Constituents of Cinnamon Inhibit Bacterial Acetyl CoA Carboxylase

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Key words

- *Cinnamomum zeylanicum*
- Lauraceae
- *trans*-cinnamaldehyde
- acetyl-CoA carboxylase
- carboxyltransferase

Abstract

Cinnamon bark (*Cinnamomum zeylanicum*) is used extensively as an antimicrobial material and currently is being increasingly used in Europe by people with type II diabetes to control their glucose levels. In this paper we describe the action of cinnamon oil, its major component, *trans*-cinnamaldehyde, and an analogue, 4-hydroxy-3-methoxy-*trans*-cinnamaldehyde against bacterial acetyl-CoA carboxylase in an attempt to elucidate the mechanism of action of this well-known antimicrobial material. These natural products inhibited the carboxyltransferase component of *Escherichia coli* acetyl-CoA carboxylase but had no effect on the activity of the biotin carboxylase component. The inhibition patterns indicated that these products bound to the biotin binding site of carboxyltransferase with *trans*-cinnamaldehyde having a K_i value of 3.8 ± 0.6 mM. The inhibition of carboxyltransferase by 4-hydroxy-3-methoxy-*trans*-cinnamaldehyde was analyzed with a new assay for this enzyme based on capillary electrophoresis. These results explain, in part, the antibacterial activity of this well-known antimicrobial material.

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Introduction

Sri Lankan cinnamon or sweet cinnamon (*Cinnamomum zeylanicum* Breyne) is one of the most ancient medicinal plant materials and is mentioned in the Bible [1]. The Greek physician Dioscorides mentions the use of cinnamon as an anti-inflammatory preparation and in combination with honey for use as an antibacterial material to remove spots from the skin [2].

Powdered cinnamon bark has recently gained popularity in Europe as a Herbal Medicinal Product (HMP), particularly amongst patients with type II diabetes, and this has led to a number of recent articles on its use [3–8]. Sweet cinnamon bark is rich in an essential oil which constitutes 0.5–2.5% w/w, and this oil is produced in considerable quantity for the flavor and fragrance markets. The European Pharmacopeia states that cinnamon oil should be comprised of 55–75% of *trans*-cinnamaldehyde with less than 0.5% of coumarin, which is also present in other species of *Cinnamomum* such as Chinese cinnamon (*Cinnamomum cassia* Blume, syn. *C. aromaticum*), which is less valuable and less commercially important than the oil of the bark of sweet cinnamon.

Cinnamon oil and the main constituent of the oil, *trans*-cinnamaldehyde (**1**), have been studied intensively for their many pharmacological effects, which include antifungal activity [9, 10], antibacterial activity [11–13], food preservation [14], antityrosinase activity [15], anti-inflammatory action [16], potential in slowing the onset of Alzheimer's disease [17], and the regulation of glucose and fatty acid metabolism [18–20].

Here we show that cinnamon oil and its main component, *trans*-cinnamaldehyde, inhibit bacterial acetyl-CoA carboxylase (ACC), and that these findings may, in part, explain the activity of these agents against bacteria and other microbes.

Materials and Methods

Materials

Coupling enzymes and substrates for the assay of *E. coli* biotin carboxylase and carboxyltransferase were from Sigma. *trans*-Cinnamaldehyde (> 99% by GC) and 4-hydroxy-3-methoxy-cinnamaldehyde (98%) were obtained from Sigma-Aldrich and cinnamon oil (batch number CIN0006/1000) was obtained from Botanicals and Natural Products, Ltd. Moiramide B was a gift from Pfizer. Nor-

floxacin (98% by TLC) was obtained from Sigma-Aldrich and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide was obtained from MTT. Tris Buffer was from Acros Organics.

Bacterial strains and chemicals

Methicillin-resistant *Staphylococcus aureus* (MRSA) strain XU212, a clinical isolate [21] which possesses the TetK efflux pump, and the standard ATCC25923 strain was obtained from Dr. E. Udo. Strain RN4220, which has the MsrA macrolide efflux protein, was provided by J. Cove [22]. EMRSA-15 and EMRSA-16 [23] were obtained from Dr. Paul Stapleton. Strain SA1199B, which overexpresses the NorA MDR efflux pump, was the gift of Professor Glenn W. Kaatz [24]. Mueller-Hinton broth (MHB; Oxoid) was adjusted to contain 20 mg/L Ca^{2+} and 10 mg/L Mg^{2+}.

GC-MS analysis of the cinnamon oil

The oil was analyzed by GC-MS on an Agilent 6890 N GC system coupled directly to a 5973 mass spectrometer. A volume of 1 microliter was injected using a split ratio (200 : 1) with an autosampler at 24.79 psi and an inlet temperature of 250 °C. The GC system was equipped with an HP-Innowax polyethylene glycol column of dimensions 60 m × 250 µm with an internal diameter of 0.25 µm film thickness. The oven temperature program was 60 °C for the first 10 minutes, rising to 220 °C at a rate of 4 °C/min and held for 10 min, and then rising to 240 °C at a rate of 1 °C/min. Helium was used as carrier gas at a constant flow of 1.2 mL/min. Spectra were obtained by electron impact at 70 eV, scanning from 35 to 550 *m/z*. The percentage compositions of the individual components were obtained from electronic integration measurements using flame ionization detection (FID, 250 °C). *n*-Alkanes were used as reference points in the calculation of relative retention indices (RRI). The identification of the compounds was carried out using NIST®, Mass Finder® and Flavour® libraries by comparing mass spectra and retention indices.

Antibacterial assays

Overnight cultures of each strain were made up in 0.9% saline to an inoculum density of 5×10^5 cfu by comparison with a MacFarland standard. Norfloxacin was dissolved in DMSO and then diluted in MHB to give a starting concentration of 512 µg/mL. Using Nunc 96-well microtiter plates, 125 µL of MHB were dispensed into wells 1–11. A volume of 125 µL of the test compound or the antibiotic norfloxacin was dispensed into well 1 and serially diluted across the plate, leaving well 11 empty for the growth control. The final volume was dispensed into well 12, which being free of MHB or inoculum, served as the sterile control. Finally, the bacterial inoculum (125 µL) was added to wells 1–11, and the plate was incubated at 37 °C for 18 h. A DMSO control (3.125%) was also included. All MICs were determined in duplicate. The MIC was determined as the lowest concentration at which no growth was observed. A methanolic solution (5 mg/mL) of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliium bromide was used to detect bacterial growth by a color change from yellow to blue.

Assay of *E. coli* biotin carboxylase

Biotin carboxylase from *E. coli* was isolated from a strain of *E. coli* that was engineered to overexpress the gene (*accC*) coding for the enzyme [25]. The activity of the purified enzyme was determined by measuring the production of ADP using a coupled enzyme assay of pyruvate kinase and lactate dehydrogenase as described by Blanchard et al. [25].

Assay of *E. coli* carboxyltransferase

The carboxyltransferase component of *E. coli* ACC was isolated from a strain of *E. coli* that was engineered to overexpress the genes (*accA* and *accD*) coding for the enzyme [26]. The activity of the enzyme was determined in the reverse direction using a coupled enzyme assay where the production of acetyl-CoA was coupled to the citrate synthase-malate dehydrogenase reactions requiring NAD^+ reduction [26]. The cinnamon constituents did not inhibit either of the coupling enzymes.

Data analysis

Data for competitive and uncompetitive inhibition were fitted to equations 1 and 2, respectively, using the programs of Cleland [27]. In equations 1 and 2, v is the initial velocity, V_{max} is the maximal velocity, A is the substrate concentration, I is the concentration of inhibitor, K_m is the Michaelis constant, and K_i is the inhibition constant.

$$v = V_{max} \cdot A/[K_m(1 + I/K_i) + A] \quad (1)$$

$$v = V_{max} \cdot A/[K_m + A(1 + I/K_i)] \quad (2)$$

Capillary electrophoresis assay of *E. coli* carboxyltransferase

Capillary electrophoretic separations were performed using a P/ACE MDQ with 32 Karat version 5.0 software from Beckman Coulter, Inc. This instrument was equipped with a D_2 lamp and a photodiode array detector. Electropherograms for this work were plotted at a wavelength of 260 nm. The enzyme assay was performed off-line, and a sample of the reaction mixture was injected hydrodynamically for 0.5 s at 5 psi. The separation was performed in 50.0 mM Tris Buffer, pH 8.85 with an applied voltage of 20.0 kV (322 V/cm) resulting in a current of 4.4 µA. The fused silica capillary was purchased from Polymicro with an inner diameter of 50 µm and a 360 µm outer diameter. The total length of the capillary was 62 cm with a 50-cm length to the detection window. The detection window was made by removing a short section, less than 5 mm, of the polyimide coating from the capillary with a MicorSolv CE window maker.

Results and Discussion

Whilst there is a plethora of literature concerning the use of cinnamon and its corresponding volatile oil as an antimicrobial substance, there is surprisingly little known about the mechanism of the antibacterial action of the oil or the major natural products. We therefore set out to investigate the oil (CO), its main constituent, *trans*-cinnamaldehyde (CA) (**1**) and the related commercially available natural product analogue, 4-hydroxy-3-methoxy-*trans*-cinnamaldehyde (**2**) (coniferaldehyde), as potential inhibitors of acetyl-CoA carboxylase (**Fig. 1**). A commercially available batch of cinnamon oil was analyzed using GC-MS and the composition and chromatogram are given in **Table 1** and **Fig. 2**, respectively. These show the characteristic major constituent *trans*-cinnamaldehyde, which is consistently present in the bark of sweet cinnamon (*Cinnamomum zeylanicum*) and along with the absence of coumarin is a diagnostic feature of this crude plant drug.

We first investigated the activity of the three components against a panel of *Staphylococcus aureus* strains with all three natural products displaying antibacterial activity with minimum inhibitory concentration (MIC) values ranging from 64–512 mg/L (**Ta-**

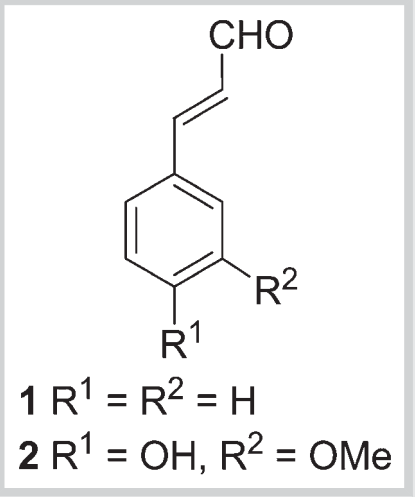

Fig. 1 Structures of *trans*-cinnamaldehyde (**1**) and 4-hydroxy-3-methoxy-*trans*-cinnamaldehyde (**2**).

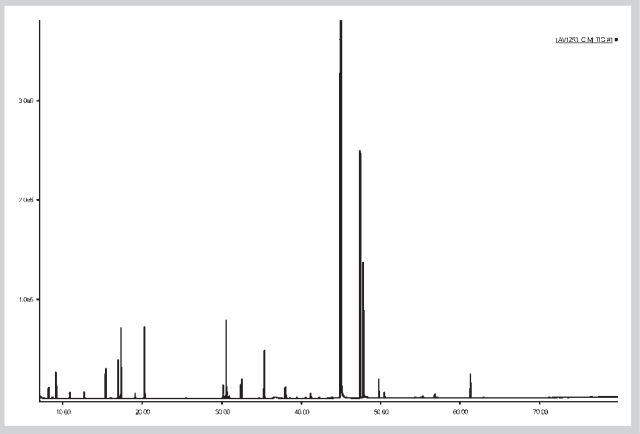

Fig. 2 GC chromatogram of the cinnamon oil used in this study.

Table 1 Relative retention indices and percentage area of cinnamon oil.

RRI	Compounds	% area
1000	Decane	0.2
1018	α-Pinene	0.4
1061	Camphene	0.1
1104	β-Pinene	0.1
1161	α-Phellandrene	0.6
1194	Limonene	0.9
1202	1,8-Cineole	1.6
1244	γ-Terpinene	0.1
1272	*p*-Cymene	1.4
1531	Dihydrolinalool	0.1
1534	Benzaldehyde	0.4
1545	Linalool	2.4
1556	*trans*-*p*-Menth-2-en-1-ol	0.1
1601	B-Caryophyllene	0.3
1603	Terpinen-4-ol	0.5
1701	α-Terpineol	1.4
1794	Benzenepropanal	0.7
1847	Geraniol	0.1
1889	Benzyl alcohol	0.1
1955	Benzene-2-propenyl	0.1
2067	*E*-Cinnamaldehyde	70.9
2170	Cinnamyl acetate	9.1
2186	Eugenol	5.4
2298	Cinnamyl alcohol	0.4
2587	2-Methyl-naphthalen-1-ol	0.2
2798	Benzyl benzoate	1.3
		98.9

ble 2). These strains included a standard laboratory strain (ATCC 25923) and several strains which are clinical isolates and resistant to several antibiotics such as the multidrug-resistant SA 1199B (fluoroquinolone-effluxing), XU212 (tetracycline-effluxing) and EMRSA-15 and 16, the two most prevalent epidemic methicillin-resistant strains from UK hospitals [23]. Given the activity of *trans*-cinnamaldehyde (**1**) it is likely that it is the major compound responsible for antibacterial action in cinnamon oil, demonstrating the highest activity against EMRSA-16 with an MIC of 64 mg/L. To investigate the possible mechanism of action for the antibacterial activity of *trans*-cinnamaldehyde (**1**) we examined the effect of cinnamon oil (CO), *trans*-cinnamaldehyde (CA) (**1**) and 4-hydroxy-3-methoxy-*trans*-cinnamaldehyde (**2**) on the activity of the antibiotic target acetyl-CoA carboxylase.

Acetyl-CoA carboxylase (ACC) catalyzes the first committed step in fatty acid biosynthesis in all animals, plants and bacteria. The enzyme requires the cofactor biotin and utilizes a two-step reaction sequence shown in **Fig. 3**.

In the first half-reaction, biotin carboxylase catalyzes the ATP-dependent carboxylation of biotin. In the next reaction, catalyzed by the carboxyltransferase subunit, the carboxyl group is transferred to acetyl-CoA to make malonyl-CoA. *In vivo*, biotin is covalently attached to the biotin carboxyl carrier protein (designated as enzyme-biotin in **Fig. 3**). In bacteria each of the three components of ACC are separate proteins where biotin carboxylase and carboxyltransferase retain their enzymatic activity after purification and will utilize free biotin as a substrate in place of the carrier protein [28]. In eukaryotes, all three functions of ACC are combined within a single polypeptide.

The biotin carboxylase component of *E. coli* ACC was not inhibited by the cinnamon oil, *trans*-cinnamaldehyde, or the cinnamaldehyde analogue 4-hydroxy-3-methoxy-*trans*-cinnamaldehyde.

In contrast to the biotin carboxylase component, the carboxyltransferase component of *E. coli* ACC was inhibited by all three materials tested. To test the inhibitory properties of cinnamaldehyde (**1**) and the cinnamon oil, a coupled enzyme assay was used where the reduction of NAD^+ can be measured based on the absorbance at 340 nm [26]. The reaction is run in the non-physiological direction, which means that malonyl-CoA and biocytin are the substrates. Biocytin is an analogue of biotin where lysine is attached to the valeric acid carboxyl group. Biocytin has utility in place of biotin for the coupled enzyme assay as it is more reactive [26].

Using this assay, both cinnamaldehyde and cinnamon oil exhibited competitive inhibition with respect to biocytin and, therefore, presumably bind in the biotin binding site (**Fig. 4A, B**). Due to the high absorbance of both **1** and **2** at 340 nm, the kinetics at only one concentration of inhibitor could be measured (i.e., higher concentrations of **1** and cinnamon oil masked the absorbance due to NADH production). The K_i for **1** was 3.8 ± 0.6 mM. By GC-MS, the *trans*-cinnamaldehyde (CA) constitutes 70.9% of the cinnamon oil by mass. Therefore, the Ki for **1** in the context of CO was calculated to be 5.6 ± 0.8 mM which is very close to the K_i value determined for pure *trans*-cinnamaldehyde. In contrast to biocytin, both **1** and cinnamon oil exhibited uncompetitive inhibition with respect to malonyl-CoA (**Fig. 4C, D**). The

Table 2 Minimum inhibitory concentrations of *trans*-cinnamaldehyde (**1**), cinnamon oil (CO), 4-hydroxy-3-methoxycinnamaldehyde (**2**) and norfloxacin in mg/L against multidrug-resistant (MDR) and methicillin-resistant *Staphylococcus aureus* strains. Resistance mechanisms for each strain are given in parentheses.

Compounds	SA 1199B (NorA)	RN4220 (MsrA)	EMRSA-15 (mecA)	ATCC 25 943	XU212 (TetK)/(mecA)	EMRSA-16 (mecA)
1	128	128	256	126	128	64
CO	256	128	256	256	256	128
2	256	256	512	256	256	128
Norfloxacin	32	1	0.5	0.5	8	256

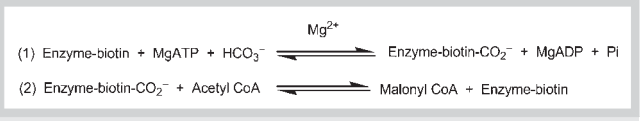

Fig. 3 Two-step reaction catalyzed by acetyl-CoA carboxylase.

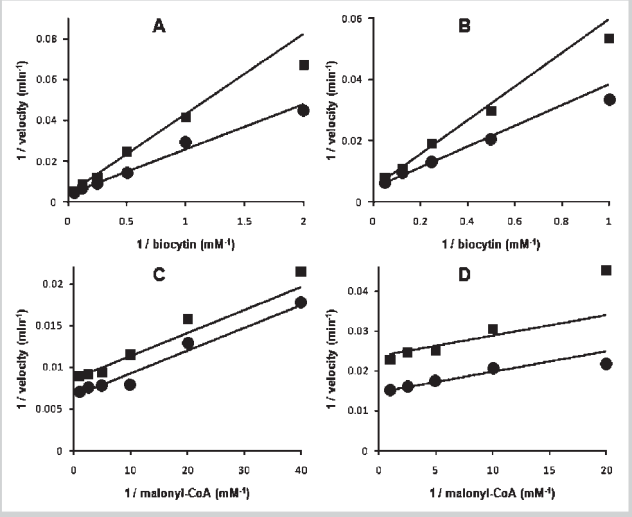

Fig. 4 Inhibition of carboxyltransferase by *trans*-cinnamaldehyde (**A, C**) and cinnamon oil (**B, D**). When malonyl-CoA was the variable substrate (**A, B**), biocytin was held constant at 2.0 mM, and when biocytin was the variable substrate (**C, D**), malonyl-CoA was held constant at 0.1 mM. The points are the reciprocal of the experimental velocities, and the lines are derived from the best fit of the data to either competitive inhibition (Equation 1) (**A, B**) or uncompetitive inhibition (Equation 2) (**C, D**). Circles represent no inhibitor present; squares represent the presence of either 2.0 mM cinnamaldehyde (**A, C**) or 0.8 µg/mL cinnamon oil (**B, D**).

uncompetitive inhibition pattern with respect to malonyl-CoA is consistent with *trans*-cinnamaldehyde (**1**) which is also present in cinnamon oil binding in the biocytin binding site, given that carboxyltransferase has an ordered addition of substrates with malonyl-CoA binding first [26, 29]. Since *trans*-cinnamaldehyde binds in the biocytin binding site, malonyl-CoA must have previously bound to the enzyme before *trans*-cinnamaldehyde can bind and inhibit turnover. Therefore, only at a saturating level of malonyl-CoA (i.e., the y intercept) do the cinnamon derivatives exhibit inhibition, whereas at a very low level of malonyl-CoA (i.e., the slope) the cinnamon derivatives do not bind, and no change is observed. As a positive control, the known carboxyltransferase inhibitor moiramide B [38] was used to exhibit inhibition of activity in the same assay used to determine inhibition by **1**. Carboxyltransferase in the presence of 600 nM moiramide B shows 19% activity compared to uninhibited enzyme, and 0.9% activity with 6 µM moiramide B present.

It is important to note that the inhibition of carboxyltransferase by *trans*-cinnamaldehyde was not irreversible. The inhibition of carboxyltransferase could have been the result of covalent modification of essential residues on the enzyme by the aldehyde group of **1**. This could feasibly occur through a classical "Michael-type" addition to the double bond which is alpha to the aldehyde. However, when 2 mM of cinnamaldehyde were incubated with carboxyltransferase, the activity was found not to decrease with time for a period of 30 min. The lack of a time dependence suggested that inhibition of carboxyltransferase by *trans*-cinnamaldehyde is not due to covalent modification but instead is simple reversible binding to the biocytin binding site. Moreover, if **1** inhibited by randomly modifying amino acids in the enzyme then biotin carboxylase would have been just as susceptible as carboxyltransferase.

The *trans*-cinnamaldehyde analogue 4-hydroxy-3-methoxy-*trans*-cinnamaldehyde (**2**) (coniferaldehyde) could not be analyzed using the conventional coupled enzyme assay described above because of its strong absorbance at 340 nm, which interferes with the absorbance of NADH from the coupled reaction. Therefore, to determine if **2** inhibited carboxyltransferase, an assay using capillary electrophoresis was developed. Capillary electrophoresis is a separation technique based on differences in the ratio of charge to hydrodynamic radius for analytes in a conductive solution. Capillary electrophoresis can be used to analyze enzyme kinetics and perform assays both on- and off-column [30, 31]. In this work the assay was performed off-column by sampling the reaction mixture at intervals. Reaction mixtures of 20 µM malonyl-CoA, 4 mM biocytin, and 114 µg/mL ACC were sampled and analyzed at 0, 30, 60, and 90 min in both the absence and presence of 20 µM of the inhibitor 4-hydroxy-3-methoxy-*trans*-cinnamaldehyde. The mixture was sufficiently separated such that malonyl-CoA, acetyl-CoA and 4-hydroxy-3-methoxy-*trans*-cinnamaldehyde, when included, were baseline resolved in less than 10 min. The reaction progress can be monitored by the increase in the acetyl-CoA product peak and the depletion of the substrate peak, malonyl-CoA, as seen in **Fig. 5**, which shows the reaction progress in the absence of 4-hydroxy-3-methoxy-*trans*-cinnamaldehyde. When this inhibitor was present in the reaction mixture, less product formation and less substrate depletion were observed (**Fig. 6**) compared to reactions without the inhibitor. These results indicate that 4-hydroxy-3-methoxy-*trans*-cinnamaldehyde is also an inhibitor of the carboxyltransferase component of *E. coli* ACC.

At first glance it is not immediately apparent why *trans*-cinnamaldehyde would inhibit the carboxyltransferase component of ACC. However, if this inhibition is examined from the context of enzyme structure, it becomes obvious why it inhibits. Carboxyltransferase belongs to the crotonase superfamily of enzymes whose members all catalyze reactions that generate enolate

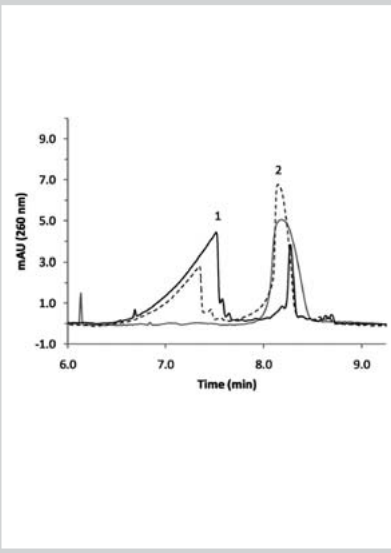

Fig. 5 Electropherograms of reaction mixture without inhibitor at times 0 (grey trace), 30 (dash), and 60 min (black) showing the increase of (1) acetyl-CoA product peak and the decrease of the (2) malonyl-CoA substrate. At each time interval a sample of the reaction mixture was injected for 5.0 s at 0.5 psi into the column, which contained a separation buffer of 50 mM Tris, pH ;8.85 and was separated with a 20.0 kV applied voltage.

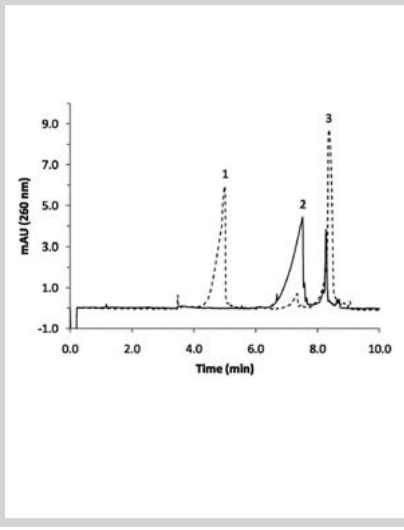

Fig. 6 Electropherograms of reaction mixtures with (dash) and without (black) inhibitor after 60 min incubation. 1.4-hydroxy-3-methoxy-*trans*-cinnamaldehyde (inhibitor), 2. acetyl-CoA (product), 3. malonyl-CoA (substrate). A sample of the reaction mixture was injected for 5.0 s at 0.5 psi into the column, which contained a separation buffer of 50.00 mM Tris, pH 8.85 and was separated with a 20.0 kV applied voltage.

anion intermediates [32,33]. In carboxyltransferase the transfer of the carboxyl group from carboxybiotin to acetyl-CoA involves formation of enolate or enolate-like anions for both carboxybiotin and acetyl-CoA. To this end, both the α and β subunits of carboxyltransferase have evolved tertiary folds that stabilize oxyanions. Acetyl-CoA binds to the β subunit, while carboxybiotin binds to the α subunit, and the tertiary structure of both the α and β subunits contain a similar α/β spiral core of two β-sheets surrounded by α-helices, suggesting a gene duplication event. Within the spiral core of both the α and β subunits of carboxyltransferase are oxyanion holes formed by main chain amides from glycine residues [34]. The inhibition patterns indicate that *trans*-cinnamaldehyde (**1**) binds to the biotin/biocytin binding site. The binding is likely to involve the interaction of the carbonyl oxygen of **1** with the oxyanion hole in the α subunit.

Further support for this mechanism of inhibition of carboxyltransferase comes from the fact that other members of the crotonase superfamily of enzymes utilize cinnamaldehyde derivatives as substrates. For example, hydroxycinnamoyl-CoA hydratase lyase plays a role in the microbial degradation of phenolic compounds by catalyzing the conversion of feruloyl-CoA into vanillin [35]. Moreover, the crystal structure of enoyl-CoA hydratase (i.e., crotonase) was solved with 4-(*N*,*N*-dimethylamino)-cinnamoyl-CoA bound in the active site [36]. The three-dimensional structures of both of these enzymes showed that the carbonyl oxygen of the thioester, which is equivalent to the carbonyl oxygen in the cinnamon derivatives used in this study, bound in the oxyanion hole. Thus, the ability of other members of the crotonase superfamily of enzymes to bind structural analogues of *trans*-cinnamaldehyde certainly makes the observation of cinnamaldehyde binding weakly to carboxyltransferase not unexpected and also suggests a possible mechanism of interaction.

The finding that cinnamon inhibits bacterial growth and the carboxyltransferase subunit of *E. coli* ACC does have implications for pharmaceutical development. Both the biotin carboxylase [37] and carboxyltransferase [38] subunits have been shown to be targets for antibiotic development. In fact, the natural product moiramide B inhibits carboxyltransferase with a K_i value of 5 nM and has structural features similar to those of cinnamaldehyde [38]. However, moiramide B was too toxic for pharmaceutical use. Cinnamaldehyde derivatives on the other hand could be used as an initial lead for fragment-based drug design. Fragment-based drug design has been used recently with increasing success to develop pharmaceuticals [39]. The basic approach involves identifying small molecules (< 250 Da) that bind weakly to a target molecule, and then through an iterative process of synthetic modification and structure determination a more potent derivative of the initial fragment is developed. *trans*-Cinnamaldehyde could therefore be a promising starting fragment especially considering that it is nontoxic.

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