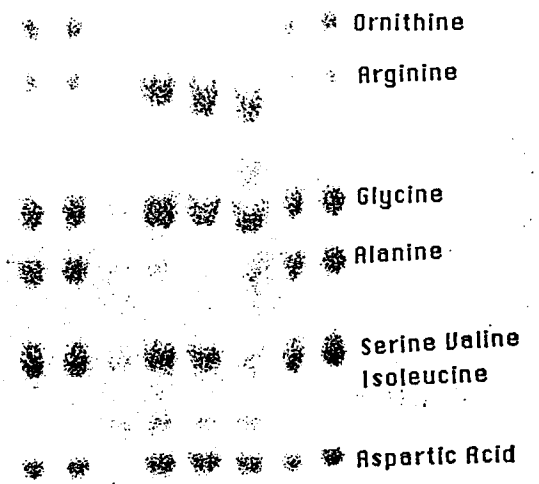

Aspartic acid
Isoleucine
Valine
Serine
Alanine
Glycine
Arginine
HVPE
PC
Isoleucine
Valine
Alanine
Aspartic acid Glycine
Serine Arginine
ORIGIN

FIGURE 4.1.2

Complete acid hydrolysate of blue fluorescent components isolated from protease treatment of trifolitoxin , run by HVPE pH1.7 and stained with ninhydrin.


Ornithine
Arginine
Glycine
Alanine
Serine Valine
Isoleucine
Aspartic Acid

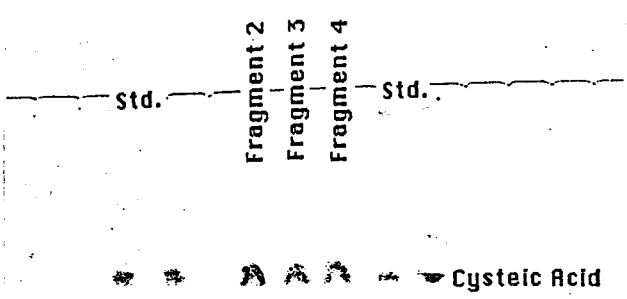

Std.
Fragment 2
Fragment 3
Fragment 4
Std.
Cysteic Acid

FIGURE 4.1.3

300 MHz proton N.M.R. of protease fragment 3.

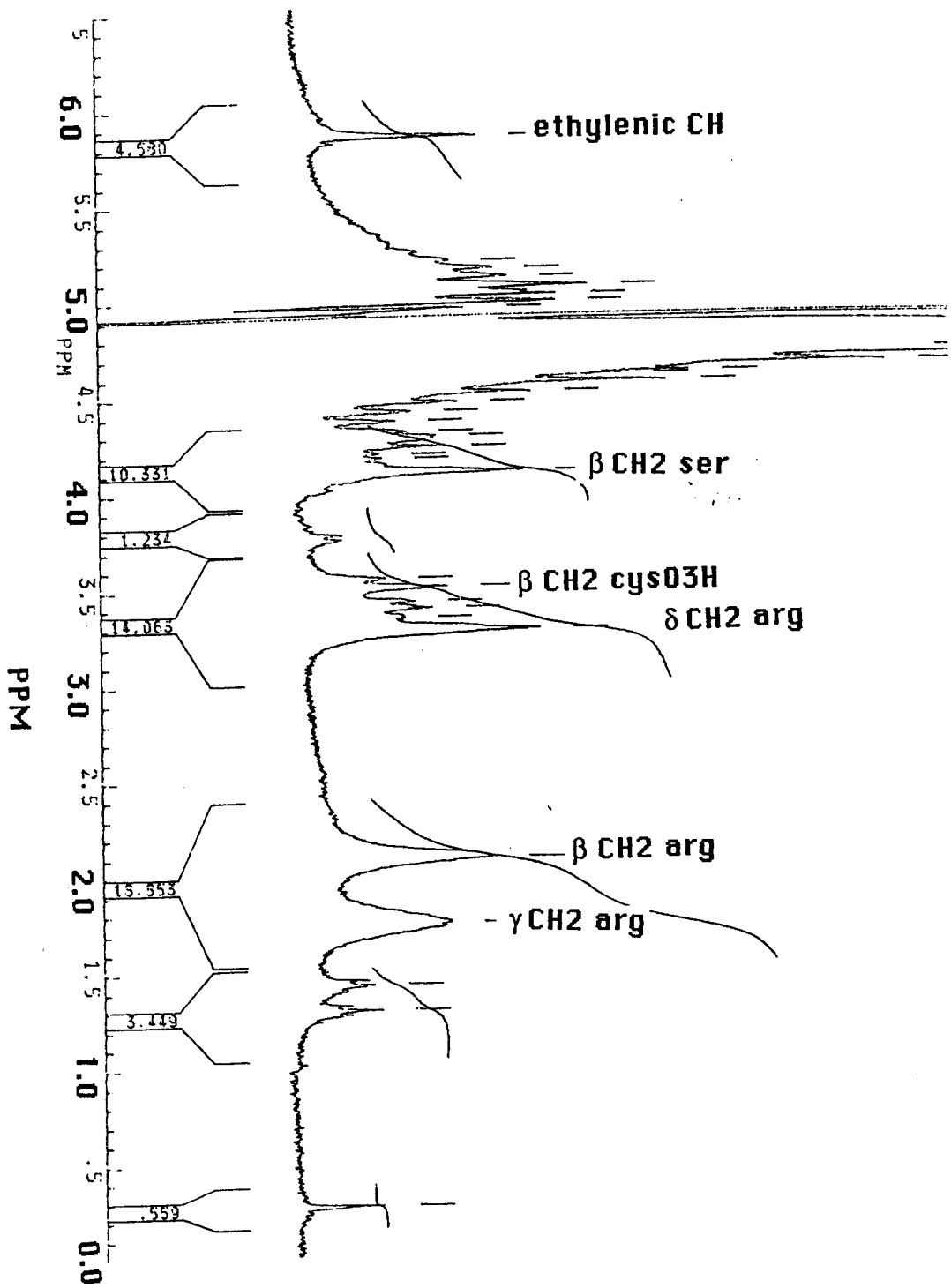

ethylenic CH
β CH2 ser
β CH2 cysO3H
δ CH2 arg
β CH2 arg
γ CH2 arg
4.530
10.331
1.234
14.065
15.553
3.449
.559
6.0
5.0
4.0
3.0
2.0
1.0
0.0
PPM

FIGURE 4.1.4

Hydrazinolysis of trifolitoxin sulphonic acid.

Dry trifolitoxin sulphonic acid (0.25 ODs 302 nm.) was heated with anhydrous hydrazine (50 microlitres) for 48 hrs. at 80° C ,lyophilized and subjected to two dimensional HVPE/PC and stained with ninhydrin.

Cysteic acid
Origin
Aspartic acid
Glutamic acid
Serine Valine
Isoleucine
Alanine
Glycine
Arginine
Ornithine
Serine
Alamine
Valine
Isoleucine

FIGURE 4.1.5

Biological activity of acetylated trifolitoxin

Trifolitoxin (0.6 ODS 302 nm., dry) was acetylated with a 1:1 mixture of acetic anhydride and glacial acetic acid (30 mins 25 ^{o}C). Control samples were incubated in glacial acetic acid under the same conditions.

The samples were dried under vacuum and electrophoresed by HVPE pH 5.0 (50 mM citrate/NaOH, 45 mins. 1500 V)) sections were cut (0.5 cms), eluted (300 microlitres H_2O) and monitored at 302 nm. Samples (10 microlitres) were respotted on 1 cm. square papers and bioassayed.

a: Biological activity (inhibition diameter) vs RM_{OG} by HVPE pH 5.0

Acetylated trifolitoxin (RM_{OG} pH 5.0 = 0.4) —◆—

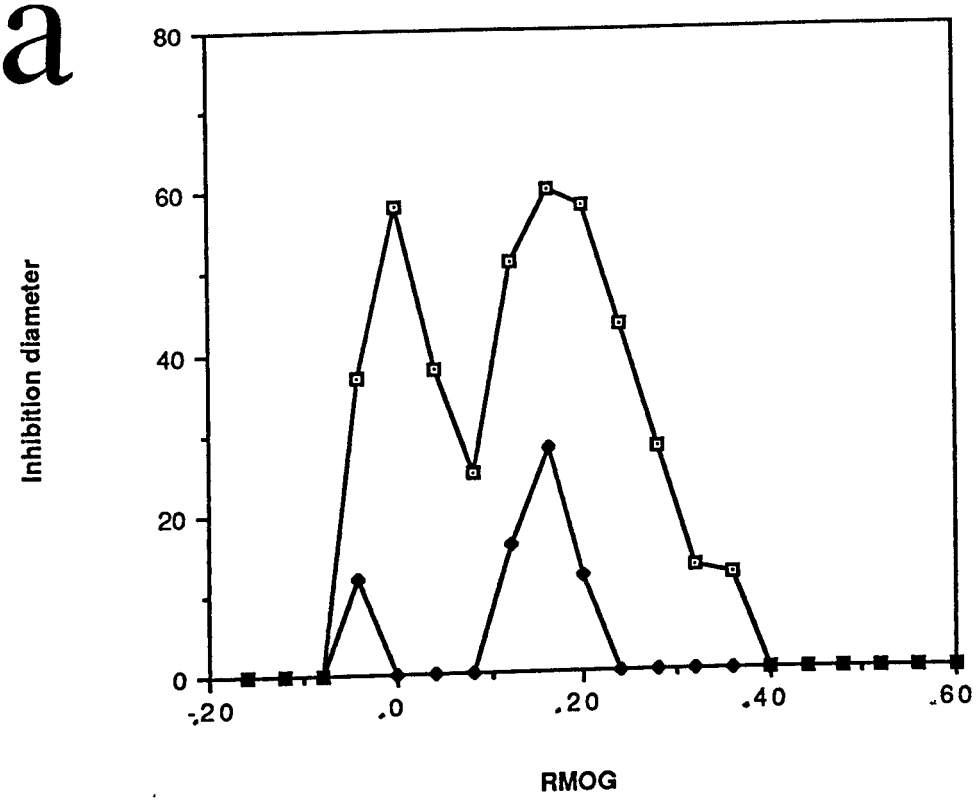
Control trifolitoxin (RM_{OG} pH 5.0 = 0.2) —□—

b: Absorbance at 302 nm. vs RM_{OG} by HVPE pH 5.0

Acetylated trifolitoxin (RM_{OG} pH 5.0 = 0.4) —◆—

Control trifolitoxin(RM_{OG} pH 5.0 = 0.2) —□—

a


80
60
40
20
0
-.20
.0
.20
.40
.60
Inhibition diameter
RMOG

b

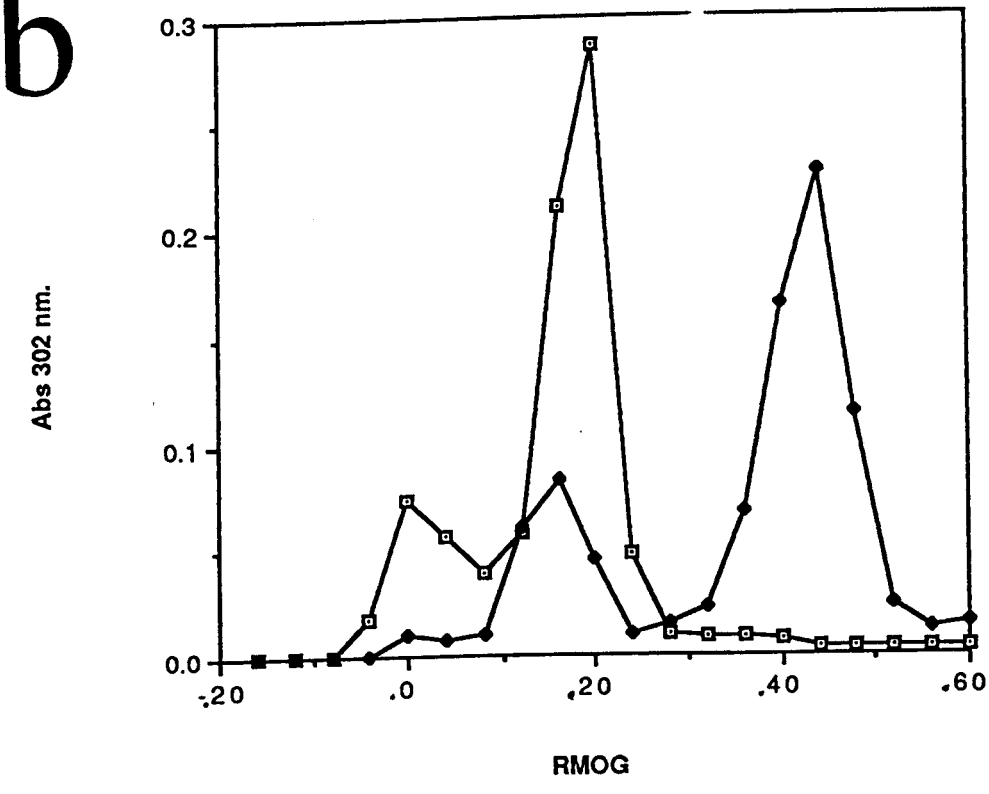

0.3
0.2
0.1
0.0
-.20
.0
.20
.40
.60
Abs 302 nm.
RMOG

FIGURE 4.1.6

Amino acids in protease treated acetylated trifolitoxin

A portion of the incubation mixture of the protease treated acetylated trifolitoxin (see Figure 4.1.5 for the origination of this sample) was electrophoresed by HVPE pH 1.7 (formic/acetic acid and stained with ninhydrin.
Note the absence of ninhydrin positive aspartic acid in the protease products of acetylated trifolitoxin.

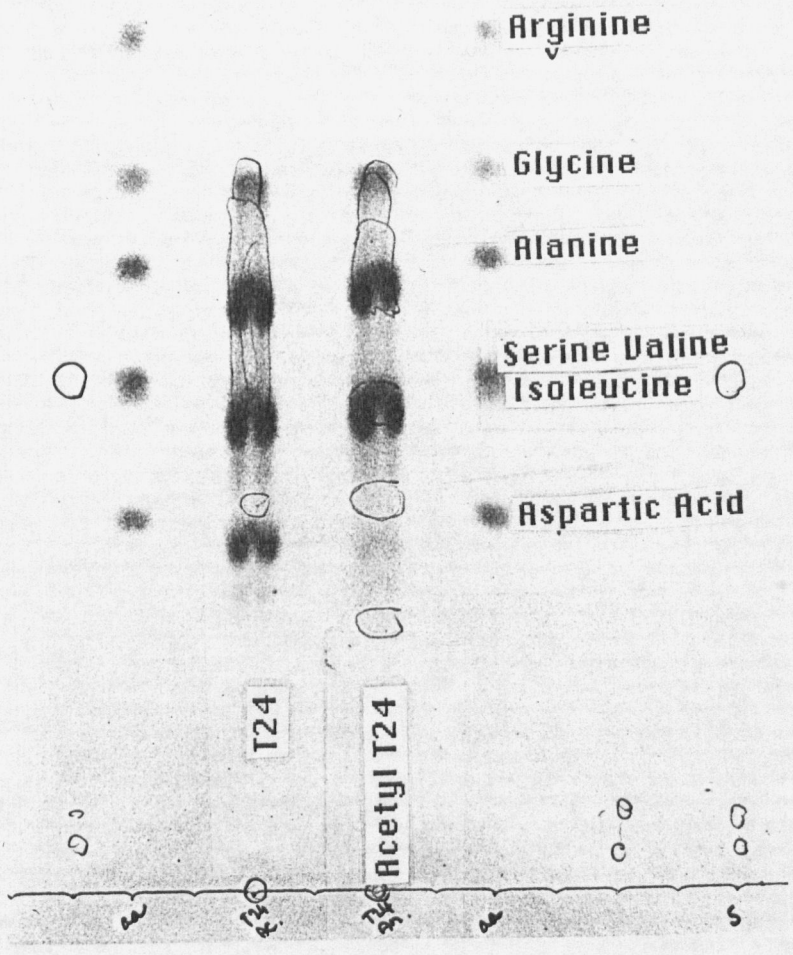
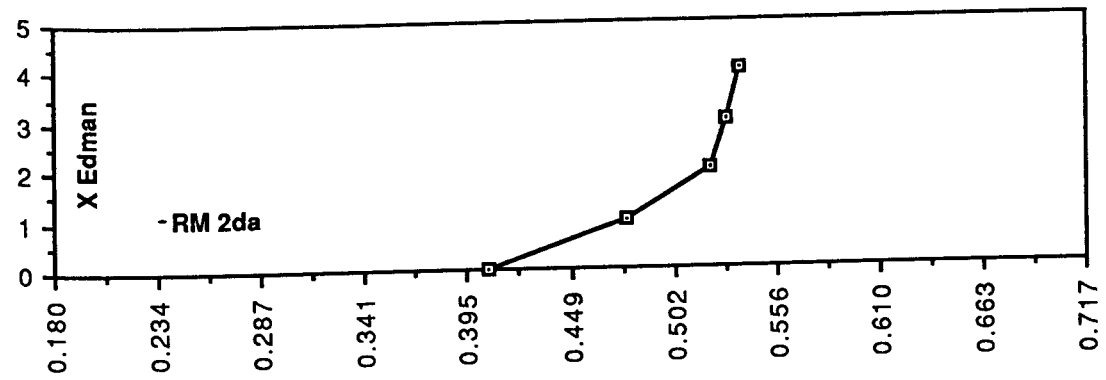
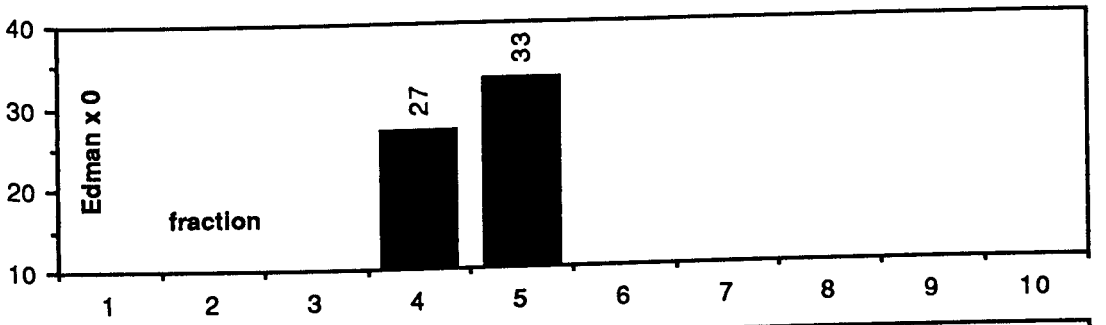

Arginine
Glycine
Alanine
Serine Valine
Isoleucine
Aspartic Acid
T24
Acetyl T24

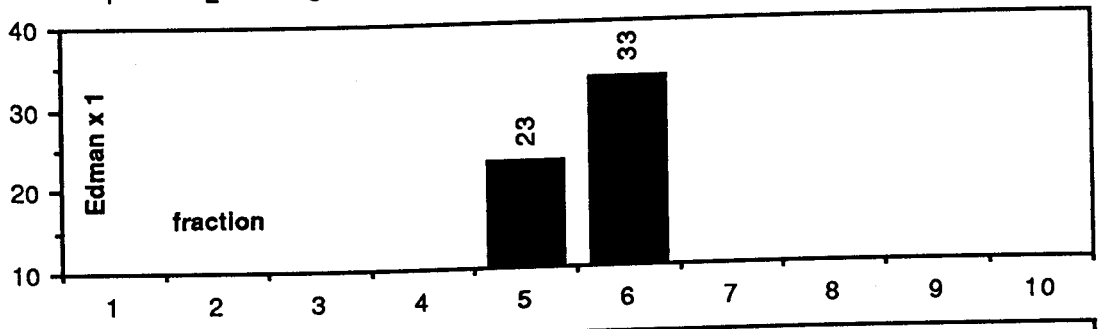
FIGURE 4.1.7

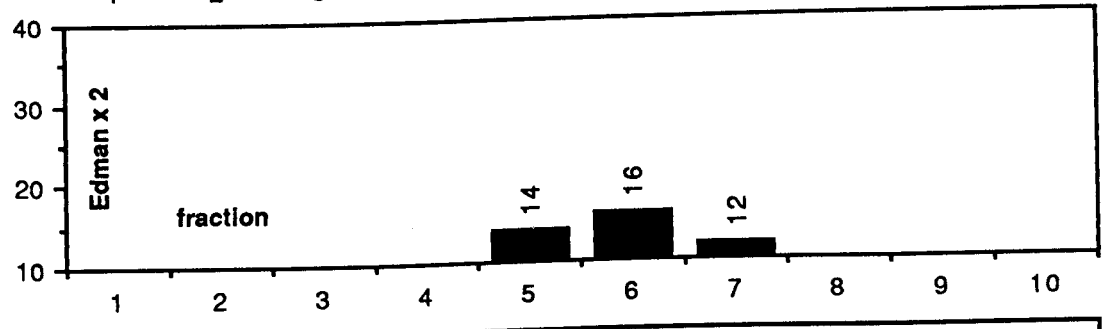
Biological activity of N terminal amino acid deficient trifolitoxin sulphonic acid

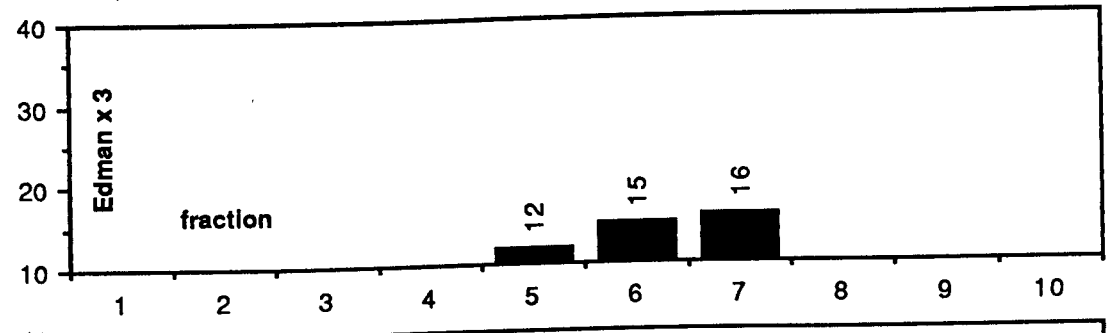
Trifolitoxin sulphonic acid (1.0 OD 302 nm..) was subjected to four rounds of manual Edman degradation (see materials and methods)
A sample of the degraded peptide was taken at each step (0.2 ODs 302 nm.) for HVPE pH 1.7 ,followed by bioassay. The peptide fragment of interest was visualized by its blue fluorescence under short wave (254 nm.) UV light after electrophoresis.
The upper five point plot represents the mobility relative to 2 deoxy adenosine of the original trifolitoxin sulphonic acid and its four Edamn degradation fragments. The lower plots show the corresponding increasing cationic nature of the biological activity at each round of Edman degradation.


X Edman
- RM 2da
0.180
0.234
0.287
0.341
0.395
0.449
0.502
0.556
0.610
0.663
0.717


Edman x 0
fraction
27
33


Edman x 1
fraction
23
33


Edman x 2
fraction
14
16
12


Edman x 3
fraction
12
15
16

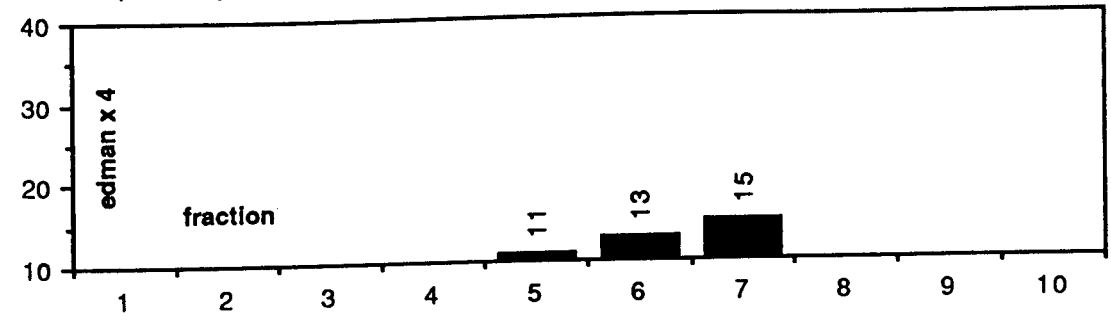
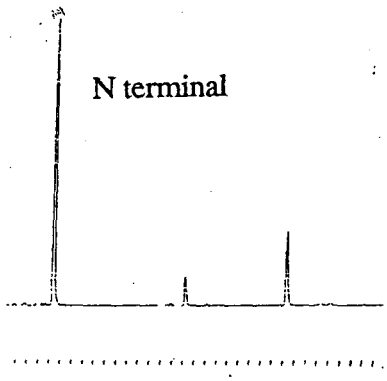

edman x 4
fraction
11
13
15

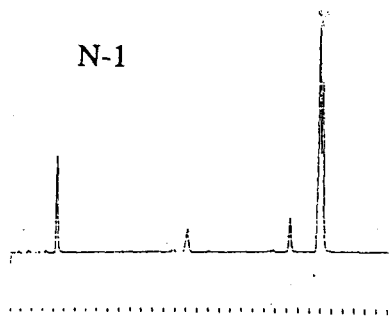
FIGURE 4.1.8

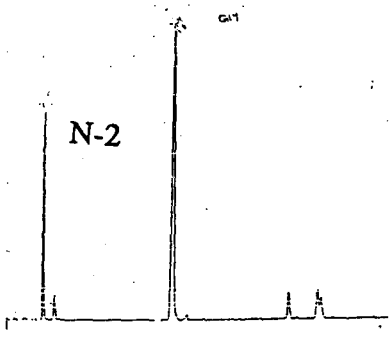
Automated Edman degradation of trifolitoxin sulphonic acid

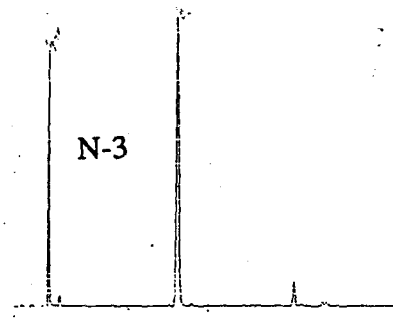
Sequential Edman degradation of trifolitoxin sulphonic acid. The overlays show HPLC traces of the PTH amino acids from each round of Edman degradation (the N terminal being the first)

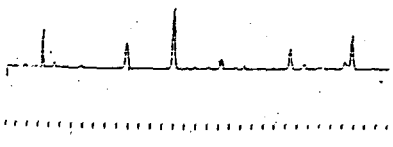
N terminal - aspartic acid
N-1 - isoleucine
N-2 - glycine
N-3 - glycine
N-4 - absent
N-5 - absent

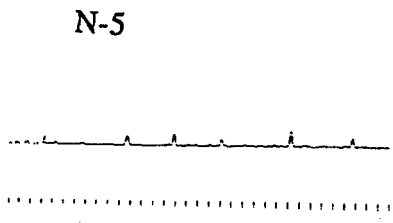

N terminal


N-1


N-2


N-3


N-4


N-5

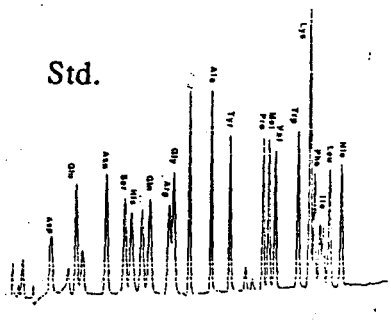

Std.

FIGURE 4.1.8a

Manual subtractive Edman degradation of the C terminal peptide of trifolitoxin.

The ninhydrin positive components with RM_{OG} pH1.7 = 0.0 from partial acid hydrolysis of trifolitoxin sulphonic acid (cysteic acid containing peptides) were subjected to one round of Edman degradation , the products were completely hydrolysed and the amino acids were visualized by HVPE pH 1.7 and ninhydrin staining.

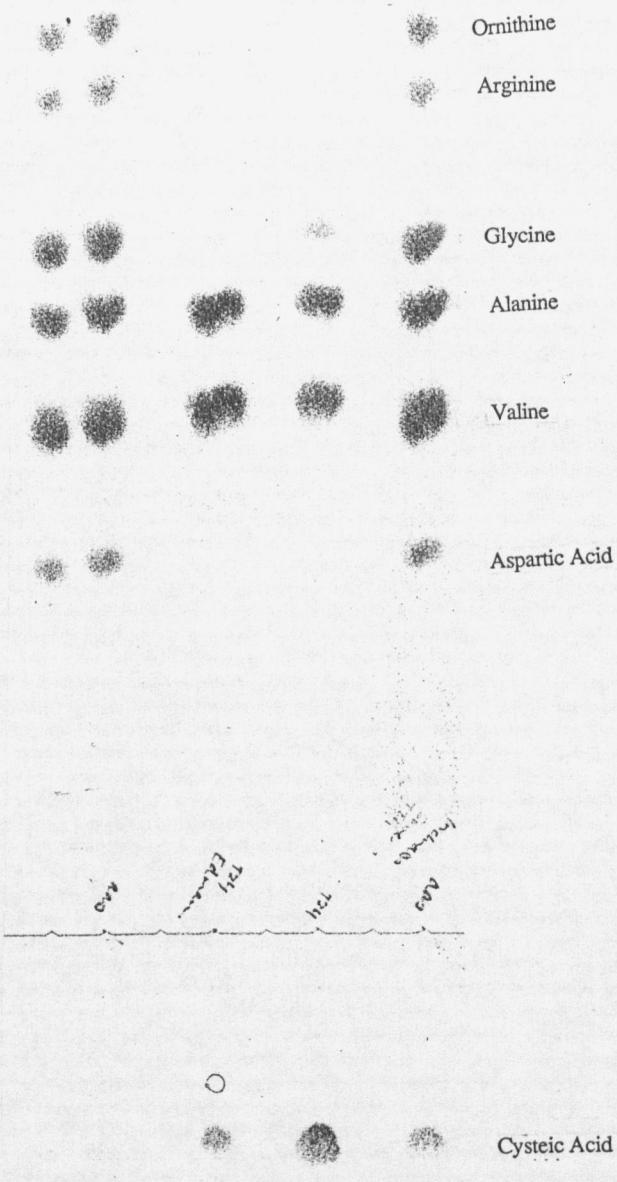

Ornithine
Arginine
Glycine
Alanine
Valine
Aspartic Acid
Cysteic Acid

FIGURE 4.1.9

Two dimensional HVPE/PC of partial acid hydrolysis products of trifolitoxin sulphonic acid

Trifolitoxin (0.1 OD) sulphonic acid (50 uls)was partially hydrolysed (5N HCl ,5 mins. ,110 ^{o}C ,under N2) dried under vacuum and run by 2D HVPE/ PC

1st dimension HVPE pH 1.7 (formic/acetic acid)
2nd dimension PC (butanol/acetic acid/water)
(see materials and methods)

Peptides were visualized by ninhydrin.

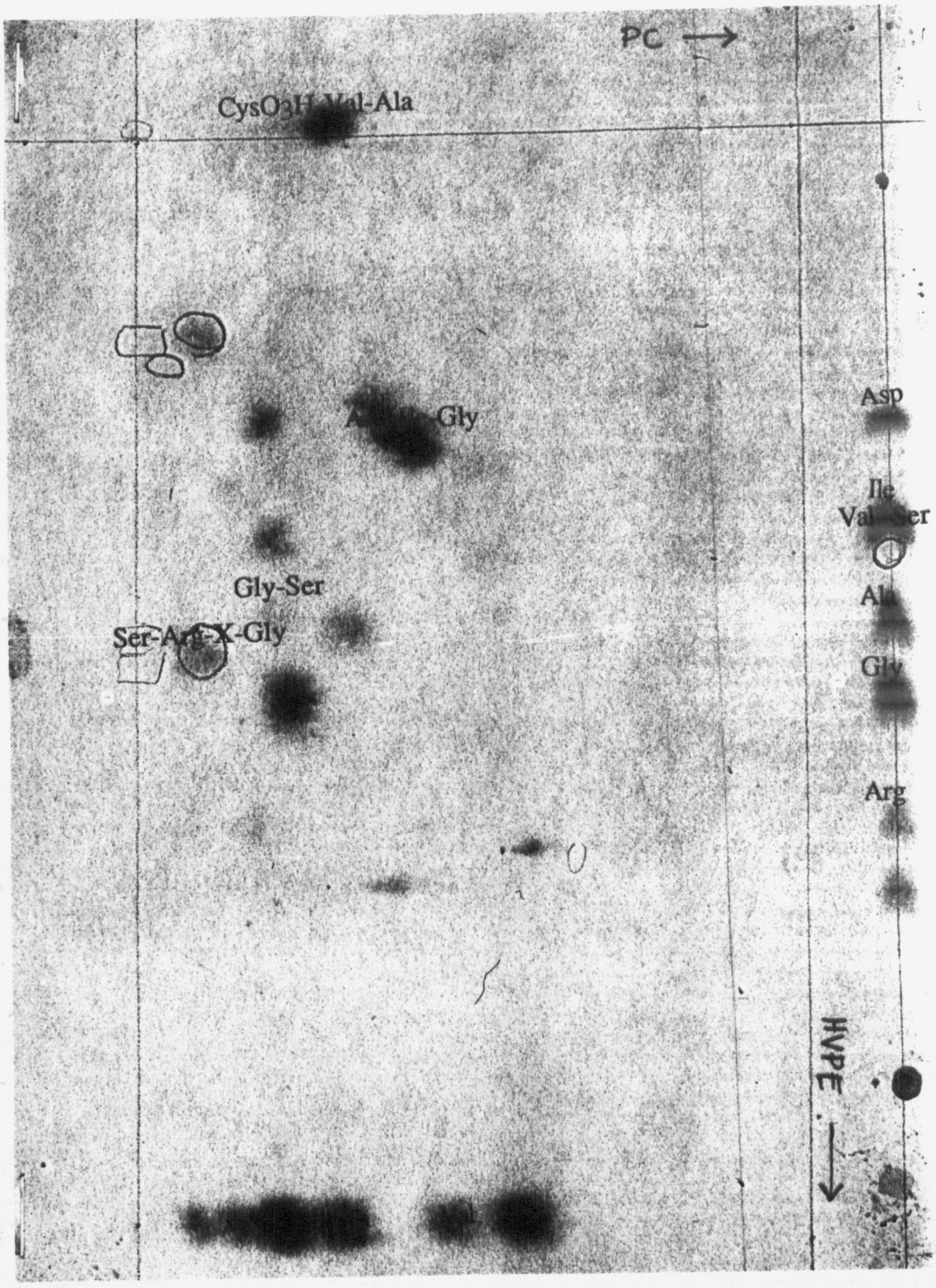

PC
Gly
Asp
Gly-Ser
Arg
HVPE

FIGURE 4.1.10

Amino acid composition of peptides isolated from a partial acid hydrolysate of trifolitoxin sulphonic acid.

Fragment	Amino acids
1#	ser, arg , gly ,(asp)
2	gly, ser
3	asp, ile,gly
4	(gly) cyso3h, val, ala
5	asp,ile,gly,ser,arg,cyso3h,val,ala

blue fluorescent

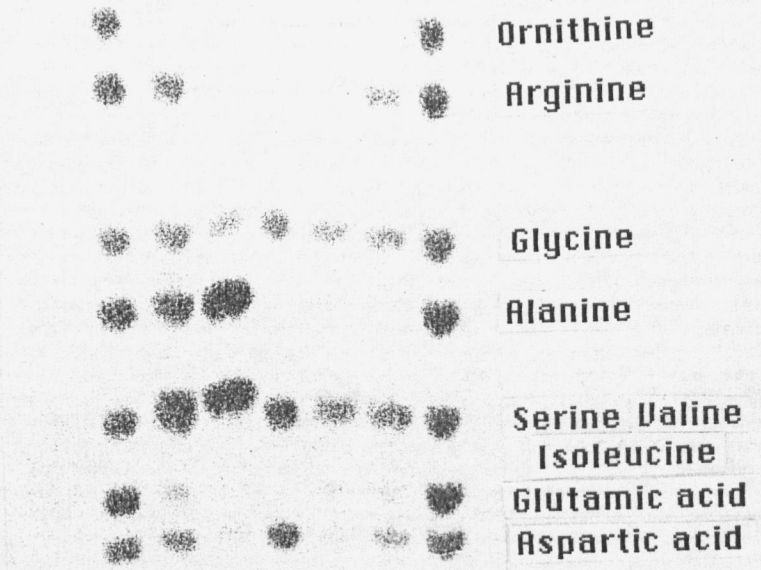

Ornithine
Arginine
Glycine
Alanine
Serine Valine
Isoleucine
Glutamic acid
Aspartic acid
Std. 5 4 3 2 1 Std.
Cysteic acid

FIGURE 4.1.11

Two Dimensional HVPE/PC of amino acids from partial acid peptides of trifolitoxin sulphonic acid.

The same numbering as applies to Figure 4.1.10

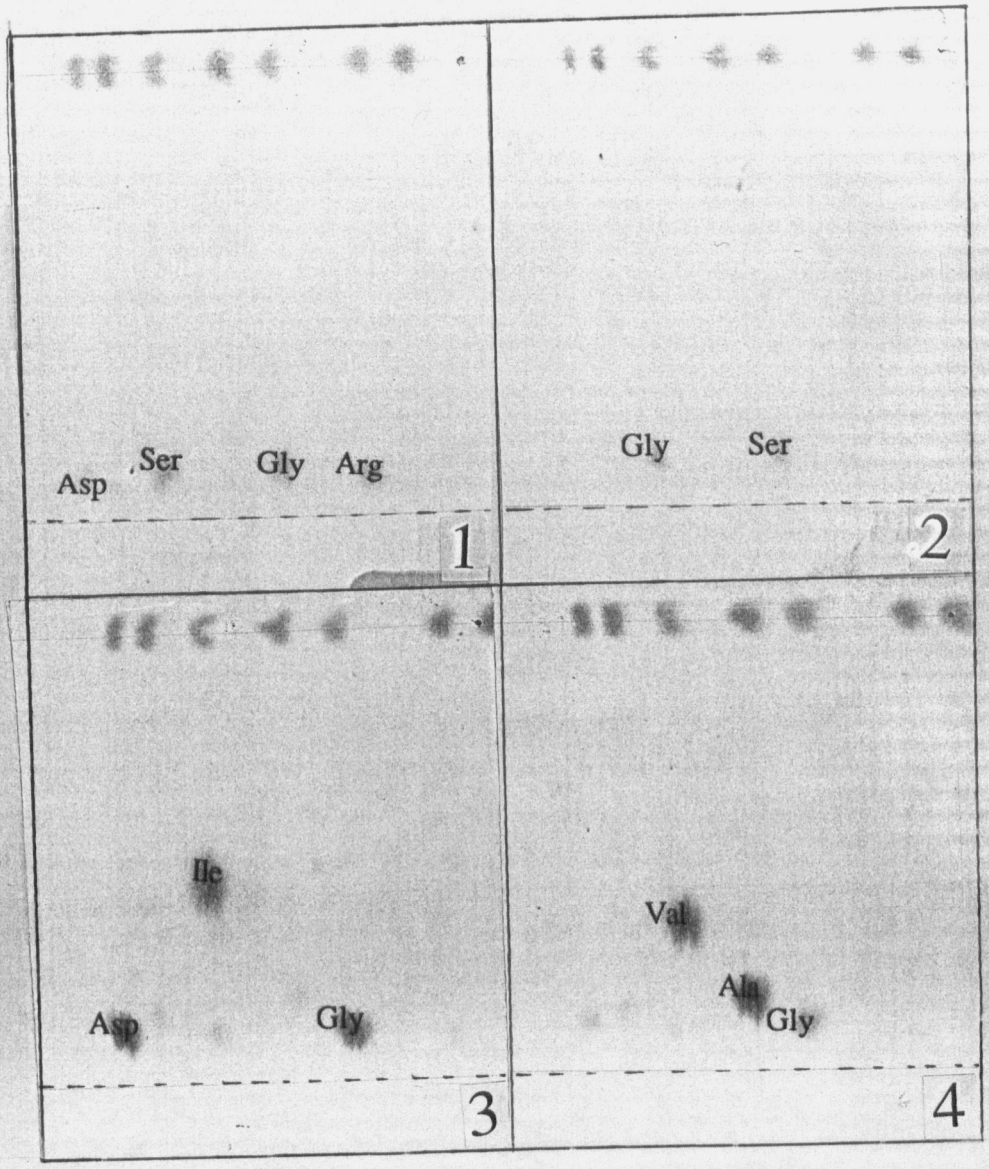

Asp
Ser
Gly
Arg
1
Gly
Ser
2
Ile
Asp
Gly
3
Val
Ala
Gly
4

FIGURE 4.1.12

pH mobility profile by HVPE of the guanidine positive ninhydrin positive blue fluorescent product from a partial acid hydrolysis of trifolitoxin sulphonic acid .

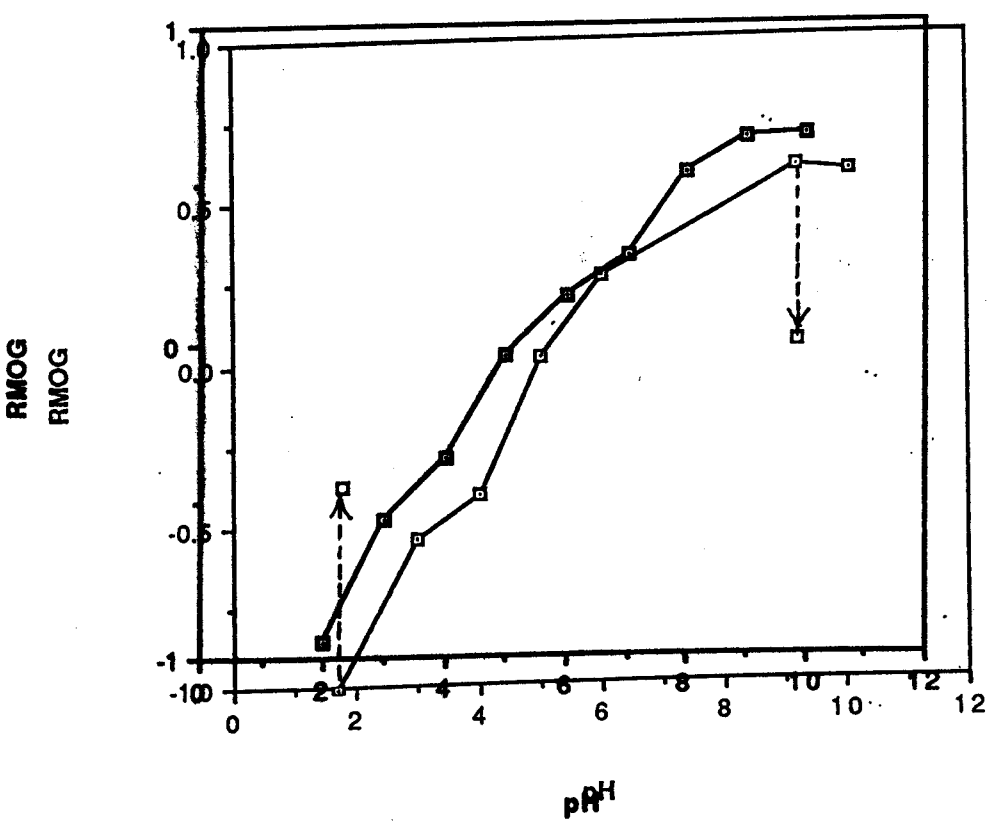
This blue fluorescent fragment of trifolitoxin was isolated from partial acid hydrolysates (5N HCl 110 degrees C 5 mins.) of trifolitoxin sulphonic acid .It was purified by HVPE pH 1.7 (RMOG =-1.0) (see Figure 4.1.10)

Mobility of peptide fragment containing chromophore

Acetylation product Mobility at pH 1.7 ↑

Methylation product Mobility at pH 9.2 ↓

A theoretical curve has been fitted for the assumed plateaus of RMOG values of 0.6 for the mono anion ,-0.5 for the mono cation and approximately -1.2 for the dication with apparent pKa_1 = 2.3, pKa_2 = 5.1 and pKa_3 = 7.5
pKa_1 is consistent with C terminal carboxyl suggested by the change in mobility after methylation.
pKa_2 is consistent with the chromophore anionic ionization.
pKa_3 is consistent with the presence of an N terminal serine amino group as suggested by the change in mobilty after acetylation.
The protonation /deprotonation of the remaining nitrogens are outside the measured range.


RMOG
RMOG
1
1.0
0.5
0
0.0
-0.5
-1
-100
0
2
4
6
8
10
12
pH
pH

Stabilization of the linkage of arginine to the blue fluorescent chromophore by hydrogenation.

Complete hydrogenation and acid hydrolysis of trifolitoxin sulphonic acid showed that no ninhydrin stainable arginine moiety was present.(Figure 4.1.13) 2D, HVPE/PC of partially hydrogenated, completely hydrolysed trifolitoxin sulphonic acid indicated that multiple phenanthrene quinone staining material was present, one of these components was also blue fluorescent.(Figure 4.1.14). This would be consistent with hydrogenation of trifolitoxin sulphonic acid creating a relatively stabilised linkage between arginine and the chromophore.

Hydrogenation of the blue fluorescent chromophore. Loss of a pKa at 5.1

Comparison of the pH mobility profiles of trifolitoxin sulphonic acid and the hydrogenated product of trifolitoxin sulphonic acid (Figure 4.3.15) shows that a plot of the difference between the two profiles is similar to the pK calculated by photospectrometric methods for the blue fluorescent chromophore (Figure 4.3.16). It is apparent from the HVPE pH profiles of $T24SO_3H$ and $T24SO_3H$ H_2/Pt product that the change in mobility is due to the loss of a negative charge with a pKa of approximately 5.1, consistent with the data obtained from the pH mobility profile of the blue fluorescent partial acid hydrolysis product (Figure 4.1. 12).

Figure 4.1.18 demonstrates that no biological toxicity is associated with the hydrogenated trifolitoxin sulphonic acid (pH 9.2 RM_{OG} = 0.65), the activity present is coincident with a control sample (pH 9.2 RM_{OG} = 0.83) and represents residual non reduced product.

The racemic nature of arginine

The alpha proton of arginine determines its chirality. The inability to detect the alpha proton of arginine by N.M.R. and the unusual linkage of arginine to the chromophore lead to the question of the enantiomeric form of arginine in trifolitoxin. Analysis of arginine obtained from complete acid hydrolysates of trifolitoxin sulphonic acid, using the specific conversion of L-arginine to L-ornithine by arginase indicated that equal proportions of the D and L isomer (a racemic mixture) of arginine were present, acid hydrolysis of control L-arginine showed no significant racemization. (Figure 4.1.19).

Spontaneous hydrolysis degradation products.

An aged N.M.R. sample which was originally electrophoretically homogeneous which had been transferred from D_2O to dimethyl sulfoxide and back again showed by electrophoresis several new species with altered UV spectra and mobility by HVPE. The UV spectra, staining properties mobilities by HVPE and FAB MS spectra of these products are presented in Figures 4.1.20, 4.1.21, 4.1.22 and Table 4.1.4. Analysis of the amino acid composition of these products indicate degradation products 2 and 3, although

FIGURE 4.1.13

Ninhydrin detectable amino acids in hydrolysates of hydrogenated trifolitoxin sulphonic acid provides evidence for the stabilisation of a saturable bond between arginine and the chromophore

Trifolitoxin sulphonic acid was hydrogenated with H_2/PtO_2 for 24 hrs. and purified by HVPE pH 9.2 ($RM_{OG} = 0.65$) .The reduced product was hydrolysed (5N HCl ,110 degrees C ,3 hrs. ,under N2) ,dried under vacuum, and electrophoresed by HVPE pH 1.7 . The amino acids were visualized by ninhydrin staining.

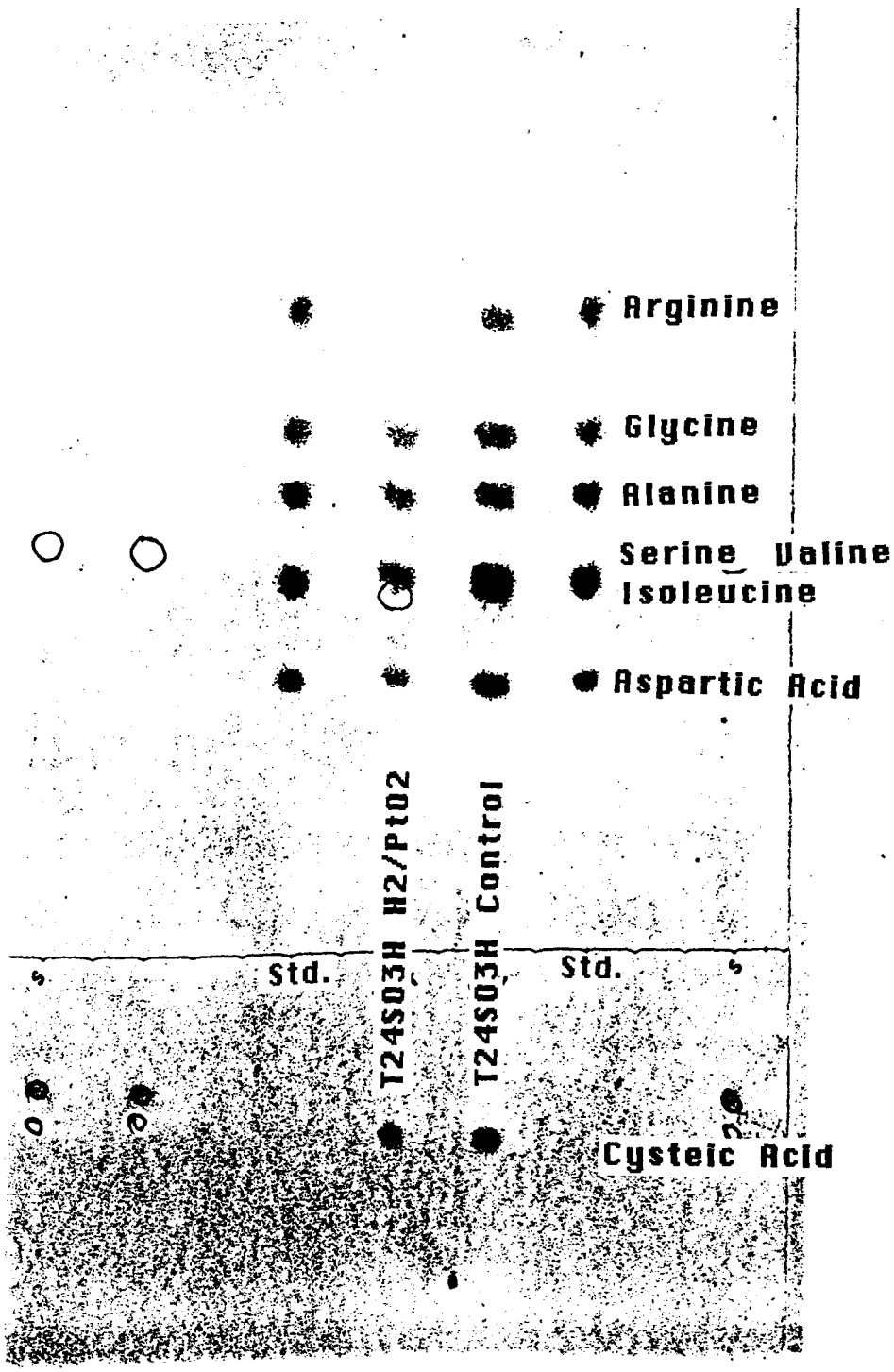

Arginine
Glycine
Alanine
Serine Valine
Isoleucine
Aspartic Acid
Std.
T24S03H H2/Pt02
T24S03H Control
Std.
Cysteic Acid

FIGURE 4.1.14

2D HVPE/PC of hydrolysate of a partially hydrogenated trifolitoxin sulphonic acid.Stained for guanidines. Note the coincidence of blue fluorescence and guanidine staining of one of the components.

Trifolitoxin sulphonic acid was partially hydrogenated with H_2/PtO_2 (24 hrs.) This sample was completely hydrolysed (5N HCl 110 degrees C 18 hrs) dried under vacuum and subjected to 2D HVPE/PC
The chromatogram was visualized under UV light (long wave (340nm.) and short wave (254 nm.)) and stained with the guanidine reagent to visual arginine derivatives.

The blue fluorescent component has been cross hatched and the remaining guanidine positive components are shown in outline.

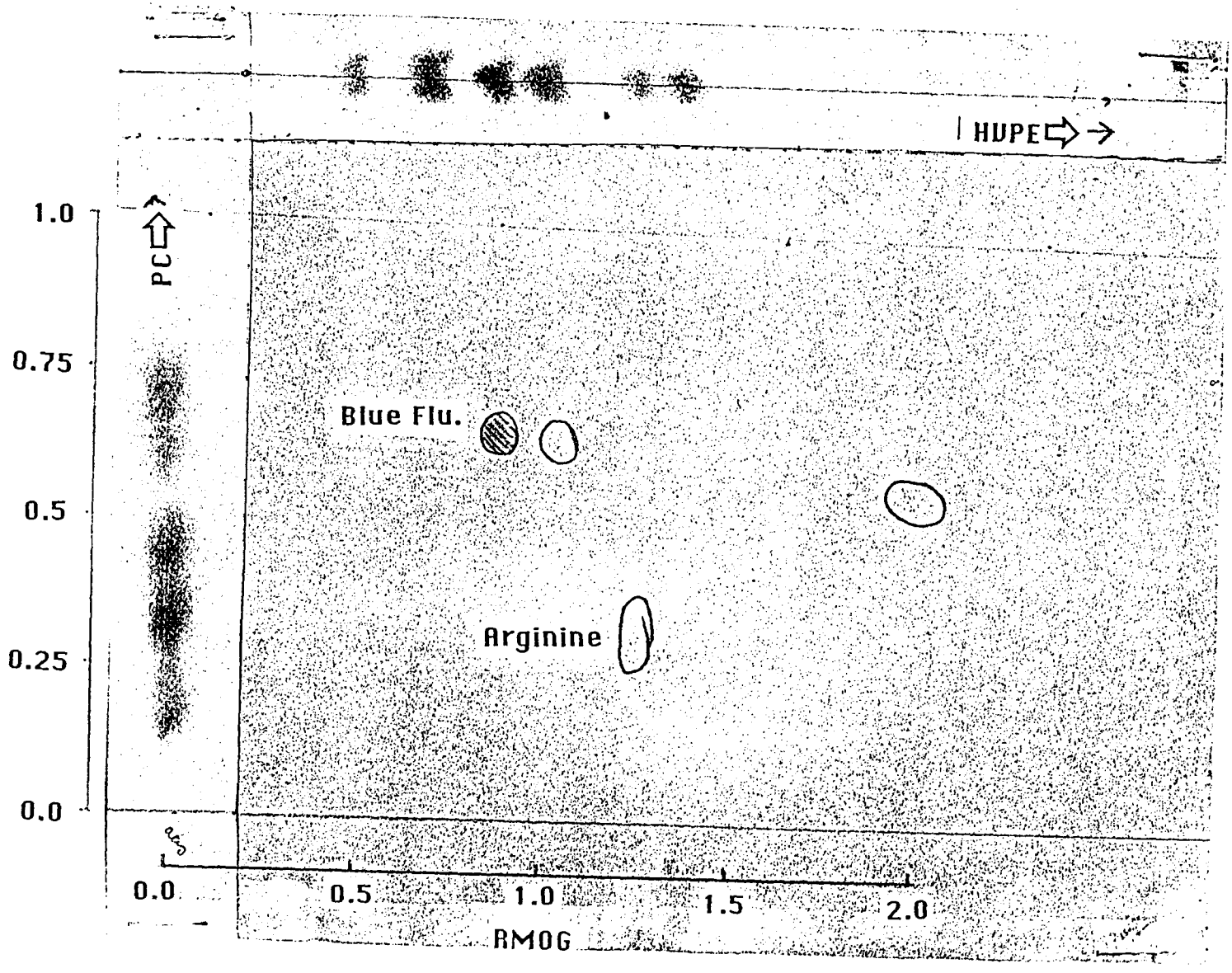

HDPE
PC
1.0
0.75
0.5
0.25
0.0
Blue Flu.
Arginine
0.0
0.5
1.0
1.5
2.0
RMOG

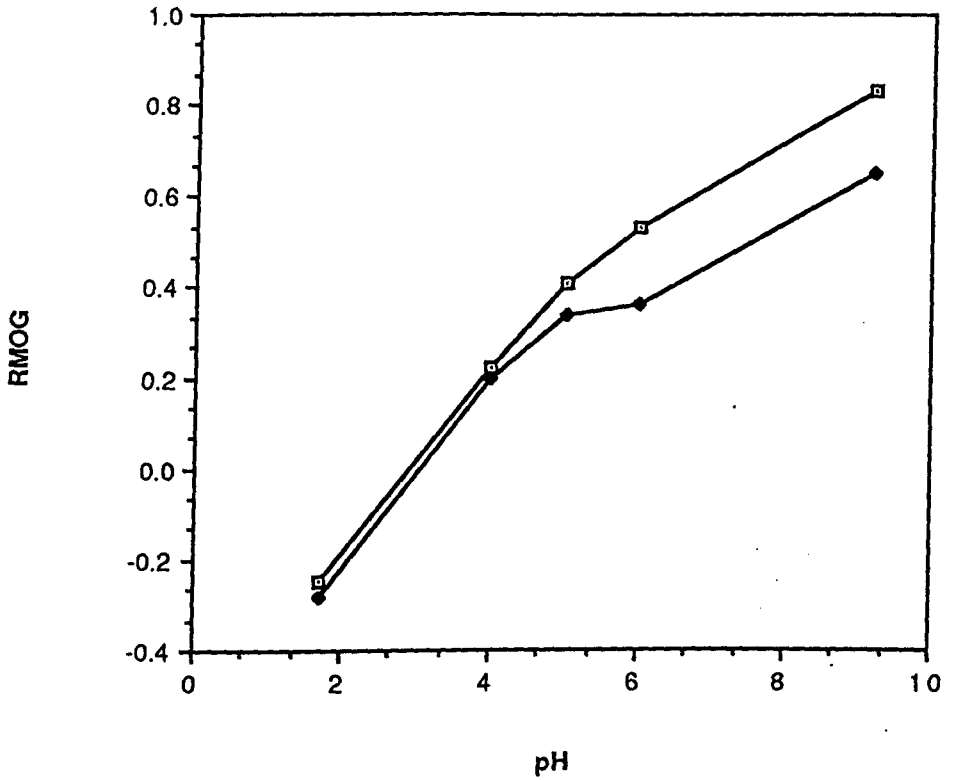
FIGURE 4.1.15

pH Mobility profile by HVPE (for details see materials and methods) of trifolitoxin sulphonic acid and hydrogenated trifolitoxin sulphonic acid

trifolitoxin sulphonic acid 

trifolitoxin sulphonic acid H_2/PtO_2 

RM_{OG} of trifolitoxin sulphonic acid and hydrogenated trifolitoxin sulphonic acid are detailed below this figure.



pH HVPE	T24SO3H RMOG	T24SO3H H2 RMOG	△ RMOG
1.7	-0.25	-0.28	0.03
4.0	0.22	0.20	0.02
5.0	0.41	0.34	0.07
6.0	0.53	0.36	0.17
9.2	0.83	0.65	0.18
11.25	0.94	0.73	0.21
13.0	0.96	0.80	0.16

FIGURE 4.1.16

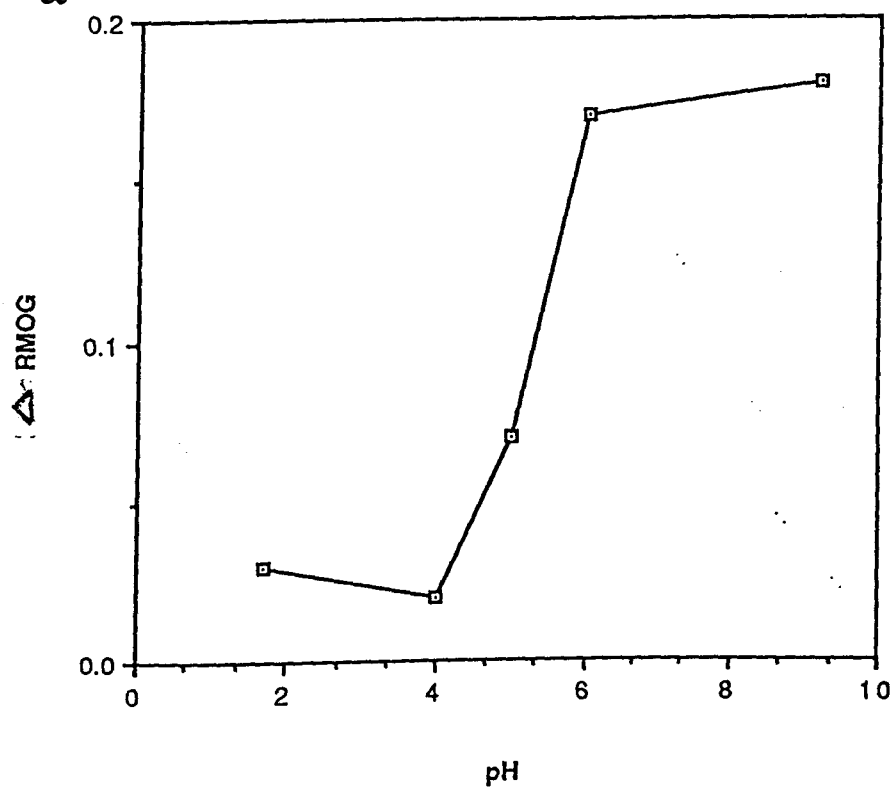
△ RM_{OG} versus pH of Figure 4.1.15

a: Plot of RM_{OG} T24SO3H minus RM_{OG} T24SO3H H_2/PtO_2

b: Plot of trifolitoxin UV spectrum vs pH 239 nm./ 228 nm. ratio (Figure 3.12 replotted)

The plots provide evidence for the saturation of a photometrically active pKa near 5.1 of the chromophore

a


0.2
0.1
0.0
0
2
4
6
8
10
Δ RMOG
pH

b

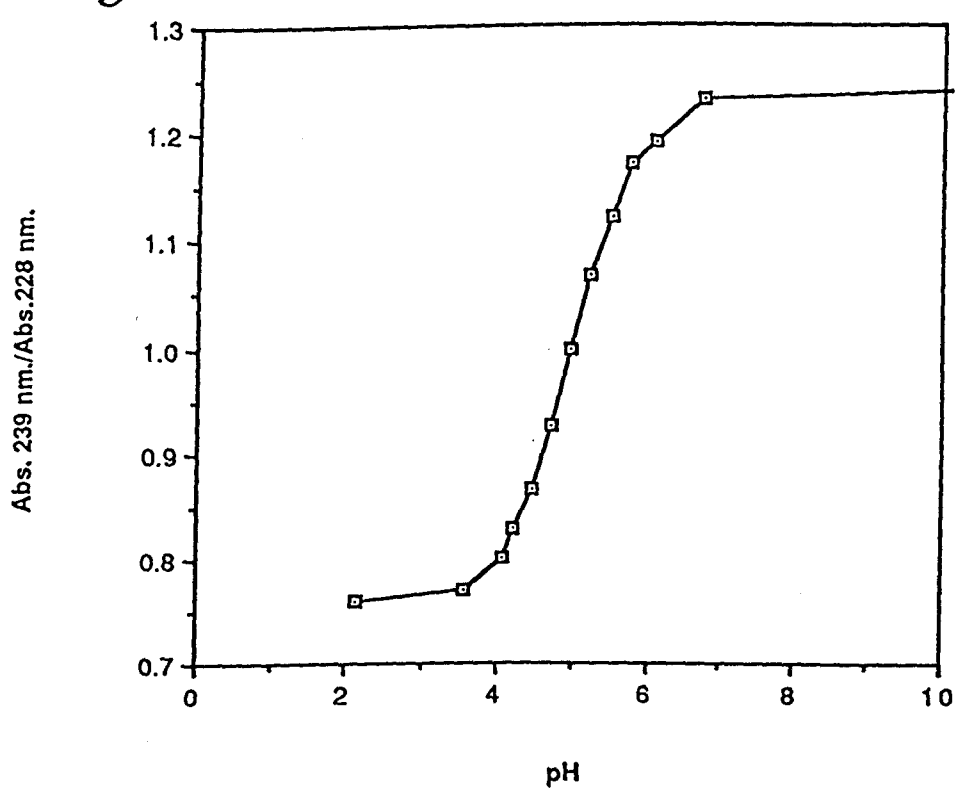

1.3
1.2
1.1
1.0
0.9
0.8
0.7
0
2
4
6
8
10
Abs. 239 nm./Abs.228 nm.
pH

FIGURE 4.1.17

Proton N.M.R. of hydrogenated trifolitoxin sulphonic acid.

a: Figure 3.14

b: hydrogenated trifolitoxin suphonic acid

Note the loss of the signal at near 6.0 ppm.

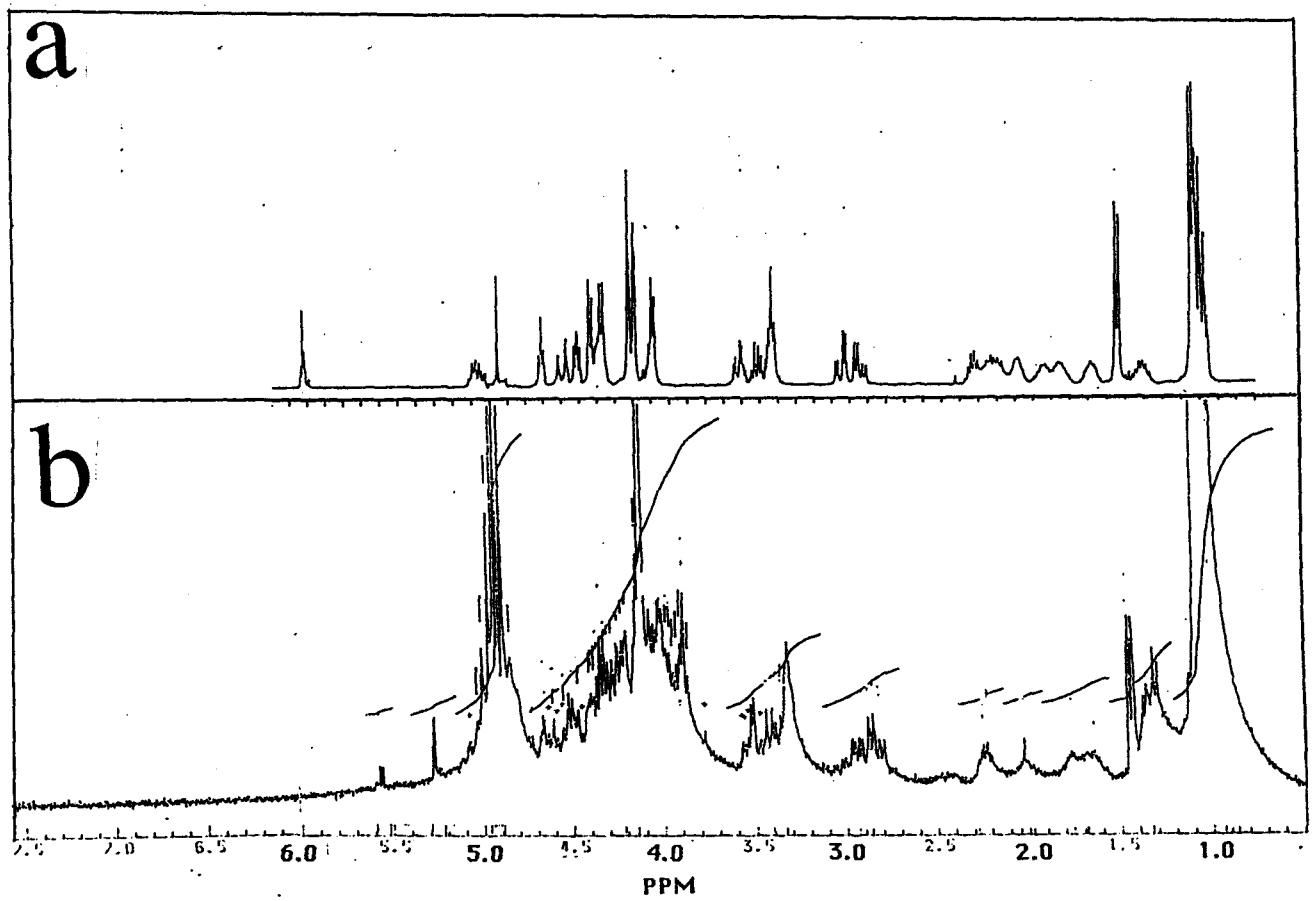

a
b
6.0
5.0
4.0
3.0
2.0
1.0
PPM

FIGURE 4.1.17a

Infrared spectrum of trifolitoxin sulphonic acid and hydrogenated trifolitoxin sulphonic acid.

a: hydrogenated trifolitoxin sulphonic acid

b: trifolitoxin sulphonic acid

No major alterations are apparent

4000
3000
2000
1600
1200
800
b
a

FIGURE 4.1.18

Biological toxicity of hydrogenated trifolitoxin sulphonic acid. Trifolitoxin sulphonic acid was hydrogenated for 24 hrs and lyophilized.
The hydrogenated sample and a control trifolitoxin sulphonic acid sample were electrophoresed by HVPE pH 9.2

RM_{OG} pH 9.2 = 0.65 is the location of the major hydrogenation product and decreased inhibition zone diameter of residual trifolitoxin sulphonic acid RM_{OG} pH 9.2 = 0.83

Trifolitoxin sulphonic acid —◆—
Hydrogenated trifolitoxin sulphonic acid —□—

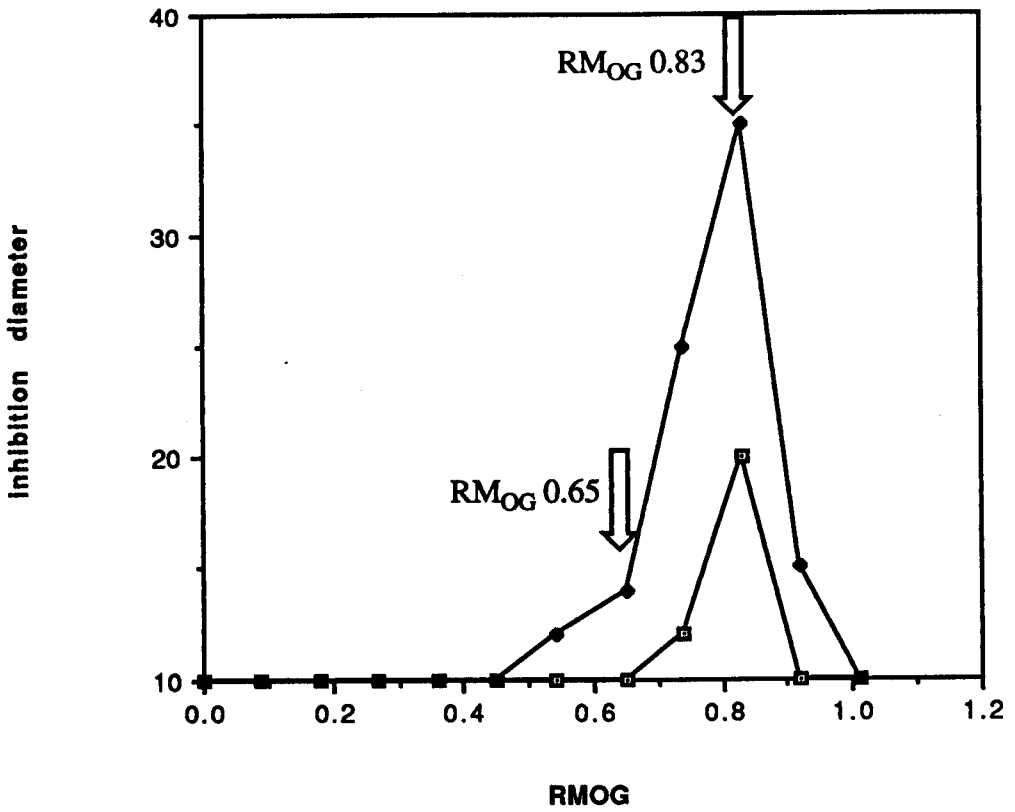

40
30
20
10
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Inhibition diameter
RMOG
RMOG 0.83
RMOG 0.65

FIGURE 4.1.19

Arginase treatment of the amino acid hydrolysis products of trifolitoxin sulphonic acid provides evidence for the racemic nature of the isolable arginine

Samples of amino acid standards and acid hydrolysates of trifolitoxin sulphonic acid (1 microlitre) were incubated with arginase(1 mg/200 microlitres , 50 mM Na_2HPO_4 pH 9.7) (2 microlitres) for the times indicated (mins) . The incubation mixture was spotted onto paper ,thoroughly dried , and electrophoresed (HVPE) at pH 1.7 (formic/acetic acid).15 mins ,3500 V. The amino acids were visualized by ninhydrin staining.Zero time samples contained the arginase preparation adjusted to pH 3.0 with formic /acetic buffer.

L aa mix.- A mixture of L amino acids in the proportions found in trifolitoxin sulphonic acid hydrolysed under the same conditions as trifolitoxin sulphonic acid.

T24SO3H- Trifolitoxin sulphonic acid hydrolysed in 5N HCl (110 degrees C ,3 hrs, under N2), the hydrolysate is dried under vacuum and a small proportion of 30% ammonia solution is added to neutralize the preparation, redried under vacuum and resuspended in water .

L-arg.- L- arginine

D-arg.- D- arginine

D,L-arg.- A racemic mixture of arginine

Std.- A mixture of amino acids found in trifolitoxin sulphonic acid plus ornithine

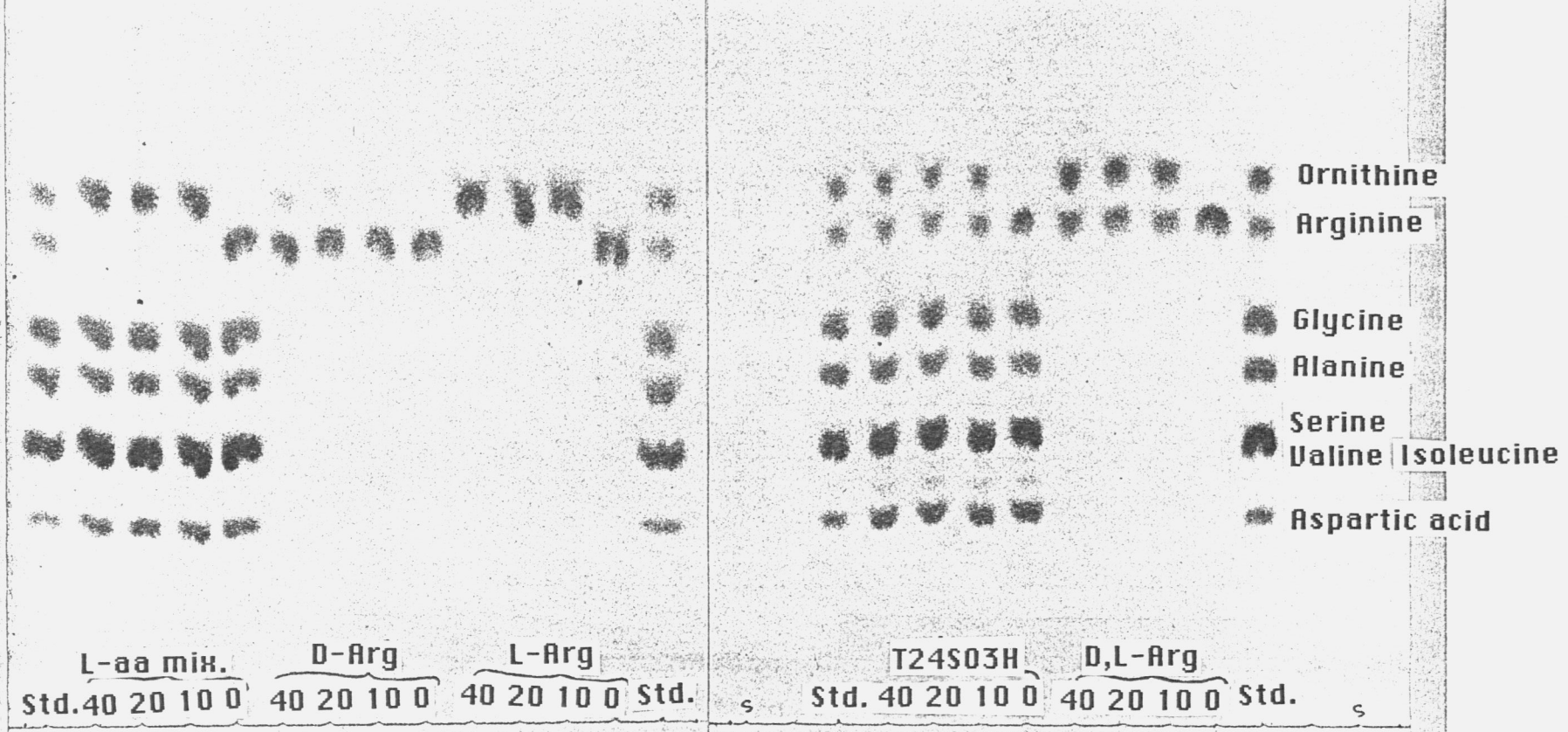

Ornithine
Arginine
Glycine
Alanine
Serine
Valine
Isoleucine
Aspartic acid
L-aa mix.
Std. 40 20 10 0
D-Arg
40 20 10 0
L-Arg
40 20 10 0
Std.
T24S03H
Std. 40 20 10 0
D,L-Arg
40 20 10 0
Std.
Cysteic acid

FIGURE 4.1.20

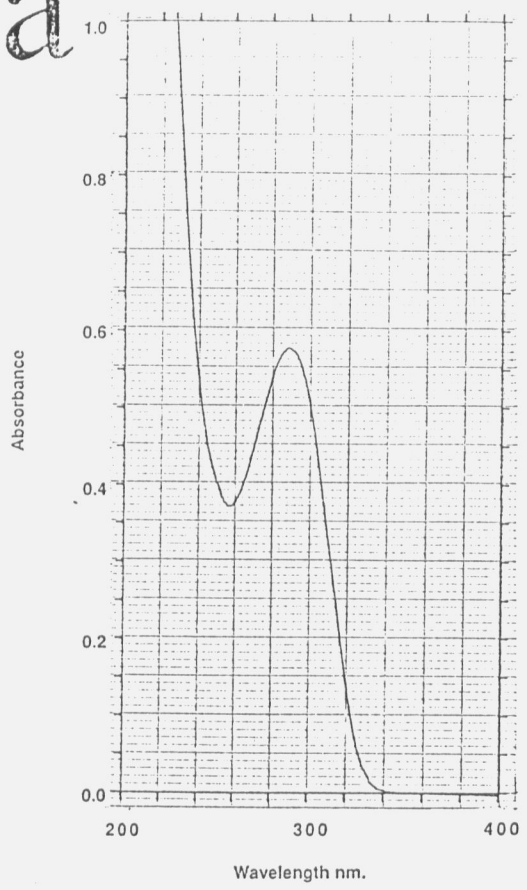
pH dependence of UV spectra of one of the two UV absorbing spontaneous hydrolysis fragments from an aged N.M.R. sample RM_{OG} pH 1.7 = -0.12

a : pH 7.0 (λ max 289 nm.)

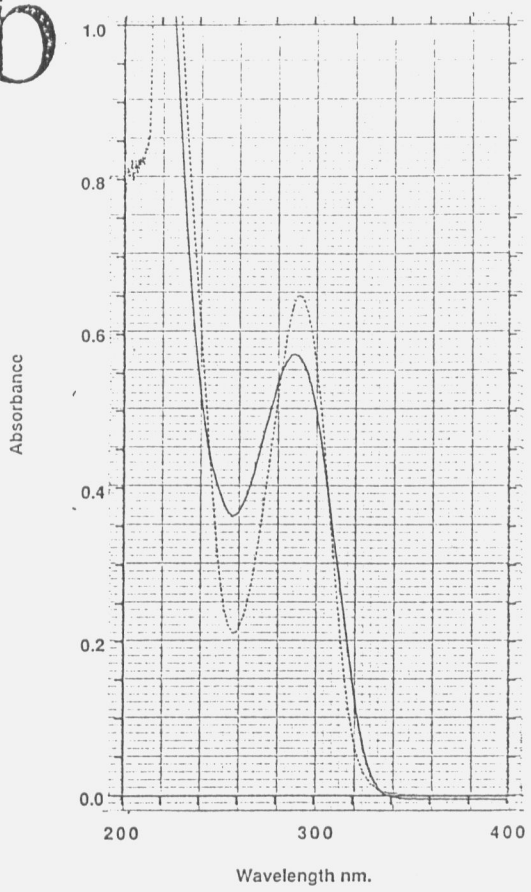
b:_____ pH 7.0 (λ max.289 nm.)
--------- 0.1 N HCl (λ max 298 nm.)

c: _____ pH 7.0 (λ max 289 nm.)
--------- 0.1 N NaOH (λ max 292 nm.)

a


1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
200
300
400
Wavelength nm.

b


1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
200
300
400
Wavelength nm.

c

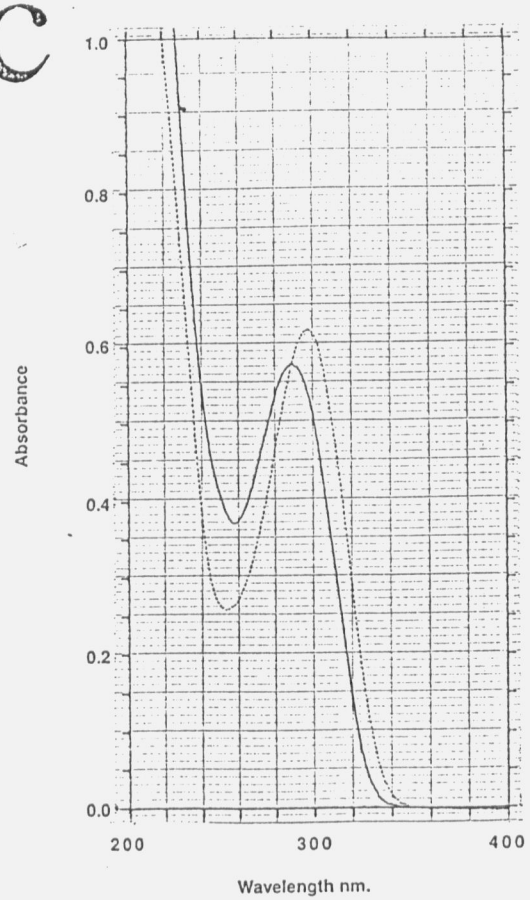

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
200
300
400
Wavelength nm.

FIGURE 4.1.21

pH dependence of the UV spectra of the anionic yellow fluorescent spontaneous hydrolysis product RM_{OG} pH 1.7 = 0.46 derived from an aged N.M.R sample .

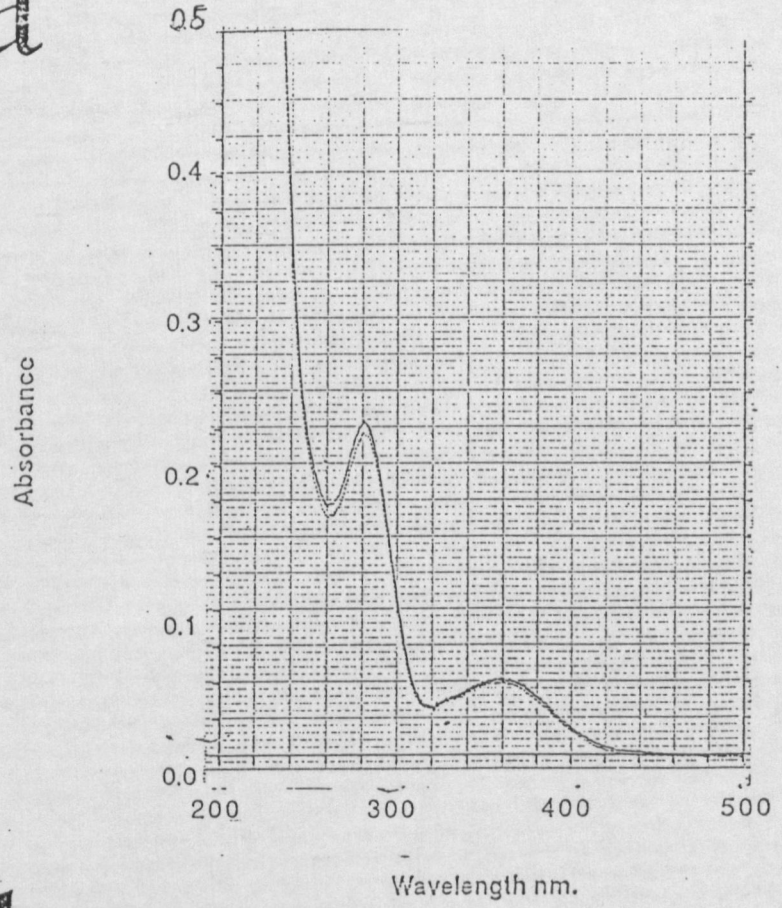
a: _____ pH 7.0 (λ max 281,360)

---------- 0.1 N HCl (λ max 281,360)

b: _____ pH 7.0 (λ max 281,360)

---------- 0.1 N NaOH (λ max 281)

a


0.5
0.4
0.3
0.2
0.1
0.0
Absorbance
200
300
400
500
Wavelength nm.

b

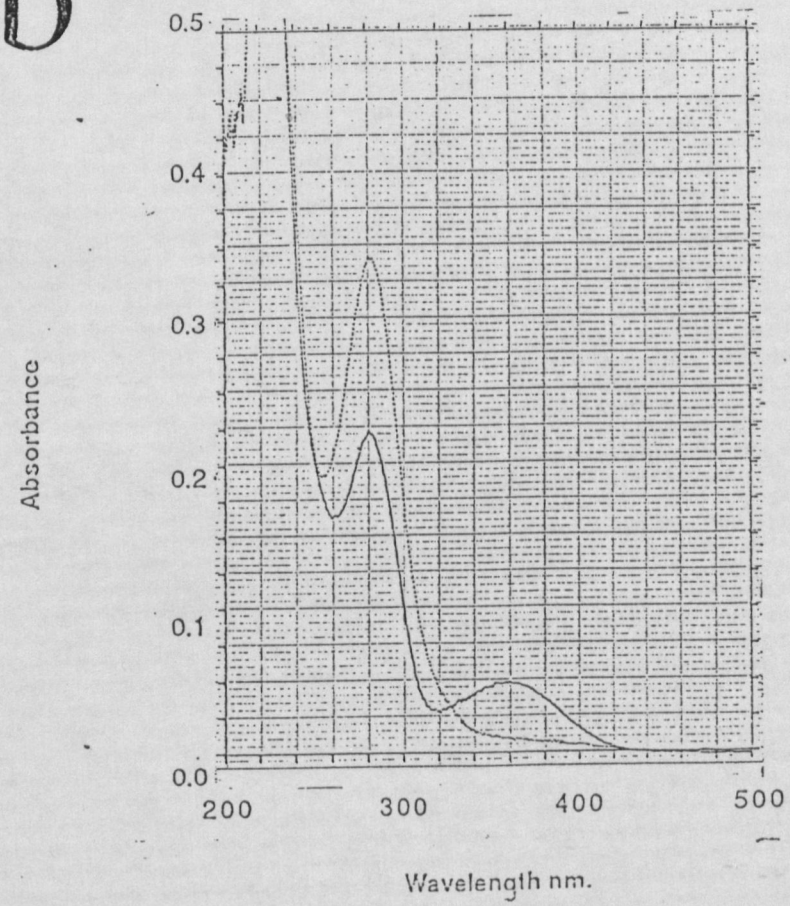

0.5
0.4
0.3
0.2
0.1
0.0
Absorbance
200
300
400
500
Wavelength nm.

FIGURE 4.1.22

FAB MS spectra of spontaneous hydrolysis derived from trifolitoxin sulphonic acid

a: UV absorbing ninhydrin positive component
RM_{OG} pH 1.7 = -0.75
positive ion spectra

b: Non UV absorbing , ninhydrin positive component
RM_{OG} pH 1.7 = -0.12
positive ion spectra

c: yellow fluorescent ninhydrin negative component
RM_{OG} pH 1.7 = 0.46
negative ion spectra

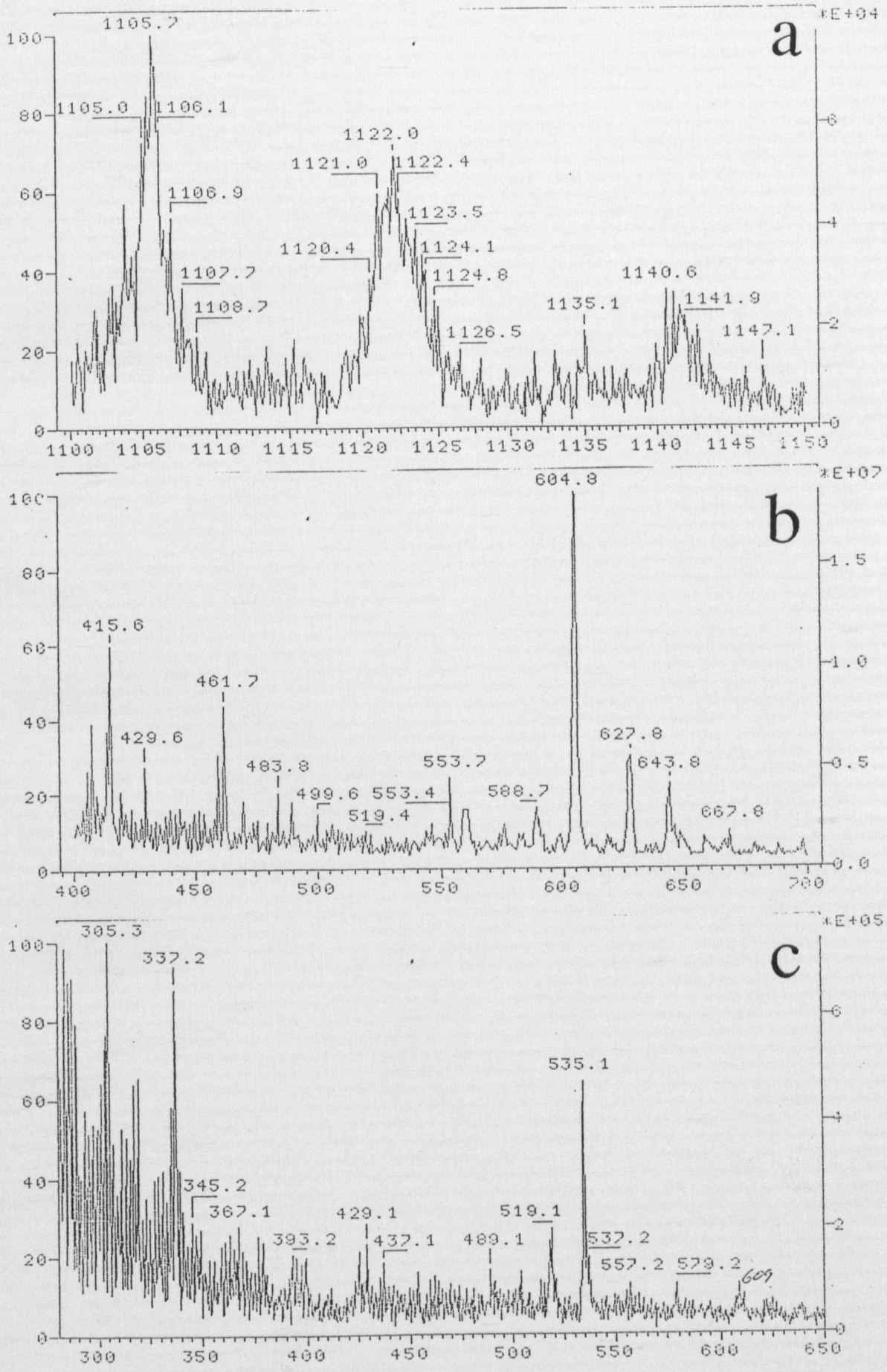

a
1105.7
1105.0
1106.1
1106.9
1107.7
1108.7
1122.0
1121.0
1122.4
1123.5
1120.4
1124.1
1124.8
1126.5
1135.1
1140.6
1141.9
1147.1
*E+04
b
604.8
415.6
461.7
429.6
483.8
499.6
553.4
519.4
553.7
588.7
627.8
643.8
667.8
*E+07
c
305.3
337.2
345.2
367.1
393.2
429.1
437.1
489.1
519.1
535.1
537.2
557.2
579.2
609
*E+05

FIGURE 4.1.23

Complete acid hydrolysis of spontaneous hydrolysis fragments.

HVPE pH 1.7 and ninhydrin stain. RMOGs as assigned in table 4.1.4

This result provides clear evidence for the spontaneous cleavage between arginine and the chromophoric C terminal sequence.

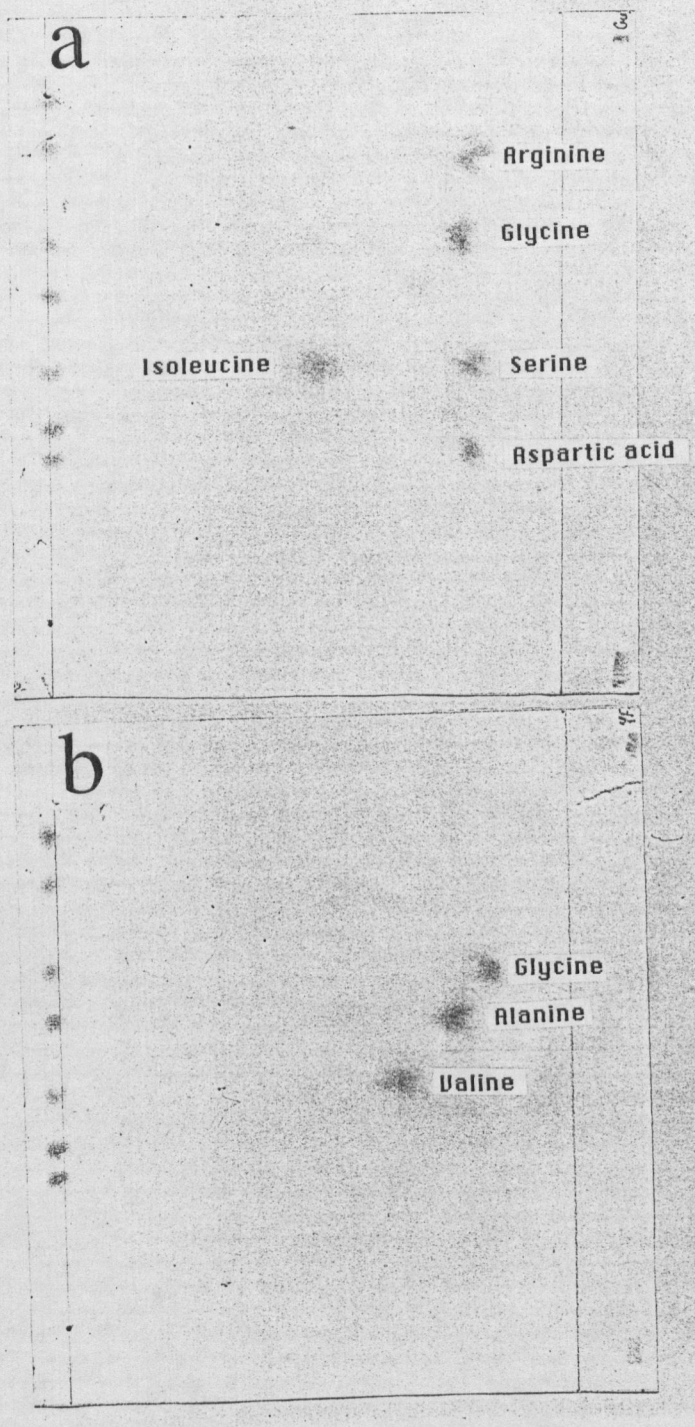
Orn
Arg
Gly
Ala
Val Ser
Ile
Glu
Asp
RMOG -0.26
RMOG -0.12
RMOG 0.46
RMOG -0.75
CysO3H

FIGURE 4.1.24

2D HVPE/PC of complete acid hydrolysates of spontaneous hydrolysis products of trifolitoxin sulphonic acid. Cationic amino acids (pH 1.7)

a: Non UV absorbing RM_{OG} pH 1.7 = -0.75

b: Yellow fluorescent RM_{OG} pH 1.7 = 0.46


a
Arginine
Glycine
Isoleucine
Serine
Aspartic acid
b
Glycine
Alanine
Valine

indistinguishable from trifolitoxin sulphonic acid in amino acid composition show different UV spectra indicating that the chromophore has been modified. Fragment 1 contains asp, ile, gly, ser and arg, is ninhydrin and guanidine positive, but shows no UV activity above 220 nm, fragment 4 is complementary to fragment 1 containing gly, cysO3H, val and ala is guanidine and ninhydrin negative and yellow fluorescent indicating the absence of an N terminal amino group and the presence of the chromophore. The amino acid composition of these fragments (Figure 4.1.23 and Figure 4.1.24) would indicate on the basis of the amino acid sequence that trifolitoxin sulphonic acid has cleaved at the linkage connecting arginine to the chromophore, the chromophore now being linked to the C terminal peptide only.

Table 4.1.4

Fragment	RMOG pH 1.7	Ninhydrin	UV(Flu.)	FAB MS
1	-0.75	+	-	604
2	-0.26	+	300 (b.)	1103
3	-0.12	+	288 (no.)	1121
4	0.46	-	280,360 (y.)	536

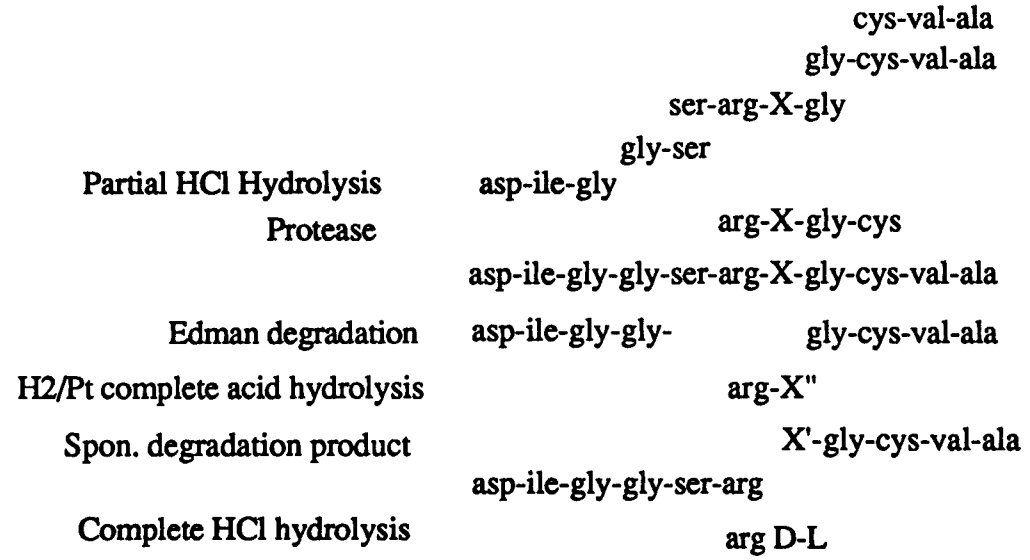
(b.) blue fluorescent, (y.) yellow fluorescent,(no.)UV absorbing only
(-) no UV activity

DISCUSSION.

Amino acid sequence

The most consistent amino acid sequence for trifolitoxin is a linear peptide with the sequence asp-ile-gly-gly-ser-(arg-X-gly)-cys-val-ala.The possibility of a cyclic structure involving the beta carboxyl of aspartic acid cannot at present be ruled out. The evidence presented is consistent with the presence of a C terminal carboxyl group and N terminal amino group and that part of the sequence not determined by Edman degradation has been demonstrated by derivatization of the terminal functionalities being consistent with a linear molecule. The peptide fragments obtained from partial acid hydrolysis would fit that expected , glycine amide bonds being most susceptible to hydrolysis (Light 1967), the side chains of aspartic acid and cysteic acid also playing a catalytic role in hydrolysis (Schultz 1967). The inability of the Edman reaction to cleave the serine residue from the peptide is unusual and no obvious explanation is immediately apparent but the proximity of the blue fluorescent chromophore with its unusual bonding to arginine may be involved. The presence of small amounts of aspartic acid in the blue fluorescent protease fragments and the blue fluorescent partial hydrolysis fragments is also unusual. It has been clearly demonstrated by two

independent methods that aspartic acid is N terminal and only one residue of aspartic acid is detectable in the acid hydrolysates of trifolitoxin. One explanation is a beta aspartyl amide linkage generating a lactam ring, to some component in the peptide chain close to or including the chromophore, which is only partially cleaved by protease. The pH mobility profile of the blue fluorescent peptide obtained from partial acid hydrolysates and the absence of detectable aspartic acid protons in the N.M.R. of the protease fragment would indicate that such a linkage of aspartic acid to the chromophore is unlikely.



Some antibiotics contain cyclic structures (Kleinhauf 1987), this is possible because they are synthesized, not ribosomally, but by multi enzyme complexes. D amino acids are also a common feature of these types of peptides and the presence of this enantiomer has been used to explain the stability of these peptides to proteolytic enzymes. In the case of trifolitoxin only the linkages of arg-X-gly-cysO3H are not susceptible to proteolytic cleavage indicating that all the other amino acids contributing to the cleaved amide linkages contain the L configuration. It is surprising then that arginine recovered from acid hydrolyses should consist of a racemic mixture, probably as the result of the unusual linkage to the chromophore.

Arginine

It is evident from the results that a racemic mixture of arginine is obtained from acid hydrolyses of trifolitoxin. The optical isomerism of amino acids in peptides is usually defined, either D or L but not both (Kleinhauf 1987). There is one circumstance as a consequence of the thiazoline in bacitracin where racemization of the N terminal amino acid occurs (Konisberg and Craig 1961) but the conditions of this reaction are not present in trifolitoxin.

Peptides decompose at neutral pH from the N terminal by diketopiperazine formation, this transformation is accompanied by extensive racemization due to the rapid rate of racemization of amino acid residues in diketopiperazines (Steinberg *et al.*, 1983). No

significant racemization of amino acids has been noted under acidic conditions, like those employed here (Greenstein and Winitz 1961), and considering that the N terminal group of arginine is not free then conditions favouring diketopiperazine formation probably do not occur.

Considering that the alpha proton of arginine is undefinable in the proton N.M.R. then one possible explanation is that the alpha carbon in trifolitoxin is non chiral and an ethylenic linkage to this carbon as an enolic bond could be present. Hydrolysis of this type of structure would yield a racemic mixture of arginine. Alternatively this proposed ethylenic bond with the alpha carbon of arginine may be a transient structure facilitated by the linkage to the blue fluorescent chromophore as is the case of the racemization of the the N terminal isoleucine of bacitracin.(Konisberg and Craig 1961).

Hydrogenation of trifolitoxin sulphonic acid stabilizes the linkage between arginine and the chromophore an enolic linkage responsible for this stabilization of the linkage of arginine to the chromophore by reduction may also be a component of the racemization reaction.

The N-terminus

The evidence presented here indicates that trifolitoxin contains a free N- terminal amino group, distinguishing it from the gramicidins and etamycin. (Sarges *et al.*, 1964, Synge 1945, Maeda 1957). Two pieces of data indicate that aspartic acid is N terminal. The most obvious is the Edman degradation data. The other is the absence of free aspartic in the amino acids recovered from the protease treatment of acetylated trifolitoxin. Protease cleaves the N-acetyl aspartic acid but will not cleave the acetyl group. This N-acetyl group prevents the reaction with ninhydrin. Such evidence is independent confirmation of the Edman sequencing.

Acetylation of the N terminal group of trifolitoxin clearly alters the charge properties and also eliminates toxicity. This evidence alone would indicate that an N-terminal group is a necessary component of trifolitoxin activity and the possibility that it is an isolation artifact due to the catalytic hydrolysis of aspartic acid amide bond can be ruled out.(Light 1967). The possibility that O-acetylation of the side chain hydroxyl of serine is responsible for the loss of biological activity cannot be excluded but no divergent amino acids (ie O-acetyl serine) were detected in the protease digestion by HVPE at pH 1.7, it is not known whether O-acetyl groups are cleaved by protease.

The loss of biological activity on N-terminal acetylation is an interesting result when taken in conjunction with the fact that trifolitoxin sulphonic acid minus the N terminal aspartic acid is as toxic as the parent trifolitoxin sulphonic acid. Removal of the isoleucine residue substantially reduces activity. Clearly it is not the N-terminal amino group that is required for toxicity but possibly the presence of a optically active N terminal amino group. The partial loss of aspartic acid in native trifolitoxin may explain the more cationic active component by HVPE at pH 5.0 in the original acetylation experiment.

The inability of leucine aminopeptidase to eliminate toxicity can be explained by the very low specificity of this enzyme for N terminal aspartic acid. (Greenstein and Winitz 1961, Smith 1969).

The C terminus

The sensitivity of trifolitoxin activity to carboxypeptidase indicates the essential nature of the C terminal amino acids. The recovery of alanine from hydrazine lysates confirms the presence of a C terminus. Such data is supportive of the 1600 cm^{-1} peak in the infrared spectrum of trifolitoxin representing the carbonyl stretch of the C terminal carboxylate anion. This peak and group is absent in bacitracin, a consequence of the branched cyclic structure.

The complete peptide sequence

The current evidence is consistent with the amino acid sequence for trifolitoxin being asp-ile-gly-gly-ser-(arg-X-gly)-cys-val-ala. The C terminal and N terminal tetrapeptides have been positively identified, gly-ser and a peptide containing ser-arg-X-gly have been identified by partial acid hydrolysis. No conclusive evidence has been obtained for the arg-gly sequence.

Hydrogenation and acid hydrolysis of T24SO3H leads to the recovery of a guanidine positive ninhydrin negative blue fluorescent product consistent with an arginine chromophore conjugate. A ninhydrin and guanidine positive UV inactive peptide containing the proposed N terminal peptide asp-ile-gly-gly-ser-arg and the complementary ninhydrin, guanidine negative, yellow fluorescent peptide containing the C terminal peptide gly-cysO3H-val-ala has fortuitously arisen in an aged N.M.R. sample. The logical inference is that arginine and glycine are directly linked to the chromophore X which intervenes between them.

Blue fluorescent peptides usually contain arginine and glycine pointing to an association between these amino acids and the chromophore. This unusual linkage appears to interfere with Edman sequencing and enzymic proteolysis and is consistent with the unusual environment of arginine and glycine in the proton N.M.R.as indicated by the lack of recognizable alpha proton of arginine and the down field displacement of the beta protons of arginine and the two geminal coupled protons assignable to the third glycine. Proteolytic fragments of trifolitoxin are currently under investigation by FABMS to confirm these findings.

CHAPTER 4.2
THE THIAZOLINE RING

RESULTS

Biological activity and low pH incubation

Schwinghamer and Belkengren (1968) experiments on trifolitoxin indicated that this bacteriocin was very unstable to acid pH incubation. Figure (4.2.1) shows comparable results to Schwinghamer's observations and provides confirmation of the bacteriocins instability at low pH

Reaction of trifolitoxin with thiol reagents and low pH incubation

The inability of trifolitoxin, a cysteine containing peptide, to react with the thiol reagents NEM or Ellman's reagent in its native form indicated that the thiol group of the cysteine was not free. Coincident with the loss of toxicity with low pH incubation there is an increase in free thiol, and a characteristic rise in absorbance at 410 nm. on reaction with Ellman's reagent (Figure 4.2.1).

Affect of low pH incubation on the UV chromophore

Another feature which is affected by low pH incubation is a UV chromophore (maxima 270 nm.) which becomes apparent only when spectra are obtained in highly acidified solution.(10 N HCl) (See Figure 3.11). Preincubation of trifolitoxin at acid pH completely eliminates this peak (Figure 4.2.2) resulting in UV spectrum similar to that obtained at pH 1 attributable to the blue fluorescent chromophore.(See Figure 3.11). The loss of the 270 nm. peak on incubation at low pH is presented in Figure 4.2.1.

Coincidence of Biological activity, UV absorbance, free thiol at low pH

Calculation of the percent native molecule using the results in Figures(4.2.1 a,b,c and 3.7) of thiol, UV and biological activity respectively, indicate that the rates of appearance of free thiol, loss of biological activity and decreasing absorbance at 270 nm. are closely related (Figure 4.2.3).

Oxidation and reduction of the free thiol leading to multiple forms

Prolonged acid incubation of trifolitoxin does not completely eliminate toxicity (approximately 2% is retained). Multiple active forms of trifolitoxin have been detected in partially purified extracts with varying specific activity (Figure 3. 5). Analysis of the performic acid treated product (Figure 3.6) which converts the cysteine derivative to cysteic acid illustrates that the multiple forms detected by HVPE could all be converted to the one trifolitoxin sulphonic acid derivative. This indicates that the observed multiplicity of forms of trifolitoxin is a

FIGURE 4.2.1

Trifolitoxin (0.22 OD, 302 nm./100 microlitres)incubated in 25 mM Citrate/NaOH at the pH indicated for 40 mins. at 25° C.

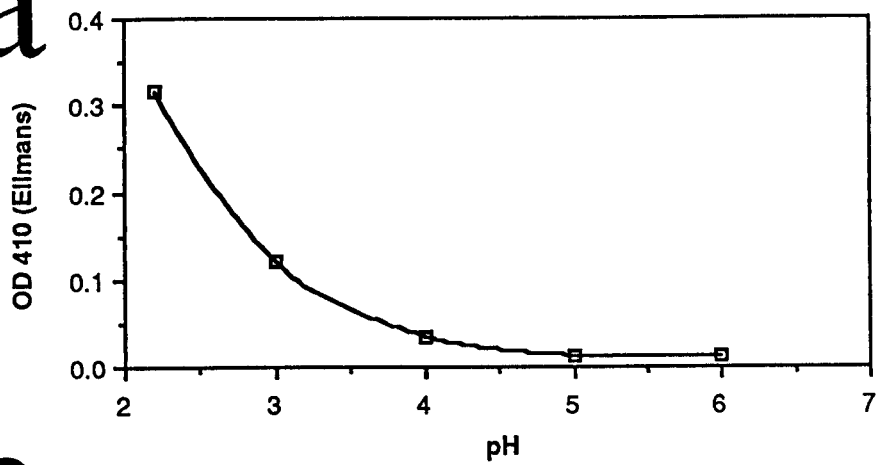
a: The product (100 microlitres) treated with Ellman's thiol reagent (Materials and Methods) and monitored by light absorption at 410 nm.

b: The product (100 microlitres) added to 10 N HCL (900 microlitres) and monitored by UV absorption at 270 nm.

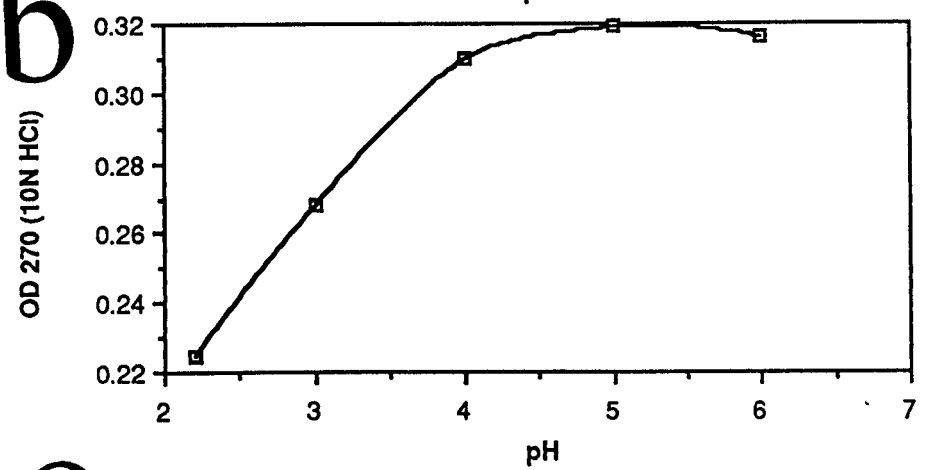
c: The product (10 microlitres) placed on 1 cm square paper dried and immediately placed on the buffered agar for the bioassay. Inhibition diameters (mm.) were measured after 48 hrs growth.

These results show the correlation of the appearance of free thiol, a loss of λ^{max} 270 nm. chromophore and a loss biological activity , evidence for the hydrolysis of a thiazoline in trifolitoxin at low pH. Figure 4.2.3 shows the quantitation of this data.

a


OD 410 (Ellmans)
0.4
0.3
0.2
0.1
0.0
2
3
4
5
6
7
pH

b


OD 270 (10N HCl)
0.32
0.30
0.28
0.26
0.24
0.22
2
3
4
5
6
7
pH

c

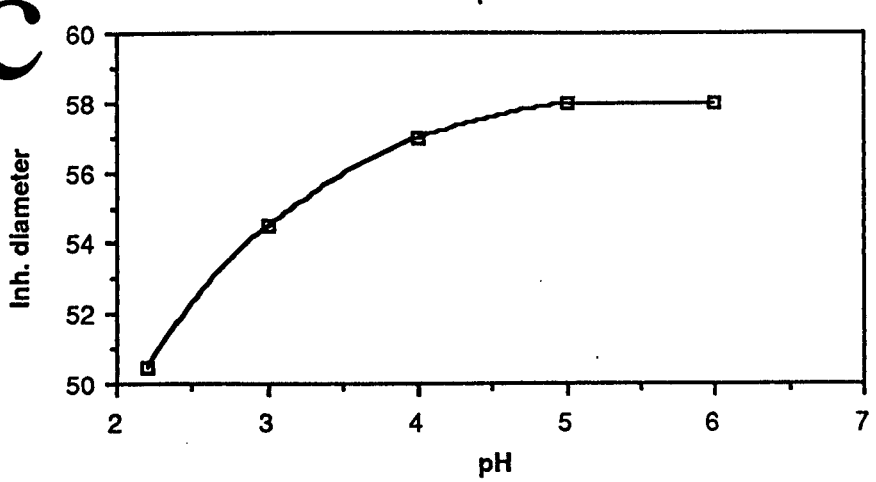

Inh. diameter
60
58
56
54
52
50
2
3
4
5
6
7
pH

FIGURE 4.2.2

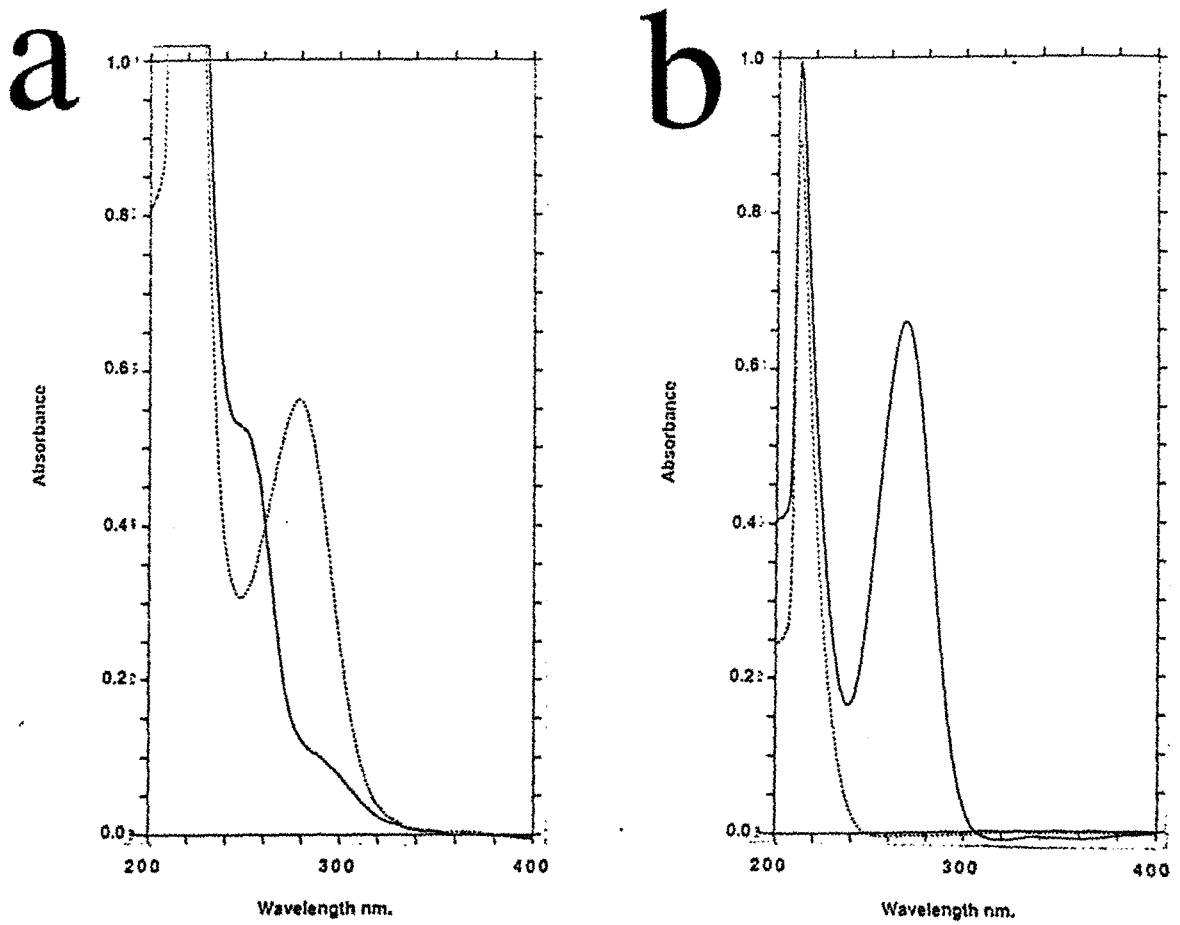
UV spectra of thiazolines in bacitracin , glutathione, 2-methyl thiazoline , trifolitoxin

a: ___ bacitracin pH 7
---- bacitracin 10 N HCl (max. 278 nm.)

b: ---- glutathione 10 N HCl 0 Hrs. ,
_____ glutathione 10 N HCl 18 Hrs.(max 268 nm.)

c: ---- 2 methyl thiazoline pH 7. ,
___ 2 methyl thiazoline 10 N HCl (max. 261 nm.)

d: ___trifolitoxin 10 N HCl (max. 270 nm)
---- trifolitoxin pretreated with 0.1 N HCl for 1hr. Spectrum in 10 N HCl


a
b
Absorbance
Wavelength nm.
1.0
0.8
0.6
0.4
0.2
0.0
200
300
400

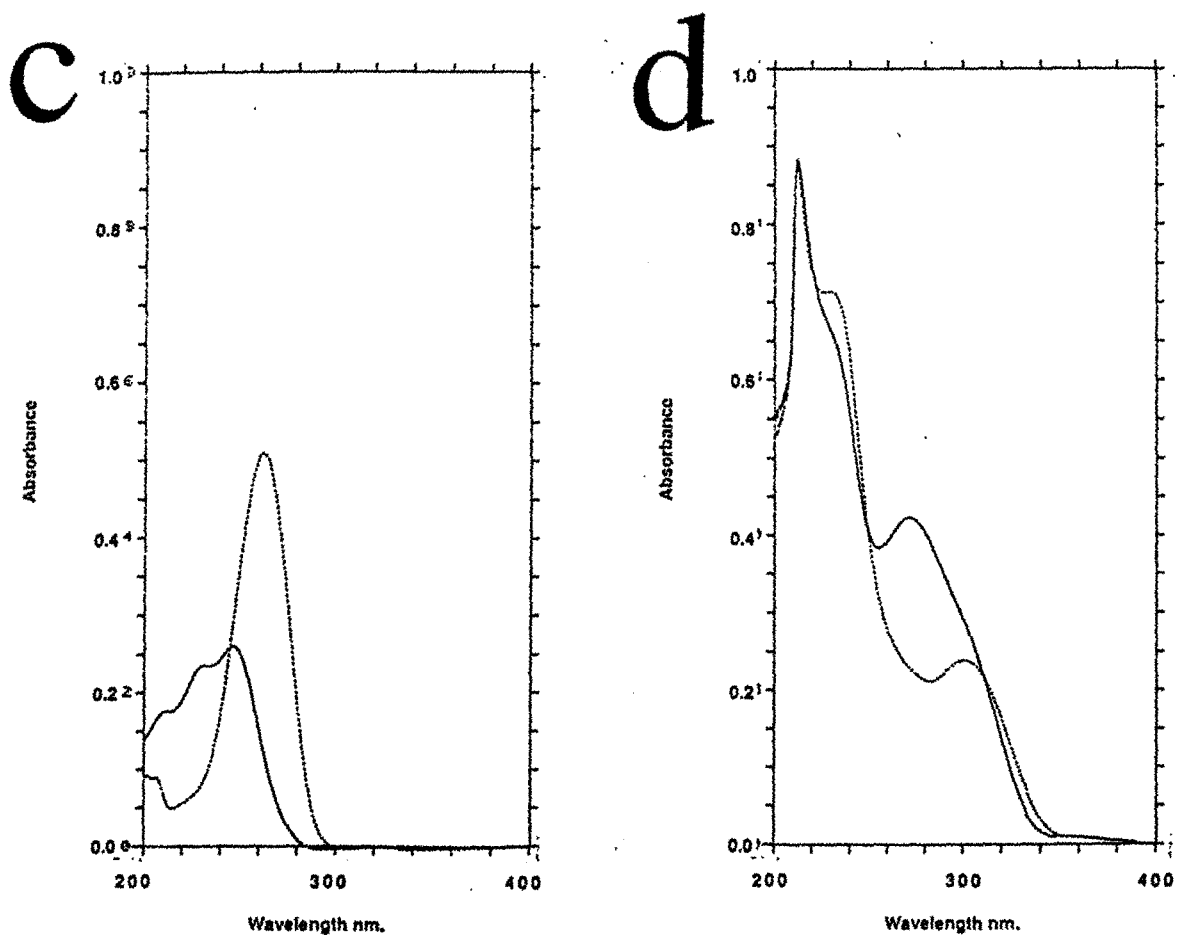

c
d
Absorbance
Wavelength nm.
1.0
0.8
0.6
0.4
0.2
0.0
200
300
400

FIGURE 4.2.3

Calculated percent residual intact trifolitoxin as a function of pH after 40 mins. incubation at 25° C.

Bioassay- from standard curve (Figure 3.7)

Ellman's- 0% ,0.0 ODs 100 % ,0.414 ODs 410 nm..

OD 270 nm..- 0% 0.18 ODs 100% 0.32 ODs

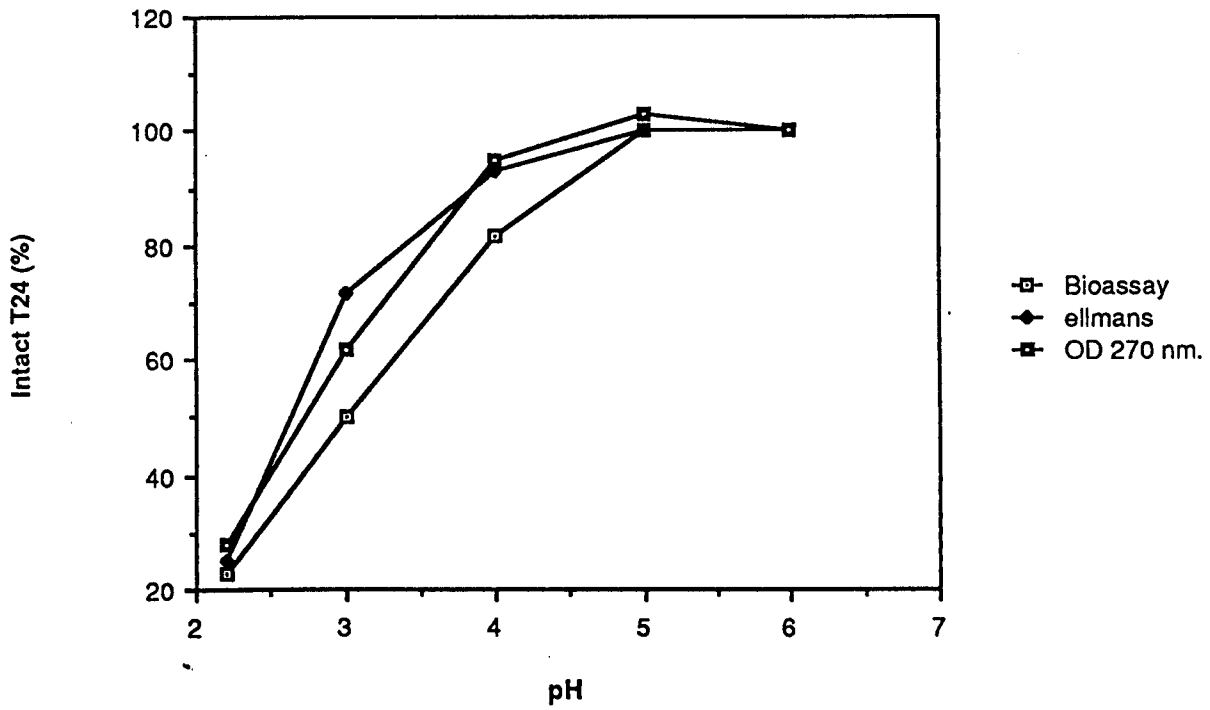

120
100
80
60
40
20
Intact T24 (%)
2
3
4
5
6
7
pH
Bioassay
ellmans
OD 270 nm.

FIGURE 4.2.4

Gel Filtration Chromatography (G15 sephadex) of trifolitoxin and derivatives

Trifolitoxin or derivatives were chromatographed on a G15 Sephadex column (1.5 cm diameter 25 cm length) in 30 mM phosphate pH6.8, flow rate 0.5 ml/min. , 1 ml fractions collected and monitored in the UV at 302 nm.

The samples were prepared as follows

a: trifolitoxin (1 OD),dried and resuspended in elution buffer

b: a treated with 0.1 N HCl (30 mins 25 degrees C) dried under vacuum, resuspended in elution buffer

c: b (minus elution buffer) electrophoresed pH 9.2 (HVPE 0.2 M NH_4HCO_3/ NH_4OH) 30 mins. The paper was dried on each side for 20 mins under a warm fan (surface temp. 65 degrees C) eluted , dried under vacuum and resupended in elution buffer.

d: b (minus elution buffer) treated with performic acid (500 uls ,20 mins), dried under vaccuum and resuspended in elution buffer

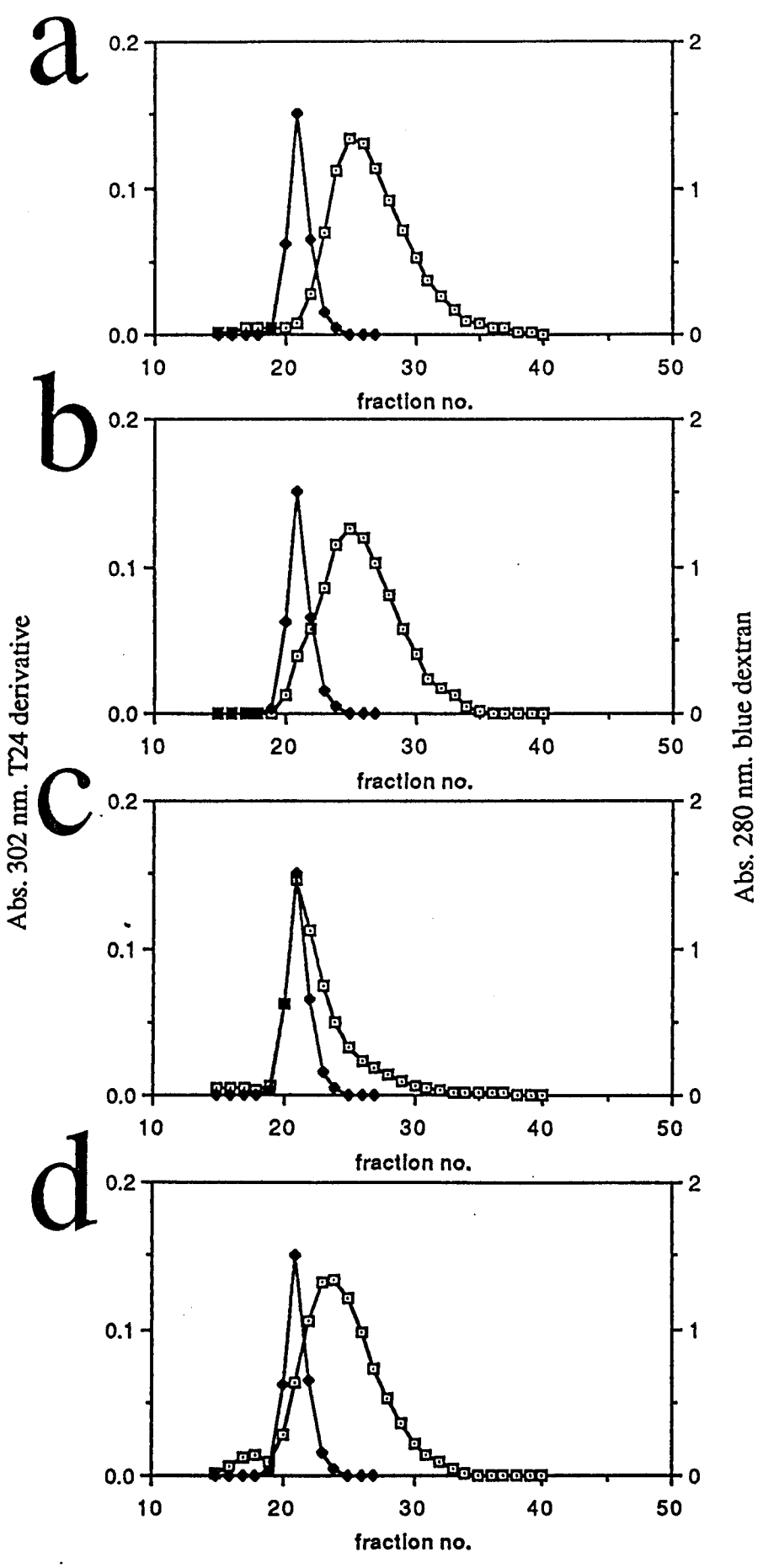

a
b
c
d
0.2
0.1
0.0
2
1
0
10
20
30
40
50
fraction no.
Abs. 302 nm. T24 derivative
Abs. 280 nm. blue dextran

FIGURE 4.2.5

Inhibition zones produced on the oxidation of T24S-ST24

F: formic (control)

PF: performic (oxidation)

Conditions presented in figure 4.2.6

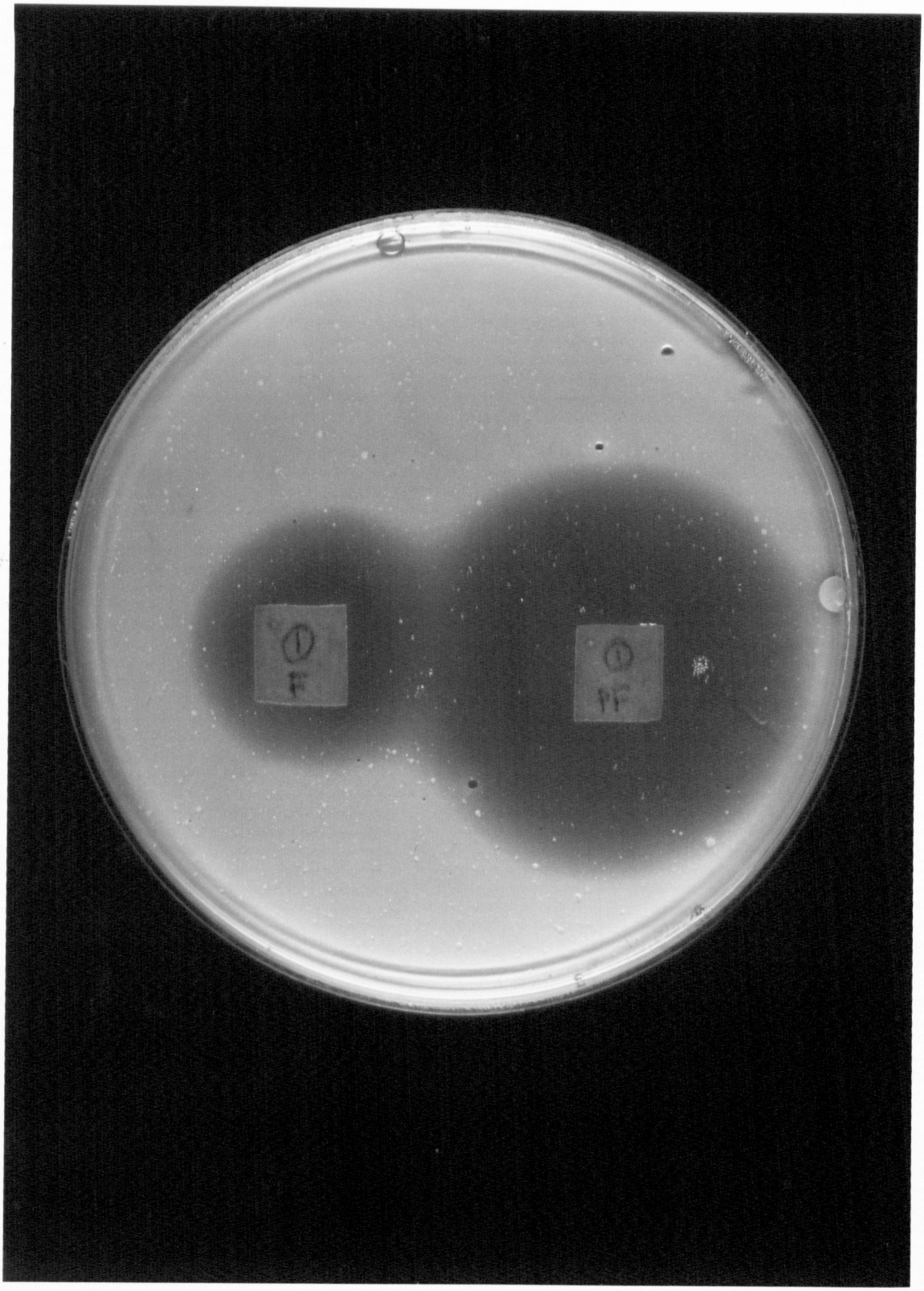

①
F
①
PF

FIGURE 4.2.6

Reduction and oxidation of a derivative of trifolitoxin

Trifolitoxin (1 OD 302 nm.) was dried under vacuum and incubated in 0.1 N HCl (200 microlitres) for 1 hr. The hydrolysed sample was dried under vacuum, electophoresed (HVPE) at pH 9.2 (0.2 M NH_4HCO_3 / NH_4OH) for 1 hr. The paper was dried (both sides, 20 mins, warm fan , temperature just above paper surface 65 degrees C) and the blue fluorescent material corresponding to T24SH was eluted and divided into 4 equal portions and dried under vacuum.

The samples were treated as follows

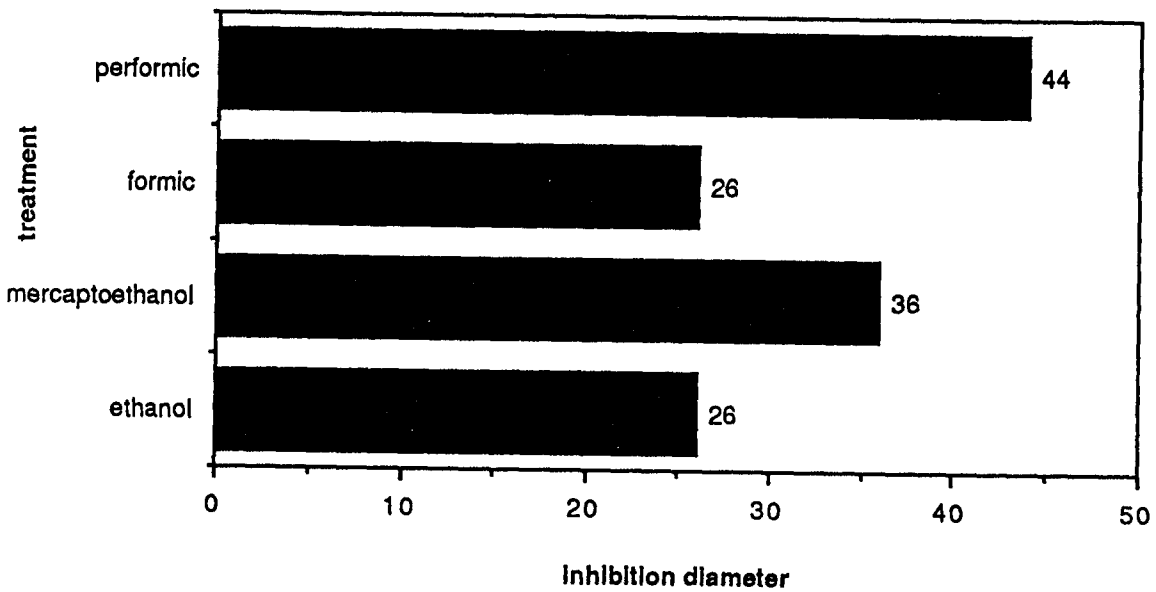
Performic- 9:1 ,formic acid : 30 % H_2O_2 , 20 mins (200 microlitres)

Formic- 90 % formic acid ,20 mins.(200 microlitres)

Mercapto-ethanol- 2% mercapto-ethanol,80 mins. (200 microlitres)

Ethanol- 2% ethanol ,80 mins. (200 microlitres)

The treated samples were dried under vacuum to remove reagents, resuspended (10 microlitres H_2O) and spotted on 1 cm. square papers dried briefly and bioassayed .Inhibition zones were measured after 48 hrs. Control experiments showed no toxicity to the assay organism.


performic
formic
mercaptoethanol
ethanol
44
26
36
26
treatment
0
10
20
30
40
50
Inhibition diameter

consequence of the multiple oxidation states of the acid released thiol of cysteine in trifolitoxin. They probably represent isolation artifacts but could also be expected to arise in aerobic environments and in that situation may have biological significance which has not been determined.

Trifolitoxin (T24) was converted to trifolitoxin thiol (T24SH), trifolitoxin disulfide (T24S-ST24) and trifolitoxin sulphonic acid (T24SO3H) and gel filtration chromatography (G15 Sephadex) (Figure 4.2.4) demonstrates the expected elution profiles, based on molecular size. The disulfide form of trifolitoxin eluting in the void volume coincident with blue dextran (Ve/Vo 1.00), ahead of the monomeric forms which are partially included in the gel matrix and elute later (Ve/Vo T24, 1.23, T24SH, 1.23, T24SO3H,1.18, the exclusion limit for G15 Sephadex is approximately 1500 MW). Oxidation or reduction of trifolitoxin disulfide to either the corresponding sulphonic acid or thiol respectively, results in an increased specific activity (Figure 4.2.5 and Figure 4.2.6), i.e. increased inhibition zones for an equal amount of starting material. These data are consistent with the specific activity and mobility by HVPE of the components isolated from crude sources.(See Table 4.2.1, Figure 3.5 and Figure 3.6).

Table 4.2.1

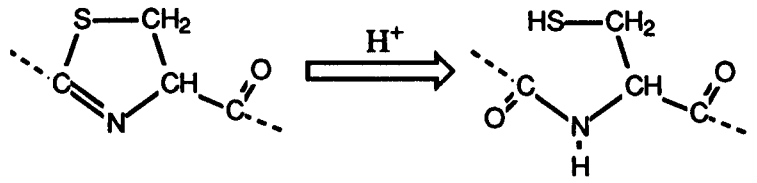
Toxin	Toxicity	RMOG 9.2	RMOG 1.7	G15 Ve/Vo
T24	100%	0.55	-0.62	1.23
T24SH	1-2%	0.62	-0.92	1.23
T24S-ST24	0.05-0.10%	0.62	-0.73	1.00
T24SO3H	1-2%	0.76	-0.31	1.18

DISCUSSION

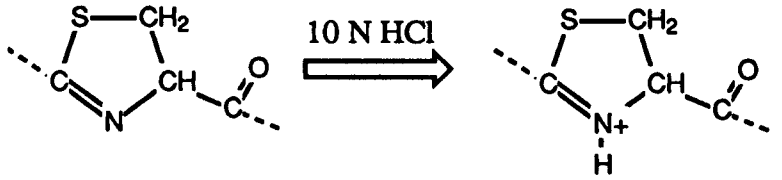
Coincidence of loss of biological activity with loss of UV chromophore and increase in free thiol is consistent with the presence of a thiazoline ring system.

The results indicate that the cystine detected in amino acid hydrolyses of trifolitoxin (Figure 3.9) is present as a thiazoline in the intact trifolitoxin , three independent pieces of data support such a conclusion.

1) A thiol group is detected only after mild acid hydrolysis (Martin and Parcell 1961). The reduction in biological activity of the acid treated bacteriocin is closely aligned with the appearance of a free thiol



(2) The ultraviolet chromophore detected in 10 N HCl solution



has similar spectra to the protonated forms of thiazoline in bacitracin, glutathione and 2 methyl thiazoline (Figure 4.2.2 b,c,d respectively). An approximate pK value of -1 for trifolitoxin for the nitrogen protonation (Figure 4.2.7) is similar to a pK of -1.8 for the bacitracin thiazoline (Martin and Parcell 1961). The loss of this chromophore by preincubation in mildly acidic solution coincides with the loss of biological activity and appearance of free thiol.

(3) The proton n.m.r.signals at 3.6,3.8 and 5.2 ppm (J.Sims personal communication) also support such a structure.

Presence of thiazolines in other peptides?

Drastic *in vitro* conditions are needed to synthesize thiazolines, Jocelyn (1972) suggests that this will only occur if there are special facilitating structural features in the peptide, in the case of trifolitoxin the close proximity of the blue fluorescent chromophore may be important. The best natural product example and also most relevant in the current context is the thiazoline of bacitracin a polar peptide antibiotic produced by a *bacillus* species active against a broad range of gram positive bacteria.(Johnson 1945). The thiazoline structure found in bacitracin was also isolated as cysteine after acid hydrolysis (Lockhart, Abraham and Newton 1955).

Cyclic structures of this nature also occur in other microbial toxins. Thiazoline or derivatives thereof (thiazole, thiazoline, thiazolidine, differing only by the ring saturation) have also been shown to be present in thiostrepton (Anderson *et al.*, 1970) microcossin P, botromycin, and penicillin and as well as glutathione and thiamine (Waley 1966, Cecil and McPhee 1959, Calvin 1952) and speculated to be present in some proteins (Linderstrom Lang *et al.*, 1941).

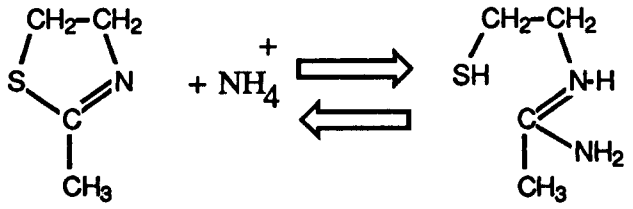
The UV spectra of thiazolines all show the characteristic appearance of a maxima between about 270 nm. and 280 nm. in strongly acidic conditions due to the protonation of the ring nitrogen of the thiazoline.(Jocelyn 1972). The hydrolysis of thiazolines occurs under

mildly acidic conditions as demonstrated for trifolitoxin (Figure 4.2.2), bacitracin (Newton and Abraham 1953), 2-methyl thiazoline (Martin and Parcell 1961) and glutathione (Calvin 1952).

Possible biological implications of the thiazoline in trifolitoxin

A potentially limiting factor to the usefulness of trifolitoxin is the stability of the thiazoline in low pH conditions. The pH buffering capacity of the rhizosphere of leguminous plants could be a factor in modifying the nodulation competitiveness of trifolitoxin producing strains in the proximity of nodulation sites. The pH of the surrounding soil may also be significant in the absence of a host plant. Reduction in soil pH under legume based pastures as a consequence of nitrogen fixation and organic acid accumulation (Israel and Jackson 1978, Ngatsamya and Pierre 1973) may be of significance to the competitiveness of strain T24.

Another interesting feature of thiazolines is the reversible cleavage with ammonium ions (Linderstrom Lang 1941).



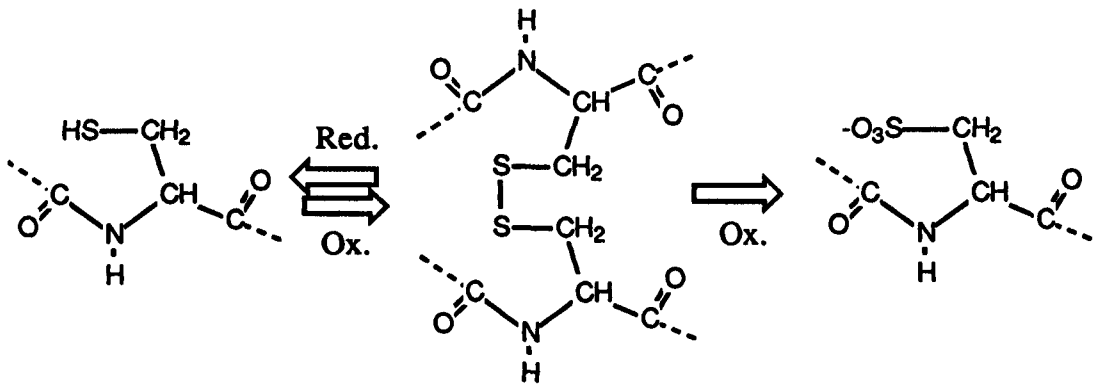
It is not known whether trifolitoxin is produced in nodules containing strain T24 but because NH_4^+ ions are a product of the nitrogenase enzyme present in effective stains (Strain T24 is non effective) it is feasible that the concentration of ammonium ions or other basic metabolites present in the nodule may regulate the toxicity of trifolitoxin produced. The observed inhibition reversal of trifolitoxin by strongly basic compounds (Schwinghamer and Belkengren 1968) may work through this reversible cleavage of thiazolines. Alternatively the thiazoline component of trifolitoxin may be involved in the reversible covalent binding of this toxin to the site of action, other mechanisms cannot be excluded.

Plant defence mechanisms are induced in the presence of reduced glutathione (Wingate *et al.*, 1988) or by infection by an incompatible rhizobium strain (Keen and Staskawicz 1988) Hence it is possible that bacteriocins containing glutathione like structures (thiazolines) (Calvin 1954) released by rhizobia in the root hairs of the leguminous plant could elicit a plant response detrimental to the nodulation process of competing strains.

Further experiments will need to be conducted to establish the importance of the thiazoline ring opening or structure on the regulation of the biological role of trifolitoxin in the sensitive rhizobia, soil, rhizosphere and nodules of leguminous plants.

Biochemical consequences of the hydrolysis of the thiazoline

The finding (Table 4.2.1) that hydrolysis of the thiazoline ring markedly diminishes activity (Figure 4.2.1) but does not completely eliminate activity indicates that the thiazoline ring is not absolutely essential to toxicity. Since this structure appears to be the most labile component of trifolitoxin the consequences of this hydrolysis may be important. By contrast hydrolysis of the thiazoline ring of bacitracin eliminates activity completely (Goldberg 1959). The thiazoline in trifolitoxin could conceivably stabilize a structural conformation necessary for toxicity or uptake which may be found only transiently in either the cysteine or cysteic acid form of trifolitoxin. The oxidation of this thiol to the disulfide and consequent dimerization of trifolitoxin results in a large reduction in specific activity and early elution by gel filtration columns. As Figure 4.2.5 shows complete oxidation of the disulfide to the sulphonic acid derivative restores some activity and has been a key feature in simplifying the current structural studies. Disulfide formation may prevent the correct folding of the molecule or somehow sterically limit the toxicity of the molecule at the receptor site.



Because the bioassay is dependent on the diffusion of the toxic component in the agar, part of the reduced zone size could be also be the consequence of reduced diffusion. The observed toxicity of the dimeric form of trifolitoxin may also be due to partial *in vivo* reduction or oxidation to the active monomer form.

In summary hydrolysis of the thiazoline ring and its subsequent oxidation products now provide a rational explanation for the initially puzzling multiplicity of biological active forms in the crude spent media from strain T24.

CHAPTER 4.3
THE BLUE FLUORESCENT CHROMOPHORE

RESULTS

This section investigates the nature of the unusual blue fluorescent chromophore linked to the peptide of trifolitoxin. Strong acid (5N HCl) or strong base (2N NaOH) hydrolysis failed to yield detectable UV absorbing material. This blue fluorescent chromophore modifies the chemical shifts of the N.M.R of the protons of arginine and the third glycine residue. Direct linkage to these amino acids has been confirmed during the sequencing experiments.

Properties of the partially hydrogenated chromophore arginine conjugate

Complete acid hydrolysis of partially hydrogenated trifolitoxin sulphonic acid allowed recovery of a guanidine positive ninhydrin negative blue fluorescent component. The arginine chromophore conjugate shows a UV maxima at 316 nm. (pH 7.0) which shifts to 335 nm. after acidification (0.1 N HCl) (Figure 4.3.1). The reduction hydrolysis product also shows a strong infrared absorption at 1600 cm^{-1} (Figure 4.3.2).

The comparison of the proton N.M.R. spectra (Figure 4.3.3) with arginine indicates that whilst the chemical shifts for the delta (3.0 ppm) and gamma (1.8 ppm) protons are unchanged the alpha and beta protons are either missing or displaced. The additional protons at 1.1 ppm. 2.1 ppm.and 2.5 ppm. must represent the protons added as a consequence of the reduction of the chromophore. Arginase failed to convert the hydrogenated arginine chromophore conjugate to the ornithine derivative suggesting that arginine plus the free alpha carboxylic acid required for arginase activity (Barman 1969) is absent.

Weak acid hydrolysis of trifolitoxin sulphonic acid

Formic acid hydrolysis (90 % 110°C 18 hrs) also gives a blue fluorescent component (yield 5% based on UV) with a UV maxima at 320 nm Figure 4.3.4b). A pH mobility profile of this product shows an apparent pK near 6 (Figure 4.3.4a). This product is always cationic indicating that a very strong base is also a component of this product. Other unidentified yellow fluorescent products were also present.(RM_{OG} -0.47 (UV λ max 288, approx. 360) and RM_{OG} 0.11) (Figure 4.3.5). Lower formic acid concentrations or reduced times did not increase the recovery of these fragments. Two dimensional HVPE/PC (Figure 4.3.5) indicated that this blue fluorescent compound has similar Rf (coincident with valine) to the reduced chromophore arginine conjugate described above, although the formic acid hydrolysis products were slightly more anionic with RMOG -0.75 compared to RMOG -0.91 by HVPE at pH 1.7 for the hydrogenation product. Due to the limitations of sample size no satisfactory structural explanation for these degradation products can be advanced at this stage.

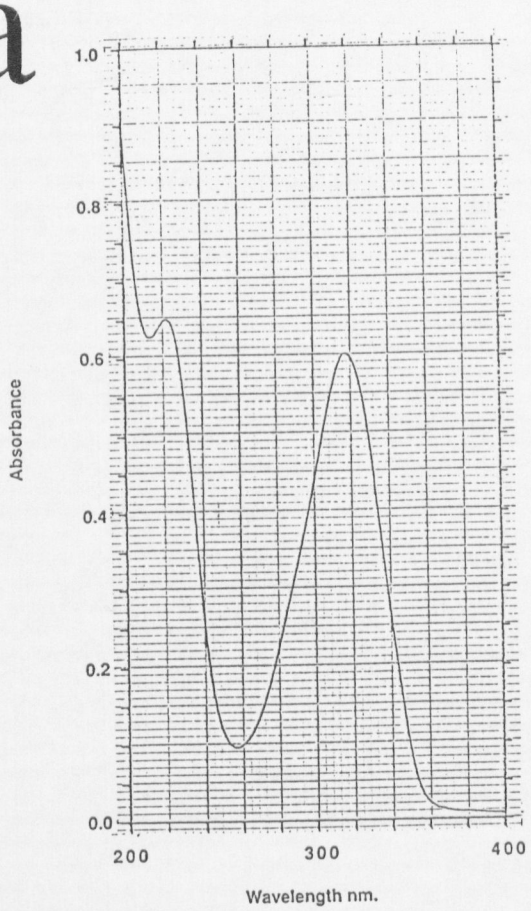
FIGURE 4.3.1

UV spectrum of guanidine positive blue fluorescent product from complete hydrolyses of partially hydrogenated trifolitoxin sulphonic acid, isolated by HVPE pH 9.2 and detected by blue fluorescence.

a :_____ pH 7.0 (λ max 316 nm.)

b:---------- pH 1.0 (λ max 335 nm.)
_____ pH 13.0 (λ max 316 nm.)

a


1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
200
300
400
Wavelength nm.

b

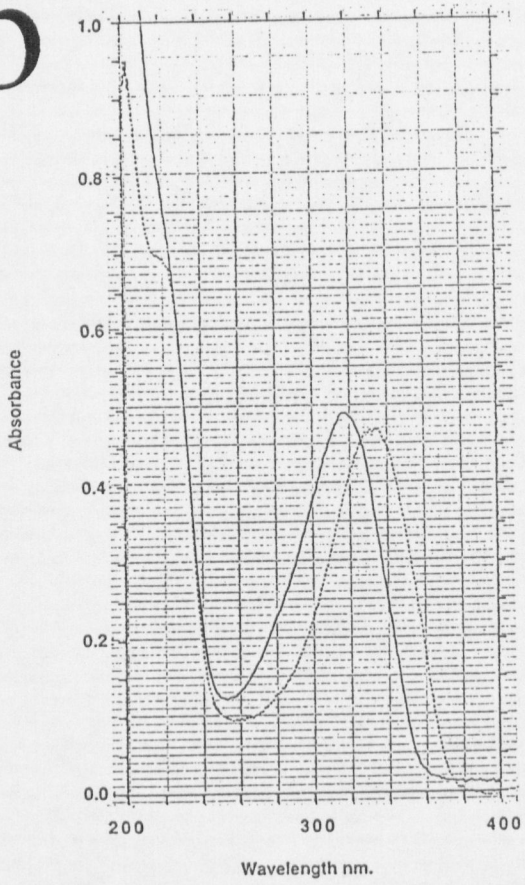

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
200
300
400
Wavelength nm.

FIGURE 4.3.2

Infrared spectra of the guanidine positive blue fluorescent component from a completely hydrolysed ,hydrogenated trifolitoxin sulphonic acid.

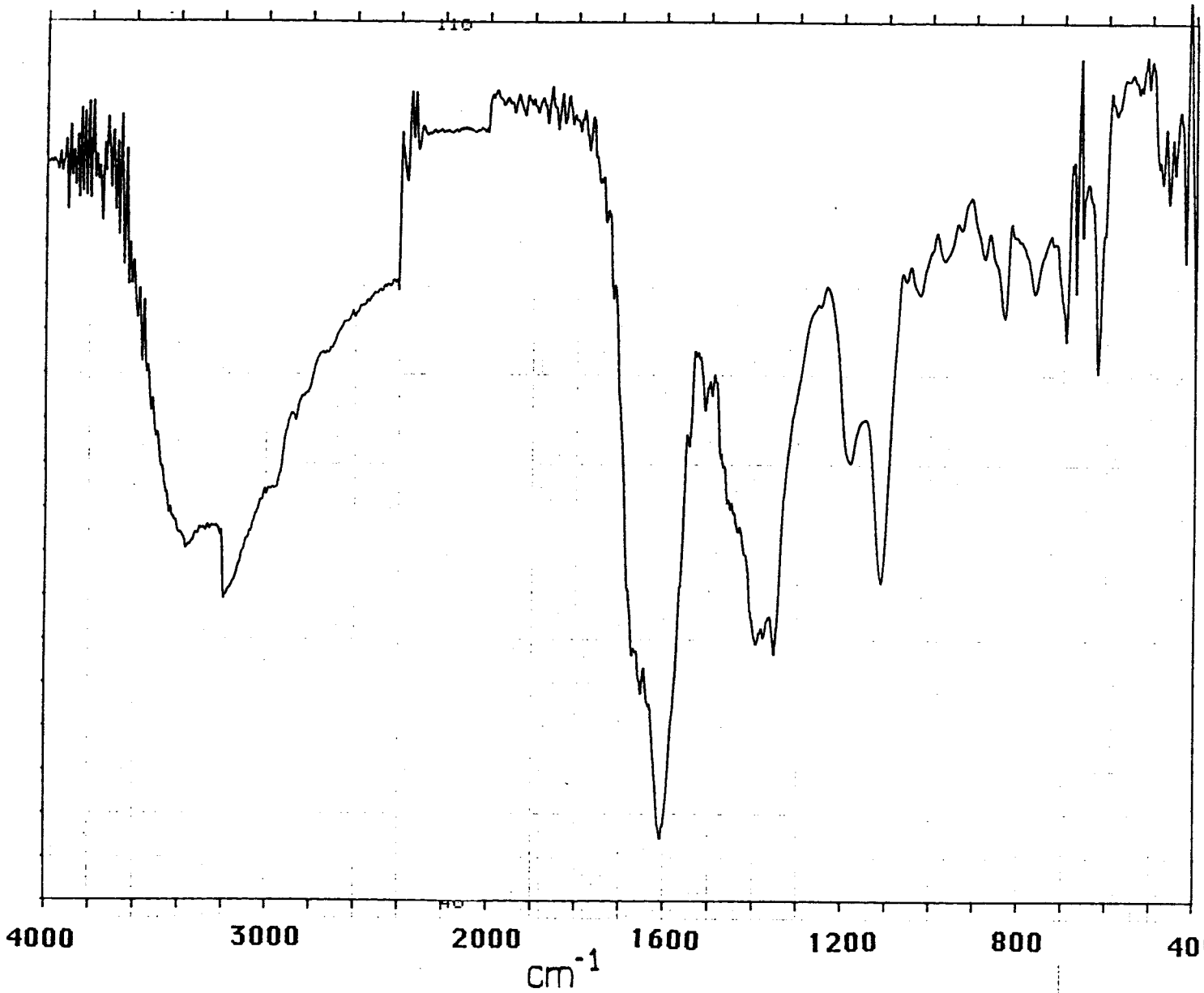

4000
3000
2000
1600
1200
800
400
cm-1

FIGURE 4.3.3

Proton N.M.R. of the Guanidine positive blue fluorescent component from completely hydrolysed , hydrogenated trifolitoxin sulphonic acid.

INTEGRAL
PPM
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
6.624
8.332
12.934
14.023
7.211
2.1214
3.0931
2.4011
2.4411
2.1541
1.6144
1.2571
1.1761
BLUE FUU SAMPLE 1

FIGURE 4.3.5

Two dimensional HVPE/PC of a 90% Formic acid hydrolysis (18 hrs. 110° C) of trifolitoxin sulphonic acid.
B represents the blue fluorescent component with pK 6 of figure 4.3.4 . Y represents yellow fluorescent material.The absence of serine in the hydrolysates can be attributed to its ready N formylation , but the absence of arginine signifies its continued conjugation to the chromophore . Cysteic acid is present in these hydrolysates but is not shown on this cationic portion of the HVPE/ PC

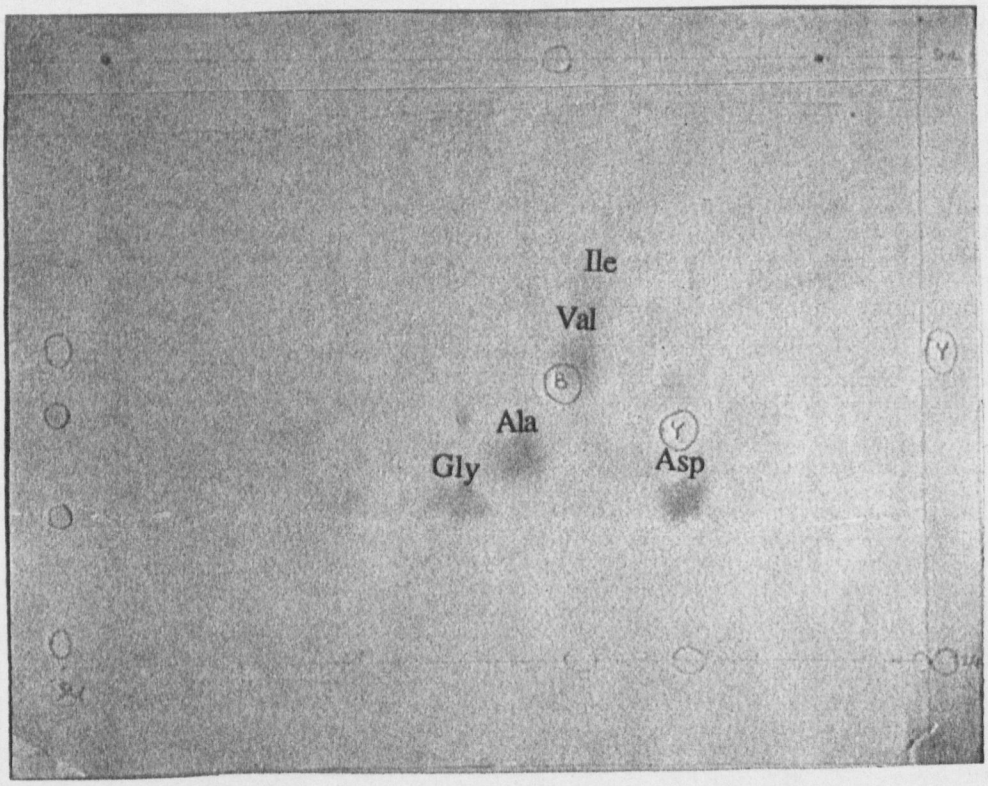

Ile
Val
Ala
Gly
Asp

FIGURE 4.3.4

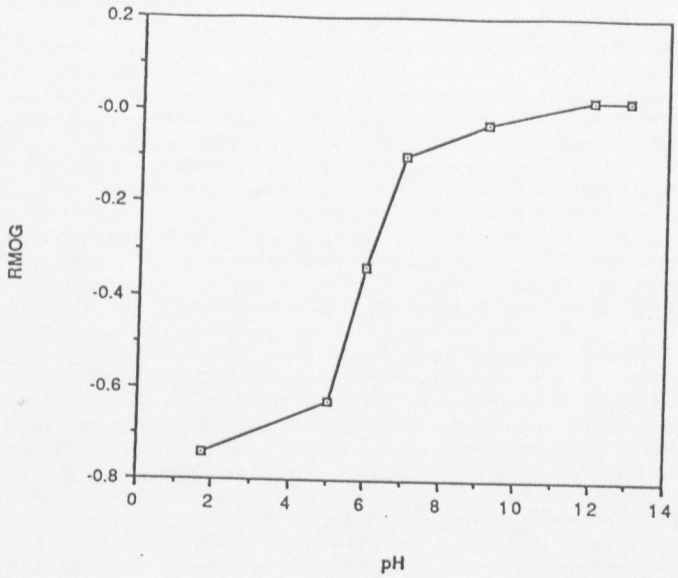
Formic acid hydrolysis of trifolitoxin sulphonic acid yields an incompletely characterized blue fluorescent fragment with pK of approximately 6.

a: pH mobility profile by HVPE of a blue fluorescent component isolated from 90% formic acid hydrolysis (110 degrees C ,18 hrs ,under N2) of trifolitoxin sulphonic acid.

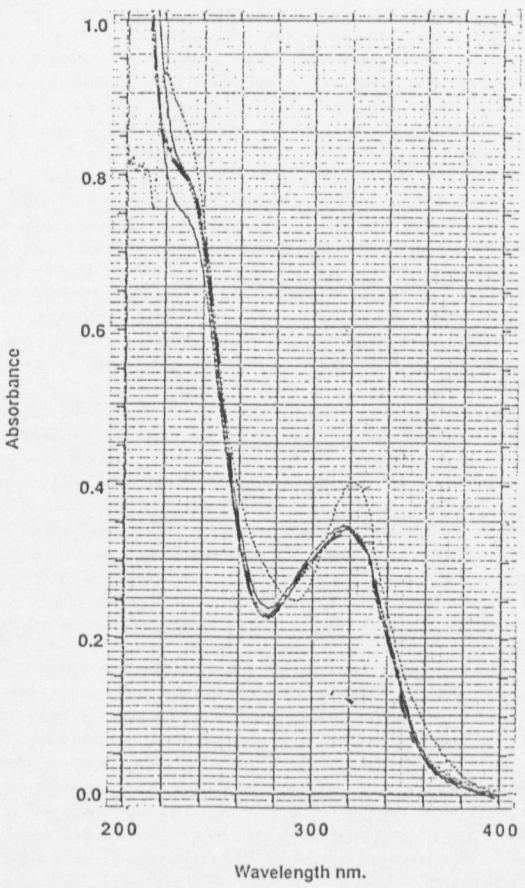
b: UV spectrum of the blue fluoresecent component as a function of pH

-·-·-·- pH 1.0 (λ^{max} 320 nm.)
_____ pH 7.0 (λ^{max} 320 nm.)
--------- pH 13.0 (λ^{max} 320 nm.)

a


0.2
-0.0
-0.2
-0.4
-0.6
-0.8
RMOG
0
2
4
6
8
10
12
14
pH

b


1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
200
300
400
Wavelength nm.

Weak base hydrolysis of trifolitoxin sulphonic acid

An ammonolysis time course of trifolitoxin sulphonic acid (3M NH3 110°C, 0 to 3 hrs.) (Figure 4.3.6) modifies the UV spectrum of trifolitoxin sulphonic acid giving rise to new peaks at 360 and 280 nm. the longer wavelength peak shifting to 380 nm. on acidification (pH 1.0) with an irreversible time dependent loss in intensity at low pH. (Figure 4.3.6). This chromophore could not be extracted into organic solvents at any pH or isolated from HVPEs. Again the paucity of the sample recovery has provided no satifactory data.

Spontaneous hydrolysis products

The fragments of trifolitoxin sulphonic acid fortuitously obtained from an aged N.M.R. sample has provided important structural insights into the T24 structure. These are presented in Table 4.1.4, Figure 4.1.20 and Figure 4.1.21. From the FAB mass spectral data in Table 4.3.1 the difference in the molecular weights of T24SO3H (component 2, MW 1103) and component 3, MW 1121, suggests that the conversion of the blue fluorescent chromophore to the UV absorbing component is the result of an intramolecular hydration of the chromophore which does not alter the amino acid composition of the the peptide.(Figure 4.1.23). From the amino acid composition of components 4 and 5 (Figure 4.1.23) it can be deduced that hydrolysis of the arginine chromophore linkage cleaves the molecule in two to give a non UV absorbing peptide with MW 603.8 consistent with a simple linear peptide and a yellow fluorescent strongly anionic component with MW 536.1. The UV spectra of which shows striking similarity to the UV chromophore detected in the ammonolysis of trifolitoxin sulphonic acid.(Figure 4.1.20).

Table 4.3.1

Component	M+H	M-H	MW
1.T24	1038		1037
2.T24SO3H	1104.3	1102.3	1103.3
3.partly degraded T24SO3H	1122		1121
4.N terminus hexa peptide	604.8		603.8
5.X + C terminus tetra peptide		535.1	536.1
Calc. peptide Mass ,formula		Difference	
1. 915.4 , $C_{36}H_{61}N_{13}O_{13}S$		121.6	
2. 981.4, $C_{36}H_{63}N_{13}O_{17}S$		121.9	
3. 981.4 , $C_{36}H_{63}N_{13}O_{17}S$		139.8	
4. 603.1 , $C_{23}H_{41}N_9O_{10}$		0.7	
5. 396.1, $C_{13}H_{24}N_4O_8S$		140.0	

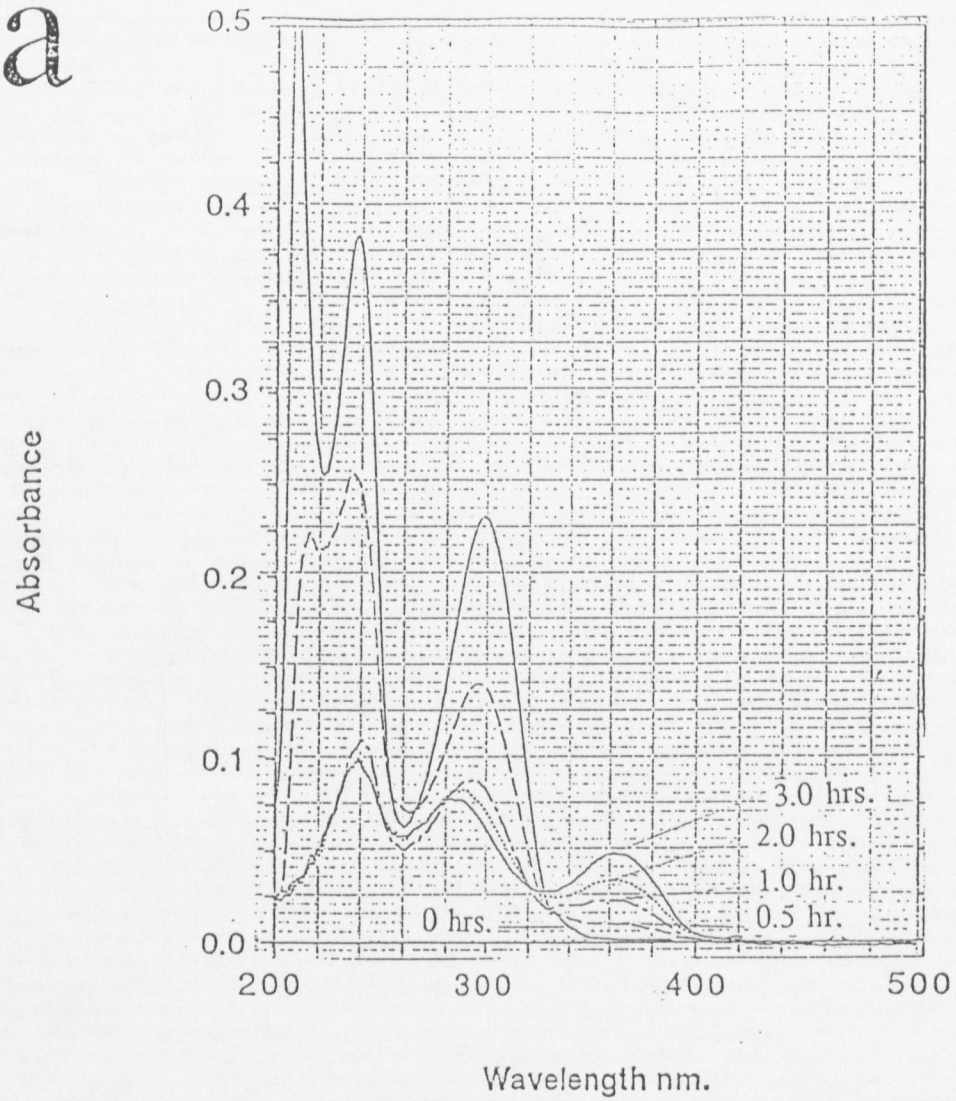
FIGURE 4.3.6

Time course ammonolysis of trifolitoxin sulphonic acid .

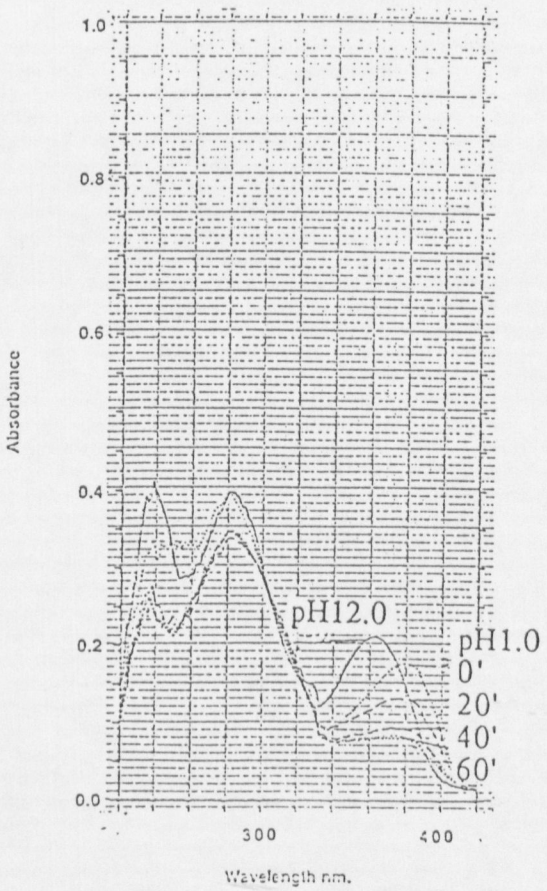
a: Trifolitoxin sulphonic acid (022 ODs 302 nm.) was hydrolysed in the presence of 3M NH_4OH(100 microlitres)(110 degrees C ,under N_2, for the times indicated)
UV spectra were recorded in 0.3 M NH_4OH. Note the increase in the 360 nm. peak with time.

b: pH dependence of the UV spectrum of trifolitoxin sulphonic acid after 3 hrs. ammonolysis (as above).Note the diminution of the 380 nm. peak at pH 1 with time.

a


0.5
0.4
0.3
0.2
0.1
0.0
Absorbance
200
300
400
500
Wavelength nm.
3.0 hrs.
2.0 hrs.
1.0 hr.
0.5 hr.
0 hrs.

b


1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
300
400
Wavelength nm.
pH12.0
pH1.0
0'
20'
40'
60'

Estimate of size

After subtraction of the sum total of the appropriate amino acid residues plus 18 for a linear peptide or zero for a peptide containing a cyclic structure then residual values of 122 for T24 or $T24SO_3H$ are obtained for the chromophore (Table 4.3.1). From degraded $T24SO_3H$ sample with a modified chromophore (component 3 Table 4.3.1) and for the yellow fluorescent chromophore from the fortuitous cleavage fragment containing X, gly, $cysO_3H$, val and ala (component 5 Table 4.3.1) a residue value of 140 daltons is obtained indicating that the chromophore has been hydrated in these components. These data indicate that the "free" chromophore has an integer mass of 158 (140 + H_20) and incorporation into T24 or $T24SO_3H$ involves loss of two molecules of water.

Table 4.3.2

Table of identifiable features of the chromophore residue in trifolitoxin sulphonic acid.

1. Acidic pK 5.1
2. UV spectrum pK 5.1,λ max pH 7.0, 239 nm., 302 nm. ($\varepsilon^{302\ nm}$ =5000)
3. Proton N.M.R. ethylenic proton 5.8 ppm
4. ^{13}C N.M.R. 81 ppm, 112 ppm , plus unassigned carbonyls
5. FAB MS Residue Molecular Weight 122

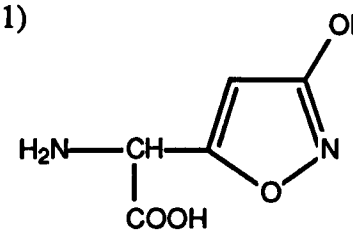
Formula.

In the absence of high resolution mass spectrometric data, the even integer molecular weight of 158 for the "free" chromophore indicates either zero or an even integer number of nitrogen atoms. Because the nett contribution of the chromophore to the charge properties of T24 or fragments is negative, the nitrogens must be non basic entities in the range pH 1.7 to pH 9.2. For a residue of 140 daltons the non nitrogenous formulae and double bond equivalents in brackets may be calculated (Williams and Fleming 1987), C_5O_5 (6), $C_6H_4O_4$ (5), $C_7H_8O_3$ (4), $C_8H_{12}O_3$ (3), $C_9H_{16}O$ (2), $C_{10}H_4O$ (9), $C_{11}H_8$ (8) $C_{10}H_{20}$(1). The corresponding dinitrogen formulae are $C_5H_4N_2O_3$ (5) , $C_6H_8N_2O_2$(4), $C_7H_{12}N_2O$(3), $C_8H_{16}N_2$(2) $C_9H_4N_2$(9) and tetranitrogen $C_4H_4N_4O_2$ (5),$C_5H_8N_4O$(4), $C_6H_{12}N_4$(3). Consideration of the N.M.R data indicates that both the non assignable carbons (carbonyls excluded) and non exchangeable proton are limiting this implies that a structure containing an even number (non zero) of nitrogens would be favoured. Similarly the long wavelength 300 nm.UV spectrum of trifolitoxin would indicate that the level of conjugation should not be less than 3 and consequently the double bond equivalents cannot be less than 3.

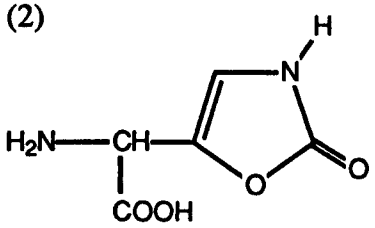
Applying these constraints restricts the residue formula to either $C_6H_4O_4$ or $C_5H_4N_2O_3$, with the nitrogen structure favoured.The fact that the chromophore lies between arginine and glycine in what is otherwise a normal peptide sequence argues for the presence of

an unusual amino acid with a formula of $C_5H_4N_2O_3$. Two known non protein amino acids ibotenic acid (1) and muscazone (2) possess this formula .

(1)



(2)



One of the reported pKa's for ibotenic acid of 5.04 (Eugster 1969) for the enol and single ethylenic proton at 6.2 ppm are in reasonable agreement with the properties of the T24 chromophore. The ibotenic U.V. spectrum at 240 nm. is not consistent with the properties of the T24 chromophore, but if conjugation of the isoxazole ring system is extended by formation of an oxazole ring system involving an exocyclic double bond comprising a C1 and C2 of arginine as shown on page 46 ,then the residue formula of 122 is satisfied, the absence of an alpha proton of arginine is explained and although no valid calculation using Woodwards (Williams and Fleming 1987) or Scotts (Scott 1964) formula seems appropriate it is reasonable to assume that the observed chromophore at 300 nm. is not to be unexpected.

DISCUSSION

The linkage of the blue fluorescent chromophore to arginine is stabilized by hydrogenation, this facilitates the recovery of a component from acid hydrolysates containing the reduced chromophore arginine conjugate. The nature of the stabilization is unknown , but the reduction of a double bond to the alpha carbon of arginine would represent a good explanation of the observed stabilization of the chromophore arginine conjugate and the observed racemization of arginine obtained from complete acid hydrolysates of trifolitoxin sulphonic acid.

The reduction of the chromophore eliminates an anionic pK of 5.1 detected by HVPE, a similar pK as detected by spectrophotometric means for the UV chromophore.The exact nature of the reduction will become apparent with FAB MS analysis of this fragment.(currently under investigation).

Mild acid hydrolysis of trifolitoxin sulphonic acid using 90% formic acid gives a blue fluorescent product which is always cationic or neutral within the pH range of 1.7 to 12 with a pKa of approximately 6. This pKa is consistent with the ionization of the chromophore detected by photospectrometric means on intact trifolitoxin.(pK 5.1). To account for the mobility profile a strong base must also be present. The guanidine group of arginine would be a suitable candidate and would be consistent with the conclusion that arginine is linked to the chromophore, a product isolated by the hydrogenation and hydrolysis of trifolitoxin sulphonic

acid. The formic acid hydrolysis product also shows a similar UV spectral maxima to the arginine chromophore conjugate hydrogenation product of 320 nm at neutral pH. Undoubtedly these spectra of these fragments will be of value in correctly identifying the chromophore, but in the absence of a suitable model they must remain unexplained.

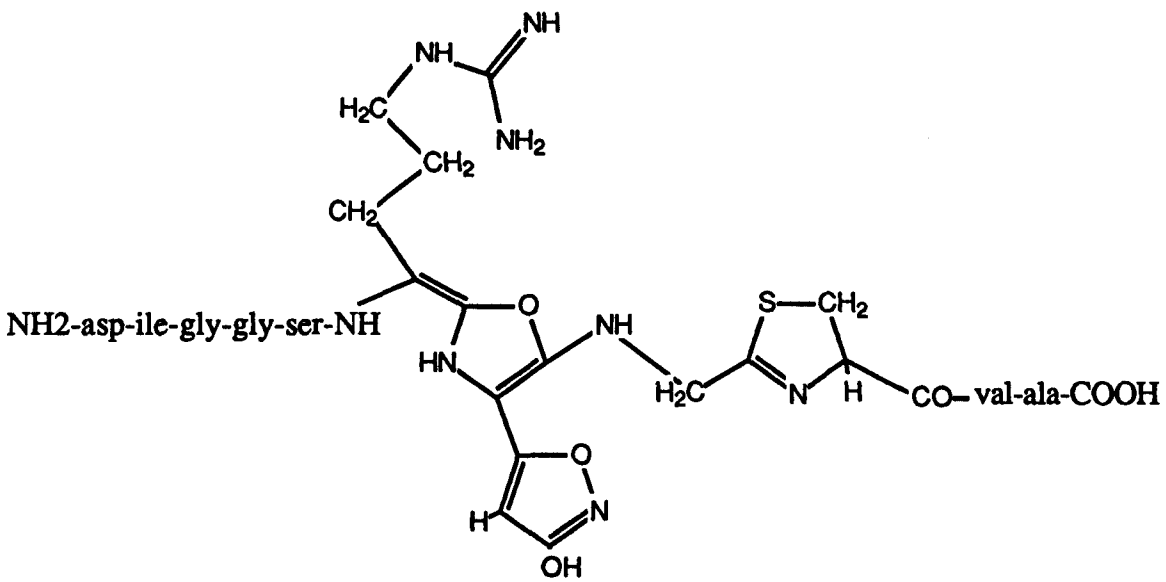
A major problem of the isolation of the chromophore component is the instability of this component to acid or base hydrolysis. Isolation of the arg-X-gly-cysO3H fragment utilizing protease digestion, currently in progress, will allow the simplification of the complex regions of the N.M.R. spectra to be more thoroughly explored i.e. the carbonyl region of the ^{13}C spectra. The amide proton region of the NH coupled proton NMR will similarly become simplified.(J Sims personal communication). This sort of data should allow us to decide more clearly the type of linkage of the chromophore to the peptide. Any extra protons or carbons that can be unambiguously assigned will allow a better understanding of the chromophore. With FABMS added to the arsenal these protease fragments, spontaneous hydrolysis fragments and hydrogenation products should reveal meaningful fragmentation data, and allow a better definition of a model chromophore in terms of molecular formula and weight.

The current evidence still does not allow an unequivocal structure for the chromophore to be obtained. Searches for relevant chromophores based on UV properties initially centred on benzene, pyridine, pyrone and pyrimidine compounds but these types of chromophores have been ruled out because of a lack of supportive data. Some dinitrogen compounds such as the pyrimidine, pyridazine, pyrazine (Silverstein and Bassler 1963) bases show a molecular weight or UV spectra consistent with some pieces of data already available but their charge properties are not in accord with the current data for trifolitoxin sulphonic acid. Dihydro-orotic acid, a pyrimidine, has a molecular weight of 158 consistent with the molecular weight calculated for one of the hydrated products of the chromophore, this model compound yields aspartic acid on hydrolysis, but only one mole of aspartate has been detected in hydrolysates of trifolitoxin and Edman degradation data clearly points to the presence of aspartic acid as the N terminal amino acid.

In the absence of the afore mentioned data the best model currently available is ibotenic acid (Eugster 1969) linked in an unusual way the to arginine and the third glycine residue. Proton , ^{13}C N.M.R. and a calculated molecular weight of 158 would be most consistent with a hypothesis for a linkage between glycine and the C1-C2 enolic form of arginine. The exact form of bonding is uncertain but presumably would give rise to the unique chromophore, ibotenic acid showing a λ max at 240 nm.(Eugster 1969). The enolic amide group of ibotenic acid shows an acidic pKa near 5.04 consistent with acidic pKa of 5.1 detected for the trifolitoxin chromophore. There would be two dehydrations to reduce the molecular weight from 158 d.to 122 d.in T24SO3H or one dehydration to reduce it to 140 d. for the yellow fluorescent anionic spontaneous hydrolysis product. The methine carbon at 81.285 ppm is

probably the ethylenic C4 carbon and the quaternary carbon at 112.074 ppm would be the C3 carbon, the remainder of the carbons masked in the carbonyl region of the spectrum.

The ibotenic acid model is currently awaiting the arrival of an authentic reference sample of ibotenic acid for comparison. The model presented below summarizes the best fit to the currently available data.



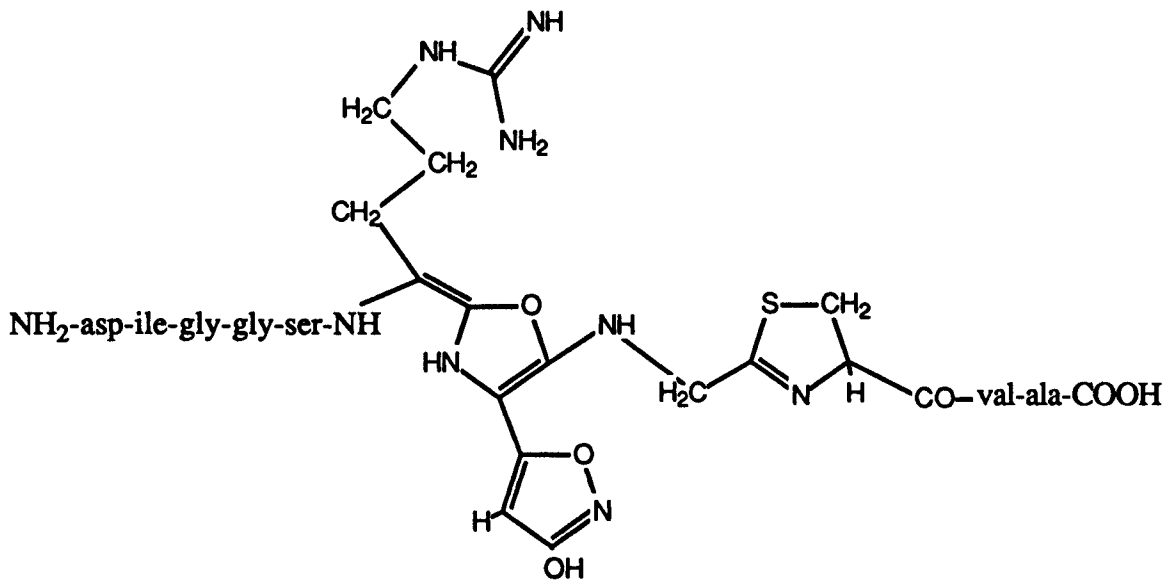
CHAPTER 5
GENERAL DISCUSSION

Structure

Trifolitoxin, the strain T24 bacteriocin, is the first bacteriocin in rhizobia to be investigated in substantial detail. Prior to this study bacteriocins from rhizobia had been classed only in the most general terms, most of these being high molecular weight proteins, with one or two exceptions (Hirsch 1979, Van Brussel *et al* .1985). Our starting hypothesis was based on the observed 260 nm./280 nm. ratio of the T24 bacteriocin (Schwinghamer and Belkengren 1968) suggesting that this low molecular weight rhizobial bacteriocin was akin to the nucleotide bacteriocins from agrobacteria (Kerr and Tate 1984). The work presented in this thesis indicates that the original U.V. data and consequently the hypothesis derived from it are incorrect. Nevertheless the general peptide nature of trifolitoxin as originally proposed by Schwinghamer and Belkengren (1968) has been confirmed.

The current proposed structure of trifolitoxin is a linear peptide with the sequence asp-ile-gly-gly-ser-(arg-X-gly)-cys-val-ala. The cysteine residue is present as a thiazoline ring system and the arginine and third glycine residues are linked to a blue fluorescent chromophore (X), this chromophore has been characterized but the structure has not yet been determined. This blue fluorescent chromophore appears to be linked within the peptide chain. The thiazoline formation may be facilitated by the close proximity of the arginine-chromophore-glycine complex, because it is known that unusual conditions are required for the formation of thiazolines (Jocelyn 1972). The current model of trifolitoxin is presented below. The model chromophoric group is based on ibotenic acid (Eugster 1969) linked into the peptide in an unusual way.

Schwinghamer's and Belkengren's (1968) conclusion that trifolitoxin belongs to the class of polar peptide antibiotics containing bacitracin is certainly appropriate and their use of bacitracin as a standard for comparison with trifolitoxin was most fortuitous. The pH experiments presented by Schwinghamer and Belkengren (1968) indicated an acid labile group and the present study has now established the importance of the thiazoline structure in trifolitoxin which is responsible for the acid lability.



Current model of trifolitoxin.

The evidence presented here indicates that trifolitoxin is linear (excluding the thiazoline and the nature of the linkage of the blue fluorescent chromophore to the peptide) whereas bacitracin is a branched cyclic peptide. Bacitracin consists entirely of alpha amino acids or structures derived directly from amino acids. The model trifolitoxin contains a blue fluorescent chromophore derived from a non protein amino acid. The blue fluorescence of an non protein component of trifolitoxin is similar to etamycin a peptide antibiotic containing a pyridine nucleus linked to the N terminal of the peptide chain (Arnold and Johnson *et al.*, 1958, Eastwood and Snell *et al.*, 1958).

Suggestions for the future study of the biochemical mode of action.

Schwinghamer and Belkengren (1968) observed clumping and cell distortion of sensitive rhizobial cells when treated with trifolitoxin and suggested a possible surface disruptive effect on the cell wall. If the analogous structures of trifolitoxin and bacitracin indicate a similar mode of action then Schwinghamer and Belkengren (1968) suggestion is quite a reasonable one. Bacitracin inhibits peptidoglycan synthesis, a major component of cell walls. Specifically the dephosphorylation of C55 isoprenoid pyrophosphate is inhibited. C55 isoprenoid phosphate acts as a lipid carrier on reaction with UDP Mur-N-Ac pentapeptide. The nett result is the accumulation of uridine nucleotide derivatives in the cell. Uridine nucleotide derivative accumulation appears to be a common feature of many cell wall synthesis antibiotics (eg. bacitracin, penicillin, ristocetin, vancomycin, cephalosporin, novobiocin) (Gale 1972) and would represent a plausible starting point in the analysis of the mode of action of trifolitoxin.

Schwinghamer (1967) found an association between resistance to cell wall synthesis antibiotics and partial or full loss of effectiveness in about one half of the mutants tested .Schwinghamer (1967) also postulated an alteration of cell wall characteristics being responsible for the decrease of effectiveness. Such results may have relevance to the self

immunity and modified colony morphology of strain T24 (Schwinghamer and Belkengren 1968).

Ibotenic acid is a toxic naturally occurring amino acid in the *Amanita* species.(Eugster 1969). Agrocin 84 contains a fraudulent toxic nucleoside at its core (Kerr and Tate 1984), and the model trifolitoxin contains a toxic amino acid at its core. It will be of interest to see whether whether toxic fraudulent moieties in bacteriocins holds true across species.

The proposed scheme, as presented above makes no account of the specificity of trifolitoxin. The specific recognition of trifolitoxin for fast growing rhizobia must be reflected in some structural component which mimics some naturally occurring process. Bacteriocins are highly specific agents, the bacteriocin Colicin (a class of coliform bacteriocins) and Agrocin 84 (an agrobacterial bacteriocin) each require specific absorption to membrane or periplasmic proteins respectively for specificity. The *in vivo* proteolytic cleavage of Colicin E3 allowed the definition of the functionality of this molecule. In this case the C terminal peptide showed toxicity and the N terminal peptide bound to cell wall receptors (Watson and Sherratt 1979). Agrocin 84 recognition is based on a structural similarity to the agrocinopine opines which are actively transported into the cell (Murphy 1981). It will be of interest to see if agrobacterial cells not originally sensitive to trifolitoxin when transconjugated with nodulation and/or N2 fixation plasmids (Martinez *et al.*, 1987) become sensitive to trifolitoxin.

The current work does not allow the structural component of trifolitoxin required for recognition by rhizobia to be identified, whether the blue fluorescent chromophore or the peptide or a synergistic effect between both. Techniques are presented in this thesis for analysing the N terminal and C terminal peptides, as well as the role of the thiazoline and chromophore structures in determining the selectivity of trifolitoxin for fast growing rhizobia. Radiolabelling of appropriate parts of the molecule for uptake studies as has been achieved for agrocin 84 (Murphy 1981) should help resolve this question. Identification of this recognition point in the structure of trifolitoxin will give us a unique feature of rhizobial metabolism which may be exploited in the understanding of the rhizobia bacteria.

The cloning of trifolitoxin genes has been successfully completed (Triplett 1988, Triplett *et al.*, 1989), these results coupled with the knowledge of the structure of trifolitoxin should lead to an exciting stage in the understanding and application to the ecology of *Rhizobium leguminosarum* biovar *trifolii* strain T24 and its use as a biological control agent (Vidaver 1976,Vidaver 1983, Weller 1988).

APPENDIX 1
MEDIA

TY Media

	g/litre
tryptone	0.5
yeast extact	0.3
$CaCl_2$	0.1

MMG Media

	g/litre	
$(NH_4)_2SO_4$	1.0	
sodium glutamate	1.0	
tri sodium citrate	0.5	
K_2HPO_4	1.74	
KH_2PO_4	5.45	
$Fe(NO_3)_3.9H_2O$	5 mg	
biotin	0.2	
thiamine	10mg	
glucose	2.0	(added after autoclaving)
$MgSO_4$	0.2	(added after autoclaving)
Doys trace elements	1 ml.	

pH 6.3

MMG2 Media

MMG2 Media is derived from MMG , it is essentially a half salts reduced phosphate media plus CaCl2.

	g/ litre	
$(NH_4)_2SO_4$	0.5	
sodium glutamate	0.5	
tri sodium citrate	0.25	
$K_2 HPO_4$	1.05	
KH_2PO_4	0.45	
$Fe(NO_3)_3.9H_2O$	5 mg	
Biotin	0.2 mg	
Thiamine	10mg	
glucose	2.0	(added after autoclaving)
$MgSO_4$	0.2	(added after autoclaving)
Doys trace elements	1ml.	
$CaCl_2$	0.1g	(added after autoclaving)

pH 6.8

MMG low sulphate media

MMG low sulphate media is MMG minus ammonium sulphate and $MgSO_4$ is substituted with $MgCl_2$

	g/litre	
sodium glutamate	1.0	
trisodium citrate	0.5	
K_2HPO_4	1.74	
KH_2PO_4	5.45	
$Fe(NO_3)_3.9H_2O$	5mg	
Biotin	0.2mg	
Thiamine	10 mg	
Glucose	2.0	(added after autoclaving)

$MgCl_2$	0.2	(added after autoclaving)
Doys trace elements	1ml	
$CaCl_2$	0.1 g	(added after autoclaving)

pH 6.3

Solid media contained 1.5 g agar per litre.

Soft buffered agar.

	g/ 200 mls
$Na_2HPO_4.12H_2O$	1.43
$NaH_2PO_4.2H_2O$	2.5
Agar	1.6

APPENDIX 2

HVPE Buffers

	pH	
Formic acetic buffer	1.7	56.8 mls.formic acid/ 118.4 mls. acetic acid per 2 litres
Citrate	2.2-6.5	50 mM citric acid adjusted to the required pH with solid NaOH
Ammonium Bicarbonate	9.2	32.8 g (NH_4) HCO_3 per 2litres adjusted to pH 9.2 with 30% ammonia
Ammonium Borate	9.2	0.2 M boric acid adjusted to pH 9.2 with 30 % ammonia
Na bicarbonate	10.0	0.1 M Na HCO_3 adjusted to pH 10.0 with NaOH
Phosphate	11.0-12.0	50 mM Na_2HPO_4 adjusted to the required pH with NaOH

Enzyme buffers

Phosphate	7.0	50 mM NaH_2PO_4 adjusted to pH 7.0 with NaOH (Protease, trypsin)
Pyridine acetate	5.5	(Papain)(Smith 1969)
ammonium bicarbonate	7.8	0.1 M NH_4HCO_3 (Protease,trypsin ,carboxypeptidase, aminopeptidase)(Smith 1969)
Phosphate	9.7	50 mM Na_2HP04 (arginase)(Greenstein and Winitz 1961)

APPENDIX 3

Estimation of Rhizobial cell density.

Absorbance at 640 nm was used to estimate the number of rhizobia per ml of culture. The equation is

$$\text{No. of cells} = (0.10 + (22.93 * \text{OD}_{640})) * 10^8$$

This formula was empirically determined to estimate the number of agrobacterial cells per ml, but is valid with rhizobial cells.

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