The identification and synthesis of a histapeptide from honeybee venom
by Merlin Larry Peck

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY Chemistry
Montana State University

Abstract:
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The isolation was effected by means of preparative paper chromatography. The structure of the peptide was determined by means of Edman degradation to be alanylglycylglutaminylglycylhistamine. The assigned structure was confirmed by the synthesis of the above peptide. The synthesis involved the use of the mixed anhydride and azide methods of coupling. The peptide was synthesized by three different routes, each yielding the histamine-containing peptide which was shown by chromatographic means to be identical to the peptide isolated from bee venom. In all three synthetic routes used, several non-peptide contaminants were obtained along with the desired product. The latter was hygroscopic and could not be obtained in a crystalline form.

Even though the product was obtained in small quantities the procedures used illustrate the methodology of synthesis of medium-sized histamine-containing peptides. Previous to this investigation only a few protected amino acids had been coupled with histamine. This investigation represents the first synthesis of a naturally occurring histamine-containing peptide.

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ABSTRACT

The histamine-containing peptide which had previously been reported to be present in honeybee (Apis mellifera) venom has been isolated from venom that was obtained by electrical excitation. The isolation was effected by means of preparative paper chromatography. The structure of the peptide was determined by means of Edman degradation to be alanylglycylglutaminylglycylhistamine. The assigned structure was confirmed by the synthesis of the above peptide. The synthesis involved the use of the mixed anhydride and azide methods of coupling. The peptide was synthesized by three different routes, each yielding the histamine-containing peptide which was shown by chromatographic means to be identical to the peptide isolated from bee venom. In all three synthetic routes used, several non-peptide contaminants were obtained along with the desired product. The latter was hygroscopic and could not be obtained in a crystalline form.

Even though the product was obtained in small quantities the procedures used illustrate the methodology of synthesis of medium-sized histamine-containing peptides. Previous to this investigation only a few protected amino acids had been coupled with histamine. This investigation represents the first synthesis of a naturally occurring histamine-containing peptide.

INTRODUCTION

Physiological Properties of Honeybee Venom. Venoms of stinging insects have been the subject of much superstition and folklore. This probably dates back to the period prior to the first reported death from a wasp sting in 2800 B.C. (1). Consequently, perusal of the literature shows many proposed physiological reactions and medical uses of honeybee (Apis mellifera) stings. Even in the most recent literature one can find many testimonials to its applications; however, sound experimental conditions have not been used to substantiate them. Only in recent years have techniques been available to allow insect venoms to be studied on a scientifically sound basis, and now many components can be isolated, identified structurally, synthesized and tested physiologically.

The best known physiological property of honeybee venom is the sting. The initial reaction is a sharp pricking sensation followed by pain which lasts for a few minutes. Due to the pain produced, many people faithfully believed Langer (2) when he reported the presence of formic acid in 1897. Even though its presence in any appreciable amounts was disproved in 1921 (3) and again in 1924 (4), textbooks of the 1950's were still reporting formic acid as a major constituent of bee venom.

Within a few minutes after the sting, a wheal forms as a small red area at the site of the sting, surrounded by a whitish zone and a

reddish flare. The wheal subsides in a few hours, giving way to irritation, itching and/or a burning feeling. In twenty-four hours there is usually no sign of the sting.

The normal reaction, as above, may be accounted for by the histamine and hyaluronidase (see Table II) content of the venom augmented by the histamine released in the host (5). Although several investigators have reported various amounts of histamine in bee venom (6,7,8,9), it appears that the amount of histamine is between 0.64 and 1.57 percent depending upon the strain of honeybee, its age, and the season. The histamine and 5-hydroxytryptamine (serotonin)-releasing effect of venom has been shown in several investigations (10,11,12), and was believed to be a property of the phospholipase A present. Phospholipase A has also been found in several snake venoms (13). It has since been shown that what was once thought to be a pure fraction phospholipase A from bee venom was in reality a mixture of phospholipase A and a mast cell degranulating peptide (14,15,16). Both compounds can cause the release of histamine: one by converting phospholipid constituents into surface-active, cytolytic agents of the lysolecithin type (see footnote d, Table II) and the other by direct attack of the mast cells. The mast cell degranulating agent is a polypeptide containing twenty-two amino acids (MW=2593) and is believed to be the most toxic component of honeybee venom. However, it constitutes only a small fraction of the venom (see Table II). A third

compound, melittin, is also found in bee venom and has histamine-releasing, as well as "direct" hemolyzing properties, and many other properties which will be discussed later. Hyaluronidase has been isolated from bee venom and has been shown to be unlike the hyaluronidase found in human testes or human synovial cells. Bee venom hyaluronidase is one of at least four compounds in bee venom against which the body can form antibodies (17). People who have a high titer of this antibody, such as beekeepers, do not exhibit the normal response described above, presumably due to reduced spreading of the venom in the tissue (see footnote c, Table II).

In more severe cases, the above effects from a bee sting may be accompanied by constriction of the throat and chest, swelling and itching about the eyes, massive urticaria, sneezing and wheezing, a rapid pulse, a fall in blood pressure and, if the dyspnea becomes severe, cyanosis. If death does not follow within approximately twenty minutes, the symptoms will usually begin to subside and comfort will return in two or three hours except for some urticaria which may persist a day or more.

Death from bee stings may be the result of several effects. The most common effects are asphyxia due to laryngeal edema, heart failure, bronchospasm, pulmonary edema, and the other reactions associated with anaphylactic shock. However, not all severe symptoms are the result of hypersensitive anaphylactic reactions since bee venom shows a toxicity

of approximately 6 mg (18) per kilogram body weight in mice in which hypersensitivity does not exist. Others have reported a toxicity of 0.21 mg per kilogram body weight when the mice were injected intraperitoneally (19).

Known active components which contribute to its toxicity are melittin and apamine, in addition to the previously discussed phospholipase A, hyaluronidase and the mast cell degranulating peptide. Melittin is the major component of bee venom (see Table II) and is responsible for most of its toxicity (i.v. in mice 3-4 mg/Kg) (16). The following effects have been ascribed to melittin. It is hemolytic without enzymatic activity and produces pain and inflammation (20); it disrupts mast cells so they release toxic histamine and liberates toxic 5-hydroxytryptamine from thrombocytes (21); it increases capillary permeability (22); it blocks nerve transmission and contracts the smooth muscle of isolated guinea pig ileum (23); and it uncouples oxidative phosphorylation (24).

Apamine comprises a much smaller percentage of bee venom (see Table II) and is a smaller molecule than melittin. However, it is nearly as toxic as melittin (toxicity 4 mg/Kg) (25). It exhibits two predominant effects: it raises the vascular permeability and produces various motor abnormalities localized in the central nervous system. Apamine is a very basic polypeptide (isoelectric point about pH 12)

and is reported to be the first peptide found to show a clear-cut action on brain functions (15).

Death resulting from bee stings is more frequent than many people realize. For example, in 215 fatalities from venomous animals and insects in the United States as reported by Parrish in 1959 (26), the most deadly creature was the rattlesnake, which accounted for 55 human deaths, but the honeybee was in a strong second place with 52 fatalities. The number of deaths may be much larger than indicated in the above figures since the symptoms of a severe general reaction are not easily recognized even by medical examiners or coroners. Death from the bee sting is often attributed to heart attack, heat stroke, or other causes, since the local reaction to the sting may be overlooked or not assigned any importance by the person signing the death certificate (27).

The most effective emergency treatment for a severe general sting reaction is the administration of a pressor amine such as aromine sulfate, epinephrine, isoproterenol, norepinephrine or phenylephrine during the shock reaction (28). In those cases where a venom hypersensitive condition is known, hyposensitization can be used as a preventative measure, although no completely effective procedure has yet been developed.

Bee venom has been employed as a therapeutic agent against many ailments, particularly chilblains, arthritis, neuritis, and trachoma

(29,30). It has been reported as beneficial against some arthritic conditions, and its use in these cases is common in Europe. It has also been shown that bee venom will reduce the swelling in joints of rats suffering from formaldehyde-induced arthritis (31). Further, statistics reveal that beekeepers have a very low incidence of cancer which may or may not be due to the large number of stings they receive (32). An anti-tumor activity has been observed against colchicine-induced plant tumors (33).

Bacteriostatic and bactericidal properties of bee venom have been shown by Ortel and Markwardt (34). Their results indicate a great variance depending upon the strain of bacteria used.

Shipman (18) and Ginsberg (35) have shown that mice injected with bee venom twenty-four hours before irradiation with a lethal level of x-radiation had a consistently higher survival rate than mice not so injected. This protection may be due to a general stress-like effect or to a specific component of the venom.

Composition of Honeybee Venom. Honeybee venom is a very complex mixture of carbohydrates, lipids, free amino acids, peptides, enzymes, and volatile compounds. Natural venom is approximately 88 percent water (36), and perhaps as many as twelve other volatile compounds are present (37). One of these volatile compounds is isoamyl acetate, a compound which has been shown to be an alarm substance. Isoamyl

acetate will excite bees greatly, but a second, as yet unidentified, factor is necessary to entice them to sting on "advice" of another bee (38).

The non-volatile portion of bee venom consists of approximately 60 percent protein (see Table II) and 40 percent low molecular weight compounds of various classes (see Tables I and II). There is reasonable evidence that fifty-three compounds exist in dry bee venom sac extracts, but sufficient evidence does not exist to confirm their presence in pure venom.

Of the fifty-three compounds listed in Tables I and II, at least twenty have never been obtained in pure form in large enough quantities for structure determination or study of their physiological properties. The four histamine compounds in Table II are the only histamine-containing peptides found thus far to exist in nature, and only a limited study has been performed on synthetic histamine-containing peptides.

Table I

Non-protein and non-peptide compounds found in dry honeybee venom

Compound	% of dry venom	Ref.
AMINO ACIDS	1	(9,39)
α-amino butyric acid	0.038	(9,39)
β-amino isobutyric acid	0.016	(9,39)
alanine	0.064	(9,39)
arginine	0.148	(9,39)
aspartic acid	0.015	(9,39)
cystine	0.012	(9,39)
glutamic acid	0.131	(9,39)
glycine	0.050	(9,39)
histidine	0.105	(9,39)
isoleucine	0.016	(9,39)
leucine	0.021	(9,39)
lysine	0.010	(9,39)
ornithine	0.027	(9,39)
phenylalanine	0.016	(9,39)
proline	0.022	(9,39)
serine	0.016	(9,39)
threonine	0.011	(9,39)
tyrosine	0.010	(9,39)
valine	0.021	(9,39)
FREE BASE		
histamine	1	(6,7,8,9)
SUGARS	2	(9)
fructose[a]	0.9	(9)
glucose[a]	0.5	(9)
LIPIDS	5	(9)
6 lecithin-like compounds[a]	4.3	(39)
2 steroid-like compounds	---	(39,40)

[a]Attempts to repeat these identifications by other students have as yet been unsuccessful.

Table II

Proteins and peptides found in honeybee venom

Compound	% in dry venom	Ref.
SMALL PEPTIDES (14 total)	15	(9)
aspartic acid, alanine	?	(41)
aspartic acid, alanine, proline	?	(41)
glutamic acid, alanine, valine	?	(41)
aspartic acid, valine	?	(41)
glutamic acid, alanine, proline	?	(41)
arginine, glutamic acid, lysine	?	(41)
glutamic acid, lysine, valine	?	(41)
alanylglycylprolylglutaminylhistamine[a]	1	(39)
alanylglycylglutaminylglycylhistamine[b]	1	
2 additional histamine-containing peptides	?	(39,41)
3 unstudied peptides	?	(36)
POLYPEPTIDES AND PROTEINS	60	(36)
apamine	2	(25,42)
melittin	40-50	(20,36)
hyaluronidase[c]	?	(17)
phospholipase A[d]	8-14	(15)
phospholipase B[e]	?	(43)
mast cell degranulating compound	1-2	(16)
at least three other compounds	?	(36)

[a]Originally reported to contain glutamic acid instead of glutamine (9).

[b]The indicated structure is that structure which has been determined by the research reported in this thesis.

[c]Hyaluronidase is an enzyme that depolymerizes hyaluronic acid polymers, which are present in large quantity in the intercellular cementing substance. Thus hyaluronidase can cause cells cemented together to separate.

[d]Phospholipase A is an enzyme capable of hydrolyzing the ester linkage at the β-glycerol carbon on a phospholipid.

[e]Phospholipase B is an enzyme capable of hydrolyzing the α-glycerol ester linkage of a phospholipid. However, in further research students have as yet been unable to confirm the presence of Phospholipase B in honeybee venom.

At present, research in honeybee venom is being actively conducted in two areas. Studies are constantly being conducted on the toxicological and physiological properties, and several studies are being conducted in structural determinations of the major constituents with some attention now being focused on the smaller peptides. It is the latter with which this thesis is concerned. This thesis reports the isolation, identification and synthesis of alanylglycylglutaminylglycylhistamine (see Table II).

Histamine-Containing Peptides and Compounds. In 1943 Rocha e Silva (44), in an effort to explain the inactivity of histamine present in the cell constituents and its release by proteolytic enzymes, suggested that histamine in the cell is bound by a peptide linkage and that histamine bound in a peptide should be physiologically "inactive". In an attempt to form a model of this "bound histamine", he synthesized the first histamine-containing peptides. The compounds which he prepared were N-acetyldehydrophenylalanylhistamine, N-acetyl-D,L-phenylalanylhistamine, N-benzoyl-L-tyrosylhistamine, N-benzyloxycarbonyl-L-tyrosylhistamine, and N-benzyloxycarbonyl-L-leucylhistamine. The peptide bond in the first two compounds was formed by treating histamine with the azlactone of acetylaminocinnamic acid and in the last three by treating the azide of that portion of the molecule containing the carbonyl carbon of the peptide with

histamine. These five compounds showed little or no physiological action on cat blood pressure, isolated guinea pig intestine, intact guinea pigs, and human skin. However, even though these compounds have no (or very little) histamine activity, evidence now indicates that these are not models of the state of histamine in cell constituents. Histamine is now known to be bound electrostatically in the cell rather than by means of a covalent bond (45).

Arold (46) has recently reported the synthesis of N-benzyloxycarbonylglycylhistamine, N-benzyloxycarbonyltyrosylhistamine, N-benzyloxycarbonylvalylhistamine, N-benzyloxycarbonylleucylhistamine and N-benzyloxycarbonylisoleucylhistamine. Although he reported no difficulties in the preparation of these blocked peptides, he reports the elemental analysis for only N-benzyloxycarbonylvalylhistamine. The deblocked peptides were reported to be hygroscopic and therefore were characterized by paper chromatography and paper electrophoresis only.

Much has been written about the physiological properties of non-peptide derivatives of histamine, and it is generally agreed that substituents on the imidazole ring destroy or greatly reduce the histamine activity. Nonetheless, many discrepancies exist in the reported data on N-histamine derivatives. For example, N-methylhistamine has been reported to have one-two hundredth (47) to twice (48) the activity of histamine. Bertaccini and Vitali (49) have

reported that N-methylhistamine closely resembles histamine, while dimethylhistamine has less histamine activity but shows weak "nicotinic" effects (i.e., spasmodic rather than continuous muscle contraction) and that trimethylhistamine has only about 1 percent of the activity of histamine but shows potent "nicotinic" activity.

From studies that have been made on histamine derivatives no sound prediction can be made as to the physiological properties of the histamine-containing peptides found in bee venom since no investigation has yet involved compounds containing both the peptide linkage and a free amino group.

Peptides of this nature found in bee venom may possess one or more of several biological properties such as those associated with histamine, histamine derivatives, melittin, and apamine, or they may possess bactericidal, bacteriostatic, anti-arthritic or anti-carcinogenic properties such as have been attributed to bee venom but not yet to any of its isolated components.

The only apparent practical way of determining the physiological properties of such peptides is to prepare them synthetically so they are available in sufficient quantity for testing.

Since the beginning of this century when Emil Fischer described the synthesis of peptides, investigators have reported the use of various methods of peptide synthesis. The methods devised in the early part of this century had many limitations, and until the 1950's

only peptides containing simple amino acids had been formed synthetically. During the 1950's major breakthroughs were made in the methodology of peptide synthesis with the advent of the mixed anhydride procedures, the active ester methods, new coupling reagents, and new blocking groups. Today one has a rich field of protection and coupling methods from which to choose. Each method has its own limitations (50).

The research presented in this thesis is concerned with the determination of the structure of one of the histamine-containing peptides in honeybee (Apis mellifera) venom and the confirmation of this structure by synthetic means. In doing this, this research adds to the general knowledge of the constitution of honeybee venom, presents methods for the synthesis of histamine-containing peptides, and reveals the difficulties encountered in the synthesis of histamine-containing peptides.

INVESTIGATIVE ASPECTS

Venom Source. The venom of the honeybee (Apis mellifera) used in this research was obtained from a number of sources. The purest form of venom used was obtained by electrical excitation of individual bees (51). The bulk of the venom used was obtained either from John Toenyes, Power, Montana, or Champlain Valley Apiaries, Middlebury, Vermont, (either directly or indirectly from Sigma Chemical Co.) both of whom presumably used a technique similar to that described by Benton, et al. (52).

Venom from the above sources proved satisfactory as a source of the desired peptides only when care was taken to prevent deterioration. Fresh samples were used whenever possible, and the venom was stored at all times in the dry state at 4°C.

Isolation. The histamine-containing peptide around which this research is centered was isolated from dry bee venom. This isolation involved a combination of solvent extraction and preparative paper chromatography.

Structure Determination. Edman degradation (53) was used to determine the sequence of the amino acids present in this peptide. Edman degradation involves the reaction of phenyl isothiocyanate with the N-terminal amino acid of the peptide to give the phenylthiocarbamyl peptide, which by means of anhydrous hydrogen chloride is

then converted to the phenylthiohydantoin (PTH) derivative of the N-terminal amino acid. The peptide remaining then contains one less amino acid. By repetition of this, the peptide is degraded one amino acid at a time and the PTH amino acid produced after each cycle is isolated and identified.

The Edman degradation indicated the structure of the peptide around which this research is centered to be alanylglycylglutaminylglycylhistamine (see Figure 1).

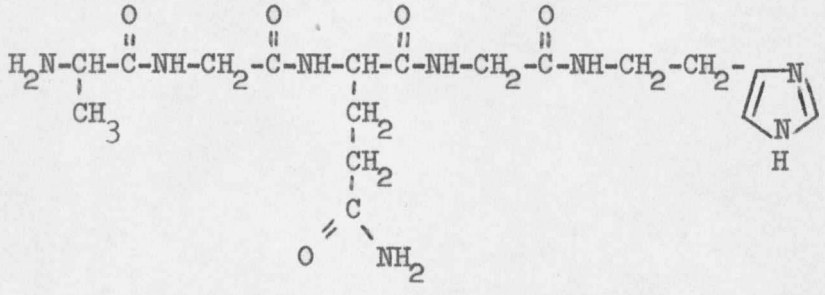

Figure 1. Compound I.

Selection of Method and Route of Synthesis. After several generally unsatisfactory attempts to couple histamine and benzyloxycarbonylglycine via means of the active p-nitrophenol ester and via direct condensation by the use of N,N'-dicyclohexylcarbodiimide, the mixed anhydride method of peptide synthesis was attempted. The former two methods were unsatisfactory in the sense that low yields resulted and purification problems were encountered. The low yields may have been due, in part, to the limited solubility of histamine in

the solvents generally used. The mixed anhydride method was investigated since it lent itself to the addition of the histamine in an aqueous solvent, thus increasing the concentration of histamine in the reaction mixture. Also, since very little was known about the properties of histamine-containing peptides, the mixed anhydride method was very attractive since the only non-volatile product produced in addition to the peptide is triethylammonium chloride (54). In this initial investigation tert-butyloxycarbonylglycine was used in preference to benzyloxycarbonylglycine due to the ease of removal of the tert-butyloxycarbonyl masking group.