Inhibition of Bacterial Urease by Autoxidation of Furan C-18 Fatty Acid Methyl Ester Products

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Autoxidation products of synthetic methyl 9,12-epoxyoctadeca-9,11-dienoate (MEFA) were investigated by gas chromatography-mass spectrometry analysis and tested for bacterial urease inhibition. A suspension of oxidized MEFA in 10% Tween 80 was an effective inhibitor for bacterial urease extract from *Helicobacter pylori* (I_{50} = 1.3 mM) and for commercial urease from *Bacillus pasteurii* (I_{50} = 0.06 mM). The urease inhibitory effect was cancelled by adding cysteine to the reaction mixture. The total content of biologically active oxidized products in the mixture was found to be 6.2%. Dioxo-ene derivatives of MEFA on the thin-layer chromatography plate surface were converted into more stable compounds, whose formation in the mixture reduced inhibition of *B. pasteurii* to about 2% of the former level. The mechanism of urease inhibition is supposed to involve the interaction of the thiol groups of the enzyme's active center with the inhibitor molecules.

KEY WORDS: Autoxidation, *Bacillus pasteurii*, furan fatty acid, *Helicobacter pylori*, methyl 9,12-epoxyoctadeca-9,11-dienoate, urease.

Long-chain fatty acids containing a furan nucleus, referred to as furan acids, have been identified in small amounts as constituents of triglycerides, phospholipids and sterol esters in certain species of fish (1,2) and plants (3), in soft corals (4), as well as in reptiles and amphibians (2). Furan fatty acids have been found to accumulate in different tissues of mammalian animals (5,6). Not much is known about the biological activity of furan fatty acids and their derivatives (7–9), and their biological role is not clear either. In the present study, the effect of the methyl ester of furan C-18 fatty acid (methyl 9,12-epoxyoctadeca-9,11-dienoate, MEFA) and its autoxidation products on bacterial urease activity was examined.

Almost a decade after the first publication on the high urease activity of *Helicobacter pylori* (10), it has been shown that this enzyme plays a major role in survival of this bacterium in the stomach and is an important factor in its virulence (11). The bacterial urease activity of certain microorganisms in the pathogenesis of pyelonephritis also has been suggested (12). Use of urease inhibitors may make it possible to promote more effective treatment and to protect the organism against urease-positive bacteria. Some problems in agriculture, concerning denitrification of fertilizers, are likewise associated with urease inhibitors whose function in this case consists of preventing rapid urease-catalyzed hydrolysis of urea to ammonium carbonate (13). Many compounds are urease inhibitors. However, most of these compounds are irrelevant to medicine and agriculture because of their insufficient effectiveness or negative secondary activities (13).

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EXPERIMENTAL PROCEDURES

Bacteria. Clinical isolates of *H. pylori* were obtained from Ezra Health Laboratories (Haifa, Israel). Primary isolation was performed on selective blood agar base No. 2 (Oxoid Ltd., Basingstoke, Hants, United Kingdom) supplemented with 5% (vol/vol) horse blood and 1 "Selectatab" tablet/500 mL (Mast Diagnostic, Merseyside, United Kingdom) with a final pH of 7.4.

Growth took place on chocolate agar under microaerophilic conditions in anaerobic jars (CampyPak, BBL, Benckton Dickinson Microbiology Systems, Cockeysville, MD) at 37°C for 3–5 d (14).

Preparation of urease. A urease preparation from *H. pylori* was obtained after breakdown of the bacterial suspension by treatment for 1 min in a 150-watt ultrasonic disintegrator at 4°C and clarification (by centrifugation at 9,000 × *g* for 10 min). In the course of the study, a solution of commercial *Bacillus pasteurii* urease (specific activity 23 mg NH_4/min/mg protein; Sigma Chemical Co., St. Louis, MO) was also used.

Measurement of urease activity. Urease activity of *B. pasteurii* was measured by alkalimetric assay as described previously (15). Briefly, the enzyme preparation (0.1 mL) was added to cuvettes containing 2.5 mL phosphate buffer (3 mM), pH 6.8, 0.1 mL of phenol red solution (0.21 mg/mL), 0.4 mL urea (0.33 mM) and 25 μL or less inhibitor suspension (15 mM in 10%, wt/vol) Tween 80). Reaction rates were followed by monitoring the increase in optical density ($O.D._{560}$) as the indicator color changed from yellow to red during the release of ammonia. Each cuvette was matched with a blank inhibitor suspension without the enzyme. *B. pasteurii* urease reaction rate in the presence of the MEFA mixture was used as indicator of the inhibitor's activity.

The crude extract of *H. pylori* urease with higher inhibitor concentration had high turbidity, and therefore its urease activity was measured by means of a Conway apparatus (16). The reaction was initiated by introducing 0.1 mL of the enzyme extract into the peripheral part of the apparatus with a mixture of 2 mL PBS (3 mM) pH 6.8, 0.4 mL urea (0.33 mM) and 0.1 mL or less inhibitor suspension (150 mM in 10%, wt/vol, Tween 80). The reactive mixture was incubated at 37°C for 30 min. The control mixture contained a complementary volume of 10% Tween 80 in water, without the inhibitor. The reaction was arrested by addition of saturated potassium carbonate and the apparatus was closed. Ammonia was removed from the reactive mixture by evaporation, absorbed in the middle part of the apparatus with boric acid, and then determined by the Berthelot method (17). The result served for evaluation of the urease activity, expressed as NH_4/min/mg protein. The protein concentration of the enzyme solution was determined by the method of Lowry *et al.* (18).

Preparation of inhibitors. The methyl ester of C-18 furan fatty acid (MEFA) was synthesized from methyl

ricinoleate according to the reported method (19) and also by esterification of the furan acid carried out by refluxing the latter in 1% sulfuric acid solution in methanol for 10 min (20). The furan fatty acid was prepared by hydrolysis of the MEFA synthesized as per Lie Ken Jie *et al.* (19).

The MEFA underwent oxidation in the course of its purification on a silica gel dry column and storage in a closed glass container for up to three weeks (20–25°C, repeated conditions). The autoxidation products were removed by silica gel thin-layer chromatography (TLC) separation (10 × 20, 0.25 mm thickness, Type Merck N 5553, hexane/ether, 10:1, vol/vol; Merck, Darmstadt, Germany) and by column chromatography separation (1.0 × 10 cm, silica gel Woelm, 100–200 mesh, Woelm Pharma, Eschwege, Germany) with the solvent system hexane/ether (90:1, vol/vol).

Gas chromatographic-mass spectrometric analysis (GC-MS). GC-MS analysis was carried out with a Finnigan (Sunnyvale, CA) ITS-40 on a DB-5 fused silica capillary column (30 m × 0.25 mm) programmed from 60 to 250°C at 10°C/min.

RESULTS

Figure 1 shows the results of inhibition of urease extract from *H. pylori* by various amounts of the unseparated autoxidized mixture. A 50% drop was observed for 1.3 mM of the mixture containing 0.08 mM active oxidation products (see below). The inhibition level of the MEFA mixture *in vitro* is of the order reported earlier for hydroxamic acid relative to *H. pylori* urease (21). This finding is surprising because hydroxamic acids were found to be highly specific inhibitors of urease (22).

The inhibition of *B. pasteurii* urease is shown in Figure 2. The inhibitory effect of the unseparated mixture on *B. pasteurii* urease was higher (I_{50} = 0.06 mM or 4 μM per oxidized products). The nonspecific interaction of the inhibitor with cytoplasmic compounds (proteins, etc.) of *H. pylori* urease crude extract, unlike the purified *B. pasteurii* urease commercial preparation, may provide an explanation for the dissimilarity in the respective inhibitory effect of MEFA oxidation products on *H. pylori*

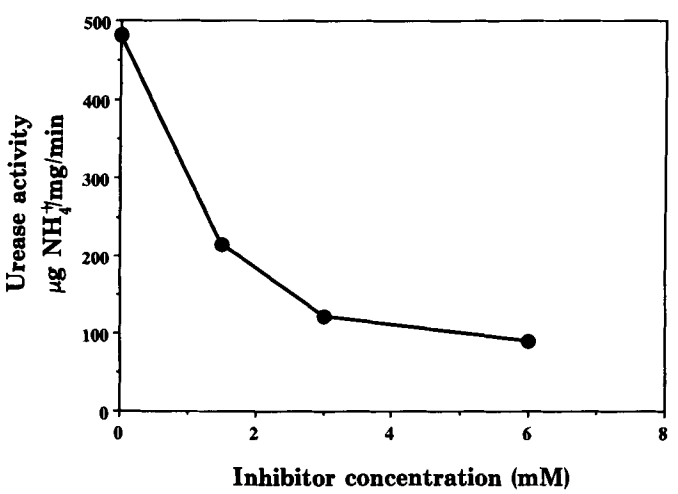

FIG. 1. Inhibition of *H. pylori* urease by oxidized 9,12-epoxyoctadeca-9,11-dienoate mixture.

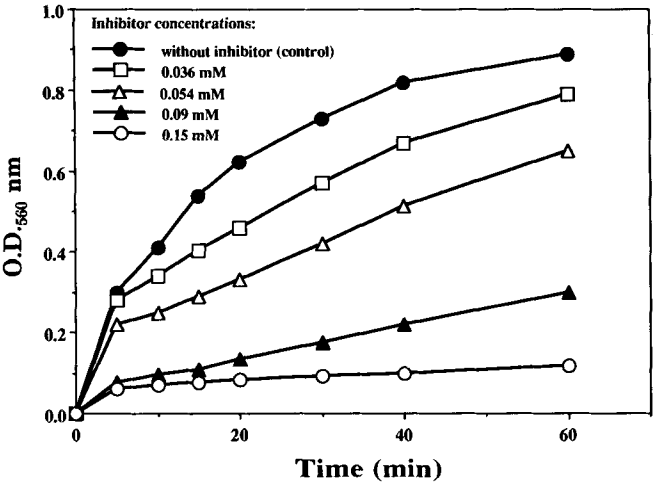

FIG. 2. Alteration of the *B. pasteurii* urease reaction rate in the presence of several concentrations of oxidized 9,12-epoxyoctadeca-9,11-dienoate mixture. Here and in Figure 3, curves indicate the rate of the urease reaction. The rates of urease reaction were followed by monitoring the increase in λ_{560} as the indicator changed from yellow to red during the release of ammonia. O.D., outside diameter.

and *B. pasteurii* urease. Addition of cysteine to the reaction mixture in advance, or in the course of the experiment, leads to loss of the inhibition effect by the oxidized MEFA mixture (Fig. 3).

GC analysis of the unseparated mixture revealed MEFA (Fig. 4, peak 1) and eight MEFA derivatives (Fig. 4). Separation of the inhibitor mixture by the chromatography column showed that the antiurease effect was connected with the fraction of the lowest polarity in the oxidized substances (active oxidation products; peaks 2, 3, 4, 5, 6), comprising 6.2% of the total weight of the mixture. Separated by column, the fraction containing these compounds had a similar urease inhibition effect as that of the original mixture, calculated for the active oxidation products (data not shown).

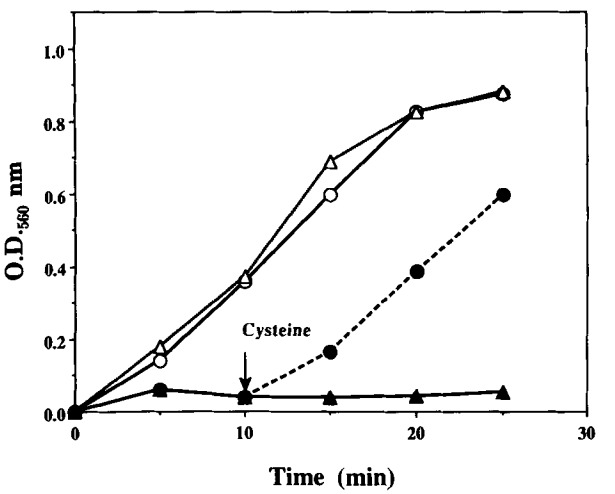

FIG. 3. Influence of cysteine addition on the inhibition of *B. pasteurii* urease by oxidized 9,12-epoxyoctadeca-9,11-dienoate mixture. The rate of the urease reaction: ○ in the presence of 0.6 mM cysteine (control); ▲ in the presence of 0.3 mM inhibitor; △ in presence of 0.3 mM inhibitor and of 0.6 mM cysteine; ● 0.6 mM cysteine was added to the reactive mixture 10 min after the start of the reaction in the presence 0.3 mM inhibitor. O.D., outside diameter.

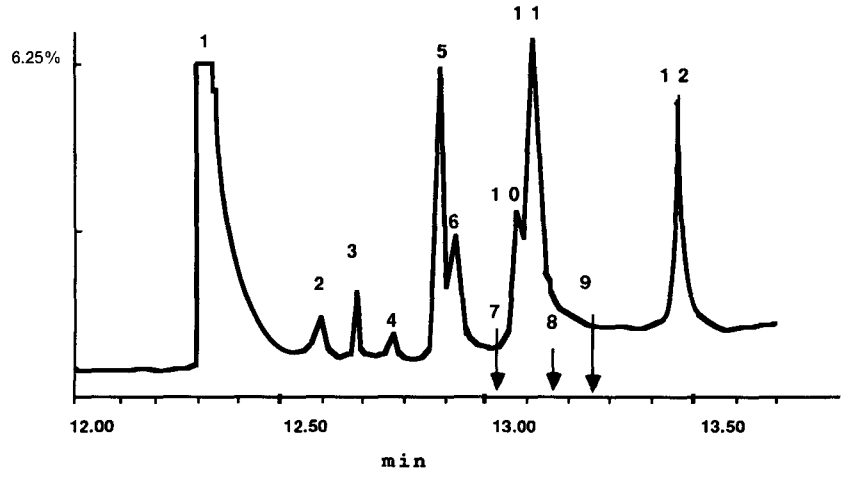

FIG. 4. Capillary gas chromatogram of oxidized 9,12-epoxyoctadeca-9,11-dienoate (MEFA) mixture. For identification of numbered peaks, see Table 1. ←, Retention time of compounds 7, 8 and 9 replacing compound 4 in gas chromatographic analysis of oxidized MEFA products preseparated by thin-layer chromatography.

The oxidized products of lowest polarity were separable from each other only by GC on capillary column, and were inseparable by ordinary chromatography column and TLC. Moreover, separation of the oxidation products fraction from MEFA on the TLC plate was accompanied by changes in their composition, and a mixture of structural and spatial isomers corresponding to peaks 7, 8 and 9, instead of to compound 4 was observed by the GC-MS method. These conversions resulted in the reduction of the inhibitory activity of the oxidation products relative to *B. pasteurii* urease ($I_{50} = 0.2$ mM instead of 4 μM, as compared with the initial suspension).

Mass spectral characteristics and putative structures of the MEFA oxidized products are shown (Table 1). Mass spectral data show that peaks 2, 4 and 6 are represented by a combination of two structural isomers of unsaturated furan acid methyl ester. Moreover, the mass spectra of peaks 2 and 3 were essentially identical with those of peaks 6 and 5, respectively, suggesting that a pair of peaks, 2,6 and 3,5, represented *cis-trans* isomers of the olefinic furan fatty acid methyl ester. These findings are essentially in agreement with the basic mass spectral data reported previously on oxidation products of natural furan acid and its esters (2,5,23).

Following removal of the oxidation products, the MEFA suspension reduced bacillus urease activity by less than 20% at 0.8 mM concentration. As freshly synthesized MEFA (by furan fatty acid acylation) caused only a slight change in the activity of the tested urease, we assume that the above inhibitory effect was due to incomplete separation of MEFA from the oxidation products.

DISCUSSION

It is known that furan fatty acid can be oxidized both by liberated enzymes and nonenzymatically to an unstable active intermediate—oxo-ene-oxo (dioxo-ene) derivatives—and other more stable products (2,23). Oxo-*trans*-ene-oxo compounds easily react with thiol anions through Michael-type addition, leading to formation of thioethers (7,23). It was reported that reaction with HS-groups may be a mechanism for highly selective inhibition of enzymatic activity by micromolar amounts of compounds containing oxo-*trans*-ene-oxo (7) or an oxo-ene and epoxy-ene structure (24). We suggest that the urease inhibitory effect may mainly be dependent on the presence in the mixture of the dioxo-ene derivatives (substance 4, Table 1) formed in the course of autoxidation and converted through cyclodehydration during GC analysis into the other compounds observed by us. Conversion of diketo-ene into the unsaturated furan acid during GC analysis was demonstrated previously for natural 10,11-dimethyl furan fatty acids (2). Reduction of the inhibitory effect of oxidized MEFA following TLC separation is attributable to conversion of the dioxo-ene on the TLC plate into the more stable derivatives 7, 8 and 9. The loss of antiurease activity of the mixture in the presence of cysteine can be the consequence of transformation of diketo-ene to thioether of furan acid (7,23), and it also can be indirectly shown that diketo-ene compounds are involved in the urease inhibition by MEFA oxidation products. One can find another explanation for the complete reactivation of the enzyme through the addition of cysteine, if it is assumed that the inhibitory effect is the result of specific oxidation of the HS-groups of the enzymes active center by the inhibitor. In this case, reduction of the oxidized HS-groups by cysteine can be suggested.

Experiments are in progress to study the urease inhibitory mechanism by oxidized MEFA mixture. Further research will determine the correctness of our reported versions.

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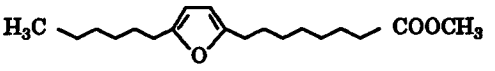
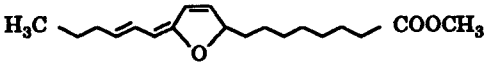
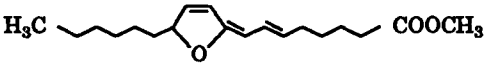
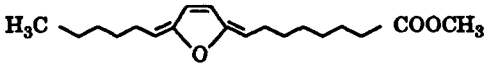
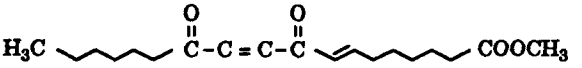
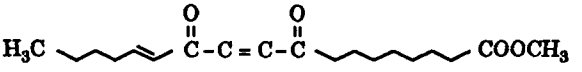
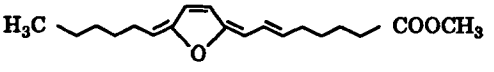
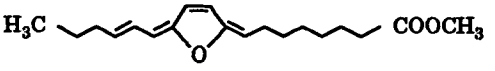
TABLE 1

Structures of 9,12-Epoxyoctadeca-9,11-Dienioate Autoxidation Products from Gas Chromatography-Mass Spectrometry Analysis Data

N peaks	Structure of substance	Major MS (*m/x*)
1		308(100), 165(77), 277(26), 145(10), 237(8)
2 and 6		2): 191(91), 232(81), 306(70), 219(12), 263(6)
		6): 191(100), 232(72), 306(45), 219(9), 263(6)
3 and 5		107(100), 163(40), 306(37), 263(20), 231(9)
4		165(98), 322(30), 179(23), 207(20), 251(10) 304(8)
		
7, 8 and 9		7): 304(100), 189(92), 230(40), 261(18)
		8): 304(100), 189(90), 230(40), 261(17)
		9): 304(100), 189(54), 230(20)

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