Antibacterial Agents That Inhibit Lipid A Biosynthesis

H. Russell Onishi, Barbara A. Pelak, Lynn S. Gerckens, Lynn L. Silver, Frederick M. Kahan, Meng-Hsin Chen, Arthur A. Patchett, Susan M. Galloway, Sheryl A. Hyland, Matt S. Anderson, Christian R. H. Raetz*

Lipid A constitutes the outer monolayer of the outer membrane of Gram-negative bacteria and is essential for bacterial growth. Synthetic antibacterials were identified that inhibit the second enzyme (a unique deacetylase) of lipid A biosynthesis. The inhibitors are chiral hydroxamic acids bearing certain hydrophobic aromatic moieties. They may bind to a metal in the active site of the deacetylase. The most potent analog (with an inhibition constant of about 50 nM) displayed a minimal inhibitory concentration of about 1 microgram per milliliter against *Escherichia coli*, caused three logs of bacterial killing in 4 hours, and cured mice infected with a lethal intraperitoneal dose of *E. coli*.

The emergence of bacterial pathogens that are resistant to major classes of commercial antibiotics has created an urgent need for novel antibacterial agents (*1–3*). Recent genetic studies have shown that biosynthesis of the lipid A anchor of the lipopolysaccharide (LPS) of Gram-negative bacteria is a suitable pharmaceutical target (*4–7*). Conditional mutants in which early steps of lipid A biosynthesis can be switched off lose several logs of viability in 3 to 4 hours (*6, 7*). Reduced lipid A biosynthesis also renders Gram-negative bacteria hypersensitive to other antibiotics (such as erythromycin) that normally are not active (*8*). Previous attempts to block LPS biosynthesis with carbohydrate analogs at the level of core oligosaccharide assembly (*9, 10*) were hampered, because such analogs do not readily penetrate bacteria and are not bactericidal within a few hours.

To identify novel inhibitors of lipid A biosynthesis, we screened for agents that selectively block [U-^{14}C]galactose incorporation into acid-precipitable material in living cells. At a concentration of 100 μg/ml, L-573,655 inhibited LPS biosynthesis by 80 to 90% of that seen in controls and had minimal effects on the synthesis of DNA, RNA, protein, and phospholipids (Fig. 1). L-573,655 is a hydroxamic acid attached to a 2-phenyloxazoline ring system that had been made previously as a precursor in the chemical synthesis of cycloserine (*11*).

The nine enzymes of lipid A biosynthesis in *Escherichia coli* extracts (*4, 5, 12*) were then assayed for sensitivity to inhibition by L-573,655. The second enzyme of the pathway, a deacetylase that acts on uridine 5′ diphosphate-3-O-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine [UDP-3-O-(*R*-3-hydroxymyristoyl-GlcNAc] (Fig. 2), was the only one inhibited by L-573,655 (100 μg/ml). The Michaelis constant (K_m; affinity for substrate) for UDP-3-O-(*R*-3-hydroxymyristoyl)-GlcNAc is 3 μM. At substrate concentrations above K_m (Fig 3A, open circles), the half-maximal inhibitory concentration (IC_{50}) was shifted to higher L-573,655 concentrations, which is consistent with a competitive inhibition model. The inhibition constant (K_i) determined for L-573,655 is 24 μM.

Analogs of L-573,655 containing a carboxylic acid function in place of the hydroxamic acid moiety were inactive at all effective concentrations of L-573,655. This suggests that there is a metal atom at the deacetylase active site, the presence of which has been confirmed (*13*). As inhibition by L-573,655 (an *RS* mixture) was stereospecific for the *R* isomer (L-159,463; Fig. 3), deacetylase inhibition may involve specific binding of L-159,463 to the enzyme. Inhibition of other metalloproteases by metal chelating compounds that bind to the active site is well documented (*14*). Inhibitors of angiotensin-converting enzyme with subnanomolar *K*'s are known (*14*) and include the commercial antihypertensives enalapril and captopril.

Over 200 analogs of L-573,655 were synthesized (*15*). Attachment of methoxy and certain other electron-donating hydrophobic groups to the phenyl moiety increased potency significantly (Fig. 3B). One of the most effective compounds identified so far is L-161,240, the K_i of which is ~50 nM. Inhibitors bearing larger hydrophobic groups, such as the (*R*,*S*)-3-benzyloxy analog of L-161,240, were comparably potent (K_i ~ 50 nM), but antibacterial activity of this and similar compounds with increased

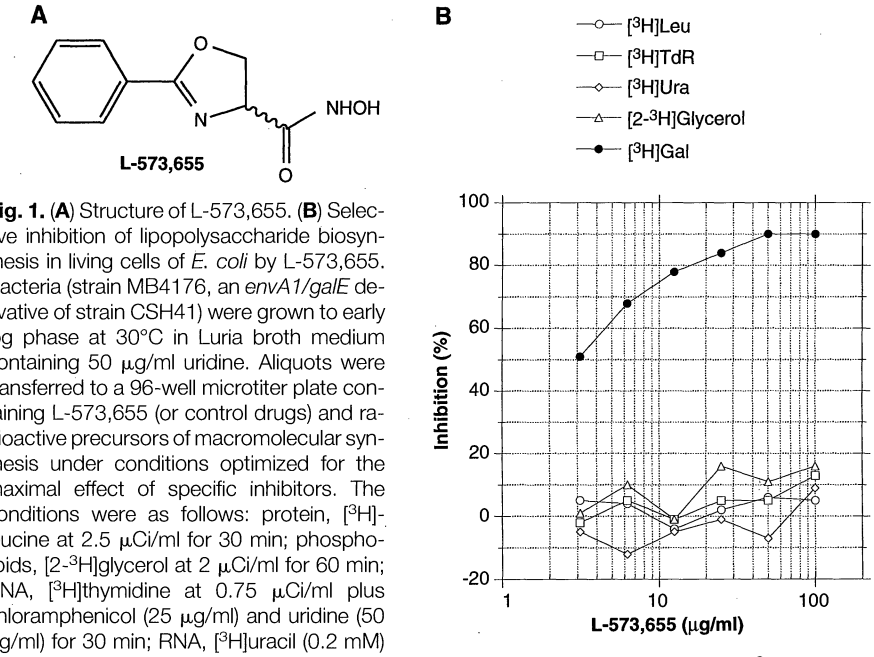

Fig. 1. (**A**) Structure of L-573,655. (**B**) Selective inhibition of lipopolysaccharide biosynthesis in living cells of *E. coli* by L-573,655. Bacteria (strain MB4176, an *envA1/galE* derivative of strain CSH41) were grown to early log phase at 30°C in Luria broth medium containing 50 μg/ml uridine. Aliquots were transferred to a 96-well microtiter plate containing L-573,655 (or control drugs) and radioactive precursors of macromolecular synthesis under conditions optimized for the maximal effect of specific inhibitors. The conditions were as follows: protein, [^{3}H]-leucine at 2.5 μCi/ml for 30 min; phospholipids, [2-^{3}H]glycerol at 2 μCi/ml for 60 min; DNA, [^{3}H]thymidine at 0.75 μCi/ml plus chloramphenicol (25 μg/ml) and uridine (50 μg/ml) for 30 min; RNA, [^{3}H]uracil (0.2 mM) at 1.5 μCi/μmol plus chloramphenicol (25 μg/ml) for 30 min; and lipopolysaccharide, [^{3}H]galactose (0.2 mM) at 20 μCi/μmol plus chloramphenicol (25 μg/ml) for 75 min. All incubations were at 30°C with shaking at 220 rpm. Chloramphenicol was present to counteract the effects of the stringent control. Incorporation was stopped with 10% trichloroacetic acid. Precipitates were collected on glass fiber filters. Uninhibited controls incorporated the following: [^{3}H]leucine ([^{3}H]Leu), 1481 cpm; [^{3}H]thymidine ([^{3}H]TdR), 9211 cpm; [^{3}H]uracil ([^{3}H]Ura), 3522 cpm; [2-^{3}H]glycerol, 17504 cpm; and [^{3}H]galactose ([^{3}H]Gal), 1323 cpm.

Departments of Microbiology, Medicinal Chemistry, and Biochemistry, Merck Research Laboratories, Rahway, NJ 07065, USA.

*To whom correspondence should be addressed. Present address: Department of Biochemistry, Duke University Medical Center, Durham, NC 27710, USA.

hydrophobicity against wild-type *E. coli* was poorer than that of L-161,240.

Minimal inhibitory concentrations (MICs) of three deacetylase inhibitors were compared to those of several clinically relevant antibiotics. A wild-type strain of *E. coli* and a mutant harboring a point mutation (*envA1*) (*16*) in the structural gene encoding the deacetylase (*4*, *5*, *17*) were used. The mutant was initially selected to eliminate the outer membrane barrier as a factor in evaluating new compounds. Strains harboring *envA1* have increased sensitivity to large or hydrophobic antibiotics (*16*, *18*), leak periplasmic enzymes (*18*), and display a 30% reduction in lipid A content as compared with wild-type strains (*19*). They are not growth-inhibited, however; lipid A content must be reduced at least 50% to block growth completely (*6*). L-573,655 had only modest activity against wild-type cells (MIC 200 to 400 μg/ml), but its antibacterial activity was two orders of magnitude greater (MIC 3 to 6 μg/ml) against strains bearing the *envA1* mutation (Table 1). This may be due to two factors: the lowering of target enzyme concentration and the permeabilization of the outer membrane. The analogs L-159,692 and L-161,240 showed enhanced antibacterial potency against both wild-type and *envA1*-bearing strains (Table 1). The antibacterial activity of L-161,240 (MIC 1 to 3 μg/ml) was comparable to that of ampicillin toward wild-type *E. coli*. However, L-161, 240 was considerably more potent than ampicillin in strains bearing the *envA1* mutation (MIC 8 to 16 ng/ml). Because L-161,240 (K_i ~50 nM) is still several orders of magnitude less potent than the best possible metalloamidase inhibitors (*14*), it may be feasible to design much more potent deacetylase inhibitors.

Treatment of *E. coli* cells with L-573,655 (Fig. 1) and the other analogs resulted in three logs of killing in 4 hours, as observed with conditional mutants in *lpxA* (*6*). This rate is comparable to that caused by ampicillin. Chloramphenicol and most antibiotics that inhibit protein syntheses do not kill bacteria in this time interval but are bacteriostatic. The frequency of L-573,655–resistant mutants was ~1 × 10^{-9}. Resistant organisms regained the capacity to make lipid A in the presence of the deacetylase inhibitors (*12*, *20*).

Despite the enhanced potency of L-161,240 in inhibiting the growth of *E. coli* and related bacteria (Table 2), no antibacterial activity was observed with *Pseudomonas* or

Table 1. Minimal inhibitory concentrations of selected deacetylase inhibitors against strains of *E. coli*. Bacterial strains used were wild-type *E. coli* strains (either MB2884 or MB5499) and isogenic *E. coli envA1* strains (MB4926 or MB5500). MICs given are the ranges of multiple tests. Stock solutions of compounds were prepared by dissolution of compounds in dimethylsulfoxide at 0.008 g/ml. For determination of the MIC, twofold serial dilutions were prepared in Mueller-Hinton broth to yield 0.05 ml of antibiotic-containing medium per well. Inocula were prepared from cultures grown overnight in trypticase-soy broth at 37°C. Cell densities were adjusted to absorbance at 660 nm (A_{660}) = 0.1; the optical density–standardized preparations were diluted 1:1000 in Mueller-Hinton broth; and wells were inoculated with 0.05 ml of the diluted bacteria, giving a final cell density of approximately 1 × 10^5 colony-forming units per milliliter. Microtiter plates were incubated at 37°C for 18 hours in a humidified incubator, and the MIC was recorded as the lowest drug concentration that inhibited visible growth.

Compound	MIC (μg/ml)	
	E. coli (*envA*$^+$)	*E. coli* (*envA1*)
L-573,655	200 to 400	3 to 6
L-159,692	25 to 50	0.25
L-161,240	1 to 3	0.008 to 0.016
Ampicillin	1 to 3	0.2
Rifampicin	6	0.003 to 0.006
Erythromycin	25 to 50	0.05
Imipenem	0.06	0.03

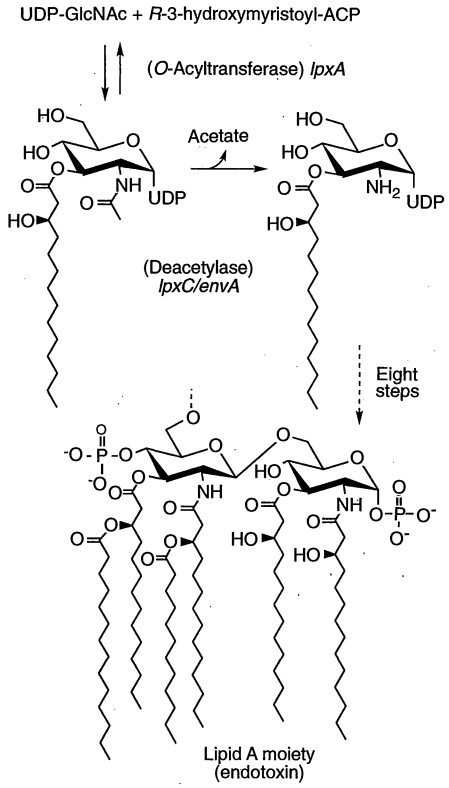

Fig. 2. A unique deacetylase catalyzes the second step of lipid A (endotoxin) biosynthesis. The enzyme recognizes UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc but not UDP-GlcNAc. The deacetylase (encoded by *lpxC/envA*) is actually the first committed step of the pathway (*4*, *5*). The LpxA-catalyzed acylation that occurs before deacetylation is reversible and has an unfavorable equilibrium constant (*23*, *24*).

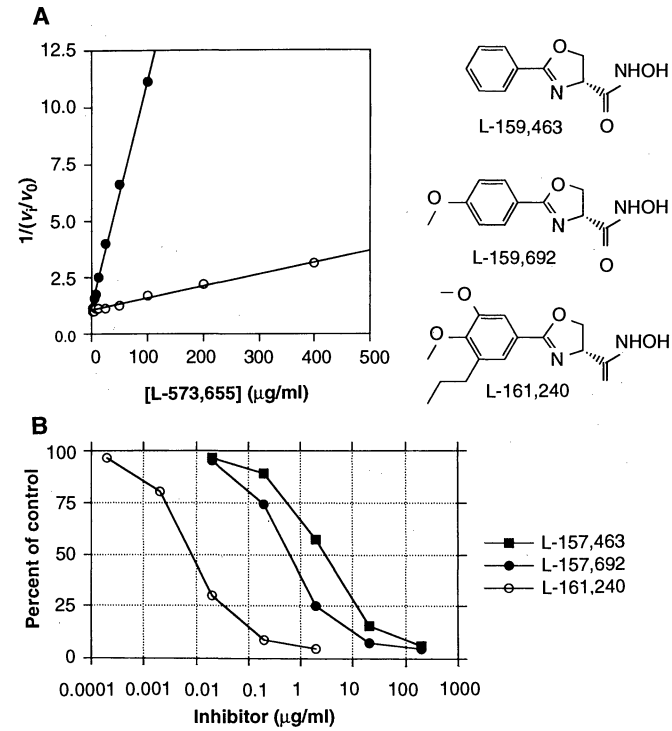

Fig. 3. Deacetylase inhibition by L-573,655 and related compounds. (**A**) Dixon plot showing competitive inhibition of *E. coli* UDP-3-*O*-acyl-GlcNAc deacetylase by L-573,655. Activity measured near K_m (3 μM substrate, filled circles) at a given concentration of inhibitor is much reduced relative to activity measured at saturation (160 μM substrate, open circles). Activity measurements were made with a radiochemical assay (*7*) in 40 mM bis-tris buffer (pH 5.5) at 30°C. The enzyme source was a membrane-free extract of *E. coli* strain JB1104 (*24*). Reactions were initiated by addition of enzyme to assay cocktail containing the indicated concentration of L-573,655. Activity measurements were made with a twofold dilution series of inhibitor in the range of 1.6 to 400 μg L-573,655 per milliliter. (**B**) Inhibition by more potent derivatives of L-573,655 at 3 μM substrate concentration. Three inhibitors related to L-573,655 were titrated with the use of deacetylase in extracts of *E. coli* strain MB2884, an infectious wild type. IC_{50}'s were as follows: L-159,463 (*R* enantiomer of L-573,655) 3 μg/ml; L-159,692, 0.6 μg/ml; L-161,240, 0.008 μg/ml. Activity measurements were made with the use of the assay in (A), with 40 mM bis-tris (pH 6.5). IC_{50} values at pH 5.5 are about twofold less than at pH 6.5.

Serratia. In extracts, however, the inhibitors were active against the *Pseudomonas* and *Serratia* deacetylases. Because the lipid A content was not reduced in living cells of *Pseudomonas* and *Serratia* treated with inhibitors (*12*, *20*), it may be that our compounds did not penetrate these bacteria or were actively extruded by them.

Our compounds were capable of curing mice infected with live *E. coli* (Table 3). The mouse model used for this purpose (Table 3) is relevant to the clinical situation of bowel surgery or bowel injury. The model can also be used to eliminate antibacterial compounds that are ineffective because they bind to animal tissues and proteins. Two intraperitoneal doses of L-159,692 or L-161,240 rescued mice [calculated median effective doses (ED_{50}'s) of 36 and 15 mg per kilogram of body weight, respectively] from an *E. coli* infection that was 100% fatal without treatment. This suggests that deacetylase inhibitors could be developed for treatment of Gram-negative infections. Deacetylase inhibitors might also lower the complications of Gram-negative sepsis by reducing the amount of endotoxin released from bacteria when other antibiotics are used as the primary therapy (*21*, *22*).

Table 2. Minimal inhibitory concentrations of deacetylase inhibitors against a panel of Gram-negative bacteria. Bacterial strains were wild-type mouse-virulent isolates. Minimal inhibitory concentrations were determined as in Table 1. In most experiments, concentrations over 100 μg/ml were not examined. The inhibitors did not kill or slow the growth of Gram-positive bacteria, yeast, or Chinese hamster ovary cells at 100 μg/ml; only bacteria that make lipid A were killed.

Bacterial strain	MIC (μg/ml)		
	L-573, 655*	L-159, 692	L-161, 240
E. coli (MB2884)	200 to 400	50	1
Enterobacter cloacae (MB2646)	>100	100	6
Klebsiella pneumoniae (MB4005)	>100	50	13
Serratia marcescens (MB3548)	>100	100	>100
Proteus mirabilis (MB3125)	>100	>100	50
Pseudomonas aeruginosa (MB3286)	>100	>100	>100

Table 3. Deacetylase inhibitors L-159,692 and L-161,240 protect mice from lethal septicemia with *E. coli* strain MB2884. A systemic infection was established by intraperitoneal (i.p.) injection of *E. coli* MB2884 diluted in brain-heart broth. Challenge doses contained 9 to 33 median lethal doses. Antibiotics were administered i.p. immediately after the infecting dose (0 hours) and again 6 hours later. Five mice per group were tested at each of several doses of inhibitor. The test was terminated 7 days after infection, and the survival records of that day were used to calculate the amount of antibiotic that should protect 50% (ED_{50}) of the infected, treated animals (*25*). Before the actual experiment, two mice were treated for 7 days with 100 mg of each compound per kilogram of body weight (a sevenfold excess over the maximal dose used in the infection model) without adverse side effects (such as seizures or lethargy) or reduced food intake.

Dose (mg/kg)	Survivors/infected mice (*n*) after 7 days	
	L-159,692 treatment	L-161,240 treatment
3.125	0/5	0/5
12.5	1/5	1/5
50	3/5	5/5

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CKI1, a Histidine Kinase Homolog Implicated in Cytokinin Signal Transduction

Tatsuo Kakimoto

Although cytokinin plays a central role in plant development, little is known about cytokinin signal transduction. Five *Arabidopsis thaliana* mutants that exhibit typical cytokinin responses, including rapid cell division and shoot formation in tissue culture in the absence of exogenous cytokinin, were isolated by activation transferred DNA tagging. A gene, *CKI1*, which was tagged in four of the five mutants and induced typical cytokinin responses after introduction and overexpression in plants, was cloned. *CKI1* encodes a protein similar to the two-component regulators. These results suggest that CKI1 is involved in cytokinin signal transduction, possibly as a cytokinin receptor.

Cytokinin regulates many physiological events such as nutrient metabolism, expansion and senescence of leaves, and lateral branching (*1*). Moreover, it induces cell division, chloroplast development, and shoot production in cells grown in culture (*2*). However, little is known about the biosynthesis and mechanism of action of cytokinin (*3*). This report describes the isolation of cytokinin-independent mutants of *Arabidopsis*, generated by activation transferred DNA (T-DNA) tagging (*4*), and the use of these mutants to identify a gene involved in cytokinin signal transduction.

Calli derived from hypocotyl segments of 50,000 seedlings of *A. thaliana* were transformed (*5*) with *Agrobacterium* containing pPCVICEn4HPT, a transformation vector with a T-DNA containing a tetramer of the enhancer of the cauliflower mosaic virus (CaMV) 35S RNA promoter (*4*). After integration of such a T-DNA into the genome, expression of genes adjacent to the integra-

Department of Biology, Graduate School of Science, Osaka University, Toyonaka, Osaka 560, Japan.