The broad spectrum antibacterial properties of 2-hydroxydiphenyl ethers have been appreciated for decades, and their use in consumer products is rapidly increasing. We identify the enoyl-acyl carrier protein reductase (FabI) component of the type II fatty acid synthase system as the specific cellular target for these antibacterials. Biologically active 2-hydroxydiphenyl ethers effectively inhibit fatty acid synthesis in vitro and FabI activity in vitro. Resistant mechanisms include upregulation of fabI expression and spontaneously arising missense mutations in the fabI gene. These results contradict the view that these compounds directly disrupt membranes and suggest that their widespread use will select for resistant bacterial populations.

The fabI gene of *Escherichia coli* encodes the NADH-dependent trans-2-enoyl-acyl carrier protein (ACP) reductase of bacterial fatty acid synthesis (1). Bacteria utilize the type II, or dissociated, fatty acid biosynthetic system (2–4), which consists of a collection of distinct polypeptides that each carry out a unique reaction in the biosynthetic cycle. FabI catalyzes the last step in each cycle of elongation and is an important regulatory point in the pathway, playing a determinant role in completing each round of elongation (5, 6). The enoyl-ACP reductase of *Mycobacterium tuberculosis*, InhA, is the target for a metabolite of isoniazid, a compound used in the treatment of tuberculosis (7–9). *E. coli* FabI is inhibited by a class of heterocyclic, boron-containing compounds (diazaborines) (1). In both cases, the drugs bind together with NAD at the active site, and resistant enzymes arise from mutations that alter the residues that form the NADH binding pocket (10, 11). The recent work identifying enoyl-ACP reductase as the target for these therapeutic agents has stimulated research into developing a second generation of antibacterial drugs that inhibit FabI and bacterial fatty acid synthesis.

2-Hydroxydiphenyl ethers are a class of compounds that exhibit broad spectrum antimicrobial activity (Fig. 1). Many of these compounds were initially used in the treatment of textiles, and there have been hundreds of patents filed worldwide for their incorporation into a diverse range of products over the last 30 years. Triclosan (VI) is the most potent and widely used member of this class in contemporary consumer products as a microicide. For example, triclosan is a component of deodorant soaps, dermatological and topical preparations for skin, oral rinses, toothpastes, and is even incorporated into the plastics of children's toys (12). Triclosan has long been thought to disrupt the cell membrane, rendering bacteria unable to assimilate nutrients and proliferate (13–15). This view of triclosan acting as a nonspecific biocide has provided the rationale for its use in consumer products and predicts that the emergence of resistant strains is very unlikely. While our work was in progress, a scientific correspondence reported that *E. coli* strains that were selected for resistance to triclosan had mutations in the *fabI* gene (16). This finding led the authors to propose that FabI was the direct target for triclosan; however, no biochemical analysis was provided and other interpretations were possible. Mutations in FabI could alter the activity of the overall pathway to compensate for triclosan inhibition of another target in fatty acid biosynthesis or FabI mutations could result in an altered membrane fatty acid composition that would blunt the proposed membrane-perturbing effects of triclosan. In this report, we demonstrate that the 2-hydroxydiphenyl ethers directly inhibit FabI enzyme activity.

**EXPERIMENTAL PROCEDURES**

**Cloning the DHDPE Resistance Gene**—A genomic library of strain MC4100 tolC::Tn10 (providing by P. Miller, Parke-Davis) was constructed as follows. Chromosomal DNA was purified from a cell lysate using a chromosomal DNA isolation kit and procedure supplied by Qiagen Inc. (Chatsworth, CA). The purified DNA was partially digested with *Sac*II, and DNA fragments estimated to range in size from 6–12 kilobases were isolated from an agarose gel using a QIAquick kit and procedure supplied by Qiagen Inc. The resulting DNA was ligated to pBR322 that had been digested with *Bam*HI and dephosphorylated with calf intestinal phosphatase. The resulting ligation mixture was used to transform strain DHDPE by a method similar to the one described by Sambrook and Russell (16). Plasmid DNA was isolated from approximately 12,000 transformants from the genomic library. Plasmids that conferred resistance to DHDPE were isolated by transforming strain MC4100 (tolC::Tn10) with the genomic library and selecting for colonies that grew on LB plates containing ampicillin (50 μg/ml) and DHDPE (2 μg/ml) at 37 °C.

**Isolation of DHDPE-resistant Mutants**—The spontaneous DHDPE-resistant (DHDPE)* mutant, strain EP1424, was isolated by plating approximately 10⁶ cells from an overnight culture of *E. coli* strain W3110 grown in LB at 37 °C onto LB plates containing 2 μg/ml DHDPE followed by incubation at 37 °C. The triclosan-resistant mutant, strain RJH108, was isolated in a similar fashion by plating strain W3110 on LB plates containing 1 μg/ml DHDPE. Strain JP1111 (Hfr *galE45 fabI392* transposon insertion) was obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, CT. The fabI gene was formerly known as *envM*. The *fabI* genes from strains W3110, EP1424, and RJH108 were amplified using polymerase chain reaction from chromosomal DNA and sequenced using *fabI*-specific primers on an ABI Model 373A sequencer.

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1 The abbreviations used are: ACP, acyl carrier protein; DHDPE, 2,2′-dihydroxydiphenyl ether; 8:1-NAC, trans-2-octadecenoyl-N-acetyl-cysteamine; MIC, minimal inhibitory concentration.
The FabI[G93S] protein was prepared by amplifying the mutant fabI gene from strain EP1424 and cloning the gene into the pET15b expression vector. The sequence of the insert DNA was verified, and purification of the FabI[G93S] protein to homogeneity was accomplished using techniques described previously for the wild-type FabI (5).

Acetate Labeling—E. coli strain UB1005 (metB1 relA1 spoT1 gyrA216 F') was grown to a density of 5 × 10⁸ cells per ml in minimal M9 medium at 37 °C. [1-¹⁴C]Acetate (specific activity 6.08 Ci/mmol) was then added to a final concentration of 0.1 mCi/ml, and the culture divided into four equal portions. DHDPE was added to a final concentration of 0, 1, 2, or 4 µg/ml, and the cultures were then incubated at 37 °C for various time intervals. 1 ml of the culture was removed and added into a 15-ml glass tube containing 2.4 ml of methanol and 100 µl of glacial acetic acid to quench further incorporation of label. Total lipids were extracted (17). An aliquot of each lipid extract was counted in Me2SO. The Me 2SO concentration in all assays was maintained at a trace. 2-Hydroxydiphenyl ethers were added to the final concentration indicated in the figure legend from serially diluted stock solutions prepared into Me2SO. Activity in the absence of drug was 0.34 mol/min/mg. Data points were within 5% of the average. Each IC₅₀ was confirmed in a separate independent assay, and the individual values were within 5% of the average. Each IC₅₀ was confirmed in a separate independent experiment and was determined from a semilog plot of the data.

RESULTS

Isolation of a DHDPE-resistant Mutant—In a screen of a chemical library, a 2-hydroxydiphenyl ether, DHDPE (I), was identified as an antimicrobial agent with a minimal inhibitory concentration (MIC) of 1 µg/ml against E. coli strain W3110 (Table I). We therefore investigated the mechanism of action of six representative members of this structural class of antimicrobial compounds (Fig. 1). First, a genetic approach was used to identify genes that when overexpressed conferred resistance to DHDPE. A genomic library was constructed and transformed into E. coli strain MC4100/tolC::Tn10, and colonies that grew on plates containing 2 µg/ml DHDPE were identified.

Plasmid DNA was isolated from two colonies and when transformed back into E. coli strain MC4100/tolC::Tn10 again gave resistant colonies whereas empty vector controls did not (Fig. 2A). The DNA sequence at both ends of two of the resistance-conferring insertions showed that they both contained the same region of the E. coli chromosome, including the complete coding sequences of fabI, ycdD, sapF, sapD, and sapC (Fig. 2A). A subclone of the resistance insert spanning fabI and a derivative that contained a 4-base pair insertion in the HindIII site situated within fabI were also tested in this assay, along with a plasmid, pfabI, containing only the fabI gene on an 0.8-kilobase insert. Only plasmids that encoded a functional fabI gene conferred resistance to 2 µg/ml DHDPE (Fig. 2A). These data, therefore, suggested that the protein product of the fabI gene was the target for DHDPE.

A spontaneous DHDPE-resistant mutant of E. coli strain W3110, termed strain EP1424 (DHDPEβ), was isolated on LB plates containing 2 µg/ml DHDPE. Based on the observation that multiple copies of fabI conferred DHDPE resistance, genetic crosses mediated by bacteriophage P1 transduction were conducted on resistant strain EP1424 to determine if it carried a mutation in the fabI gene. A derivative of strain EP1424 that carried a Tn10kan near fabI was constructed (strain EP1480) and was used as a donor in a cross with strain JP1111 which harbors a temperature-sensitive mutation in fabI and was used as a donor in a cross with strain JP1111 which harbors a temperature-sensitive mutation in fabI. Recombinants were selected on LB-kanamycin plates at 30 °C and scored independently for their ability to grow at 42 °C and for their sensitivity to DHDPE. The co-transduction frequencies between the various elements were: Tn10kan and the fabI(Ts) allele, 50%; Tn10kan and the DHDPE allele, 50%; fabI(Ts) allele and the DHDPE allele, 96%. The conclusion from these crosses is that a mutation in or very near fabI was sufficient to confer DHDPE resistance.

The DNA sequence of the fabI coding region and 230 nucleotides upstream from the fabI start codon was determined from both resistant strain EP1424 and its isogenic DHDPE parent (strain W3110). Comparison of the wild-type and mutant sequences revealed only a single base change between the two alleles. The sequence corresponding to codon 93 of the fabI gene was sufficient to confer DHDPE resistance.

DHDPE Inhibition of Fatty Acid Synthesis—We determined whether DHDPE inhibited fatty acid synthesis in intact cells by measuring the effect of the drug on the incorporation of [3H]acetate into fatty acids (Fig. 2B). In the absence of drug, de
Growth was also monitored and was not significantly decreased by the presence of any concentration of the drug during the labeling experiment.

The culture was then split into four equal portions, and drug was added to the indicated concentration. Aliquots of the cultures were removed at the times indicated, and the amount of label incorporated into the fatty acids was determined as described under “Experimental Procedures.”

Inhibition of FabI by 2-Hydroxydiphenyl Ethers—The ability of DHDPE to specifically inhibit FabI was addressed in an in vitro spectrophotometric assay using homogeneous FabI and the enoyl-ACP substrate analog, trans-2-octenoyl-N-acetylcycteamine (8:1-NAC) (Fig. 3A). Addition of increasing concentrations of DHDPE to the reaction potently inhibited the reduction of 8:1-NAC by NADH with an IC50 of 2.5 μM (0.5 μM/ml) (Fig. 3A). We expanded our analysis to include 5 additional 2-hydroxydiphenyl ethers (Table I). These data clearly show that DHDPE had a profound inhibitory effect on FabI. The ability of DHDPE to specifically inhibit FabI was a characteristic of this class of antimicrobial compounds.

The antimicrobial activity of all of these compounds was directly related to their ability to inhibit FabI. The MIC values for each compound against the wild-type strain E. coli strain W3110 (Table I) correlated with their potency in the inhibition of FabI in vitro (Fig. 3A). The three most potent compounds for the inhibition of FabI in vitro (I, III, and VI) had the lowest MICs against strain W3110, whereas the thioether analog (II) exhibited a high IC50 in the FabI assay and was inactive against E. coli. The six compounds were then screened against strain EP1424, which expresses the mutant FabI[G93S] protein (see above), and strain SJ53/pFabI, which overexpresses the wild-type FabI protein. Both strains exhibited significantly increased resistance to the entire panel of 2-hydroxydiphenyl ethers (Table I).

The 4-fold increase in the MIC for triclosan (VI) in the DHDPE-resistant strain EP1424 was the lowest among the compounds examined (Table I). These data indicated that there was another target for triclosan or that the FabI[G93S] mutant remained sensitive to triclosan inhibition. Therefore, we tested the ability of the three most potent compounds (I, III, and VI) to inhibit homogeneous FabI[G93S] in vitro (Fig. 3B). FabI[G93S] was completely refractory to inhibition by compound I (DHDPE) and was only marginally affected by compound III. However, the IC50 for triclosan was 8 μM, corresponding to a 4-fold increase in the IC50 in the mutant compared with the wild-type enzyme (Fig. 3). The ability of triclosan to inhibit fatty acid synthesis in the sensitive and resistant strains was tested at twice the MIC for the respective strains using a [3H]acetate labeling experiment. Triclosan (1 μg/ml) inhibited the incorporation of acetate into fatty acids by 80% in strain W3110, whereas a concentration of 4 μg/ml triclosan inhibited fatty acid formation by 60% in strain EP1424. As expected, DHDPE did not affect the rate of fatty acid synthesis in strain EP1424. Thus, the 4-fold increase in the IC50 of triclosan for the FabI[G93S] mutant compared with the wild-type protein correlated with the 4-fold increase in the MIC and the increased resistance of fatty acid synthesis to triclosan in strain EP1424.

Isolation of a Triclosan-resistant Mutant—These data suggested that the ability of triclosan to inhibit the growth of the DHDPE-resistant strain EP1424 was due to the residual sensitivity of the FabI[G93S] protein to triclosan. We selected a spontaneous triclosan-resistant mutant to determine if a different alteration in the fabI gene would confer a higher level of resistance to triclosan. Strain RJH108 was a spontaneously arising mutant that exhibited a 64-fold higher resistance to triclosan compared with the parental strain (Table I). DNA sequence analysis of the fabI gene in strain RJH108 showed the presence of a single missense mutation at codon 93 from GGT to GTT, resulting in a predicted change from serine to valine at this position. Strain RJH108 (FabI[G93V]) was also cross-resistant to the other 2-hydroxydiphenyl ethers (Table I). Thus, high level resistance to triclosan results from a missense mutation in the fabI gene that substitutes a bulky hydrophobic amino acid side chain for glycine at position 93 of the protein.

The antimicrobial activity of DHDPE, triclosan, and other 2-hydroxydiphenyl ethers is attributed to their ability to inhibit fatty acid biosynthesis at the FabI step. The essential function of FabI in the type II fatty acid synthase systems of bacteria suggests that inhibitors of this enzyme have the po-

Fig. 2. Identification of the fabI gene and fatty acid biosynthesis as the DHDPE target. Panel A, the E. coli chromosomal region around fabI (GenBank™ accession numbers AE000226 and AE000227) is shown at the top. Plasmids containing various DNA fragments are shown along with their ability to permit growth on LB plates containing 2 μg/ml DHDPE. The DNA inserts in the pNY plasmids were in pBR373, and pFabI is described in the legend to Table I. Panel B, E. coli strain UB1005 was grown at 37 °C to midlog phase, and [3H]acetate was added to 0.1 mCi/ml. The culture was then split into four equal portions, and drug was added to the indicated concentration. Aliquots of the cultures were removed at the times indicated, and the amount of label incorporated into the fatty acids was determined as described under “Experimental Procedures.” Cell growth was also monitored and was not significantly decreased by the presence of any concentration of the drug during the labeling experiment.
The discovery of a specific intracellular target for 2-hydroxydiphenyl ethers contradicts the prevailing view of the mechanism of action of these compounds. Triclosan (VI) is thought to attack the bacterial envelope making it more porous and preventing the uptake of nutrients and growth of the organism (13–15). This mechanism of action has been used to justify the widespread and rapidly increasing use of triclosan in personal care products since such organisms are unlikely to acquire resistance to a compound that acts by nonspecifically disrupting membrane architecture. The first indication that triclosan has a specific cellular target was provided by McMurray et al. (16) who report that triclosan-resistant strains have mutations in the fabI gene suggesting that the enoyl-ACP reductase was the target for the compound. Our biochemical analysis of enoyl-ACP reductase inhibition by these compounds provides the definitive evidence that the membrane is not the primary site of action. Indeed, the fact that triclosan-treated bacteria exhibit alterations in membrane permeability and function (13–15) is consistent with FabI as the cellular target. First, the inhibition of fatty acid biosynthesis interferes with membrane assembly and integrity by blocking phospholipid formation. Second, the temperature-sensitive fabI mutant was initially designated enuM because it was isolated through a genetic selection for strains with thermosensitive envelope permeability defects (19). Finally, the diazoborine FabI inhibitors are also known to perturb membrane functions (20). Therefore, the reported effects of triclosan and other 2-hydroxydiphenyl ethers on membrane structure and function arise secondarily from its specific inhibition of fatty acid biosynthesis at the FabI step.

Triclosan is effective against a broad spectrum of bacteria (12), including multidrug-resistant Staphylococcus aureus (21, 22) indicating that development of additional FabI inhibitors will supplement the arsenal against multidrug-resistant bacteria. However, the ability of bacteria to acquire genetic resistance to triclosan and related compounds suggests that the widespread use of these chemicals will eventually lead to the appearance of resistant organisms that will compromise the usefulness of FabI inhibitors. Prudence dictates that the uses for this class of compounds be re-evaluated in light of their specific mechanism of action.

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