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Identification of Some Antibacterial Constituents of New Zealand Manuka Honey

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Some components responsible for the exceptionally high antibacterial activity of manuka honey were isolated by testing fractions of the honey for activity against *Staphylococcus aureus*. An ethanol–ether extract of the honey was separated by preparative-layer chromatography and the fractions thus obtained were assessed for antibacterial activity. One fairly homogeneous fraction was identified as methyl 3,5-dimethoxy-4-hydroxybenzoate (methyl syringate, **1b**). Combined gas chromatography–mass spectroscopy indicated the presence of this compound in some of the other antibacterial fractions together with methyl 3,4,5-trimethoxybenzoate (**1c**) and 3,4,5-trimethoxybenzoic acid (**1a**). Authentic specimens of 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid, **1d**) and 3,4,5-trimethoxybenzoic acid (**1a**) and their methyl esters were tested against *S. aureus*. The acids and, to a lesser extent, methyl syringate were found to possess significant antibacterial activity.

The antibacterial property of honey has long been recognized in vivo (Aristotle, 350 B.C.) and more recently in vitro (Sackett, 1919), but little work has been directed toward identifying the constituents responsible for this property. The antibacterial activity was originally thought to be due the high osmolarity of honey, but some activity persisted after dilution. Dold et al. (1937) termed this activity "inhibine" and found it to be labile to light and heat. Other workers (White et al., 1962; White and Subers, 1963) concluded that inhibine was hydrogen peroxide generated by the action of the glucose oxidase in honey; however, it was found (White and Subers, 1963) that some honey samples had antibacterial activity in excess of that which could be accounted for by the action of hydrogen peroxide alone. Also, Adcock (1962) found antibacterial activity to persist after the removal hydrogen peroxide by the addition of catalase.

Other authors have suggested that there may be additional antibacterial substances in honey. Antibacterial activity was found to be extractable with ethanol, acetone, and ether (Vergé, 1951; Schuler and Vogel, 1956; Lavie, 1960). Gonnet and Lavie (1960) have reported the antibacterial extractives to be stable to light and reasonably stable to heat, while Lavie (1963) noted that some of the antibacterial substances recovered from an ether extract were volatile at 95 °C. More recently, Mladenov (1974) has reported that honey contains volatile, heavy-volatile, and nonvolatile antibacterial substances, while Dustman (1979) also noted the existence of antibacterial activity that was not due to glucose oxidase activity or the high osmolarity; however, he was of the opinion that the latter activity was only a minor portion of the total activity.

We consider it likely that the differences in opinion on the significance of the additional antibacterial activity (i.e., that not due to hydrogen peroxide or the high osmolarity) result from the differences that exist in the amount of this activity. Molan and Russell (1989) found that for a range of New Zealand honey samples the additional activity varied from nil in some samples, to almost the whole of the activity in other samples. They also noted a close correlation existed between the level of additional antibacterial activity and the overall antibacterial activity of individual honey samples. Because of this finding we undertook to assess the antibacterial activity of the organic substances present in the extractives of manuka honey, a honeytype known to possess substantial additional antibacterial activity (Molan and Russell, 1989).

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MATERIALS AND METHODS

Honey Samples. Manuka (*Leptosperunum scoparium*) honey samples were obtained from local apiarists, floral source identification being based on the location of the hives, season of collection, pollen analysis, color, taste, and thixotropic nature of the honey. The sample with the highest antibacterial activity was used for the study; this sample was 18-month-old, raw, cut comb honey that had been stored in an air-tight jar in the dark at room temperature. The honey was scraped from the comb and obvious pieces of wax were removed.

Extraction of Honey Samples. Honey samples (40 mL) were homogenized with absolute ethanol (80 mL) in a Waring blender for 2 min, then centrifuged at 8000 rpm in a bench centrifuge for 15 min after which the extract was taken to dryness under vacuum in a rotary evaporator at 37 °C, and freeze-dried. The dried extract was taken up in absolute ethanol (40 mL) and shaken for 2 min on a vortex mixer, after which diethyl ether (60 mL) was added and the shaking repeated. Precipitated sugars were removed by centrifuging at 8000 rpm for 10 min. After removal of the solvent the extractives were dissolved in ethanol–ether (3:2) (20 mL) for chromatography.

Preparative-Layer Chromatography. Preparative-layer chromatography (PLC) plates (20 × 40 cm; 2-mm layer) were prepared from Kieselgel 60 $PF_{254+366}$. Plates were activated at 120 °C for 2 h, prerun in absolute ethanol, and dried at 110 °C. The honey extract (20 mL) was applied as a narrow band across the 40-cm width of the PLC plate. Plates were developed with ethanol–ether (3:2) (first plate) or toluene–chloroform–acetone mixtures (second and third plates) and viewed after drying under UV illumination at λ = 254 and/or 350 nm. The location of antibacterial bands was determined by dividing a 5-cm portion of the 40-cm width of the plate into 30 strips (ca. 5-mm each) along its length. The silica gel scrapings from each of the 30 bands was placed in agar plate wells and assayed for antibacterial activity. Bands from the remaining 35-cm width of the PLC plate were recovered by eluting the scrapings with ethanol–ether (3:2), followed by removal of the solvent under vacuum in a rotatory evaporator.

Spectroscopy and Gas Chromatography. ^1H NMR spectra were determined in $CDCl_3$ at 22.5 MHz on a Jeol FX-90Q spectrometer; IR spectra were determined on a Perkin-Elmer 180 spectrometer, and mass spectra were determined at 70 eV on a Varian Mat CH-5 spectrometer. Gas chromatography and combined gas chromatography–mass spectroscopy were conducted as reported previously (Tan et al., 1988).

Assessment of Antibacterial Activity. An agar well diffusion assay was used with plates seeded with *S. aureus* (ATCC 25923). Plates were prepared by adding 30 mL of a 24-h culture of the bacteria in nutrient broth (Difco, 8 g/L) to 450 mL of sterilized nutrient agar (Difco, 23 g/L) cooled to 45 °C. The plates were poured immediately after mixing and were stored at 4 °C until required. Comparisons of activity were made on plates from the same batch and incubated at the same time. The microorganism used in the assays had been previously demonstrated (Molan and Russell, 1989) to be sensitive to the non-peroxide antibacterial component(s) of honey and not to be inhibited by the osmolarity or acidicity of the honey samples.

EXPERIMENTS AND RESULTS

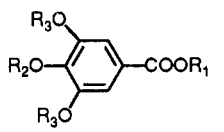
In order to obtain a solution of the antibacterial components of honey free from substantial quantities of sugar, an alcohol extract was prepared. Antibacterial activity assays showed that most of the activity was extracted by this method.

Separation of the alcohol extract by PLC on silica gel with ethanol–ether (3:2) as the developing solvent afforded 30 bands, 5 of which were found to possess antibacterial activity. These bands extended from R_f 0.83 to 0.97, with the greatest activity being observed for two bands of R_f 0.84–0.88 and 0.91–0.95, respectively. These bands exhibited strong purple colorations when viewed under UV illumination at λ = 254 nm.

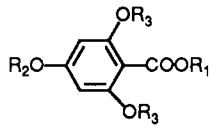
The extractives eluted from the R_f 0.83–0.97 region were dissolved in chloroform and reapplied to a PLC plate that was developed with toluene–chloroform–acetone (40:25:35). As before, small portions of the extractives were assessed for antibacterial activity by the agar well diffusion technique. When viewed under UV illumination 10 bands with R_f values ranging from 0.02 to 0.94 were observed. Modest antibacterial activity was found for bands with R_f 0.74–0.83 and for bands extending from R_f 0.53 down to the origin, while stronger activity was observed for bands in region R_f 0.53–0.65. The latter region consisted of three poorly resolved bands.

A better separation of the constituents of the R_f 0.53–0.65 region was achieved when they were recovered and chromatographed for a third time on a silica gel plate with toluene–chloroform–acetone (2:1:2) as the developing solvent. Of the three bands thus obtained, that of R_f 0.69 (strong purple coloration when viewed at λ = 254 nm) was the least active, while greater activity was found for the bands of R_f 0.90 (purple colored) and 0.84 (green fluorescing).

The band of R_f 0.69 displayed IR absorptions at 3400, 1695, 1085 and 1100 cm^{-1}, indicative of the presence of a conjugated ester, and/or hydroxyl group(s), while ^1H NMR signals at δ 7.32 (2 H, s), 3.94 (6 H, s), and 3.89 (3 H, s) indicated the presence of two stereochemically equivalent aryl protons, and a total of three methoxy and/or carbomethoxy groups. High-resolution mass spectroscopy established the molecular formula $C_{10}H_{12}O_5$ (m/e 212, M^+). From the foregoing data it can be concluded that the substance is one of 3,4,5-trimethoxybenzoic acid (**1a**), 2,4,6-trimethoxybenzoic acid (**2a**), methyl 3,5-dimethoxy-4-hydroxybenzoate (**1b**), or methyl 2,6-dimethoxy-4-hydroxybenzoate (**2b**).



1a: R_1 = H, R_2 = R_3 = CH_3
b: R_1 = R_3 = CH_3, R_2 = H
c: R_1 = R_2 = R_3 = CH_3
d: R_1 = R_2 = H, R_3 = CH_3



2a: R_1 = H, R_2 = R_3 = CH_3
b: R_1 = R_3 = CH_3, R_2 = H

Permethylation with diazomethane in a sealed tube at room temperature overnight gave a compound of molecular formula $C_{11}H_{14}O_5$ (m/e 226, M^+). Hydrolysis of the isolated compound at 130 °C for 18 h in a sealed vessel with methanol/NaOH afforded a product of molecular formula $C_9H_{10}O_5$ (m/e 198, M^+). These reactions are consistent with the presence of a carbomethoxy group together with two methoxy groups and a single hydroxyl group. Thus, it can be inferred that the isolated substance was either **1b** or **2b**, since **1a** and **2a** should be readily methylated at room temperature on reaction with diazomethane. A sample of 3,4,5-trimethoxybenzoic acid (**1a**) (Sigma Chemical Co., St. Louis, MO) when methylated at room temperature by reaction with diazomethane afforded methyl 3,4,5-trimethoxybenzoate (**1c**). This ester was found to be identical (IR, NMR, MS) with the permethylated derivative of the isolated substance. Since **1a** was not identical with the isolated substance, it follows that the latter substance is methyl 3,5-dimethoxy-4-hydroxybenzoate (**1b**). In a like manner the hydrolysis product was identified as 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid, **1d**).

Mass spectrometry of the less active bands of R_f 0.0.2–0.53, 0.74–0.83, and 0.84–0.90 recovered from the second PLC plate revealed the presence in each of fractions of substances, which possessed molecular ions of m/e 226

and/or 212. Because of the differing mobilities of the components of the faster and slower moving bands, it was thought likely that the lower R_f band might contain 3,4,5-trimethoxybenzoic acid (**1a**) (m/e 212, M^+) rather than methyl 3,5-dimethoxy-4-hydroxybenzoate (**1b**) (m/e 212, M^+). Combined capillary column gas chromatography–mass spectroscopy (Tan et al., 1988) subsequently confirmed this.

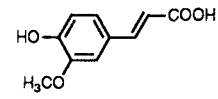
Authentic specimens of the foregoing acids and esters were tested for their activity against *S. aureus*. Esters **1b** and **1c** were prepared by methylation of syringic acid (**1d**) and 3,4,5-trimethoxybenzoic acid (**1a**), respectively. Crystals of each of the substances were ground to a fine powder and then dispersed in water with the aid of an ultrasonic probe. Solutions of 10, 5, 1, and 0.5 mg/mL were tested. Acids **1a** and **1d** were found to have detectable activity down to 0.5 mg/mL, while methyl syringate was active down to 5 mg/mL and methyl 3,4,5-trimethoxybenzoate was found to have no detectable activity at the concentrations tested.

DISCUSSION

Aromatic acids **1a** and **1d** are structurally similar to the artificial preservatives benzoic acid and 4-hydroxybenzoic acid that are often added to foodstuffs to inhibit bacterial growth. Methyl 4-hydroxybenzoate is also commonly used, it being considered more effective than the parent acid (Chichester and Tanner, 1968). That in this study the activity of the methyl esters was less than the parent acids could be a consequence of the esters' lower solubility in water.

The findings of Molan and Russell (1989) that honeys with high non-peroxide antibacterial activity are from particular floral sources would suggest that the active substances originate from a floral source, rather from the bee. It is noteworthy that the 3,5-dimethoxy-4-hydroxy-substituted aryl moiety of syringic acid is a common fragment of hard-wood lignin, while syringaldehyde is a common constituent of the sap of trees (*Merck Index*). It is possible that the aromatic acids in manuka honey originate from the sap of the tree rather than from the nectar. Manuka is often infested with a scale insect, *Eriococcus orariensis*, and this gives rise to a honeydew that could be collected by the bees. Plachy (1944) and Lavie (1960) have both reported finding honeydew to be antibacterial. The honeydew on the manuka supports the growth of sooty mold fungus (*Capnodium walteri* Sacc.), raising the possibility that an alternative source of the aromatic acids may be the degradation of plant material since enzymes produced by white rot fungi growing on sawdust have been demonstrated to release syringic acid from lignin (Walker, 1975). We consider it likely that the aromatic acids isolated in this study are derived from precursors collected by the bees from the manuka trees. Methylation or demethylation of stilbenes collected from *Eucalyptus* was thought by Ghisalberti (1979) to be the source of the pterostilbine found in propolis. Similar reactions could account for the presence of the methylated and demethylated analogues of syringic acid found in the manuka honey samples.

It is possible, but unlikely, that the antibacterial substances isolated in this study originate from propolis. Propolis, a resinous material collected by bees from buds, is known to be strongly antibacterial and is soluble in alcohol and water (Lavie, 1960). However, its antibacterial activity has been well investigated, and the components responsible have been identified as galangin, pinocembrin, caffeic acid, and ferulic acid (Ghisalbert, 1979). It is of interest to note that ferulic acid (**3**) is considered to be a soft-wood lignin precursor.



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The components of bee venom responsible for its weak antibacterial activity (Lavie, 1960) have been identified. Mellitin (the principal component) is known to be active against *S. aureus* (Fennel et al., 1968). Phospholipase A, which can degrade membranes, is also present (Orlov, 1979). Both of these components are proteins. Gel filtration chromatography (Sealey and Molan, personal communication) has established that for manuka honey the antibacterial substances have molecular weights of less than 1000 amu.

The acid fraction of royal jelly is weakly antibacterial and is soluble in water, alcohol, and ether but is very unstable (Lavie, 1960). On the other hand the antibacterial activity of manuka honey is heat-stable (Molan and Russell, 1989). Pollen present in honey could be the source of the antibacterial aromatic acids. Antibacterial activity, soluble in water and alcohol, has been found in some types of pollen (Lavie, 1960). Yet an another source of the aromatic acids could be the wax. Beeswax is known to contain heat-stable water-soluble antibacterial activity (Lavie, 1960). However, it is possible that this activity could be the result of antibacterial substances of low solubility in water diffusing into the wax from honey.

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Registry No. **1a**, 118-41-2; **1b**, 884-35-5; **1c**, 1916-07-0; **1d**, 530-57-4.

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Oxidative Damage of Protein Induced by the Amadori Compound–Copper Ion System

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Generation of oxygen radicals from the browning solution of glucose and amino acids in the presence of copper ion and their oxidative action on protein were investigated in detail. Reaction mixtures of the browning solution, bovine serum albumin (BSA) and copper(II), incubated for 24 h at 40 °C under aerated conditions markedly accelerated the oxidative depolymerization of BSA and the oxidative degradation of its histidine residue. Among the many amino acids used in these browning solutions, β-alanine, 4-aminobutyric acid, and 6-aminocaproic acid containing solutions exhibited very high activities for oxidative degradation of *N*-benzoyl-L-histidine (Bz-His). The active product in the browning solution of glucose and β-alanine was estimated to be an Amadori compound (AC), 1-deoxy-1-β-alanino-D-fructose.

Amino–carbonyl reaction, e.g., the Maillard reaction, between reducing sugars and amino acids or proteins produces the brown color and specific flavor in food processing that contribute to the development of good qualities in food. On the other hand, the formation of Amadori compounds from glycated proteins in the human body is thought to promote several diseases associated with diabetes and aging (Pongor et al., 1984; Brownlee et al., 1986; Cerami et al., 1987). In the amino–carbonyl reaction, many dicarbonyl compounds and keto amines are produced as intermediates of the browning reaction (Feather, 1985), and then these intermediates must be reversibly transformed to enediols and enaminols, respectively, in the reaction processes. Those compounds may easily form complexes with metal ions. The formation of such complexes under aerated conditions may cause one-electron transfer from enediols and enaminols to an oxygen molecule through metal ions to give a superoxide anion radical (O_2^-) (Fridovich, 1979). In general, a solution containing ascorbic acid and metal ion is well-known as a generating system for active oxygen, which means a series of oxygen radicals formed by a one-electron reduction of oxygen. The interaction of the ascorbic acid–metal ion system and some biological substrates spontaneously produced the oxidation and oxidative changes in their molecules, that is, protein (Marx and Chevion, 1986; Garland et al., 1986; Kano et al., 1987; Uchida and Kawakishi, 1988b), enzymes (Shinar et al., 1983; Levine, 1983; Nakanishi et al., 1985), polysaccharides (Matsumura and Pigman, 1965; Wong et al., 1981; Uchida and Kawakishi, 1986b, 1987), and DNA (Shinohara et al., 1983; Aronovitch et al., 1987).

The Amadori compound formed in glycated protein, N^{ϵ}-fructosyllysine, is oxidatively degraded in phosphate buffer under aerated conditions to give N^{ϵ}-(carboxymethyl)lysine residue (Ahmed et al., 1986). Kashimura et al. (1986) reported the cleavage of DNA strand and virus inactivation by the action of some Amadori compounds with copper ion.

With this background, it is considered that reaction mixtures of glucose and amino acids may generate some oxygen radicals by addition of metal ion and these radical species will oxidatively attack some biological and food constituents, if they exist together with the radical-generating system. By using the ascorbic acid–copper ion system as an oxygen radical generator, we have investigated the oxidative depolymerization of polysaccharides (Uchida and Kawakishi, 1986b, 1987) and protein (Uchida and Kawakishi, 1988b) and the oxidative degradation of oligosaccharides (Uchida and Kawakishi, 1988a) and histidine residue in protein (Uchida and Kawakishi, 1986a).

In this paper, we report the generation of active oxygen from the browning reaction mixture–copper ion system and its oxidative damage to protein and also prove the inducer of this oxidation to be mainly Amadori compounds.

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