Chapter 13

Bee Venom Composition: From Chemistry to Biological Activity

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Chapter Outline

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INTRODUCTION

Honeybee workers (*Apis mellifera* L.) secrete bee venom (BV) from a highly specific venom gland connected to the venom reservoir located in the abdominal cavity. More than 80% of BV is water, and ca. 0.1 μg of dry venom is extractable from a single bee [1]. BV is a natural toxin that contains more than 18 pharmacologically active compounds, including small peptides and enzymes, which play a major role in defending bee colonies [2]. The vasoactive and hemolytic substances of BV are responsible for the physiological effects brought on by massive bee stings [3]. BV has also been shown to have antiinflammatory [4,5] and radio-protective properties against X-ray, gamma, and microwave radiation [6–8], to alleviate rheumatoid arthritis (RA) [9], and to possess antibacterial properties as shown in Table 13.1 [10]. Additionally, BV may possess anticancer properties since it diminishes tumor size by selectively killing cancer cells. Several studies illustrated this effect on diverse tumor cell lines ranging from liver, breast, and prostate to mammary and leukemia cancer cells [50–53]. However, BV administration below cytotoxic concentrations for cancer cells has been demonstrated to induce cytotoxic and genotoxic effects in human HepG2 hepatoma cells [54], hence BV should be used with great caution.

BV attenuates neuroinflammatory events and is potentially neuroprotective as it has been shown to extend survival against amyotrophic lateral sclerosis in a mouse model [55]. Similarly, it has been utilized as an optional prescription for Parkinson's disease (PD) patients [56] and patients with inflammatory diseases, such as RA and osteoarthritis [57]. Several reports have demonstrated the positive effects of BV in arthritic rat models as an antiinflammatory adjuvant, as it reduces inflammatory edema and polyarthritis [58–60] (Fig. 13.1).

The antiinflammatory and antioxidant properties of BV led to the development of a BV-loaded hydrogel mixed with 10% polyvinyl alcohol and 0.6% chitosan that can be successfully used as a wound-healing accelerator in rat models for diabetic skin wounds. This BV preparation has been found to be advantageous for wound dressing due to its easy removal by either peeling or washing off the skin [61].

A number of registered pharmaceutical formulations available on the European and global market are BV based. For example, Forapin in Germany is indicated for rheumatism; Virapin in Slovakia for nonspecific inflammation of multiple joints (polyarthritis), inflammation of muscles (myositis), and rheumatism; Melivenon in Russia for the treatment of joint and bone pain (osteoarthritis); and Apiven in France as an antiwrinkle facial cream [62].

TABLE 13.1 Bee Venom (BV), Its Components, and Biological Activities

Component[a] (MW in Da)	Biological Activity	Dose	Reference
BV	Antimicrobial		
	Propionibacterium acnes	MIC 0.086 μg/mL	[10]
	Staphylococcus epidermidis	MIC 0.104 μg/mL	[10]
	Clindamycin-resistant *P. acnes*	MIC 0.067 μg/mL	[10]
	Streptococcus pyogenes	MIC 0.121 μg/mL	[10]
Peptides			
Melittin$^{50\%–60\%}$ (2846)	Antimicrobial		
	Escherichia coli	MIC 56.92 μg/mL	[11]
	Staphylococcus aureus	MIC 8.5 μg/mL	[11]
	Staphylococcus aureus	MIC 25 μg/mL	[12]
	Staphylococcus aureus amme	MIC 6 μg/mL	[13]
		MBC 10 μg/mL	
	Anti-MRSA/NCTC 10442	MIC 100 μg/mL MBC 30 μg/mL	[13]
	Lactobacillus casei	MIC 4.0 μg/mL	[14]
	Enterococcus faecalis	MIC 6.0 μg/mL	[14]
	Listeria monocytogenes	MIC 12.5 μg/mL	[12]
	Listeria monocytogenes	MIC_{90} 0.3 μg/mL	[15]
		MBC 3.3 μg/mL	

Continued

TABLE 13.1 Bee Venom (BV), Its Components, and Biological Activities—Cont'd

Component (MW in Da)	Biological Activity	Dose	Reference
	Candida albicans	MIC 9.961 μg/mL	[11]
	Candida krusei	MIC 30 μg/mL	
		MBC 60 μg/mL	[13]
	Arenavirus *Junin virus*	EC_{50} 2.4 μg/mL	[16]
	Effective against herpes virus-1	EC_{50} 3.8 μg/mL	[16]
	Effective against herpes virus-2	EC_{50} 5.8 μg/mL	[16]
	Other		
	Antiinflammatory	10 μg mixed with vaseline	[17]
	Antiapoptotic	1 μg/mL	[18]
	Induces apoptosis in several cancer cells and combats rheumatoid arthritis synovial fibroblasts in vitro and in vivo	10 μg/mL for 24 h	[19]
	Inhibition of tumor necrosis factor (TNF)-α/actinomycin (Act) D-induced apoptosis in hepatocytes	1 μg/mL	[20]
	As antiangiogenic in human cervical carcinoma cell lines, and inhibiting hypoxia-inducible factor-1α in CaSki cells	More than 3 μg/mL	[21]
	As wound healer via reducing the wound and scar sizes	1:1000 (melittin:vaseline) w/w for 8 days	[12]
	Stimulates the growth of thymocyte cells isolated from male Wistar rats	Below 5 μg/mL	[22]
	As neuroprotective against H_2O_2-induced apoptosis in the human neuroblastoma cell line SH-SY5Y via increased cell viability and decreased apoptotic DNA fragmentation	0.5 and 1 μg/mL	[23]
	Inhibits thymocyte apoptosis	50 μg/mL	[22]

Synthetic melittin	Genotoxic on *Daphnia magna* and *Pseudokirchneriella subcapitata*	50 and 10 μg/mL, respectively	[24]
Melittin I17K	Anti-*Listeria monocytogenes* (F4244)	MIC_{90} 0.8 μg/mL	[15]
		MBC 7.4 μg/mL	
Melittin G1I	Anti-*Listeria monocytogenes* (F4244)	MIC_{90} 0.5 μg/mL	[15]
		MBC 5.4 μg/mL	
Melittin-$S^{1\%–2\%}$ (2830)	Hemolytic effect	8.5 mg/mL	[25]
Cecropin A (1-7)-melittin (4-11)	Anti-*Escherichia coli*	100 μg/mL	[26]
Melittin-F (2208)	Undefined	ND	[27]
Apamin$^{1\%–3\%}$ (2027)	Binds to the SK channels in the brain and spinal cord	IC_{50} 4.7 ng/mL	[28]
	Improves learning and memory return in rats in vivo	0.4 mg/kg	[29]
	Neuroprotective against PD in mouse models	1.0 μg/kg	[30]
	Increases the number of dendritic spines and dendritic length	4 μg/kg for 14 months	[31]
	Inhibition of platelet-derived growth factor (PDGF)-BB-induced vascular smooth muscle cell proliferation and migration in atherosclerosis models	2 μg/mL:25 ng/mL (apamin: PDGF-BB) for 24 h	[32]
MCD$^{1\%–3\%}$ (2586)	Antiinflammatory action via inhibition of edema in vivo (rats)	0.1 mg/kg for 4 h	[33]
	Stimulates histamine release as part of the inflammatory response	ED_{50} 18.1 μg/mL	[34]
Tertiapin$^{0.1\%}$ (2459)	Potent and selective blocking of the muscarinic K^+ channel in cardiac myocytes	100 nM	[35]

Continued

TABLE 13.1 Bee Venom (BV), Its Components, and Biological Activities—Cont'd

Component (MW in Da)	Biological Activity	Dose	Reference
Secapin[1%–2%] (2866)	Injection with two doses causes marked hypothermia and signs of sedation	40 and 80 mg/kg	[36]
Secapin-1[1%] (2822)	Undefined	ND	[37]
Secapin-2 (2876)	Induces hyperalgesia and the edematogenic response	10 and 30 μg up to 24 h, respectively	[38]
Adolapin[0.1%–0.8%] (11,500 and 11,092)	Antipyretic	40 μg/kg	[39]
Minimine[2%–3%] (6000)	Stops feeding, growing, and lethargy of *Drosophila melanogaster* larvae	LD_{50} 5 ng	[40]
Polypeptides			
Api m 6 (ca. 8000)	Promising molecule for immunotherapy	ND	[41]
Cardiopep[0.7%] (1940)	Possesses antiarrhythmic properties	0.2 mg	[42]
Icarapin (24,387)	Undefined	ND	[43]
MRJP8 (45,100)	Promising molecule for immunotherapy	ND	[44]
MRJP9 (46,300)	Promising molecule for immunotherapy	ND	[44]

Enzymes			
PLA2[10%–12%] (19,000)	Antimicrobial		
	Enterobacter cloacae	MBC 10 ng/mL	[45]
	Escherichia coli	MBC 10 ng/mL for 30 min	[45]
	Citrobacter freundii	MBC 1 and 10^{-6} ng/mL at 1 and 2 h, respectively	[45]
	Trypanosoma brucei	1 mg/mL after 30 min	[45]
	Lactobacillus casei	400 μg/mL	[14]
Hyaluronidase[1%–3%] (40,700/60,000)	Spreading factor as it enhances the penetration of the venom components by its ability to activate the hyaluronic acid in the target tissue	ND	[46]
Api m 3 (45,000/96,000)	A promising molecule for immunotherapy	ND	[47]
Api m 5 (102,000/105,000)	Undefined, but the recombinant allergens represent valuable tools to improve current diagnostic tests and immunotherapy of insect venom allergy	20 μg/mL	[48]
Api m 7 (39,000)	Contributes to the total allergenic effect of BV	ND	[49]

ED_{50}, *Effective concentration for half-maximal response;* EC_{50}, *effective concentration;* MBC, *minimum bactericidal concentration;* SK channels, *small conductance* Ca^{2+}*-activated* K^+ *channels; ND, not defined;* MIC, *minimum inhibition concentration.*
[a]*Ingredient percentage/weight of bee venom.*

FIG. 13.1 Schematic drawing of the biological effects of bee venom components.

HISTORICAL USES OF BEE VENOM

BV has long been used in traditional medicine and has been considered a therapeutic modality in East Asia since the second century [63]. Countries such as China, Korea, and Japan have administered BV either directly via an acupuncture-like bee sting or via a prepared injection to combat inflammatory disorders [64]. The effects of BV depend on the injection site (i.e., acupuncture points exert much stronger effects than general injections) [65,66]. Numerous studies have analyzed the basis of BV allergy, anaphylactic reactions, effects on immune-related diseases, and more recently the application and beneficial results of BV immunotherapy (BVT). This research looked at safe use of BV by weighing up allergic reactions vs therapeutic efficacy [64,66]. BVT increasingly gained acceptance among physicians in Europe and the United States after it was approved for use in treating pathological conditions based on animal studies. It is currently used in the West to support wound healing [61] and to treat back pain, skin diseases, and rheumatism [67]. It is also used as a remedy for many ailments ranging from multiple sclerosis [68], arthritis [69], and asthma [70] to malaria [71] and epilepsy [72].

Factors Affecting Bee Venom Content

BV content varies substantially according to internal factors related to the bee itself, including age [72], strain [73] and caste, and external factors, such as seasons [25] and methods of BV collection [74].

The age of a bee largely affects the quantitative composition of BV; very low levels of melittin and histamine have been observed in young bees. However, melittin content continuously increases from the time of eclosion until 4 weeks of age when it peaks at 500 μg/sting and then gradually decreases to 250 μg/sting by the age of 5–6 weeks. Promelittin shows the highest concentration in 8- to 10-day-old bees. In contrast, high levels of histamine appear at 35 days of age [75]. Additionally, both phospholipase A2 (PLA2) and hyaluronidase content are influenced by the age of the honeybee. PLA2 is present at low levels at the time of eclosion and displays a steady increase, peaking at 7–10 days old, which is maintained from this point on [72]. In contrast, hyaluronidase is present in the venom of a honeybee worker at a very early stage (4 days prior to eclosion or −4 days), increasing in concentration between −4 to −2 days, followed by a drop at −1 day. The minimal level can be seen on the day of eclosion, followed by an increase throughout the bee's adulthood [76]. Conversely, 5-hydroxytryptamine (5-HT) is not detectable at eclosion, but rises to 2.5 and 9 ng/sting at the age of 5 and 10 days, respectively. Subsequently, the level of

5-HT increases dramatically reaching its highest value in 30-day-old bees followed by a decrease thereafter [77].

In addition to the age of a bee, seasonal changes play a major role in BV content. Melittin and PLA2 reach maximum levels in March and May, and decline to lowest levels in January [78]. Furthermore, melittin content changes during summer, peaking at the beginning of June and decreasing in August [79]. The melittin isomer, melittin-S, is present at low levels of 1%–2% in BV throughout the year, but suddenly rises to 10% during the winter months [25]. This difference is related to change in the honeybee's diet (plant source), which is influenced by seasonal conditions [80].

Along with the bee's age and seasonal influences the strain of honeybee has a major impact on specific venom content and volume. African bees (bumblebee species) contain less venom than European bees (*A. mellifera*), with a European bee sting releasing ca. five times more BV than that of African bees [81]. The levels of melittin and hyaluronidase are lower in African bees than in European strains, while the opposite is true for PLA2 levels [73,82,83].

Interestingly, there are also differences in BV composition between queens and worker bees. Relative amounts of the peptides melittin and apamin are lower in queen BV than in honeybee worker venom. Additionally, younger workers (nurses, guards, and foragers) have higher apamin and lower melittin concentrations than their older nestmates (guards and foragers) [74]. In contrast, the histamine content in queen BV exceeds the levels measured in worker BV [84].

The method of BV collection also affects its content. Indeed, the composition of BV collected manually (MBV) or by electrical shock (EBV) differs substantially. For instance, among the 17 proteins identified in EBV, 80% were toxic, while only 40% of the 43 proteins reported from MBV were toxic [85]. Analytical high-performance liquid chromatography (HPLC) can be used to detect differences between the two methods, with peak shape indicating the presence/absence of certain components and intensity illustrating the relative abundance of compounds in crude venom [78]. Some peaks were missing when using the EBV method due to loss of some constituents during collection (e.g., volatile compounds like histamine). While the protein composition of MBV generally resembled venom injected during a natural honeybee sting, EBV provides a more consistent and clear venom without killing the bees [86].

Bee Venom Collection

The procedures used to gather venom include dissection of the whole stinging apparatus, application of pressure to the abdomen, solvents, MBV, and EBV. EBV was first introduced by Markovič and Molnár who subjected honeybees to electric shocks to acquire venom [87]. Bees were caught between two spinning cylinders, where they were shocked and squeezed to make them sting. However, the disadvantage of this method was that the bees died during the process, leading to the resulting venom being contaminated with gland tissue damaged during extraction and difficulties in scraping off the pure venom. To

overcome this problem an elastic dam with filter paper underneath was used [87]. Later research replaced the paper by a sheet of silicone [88].

In 1963 Benton and coworkers made several modifications and presented a new device composed of a wooden frame over which steel wires were stretched at 6-mm intervals. Alternate wires carried an electric charge. The uncharged wires were grounded, and all the wires were open at one end. The circuit was completed when a bee came into contact with any two consecutive wires. The glass plate under the wires was covered by a tightly stretched piece of nylon. The frame was connected to a timer, and the transformer used was adjusted to 33 V. The power was turned on for 5 min, then the device was disconnected and slowly removed from the hive. This device allowed collecting pure venom from several thousand honeybees. An average of 20 hives must be milked to get 1 g of venom. Under ideal conditions, this amount of venom is produced by 10,000 worker bees milked for 5 min [89].

Products from final venom should be collected underwater to avoid the evaporation of volatile compounds [90]. This collection method seems to yield the most potent venom. To preserve more of the different compositions the frame of the device is placed either between the bottom board and brood chamber at the hive entrance or in a special box between the honey supers and the hive cover. Under these conditions, dried BV is easily scraped from the glass plate and the underside of the nylon sheet using extremely sharp steel. The venom's appearance is clear, crystalline, and free from contaminants [89]. However, some commercial preparations are brown, probably due to photo-oxidation of tryptophan sidechains in the venom proteins. To ensure the quality of biochemical and pharmacological studies, powdered venom should thus not be exposed to sunlight and must be stored in the cold to avoid oxidation.

Main Components of Bee Venom

BV is composed of a very complex mixture that contains at least 18 active components, including peptides, enzymes, and amines [27,91,92]. HPLC allows the separation and identification of many BV ingredients [93]. HPLC coupled with a diode array detector and ultraviolet detector (UVD) has been used for the detection of melittin and apamin [94]. HPLC coupled with tandem mass spectrometry has been used to analyze some of the other venom ingredients, such as apamin, mast cell-degranulating peptide (MCD), and oligosaccharides released from PLA2 [95–97]. Capillary electrophoresis (CE) using an UVD was developed to quantify PLA2 and melittin [98]. High-performance capillary electrophoresis (HPCE) is a new, precise, and accurate method recently used for quantitative analysis of four major BV compounds: melittin, PLA2, MCD, and apamin. HPCE and HPLC data do not differ significantly, but the costs are lower using HPCE than HPLC due to the latter's consumption of analytical reagents [62]. Other techniques, such as ultraviolet spectroscopy [99], infrared spectroscopy [100], spectrophotometry [101], proton nuclear magnetic resonance (^{1}H NMR) [102], differential scanning

calorimetry [103], and inductively coupled plasma mass spectrometry, have also been used to identify and test BV constituents [104]. In some cases the presence of melittin and PLA2 masked the detection of less abundant ingredients. Many techniques have been developed to solve these problems, such as solvent extraction, ultra-filtration, solid phase extraction, electrophoresis, and the combinatorial peptide ligand library strategy (CPLL). For instance, combining CPLL technology with high-performance liquid chromatography-mass spectrometry assisted the discovery of 83 new compounds, the function of 11 of which is still unknown [105]. Advances in chromatographic and spectroscopic techniques will provide the possibility to explore BV ingredients in even more detail. BV has so far been shown to represent a rich source of natural products, but a series of isolation, purification, and chromatographic processes are required to obtain the pharmacologically active ingredients [27,37,38,41,62,91,98,106].

Efficient synthesis is thus needed to obtain sufficient amounts, and in particular to produce analogue sets at high yield and high quality. Solid phase peptide synthesis (SPPS) is one of the recent methods used for peptide synthesis. The remainder of this paragraph is devoted to a description of this method. The first residue is attached to a resin support, and the other residues are sequentially coupled in the presence of dimethylformamide (DMF), an activating agent that consists of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HBTU), *N*,*N*′-diisopropylethylamine as a base, and fluorenylmethoxycarbonyl as a protecting group [107]. The completed peptide is then released from the resin, partially purified by RP-HPLC, and freeze-dried for subsequent further characterization and analysis [108].

Peptides

Melittin and Its Isoforms

Melittin is the principal toxic component of BV and belongs to a group of highly basic peptides [109]. It is a small linear alkaline peptide and a minor allergen that, according to a comparative study carried out between BV-sensitive persons and nonsensitive beekeepers, shows specific immunoglobulin E antibodies in only 28% of people [110].

The peptide is composed of 26 amino acid residues, and its conformation and aggregation in solution depend on several factors, including peptide concentration, pH, ionic strength, and nature of the ions in an aqueous medium. Melittin behaves in solution as a monomer, forming secondary or quaternary structures depending on the final concentration. For example, in low ionic strength and low concentrations, melittin does exist as a monomer [111]. Nevertheless, the tetrameric form is pH stable at high peptide concentrations [112] and predominates in the glands of bees. When the plasma is subjected to physiological ionic conditions (pH 7.0), melittin loses its tetrameric organization and changes to its monomeric form. Melittin is water soluble where the 20 N-terminal residues are hydrophobic and the 6 C-terminal residues are

basic [113,114]. In methanol, melittin is moderately soluble at 27°C [115]. The unfolding properties of melittin can be observed in a water and methanol solution [116]. Once embedded in a lipid bilayer, melittin retains its helical structure. It can also adapt to a dimeric form that destabilizes artificial lipid bilayers composed of dioleoylphosphatidylcholine, leading to membrane deformation and leakage. On the other hand, melittin in its monomeric form showed little effect on artificial lipid bilayer-like structures [117,118]. Melittin has here been shown to act as a nonselective cytolytic peptide that lyses red blood cells by destabilizing the cell membrane [119], thus occasionally causing toxicity in vivo. Attempts have been made to avoid these melittin side effects via phosphorylation of melittin in ^{10}Thr and ^{18}Ser residues. Phosphorylated melittin elicits lower allergenic responses than native melittin [3]. Moreover, studies are ongoing into incorporating the peptide into nanoparticles, which is thought to decrease the hemolyticity of melittin by 90% compared with native melittin [120]. This seems to be due to the slower release of melittin from nanoparticles and reduction in the nonspecific lytic activity of melittin.

Melittin is an attractive candidate for use in the treatment of cancers and infectious diseases (Table 13.1), and, as just mentioned, the application of melittin-based nanoparticles is thought to circumvent some of the toxic effects of systemic administration of free melittin.

Melittin was identified through X-ray [121], NMR [122], circular dichroism (CD) [123], and Raman spectroscopy [124]. Isolation and identification of the peptide indicated that it has triple-, quadruple-, quintuple-, and sextuple-charged molecular ions with *m*/*z* values of 949.8 ($[M+3H]^{3+}$), 712.5 ($[M+4H]^{4+}$), 570.2 ($[M+5H]^{5+}$), and 475.3 (Da) ($[M+6H]^{6+}$), and a molecular weight (MW) of 2846, 2868, and 2884 Da corresponding to melittin, melittin + Na^+, and melittin + K^+, respectively [74,125].

Two different variants of melittin have been identified in BV to date, as shown in Fig. 13.2. One isoform is melittin-S, which contains the ^{10}Ser substitution isolated for the first time from Africanized bees. The fragment pattern is described by *m*/*z* values of 944.6 ($[M+3H]^{3+}$), 708.67 ($[M+4H]^{4+}$), 567.16 ($[M+5H]^{5+}$), and 472.8 Da ($[M+6H]^{6+}$). The second isoform is melittin-F, which consists of 19 amino acids and is considered a melittin fragment, as the seven N-terminal residues are missing (Fig. 13.2) [27]. The fragment pattern is described by *m*/*z* values of 1105.31 ($[M+2H]^{2+}$), 737.21 ($[M+3H]^{3+}$), 553.16 ($[M+4H]^{4+}$), and 441.73 Da ($[M+5H]^{5+}$). The peptide was first identified in BV from an *Apis* species by Gauldie and Hanson [27].

The synthesis of melittin is crucial to obtaining a high yield and of course allowing the generation of desired point mutants, thus changing the peptide's physical and biological properties [126]. Melittin has been synthesized via SPPS (manual and automated techniques) [126–128] with 4-methylbenzhydrylamine resin and via the 9-fluorenylmethoxycarbonyl (Fmoc) strategy, both of which

Melittin	1	GIGAVLKVLTTGLPALISWIKRKRQQ	26
Melittin-S	1	GIGAVLKVLSTGLPALISWIKRKRQQ	26
Melittin-F	1	-------VLTTGLPALISWIKRKRQQ	19
Melittin (12-26)	1	-----------GLPALISWIKRKRQQ	15
Melittin (12-25)	1	----------GLPALISWIKRKRQ-	14
Melittin (12-24)	1	----------GLPALISWIKRKR--	13
MCF	1	----------GLPALISWIKRQQG-	14
MCFA	1	----------GLKKLISWIKRAAQQ	15
Melittin-a	1	GIGAVAKVLTTGAPALISWAKRKRQQ	26
Melittin-I	1	GIGAVLKVLTTGLPALISWLKRKRQQ	26
Melittin-f	1	GIGAVFKVLTTGFPALISWFKRKRQQ	26
Melittin-k	1	GIGAVKKVLTTGKPALISWKKRKRQQ	26

FIG. 13.2 Peptide sequences of melittin and its analogues.

have been used to achieve a sufficient yield and a high level of purity [128]. The latter strategy involves coupling Fmoc-protected amino acids to *N*,*N*-diisopropyl carbodiimide and 1-hydroxy-7-azabenzotriazole in DMF. Two melittin analogues have been identified, melittin 12-26 and melittin 12-25 (illustrated in Fig. 13.2). They have been synthesized using *t*-butyloxycarbonyl chemistry with *p*-methylbenzhydrylamine as resin and coupling by dicyclohexylcarbodiimide when added to 1-hydroxybenzo-triazole. The peptide–resin bond is cleaved by adding hydrogen fluoride (HF), and subsequent purification by HPLC yields peptides with a high level of purity (>95%). The structure can be confirmed by amino acid analysis and mass spectrometry (MS) [127,129].

Ius and coworkers tried to synthesize some analogues with activities that were less lytic against cellular membranes. Using theophylline (Th), four melittin analogues were generated: Th-K^{23}-melittin, Th-K^{21}-melittin, Th-K^{7}-melittin, and Th-G^{1}-melittin. Th-G^{1}-melittin displayed almost the same lytic activity as melittin, the other analogues (in particular Th-K^{7}-melittin) were significantly less efficient at lysing cellular membranes [130]. On the other hand, the removal of one or both immunoglobulin E residues increased the antimicrobial efficiency of synthesized melittin with lengths of 12-25 or 12-24 compared with the parent peptide at 12-26. If both glutamine residues of melittin were replaced by G (hydrophilic amino acid) or by L, the antimicrobial activity slightly increased (for G) or decreased (for L), respectively [130]. In addition, the C-terminal's 15 residues of melittin (Fig. 13.2), resulting in a peptide termed MCF, have been synthesized by manual methods using Fmoc chemistry on peptide amide linker resin. MCF harbors the most amphiphilic segment of melittin and its hemolytic effect is reduced about 300-fold compared with the parent peptide.

In addition, MCF (Fig. 13.2) did not show any potent antibacterial activity when administrated at the same doses as its parent peptide to microorganisms including *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas putida*. An analogue of MCF, termed MCFA (Fig. 13.2), exhibited antibacterial activity against *B. subtilis*, but was less active against *P. putida* than melittin [131].

Furthermore, four additional analogues of melittin, termed melittin-a, melittin-l, melittin-f, and melittin-k (Fig. 13.2.), have been synthesized on rink amide 4-methyl benzhydrylamine (MBHA) resin providing a final purity of 98% [132]. Promelittin has been synthesized by Fmoc-E (Trt) on rink amide MBHA resin. It was found to be elongated in shape under standard Fmoc-SPPS conditions, and provided the desired purity of >95% [133].

Apamin

With only 18 amino acids, apamin represents the smallest neurotoxic peptide within BV. It contains four cysteine residues with two disulfide bonds ($C^{1,11}$ and $C^{3,15}$). The cytotoxic and nociceptive nerve effects of apamin are due to its ability to potently block Ca^{2+}-activated K^+ channels [134]. Furthermore, apamin inhibits neuromuscular transmission via a mechanism independent of Ca^{2+}-activated K^+ channel blockage, which might involve the activation of inhibitory muscarinic M2 receptors on motor nerve terminals [135]. It has a compact and extremely stable 3D structure over a broad range of temperature, pH, and denaturants, as assessed by extensive CD spectroscopy investigations in a variety of different media and under experimental conditions [136,137].

Rietschoten et al. synthesized the peptide successfully using SPPS. It had the same physicochemical and chemical properties as natural apamin [138]. Fiori et al. synthesized apamin using a fully automated synthesizer at the 0.25-mmol scale with Tenla Gel S RAM and Fmoc-Ala-OH/HBTU in *N*-methylpyrrolidone/CH_2Cl_2 as the coupling agent, followed by deprotection and cleavage of the Fmoc group from resin. The peptide was purified by preparative HPLC providing a yield of 22% [139].

Mast Cell-Degranulating Peptide (MCD)

MCD peptide, also known as peptide 401, is chemically similar to apamin's secondary structure [140]. MCD is responsible for the release of histamine on exposure of the human body to BV. MCD and its analogues have been tested for their ability to release histamine in Sprague–Dawley rats when administered in the peritoneum and in pleural mast cells. The results demonstrated that MCD is more efficient at releasing histamine than its analogues [141]. MCD has unique immunologic properties, causing histamine release at concentrations of less than 0.1 μg/mL, but acting as an antiinflammatory compound at higher concentrations [141]. It is a cationic peptide of 22 amino acids with two disulfide bridges between $C^{3,15}$ and $C^{5,19}$ [141].

MCD has been synthesized according to a double-coupling protocol that lasted throughout the chain-elongation reaction. The protected peptide resin was treated with HF and anisole under reduced pressure, then the peptide-containing fractions were subjected to further purification and the structure confirmed by amino acid analysis [142]. Buku and coworkers also synthesized the peptide on a benzhydrylamine resin. Disulfide bridges between $C^{3,15}$ and $C^{5,19}$ were formed selectively using *S*-acetamidomethyl and *S*-methyl benzyl protection, respectively. After deprotection and cleavage the end product displayed physical, chemical, and biological properties identical to the parent peptide isolated from BV [143].

Tertiapin

Tertiapin is a peptide composed of 21 amino acids that is very sensitive to oxidation due to the presence of methionine residue. Note that this methionine can be synthetically replaced by glutamine to avoid oxidation-driven chemical changes, resulting in tertiapin-Q (TPNQ), a variant that displayed the same biological activity as the original peptide [144]. TPNQ is stable and functionally resembles the native peptide.

Secapin and Its Isoforms

Secapin has been identified in the venom of a European honeybee (*A. mellifera* L.). It contains one disulfide bond between $C^{9,20}$ [27] and has a unique chemical structure with no similarities to other basic peptides isolated from BV. Although secapin was first isolated 41 years ago, no detailed information is known about its biological activities [145]. Two secapin isomers, secapin-1 and secapin-2, have been purified and characterized from Africanized honeybees (*A. mellifera* L.), both harboring two amino acid substitutions compared with the original secapin. Secapin-1 and secapin-2 have been synthesized using SPPS and contain 25 and 24 amino acids, respectively (Fig. 13.3). They are rich in arginine and lysine and form a disulfide bond between $C^{9,2}$ that contributes to the stability of peptides. The secondary structure of secapin-2 was determined using CD spectroscopy [37,38].

Adolapin, Procamine, and Minimine

Adolapin is a basic polypeptide with 103 amino acids. Its amino acid sequence can be determined by sodium dodecyl sulfate electrophoresis [146]. Adolapin had an antipyretic effect on rats at 40 μg/kg causing a remarkable decrease in

Secapin	1	YIIDVPPRCPPGSKFIKNRCRVIVP	25
Secapin-1	1	YIINVPPRCPPGSKFVKNKCRVIVP	25
Secapin-2	1	YIIDVPPRCPPGSKFVHKRCRVIVP	25

FIG. 13.3 Peptide sequences of secapin and its analogues.

mean temperature, as shown in Table 13.1 [39]. Adolapin inhibited the activity of PLA2 (7 nM/mL inhibits ca. 80% of 2.5 nM/mL PLA2).

Procamines are a series of smaller peptides that possess only five residues. They were isolated from Canadian honeybee venom. The series includes procamine A, AGPQ-histamine, and procamine B (AGQG-histamine). These hydrophilic peptides have a unique feature: the histamine and Q residue are located in the C-terminus [1].

Minimine is a basic peptide with a MW of 6000 Da. It is insoluble in methanol but soluble in acetone [40]. Nothing is known about its chemical properties and biological activities to date.

Polypeptides

Api m 6

Api m 6 was isolated and fully characterized for the first time in 2001 by Kettner and coworkers. This study, involving BV-allergic patients, discovered Api m 6 had bound to IgE antibodies in more than 40% of patients. It was also positive for specific IgG and IgG4 antibodies. Api m 6 is a minor allergen containing 71 amino acid residues. Four isoforms are known, corresponding to Api m 6.01–6.04, based on variations in the number of amino acids at their N- and C-terminus. All possess five disulfide bonds and share a common central amino acid sequence of 67 residues. They differ only in their N- and C-terminus by up to six amino acids, leading to the observed MWs of 7190, 7400, 7598, and 7808 Da, respectively. Api m 6.01 and Api m 6.03 differ at the N-terminus by FGGF segment, while Api m 6.02 and Api m 6.04 possess two additional amino acids at the C-terminus (leucine and proline) [41,147,148]. The genetic mechanism behind this variation is unknown.

Cardiopep

Cardiopep is a cardioactive polypeptide and a potent, nontoxic β-adrenergic compound with a LD_{50} at 15 mg/kg in mice, compared with whole BV with a LD_{50} of 35 mg/kg [42]. It consists of low-MW compounds (ca. 50%) and total BV (0.7%).

Icarapin (Api m 10)

Api m 10 is a polypeptide that has 223 amino acids and is commonly called protein 2. The peptide's name is derived from two terms: *Icarus* (Greek: mythology) and *Apis* (genus of the honeybee). Api m 10 is a glycoprotein that was recently identified by two independent groups involved in proteomic studies [149,150]. Subsequently, it has been cloned and expressed in *E. coli* and found to bind to IgE. About four out of five patients with a compelling case history of BV allergy developed an icarapin–IgE response. Api m 10 has been found to be produced in soluble form in baculovirus-infected

insect cells as well as in *E. coli*. While the abundance of the phosphorylated peptide is very low (0.01% of total peptide), phosphorylation still affects allergenicity and the phosphorylation site represents an important allergenic epitope [3,149–151].

Major Royal Jelly Proteins (MRJPs)

MRJP8 and MRJP9, which have molecular masses of 45.1 and 46.3 kDa, respectively, were first identified by proteomic approaches. Though MRJP9 was found in low concentrations in several bee organs, it was mainly present in the venom gland. MRJP8 was identified as the second member of the MRJP protein family associated with the venom gland. It consists of 400 amino acids, while MRJP9 consists of 403 residues. Both MRJPs carry six and three putative *N*-glycosylation sites, respectively. Beyond their dietary function, glycosylated MRJP8 and MRJP9 have IgE-sensitizing potential in BV-hypersensitive patients. Due to their allergenic properties, MRJP8 and MRJP9 have been assigned as allergens Api m 11.0101 and Api m 11.020 in accordance with criteria set out by the International Union of Immunology Societies [44,152].

Enzymes

Phospholipase A2 (PLA2) or Api m 1

BV PLA2 is a polypeptide of 134 amino acids that is glycosylated at N^{13} [153,154]. PLA2 is the main immunogenic and allergenic component of BV. This enzyme contains five disulfide bonds between $C^{9,31}$, $C^{30,70}$, $C^{37,63}$, $C^{61,95}$, and $C^{105,113}$, which are critical to proper folding and stability [155]. The enzyme possesses antibacterial properties depending on its concentration, exposure time, and stage of bacterial growth, as shown in Table 13.1 [45].

PLA2 can be isolated from the mammalian pancreas (porcine or bovine) and is available commercially. However, the product has been questioned by many for religious reasons or the risk of virus transmission. This has led to the need to produce PLA2 from independent sources [156]. Markert et al. reported the synthesis of several PLA2 variants via genetic engineering and expression in *E. coli*. The enzyme accumulated in inclusion bodies and could be isolated with a purity of >90%. Purified PLA2 was then analyzed by MALDI-MS and N-terminal protein sequencing [156]. Recombinant PLA2 variants were similar to glycosylated PLA2 isolated from the venom glands of honeybees, and their thermodynamic stabilities were virtually identical. PLA2 production in *E. coli* might therefore be an appropriate future strategy for mass production due to its simplicity and high yields.

Hyaluronidase (Api m 2)

Api m 2 is a single polypeptide that has 349 amino acids and harbors a disulfide bridge between $C^{189, 201}$, which stabilizes the long loop at the C-terminus. In addition, it contains three sites for *N*-glycosylation [157] and an attached

carbohydrate content that accounts for 7% of the protein's mass. Hyaluronidase shares 30% of its amino acid sequence with human hyaluronidase and the enzyme makes up 1%–2% of the venom content. Recombinant hyaluronidase has been expressed in prokaryotic (*E. coli*) and eukaryotic (baculovirus-infected insect) cells [158]. Hyaluronidase breaks down hyaluronic acid in different tissues (e.g., in the synovial bursa of rheumatoid arthritis patients) and thus is utilized as an antiinflammatory [159].

Acid Phosphatase (Api m 3)

Api m 3 is a glycoprotein that exists as a dimer and is thought to be one of the allergens in BV [160]. Recombinant Api m 3 is utilized as a means to design more secure and more potent immunotherapies against BV hypersensitivity [47]. The complete sequence consists of 373 amino acid residues with four potential N-linked carbohydrate sites. The enzyme was purified and partially characterized more than two decades ago [161].

Dipeptidylpeptidase IV (Api m 5)

Api m 5 is also called allergen C, as it activates IgE in the majority of BV-allergic patients, even in the absence of cross-reactive carbohydrates. It is glycosylated and accounts for 1% of dry venom mass (as determined by gel electrophoresis and sequence characterization). Api m 5 belongs to the dipeptidylpeptidase enzyme class [48].

Serine Proteases

BV serine protease is composed of a single serine protease domain. It is a common allergen that has significant IgE-binding activity [162,163]. Api m 7 is a protease allergen of BV and comprises a serine protease-like (SPL) and a CUB domain connected with a linker peptide. Knowledge about the structure and function of Api m 7 is mainly limited to its amino acid sequence, which contains 405 amino acids with a MW of 39 kDa [49].

Volatile Ingredients

There are over 20 volatile components in BV, many of which are given in Fig. 13.4: isopentyl acetate, *n*-butyl acetate, isopentanol, *n*-hexyl acetate, *n*-octyl acetate, 2-nonanol, *n*-decyl acetate, benzyl acetate benzyl alcohol, and (*Z*)-11-eicosen-1-ol [164,165]. (*Z*)-11-Eicosen-l-ol has been identified by gas chromatography-mass spectrometry as a major volatile component (ca. 5 μg per bee). Isopentyl acetate and (*Z*)-11-eicosen-l-ol are known to be pheromones that warn bees of danger, thus stimulating the stinging reaction. Moreover, (*Z*)-11-eicosen-l-ol can be used to prolong the effectiveness of isopentyl acetate [164]. 2-Heptanone is another volatile component and the principal aggression-provoking factor among sting pheromones [166].

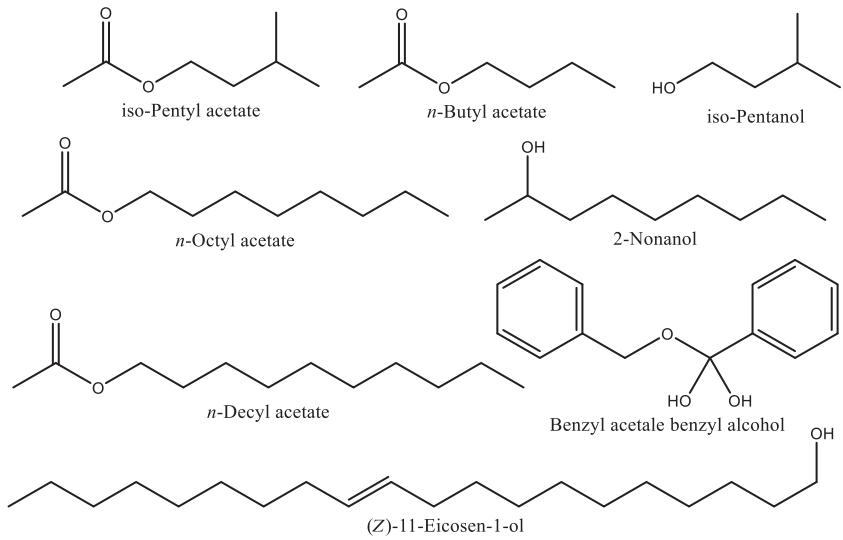

FIG. 13.4 Volatile compounds identified from bee venom.

Concluding Remarks

BV has long been widely accepted for use in the treatment of many diseases. Thousands of years ago, Hippocrates, the father of medicine, utilized honey bee stings in his restorative practices [68], and currently physicians are using BV to treat patients worldwide. BV is known for its positive effects on wound healing. In vitro and in vivo studies have provided substantial insights into the biological activities of distinct BV components and highlighted their potential for treating inflammatory diseases. Furthermore, mounting evidence has illustrated their positive effects on a variety of human diseases besides inflammatory disorders [167], such as viral and microbial infections [168,169], neurodegenerative diseases [170], diabetes mellitus [171], and cancer [172]. Despite this progress there are problems with direct application of BV or its constituents. For instance, melittin can cause mass hemolysis, is highly toxic against both cancerous and noncancerous cells with a short half-life, and has low selectivity for target cells. To overcome these problems a number of attractive strategies have already been developed to deliver melittin safely to target tissue, such as nanoparticles [173], virus vectors, targeting ligands or antibodies, and nonviral vectors. These tools can be used to improve melittin delivery, decrease its toxicity, and enhance the melittin-mediated effect in vitro and in vivo. Future studies are expected to add to these promising results [174].

Future Prospects

The remarkable diversity of BV constituents and the many different properties they exhibit explain why BV has such a wide range of applications.

Tremendous progress in the fields of chromatography, spectroscopy, proteomics, transcriptomics, and genomics has led to the identification of numerous novel compounds and potential drug lead components; many more will likely be identified. Further in-depth studies are needed to explore specific structure–function relationships and biological activities to utilize the full potential BV offers.

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ABBREVIATIONS

5-HT	5-hydroxytryptamine
Act	actinomycin
Api m 1	phospholipase A2
Api m 10	icarapin
Api m 2	hyaluronidase
Api m 3	acid phosphatase
Api m 5	dipeptidyl peptidase IV
Api m 7	CUB serine protease
BV	bee venom
BVT	BV immunotherapy
CD	circular dichroism
CE	capillary electrophoresis
CPLL	combinatorial peptide ligand library strategy
DMF	dimethylformamide
EBV	electrical collection of bee venom
EC_{50}	effective concentration
ED_{50}	effective concentration for half-maximal response
Fmoc	9-fluorenylmethoxycarbonyl
HBTU	2(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexauorophosphate
HF	hydrogen fluoride
HPCE	high-performance capillary electrophoresis

HPLC	high-performance liquid chromatography
MBC	minimum bactericidal concentration
MBV	manual collection of bee venom
MCD	mast cell-degranulating peptide
MRJPs	major royal jelly proteins
MW	molecular weight
ND	not defined
^{1}H NMR	proton nuclear magnetic resonance
PD	Parkinson's disease
PDGF	platelet-derived growth factor
PLA2	phospholipase A2
RA	rheumatoid arthritis
SK channels	small-conductance Ca^{2+}-activated K^+ channels
SPPS	solid phase peptide synthesis
TFA	trifluoroacetic acid
Th	theophylline
TNF	tumor necrosis factor
TPNQ	tertiapin-Q
UVD	ultraviolet detector

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