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Analogs of the Autoinducer 3-Oxooctanoyl-Homoserine Lactone Strongly Inhibit Activity of the TraR Protein of *Agrobacterium tumefaciens*

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The TraR and TraI proteins of *Agrobacterium tumefaciens* mediate cell-density-dependent expression of the Ti plasmid *tra* regulon. TraI synthesizes the autoinducer pheromone *N*-(3-oxooctanoyl)-L-homoserine lactone (3-oxo-C_8-HSL), while TraR is an 3-oxo-C_8-HSL-responsive transcriptional activator. We have compared the abilities of 3-oxo-C_8-HSL and 32 related compounds to activate expression of a TraR-regulated promoter. In a strain that expresses wild-type levels of TraR, only 3-oxo-C_8-HSL was strongly stimulatory, four compounds were detectably active only at high concentrations, and the remaining 28 compounds were inactive. Furthermore, many of these compounds were potent antagonists. In contrast, almost all of these compounds were stimulatory in a congenic strain that overexpresses TraR and no compound was a potent antagonist. We propose a model in which autoinducers enhance the affinity of TraR either for other TraR monomers or for DNA binding sites and that overexpression of TraR potentiates this interaction by mass action. Wild-type *A. tumefaciens* released a rather broad spectrum of autoinducers, including several that antagonize induction of a wild-type strain. However, under all conditions tested, 3-oxo-C_8-HSL was more abundant than any other analog, indicating that other released autoinducers do not interfere with *tra* gene induction. We conclude that (i) in wild-type strains, only 3-oxo-C_8-HSL significantly stimulates *tra* gene expression, while many autoinducer analogs are potent antagonists; (ii) TraR overexpression increases agonistic activity of autoinducer analogs, allowing sensitive biodetection of many autoinducers; and (iii) autoinducer stimulatory activity is potentiated by TraR overproduction, suggesting that autoinducers may shift an equilibrium between TraR monomers and dimers or oligomers. When autoinducer specificities of other quorum-sensing proteins are tested, care should be taken not to overexpress those proteins.

Many genera of bacteria use diffusible chemicals to exchange information (3, 5, 12, 18, 26, 36, 41, 45). An important class of chemical signal molecules is the family of *N*-acyl-homoserine lactones (*N*-acyl-HSLs), which are generally synthesized by an enzyme related to the LuxI protein of *Vibrio fischeri*. These compounds, called autoinducers, passively diffuse across the bacterial envelope and therefore accumulate intracellularly only at high bacterial densities (25). These chemicals are thought to bind to a protein related to the LuxR protein of *V. fischeri*, whose amino terminus contains an autoinducer binding site and whose carboxyl terminus binds to a DNA site directly upstream of the *lux* promoter (1, 7, 21, 42). Cell density-dependent gene expression is denoted quorum sensing, and this sort of regulation is used by a wide spectrum of bacteria to regulate diverse genes, including the pathogenicity genes of several plant and animal pathogens.

The Ti plasmids of several *Agrobacterium tumefaciens* strains bear genes that encode a LuxI-type protein called TraI, which synthesizes *N*-(3-oxooctanoyl)-L-HSL (3-oxo-C_8-HSL) and a LuxR-type protein called TraR, which presumably binds 3-oxo-C_8-HSL (17, 23, 30, 34, 46). Putative TraR-autoinducer complexes activate transcription of several genes required for Ti plasmid conjugal transfer as well as other Ti plasmid-borne genes. As expected, Ti plasmid conjugation occurs only at high donor population densities (16). The expression of TraR is regulated by particular opines (17). Octopine induces expression of the *traR* genes of plasmids related to pTi15955, pTiA6, and pTiR10, while agrocinopines A and B induce the *traR* gene of pTiC58 (4, 17). These observations explain older findings that Ti plasmid conjugation occurs only within crown gall tumors or in the presence of particular opines (9). The *traR* and *traI* genes are positively autoregulated (17).

All autoinducers described to date contain an HSL moiety and a fatty acyl group whose members have various lengths, saturation levels, and oxidation states. Two of these compounds have been shown to derive their fatty acids from acyl-ACP (22, 30, 38), which indicates that these fatty acids are drawn from pools of fatty acid biosynthetic intermediates. The fatty acids of acyl-ACPs always have even numbers of carbon atoms. They also have either 3-oxo, 3-hydroxyl, or fully reduced methylene groups at the C-3 position or a have a 2,3 unsaturated bond (8). Accordingly, the acyl groups of all natural autoinducers have even numbers of carbon atoms and have 3-oxo, 3-hydroxyl, or fully reduced acyl groups. Curiously, no autoinducer has been reported to contain a 2,3 unsaturated bond in its acyl moiety, although two autoinducers have 7,8 unsaturated bonds (20, 35, 39). The lengths of these side chains contain between 4 and 14 carbon atoms.

In several studies, the ability of autoinducer analogs to induce expression of quorum-sensing systems has been tested (6, 13, 19, 32, 37, 46). In general, compounds closely related to the cognate autoinducer caused weak-to-moderate gene expression while less similar compounds were less active. In some

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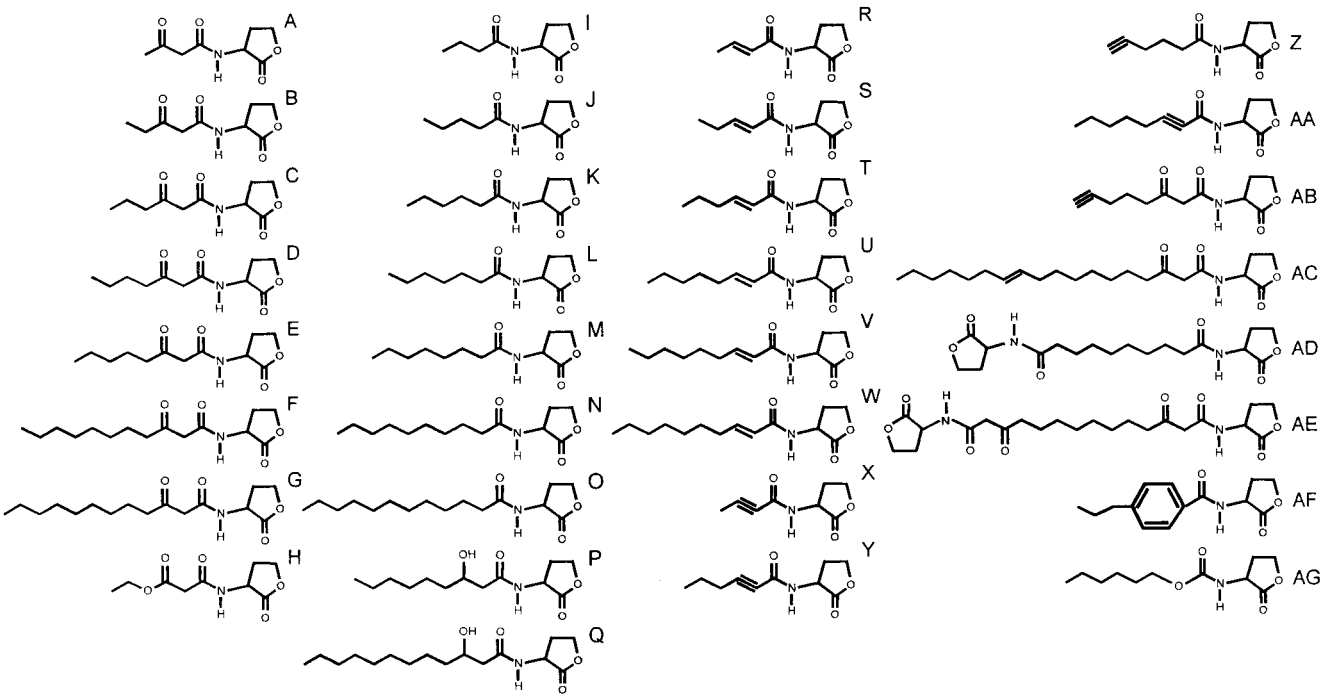

FIG. 1. Chemical structures of *N*-acyl-HSL compounds used in this study. Non-IUPAC descriptions of compounds are as follows: A, 3-oxobutanoyl-HSL; B, 3-oxopentanoyl-HSL; C, 3-oxohexanoyl-HSL; D, 3-oxoheptanoyl-HSL; E, 3-oxooctanoyl-HSL; F, 3-oxoundecanoyl-HSL; G, 3-oxododecanoyl-HSL; H, 4-oxa-3-oxohexanoyl-HSL; I, butanoyl-HSL; J, pentanoyl-HSL; K, hexanoyl-HSL; L, heptanoyl-HSL; M, octanoyl-HSL; N, decanoyl-HSL; O, dodecanoyl-HSL; P, 3-hydroxynonanoyl-HSL; Q, 3-hydroxydodecanoyl-HSL; R, 2-butenoyl-HSL; S, 2-pentenoyl-HSL; T, 2-hexenoyl-HSL; U, 2-octenoyl-HSL; V, 2-nonenoyl-HSL; W, 2-decenoyl-HSL; X, 2-butynoyl-HSL; Y, 2-hexynoyl-HSL; Z, 5-hexynoyl-HSL; AA, 2-octynoyl-HSL; AB, 3-oxo-7-octynoyl-HSL; AC, 3-oxo-11-octadecenoyl-HSL; AD, diHSL-decandioate; AE, diHSL-3,12-dioxotetradecandioate; AF, *p*-propylbenzoyl-HSL; and AG, *O*-hexyl-*N*-HSL carbamate.

cases, autoinducer analogs acted as antagonists of the native autoinducer and thereby inhibited the induction of target genes. In two studies, autoinducer analogs were demonstrated to inhibit binding of radiolabeled autoinducers (32, 37).

There are a few reports of a single LuxI-type protein synthesizing more than one autoinducer. LuxI synthesizes primarily 3-oxo-C_6-HSL (see Fig. 1, compound C) but also synthesizes small amounts of C_6-HSL (compound K; see reference 27). Similarly, an *Escherichia coli* strain expressing the LasI protein of *Pseudomonas aeruginosa* synthesizes primarily 3-oxo-C_{12}-HSL but also synthesizes trace amounts of additional compounds, including two that coelute by reversed-phase high-performance liquid chromatography (HPLC) with 3-oxo-C_6-HSL and 3-oxo-C_8-HSL (33, 44). The SwrI protein of *Serratia liquefaciens* synthesizes both C_4-HSL and C_6-HSL in a 10:1 ratio (14). HPLC fractionation of *Agrobacterium* autoinducers revealed two bioactive fractions (46). One fraction contained 3-oxo-C_8-HSL, while a second, less homogeneous, fraction contained compounds having molecular masses of 274, 214, and 200 Da. While the 274-Da compound was thought to be due to a purification artifact, the other two compounds were not since they had molecular masses identical to those of 3-oxo-C_6-HSL and C_6-HSL, respectively. That study did not determine whether all three compounds were synthesized by TraI.

Shaw and colleagues recently described an elegant method for using an *A. tumefaciens* strain to detect a broad range of autoinducers (40). The strain used in these studies was able to recognize no fewer than nine autoinducers having a wide variety of acyl side groups. However, in an earlier report, an *A. tumefaciens* strain discriminated between different autoinducers far more strongly (46). One difference between these two studies is the genotypes of the bioassay strains. In the present study, we evaluated whether differences in the *traR* genotype that affect its expression level might alter the ability of *A. tumefaciens* to detect analogs of 3-oxo-C_8-HSL. We report that TraR overproduction allows *A. tumefaciens* to detect a broad range of autoinducer analogs and abolishes the ability of autoinducer analogs to act as antagonists.

MATERIALS AND METHODS

Strains and plasmids. WCF47 is a derivative of R10 (10) containing a nonpolar internal deletion of *traI*. The *traI* deletion was constructed with two fragments generated by PCR, one containing the 5′ end of *traI* and upstream sequences and the other containing the 3′ end of *traI* and downstream sequences. These fragments were ligated together, and the resulting *traI* deletion was introduced into the suicide plasmid pKNG101 (24). The resulting plasmid, pCF393, was introduced into strain R10 by conjugation and selected with streptomycin. Streptomycin-resistant transconjugants were plated on solid medium lacking streptomycin and containing 5% sucrose. The *sacB* gene of pCF393 confers sucrose sensitivity, and sucrose therefore selects for Campbell-type excision. Sucrose-resistant derivatives were screened for the inability to produce 3-oxo-C_8-HSL. pCF218 is an IncP plasmid that expresses TraR from a vector promoter (17), while pCF372 contains a P*traI-lacZ* fusion (16).

Synthesis of autoinducer analogs. The chemical structures of autoinducer analogs used in this study are shown in Fig. 1. Synthesis of compounds A, E, I, J, K, L, M, and N was previously described (13). Synthesis of compounds B, T, and Y was as described in reference 37, while synthesis of compounds C and G was as described in references 11 and 33, respectively.

Compounds D, F, AB, AC, and AE were synthesized by the same procedures as those used to make compound B but with the corresponding acyl acid chlorides. Compounds H, O, R, S, U, V, W, X, Z, AA, and AF were synthesized by the same procedures as those used to make compound I but with the sodium salt of the corresponding fatty acid. Monoethyl malonate was used to make compound H. Compound AD was synthesized by reaction of the dicarboxylic acid chlorides with two equivalents of HSL hydrobromide in pyridine followed by adsorption to a BondElut column and purification by preparative HPLC. Compound AG was made similarly from hexyl chloroformate. Compounds P and Q were synthesized by a Reformatsky reaction followed by coupling of the resulting

3-hydroxycarboxylates with HSL hydrobromide by the same technique used to make compound I. The purity of all compounds was carefully tested by reversed-phase HPLC.

The formal International Union of Pure and Applied Chemistry (IUPAC) chemical designations for the compounds used in this study are as follows: A, 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)butanamide; B, 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)pentanamide; C, 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)hexanamide; D, 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)heptanamide; E, 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)octanamide; F, 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)undecanamide; G, 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)dodecanamide; H, 2-ethoxycarbonyl-*N*-(tetrahydro-2-oxo-3-furanyl)ethanamide; I, *N*-(tetrahydro-2-oxo-3-furanyl)butanamide; J, *N*-(tetrahydro-2-oxo-3-furanyl)pentanamide; K, *N*-(tetrahydro-2-oxo-3-furanyl)hexanamide; L, *N*-(tetrahydro-2-oxo-3-furanyl)heptanamide; M, *N*-(tetrahydro-2-oxo-3-furanyl)octanamide; N, *N*-(tetrahydro-2-oxo-3-furanyl)decanamide; O, *N*-(tetrahydro-2-oxo-3-furanyl)dodecanamide; P, 3-hydroxy-*N*-(tetrahydro-2-oxo-3-furanyl)nonanamide; Q, 3-hydroxy-*N*-(tetrahydro-2-oxo-3-furanyl)dodecanamide;R, *N*-(tetrahydro-2-oxo-3-furanyl)-2-butenamide; S, *N*-(tetrahydro-2-oxo-3-furanyl)-2-pentenamide; T, *N*-(tetrahydro-2-oxo-3-furanyl)-2-hexenamide; U, *N*-(tetrahydro-2-oxo-3-furanyl)-2-octenamide; V, *N*-(tetrahydro-2-oxo-3-furanyl)-2-nonenamide; W, *N*-(tetrahydro-2-oxo-3-furanyl)-2-decenamide; X, *N*-(tetrahydro-2-oxo-3-furanyl)-2-butynamide; Y, *N*-(tetrahydro-2-oxo-3-furanyl)-2-hexynamide; Z, *N*-(tetrahydro-2-oxo-3-furanyl)-5-hexynamide; AA, *N*-(tetrahydro-2-oxo-3-furanyl)-2-octynamide; AB, 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)-7-octynamide; AC, 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)-11-octadecenamide; AD, *N*,*N*′-bis(tetrahydro-2-oxo-3-furanyl)decandiamide; AE, 3,12-dioxo-*N*,*N*′-bis(tetrahydro-2-oxo-3-furanyl)tetradecandiamide; AF, 4-propyl-*N*-(tetrahydro-2-oxo-3-furanyl)benzamide; and AG, 1-hexoxy-*N*-(tetrahydro-2-oxo-3-furanyl)methanamide. See the legend to Fig. 1 for non-IUPAC descriptions of these compounds.

Bioassays of autoinducer activity. Autoinducer analogs were stored in ethyl acetate at −80°C. Prior to use, autoinducers were warmed to room temperature and 0.017 μmol was added in 34 μl of ethyl acetate to empty culture tubes. After the solvent was allowed to evaporate, 1.75 ml of AT medium (43) supplemented with 400 μg of octopine per ml and approximately 10^7 bacterial cells [strain WCF47(pCF372) or WCF47(pCF372)(pCF218)] was added. After the autoinducer analog was allowed to dissolve, 0.55 ml was removed and added to 1.2 ml of the same culture (a 3.16-fold dilution). This dilution was repeated serially nine times, resulting in 10 culture tubes having analogs at concentrations ranging from 10^{-1} to 10^4 to nM. These cultures were incubated with aeration for 12 h and assayed for β-galactosidase specific activity (29). The bacterial cultures remained in exponential growth phase during this interval. To assay the abilities of analogs to prevent induction, the assays described above were repeated, except that 3-oxo-C_8-HSL (final concentration, 10^2 nM) was added in addition to the indicated amounts of other compounds.

Western immunoblots of TraR. To compare intracellular concentrations of TraR in the two bioassay strains described above, we cultured each strain in 100 ml of AT broth containing 100 nM 3-oxo-C_8-HSL to an optical density at 600 nm of 0.6, concentrated the cultures by centrifugation, resuspended the cultures in 1 ml of TEDG (50 mM Tris-HCL [pH 7.9], 0.5 mM EDTA, 1 mM dithiothreitol, 5% glycerol), disrupted the cells using a French pressure cell, and ultracentrifuged the lysates at 150,000 × *g* for 30 min. The resulting cleared lysates were size fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and detected with affinity-purified polyclonal anti-TraR rabbit antiserum.

TLC analysis of autoinducers released by *A. tumefaciens*. The isolation of autoinducers by strains expressing TraI was done by culturing strains to stationary phase in 20 ml of broth, removing bacteria by centrifugation, and extracting the cell-free spent broth three times with 3 volumes of ethyl acetate. In control experiments, this procedure was shown to recover virtually all of the 3-oxo-C_8-HSL and 3-oxo-C_6-HSL, although it may not result in quantitative recovery of more polar compounds. The ethyl acetate fractions were pooled and evaporated to dryness, and each residue was resuspended in 1 ml of ethyl acetate, transferred to a 1-ml glass vial, and reevaporated. The residue was resuspended in 200 μl of ethyl acetate and stored at −80°C. Aliquots (1 μl) were applied to C_{18} reversed-phase thin-layer chromatography (TLC) plates (Whatman) and chromatographed with 60% methanol–40% water as described previously (40). After chromatography, the plates were dried and overlaid with 200 ml of agar containing AT medium, 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and approximately 10^7 bacteria per ml [strain WCF47(pCF372)(pCF218)]. TLC plates were incubated overnight at 28°C and examined for X-Gal hydrolysis.

In experiments to identify products of alkaline hydrolysis of autoinducers, 0.2 nmol of 3-oxo-C_8-HSL or 2 nmol of 3-oxo-C_6-HSL that had been dissolved in ethyl acetate was transferred to empty microcentrifuge tubes and the ethyl acetate was removed by evaporation. The dried autoinducers were resuspended in 20 μl of water, 20 μl of 0.01 M NaOH (pH 12), or 20 μl of 0.1 M NaOH (pH 13) and then incubated at room temperature for 1 h. One microliter of each was spotted directly onto a reversed-phase TLC plate, which was developed as described above.

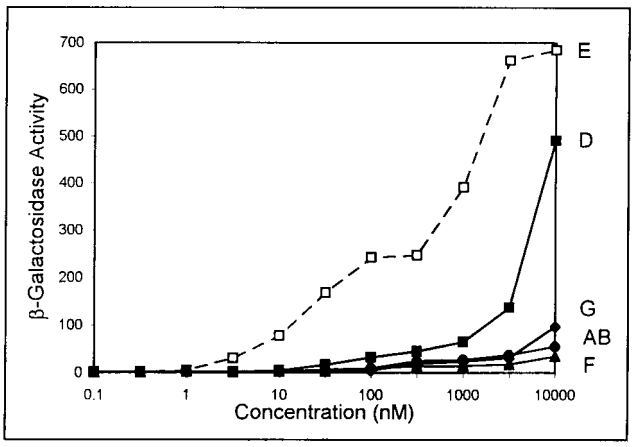

FIG. 2. Dose-response curves with *A. tumfaciens* WCF47(pCF372). Results with compounds E (□), D (■), F (▲), G (◆), and AB (●) are shown. All other compounds were inactive.

RESULTS

Recognition of autoinducer analogs by a strain expressing wild-type levels of TraR. We assayed 33 autoinducers and chemically related compounds (Fig. 1) for the ability to induce β-galactosidase activity in strain WCF47(pCF372), which lacks its own *traI* gene and which has a plasmid-borne P*traI-lacZ* fusion. Synthetic 3-oxo-C_8-HSL (compound E) detectably activated this fusion at a concentration of 3.0 nM (Fig. 2). A concentration of 10^4 nM caused production of approximately 700 U of enzyme activity. The dose-response curve shown in Fig. 2 suggests that this regulatory system was not saturated by a 10^4 nM concentration of this autoinducer. In marked contrast to these results, of the 32 other compounds tested, only four caused production of significant levels of β-galactosidase activity (Fig. 2 and Table 1). These compounds are 3-oxo-C_7-HSL (compound D), 3-oxo-C_{11}-HSL (F), 3-oxo-C_{12}-HSL (G), and 3-oxo-7-octynoyl-HSL (AB). All of these compounds closely resemble 3-oxo-C_8-HSL, differing only in the length or level of desaturation of the acyl moeity. All compounds except 3-oxo-C_8-HSL were weakly active or inactive at concentrations less than 10^3 nM (Fig. 2).

We also tested whether any of these compounds could antagonize induction by 3-oxo-C_8-HSL. We assayed cultures of strain WCF47(pCF372) in the presence of 10^2 nM 3-oxo-C_8-HSL and a 10^2, 10^3, or 10^4 nM concentration of each of the other analogs. Approximately half of these analogs inhibited induction at least fourfold when they were provided at a 100:1 molar ratio (Table 2). In some cases, activity was reduced 100-fold or more. Six compounds inhibited induction at least fourfold when they were supplied at a 10:1 molar ratio, and one compound (C_8-HSL, compound M) inhibited induction when it was provided at a 1:1 molar ratio (Table 2). The most effective antagonists included 3-oxo-C_6-HSL (compound C), C_7-HSL (L), C_8-HSL (M), C_{10}-HSL (N), and 3-hydroxy-C_9-HSL (P), all of which closely resemble 3-oxo-C_8-HSL in structure.

Autoinducer recognition by a strain that overexpresses TraR. To determine whether TraR overexpression altered the detection of autoinducers, we constructed a derivative of WCF47(pCF372) that contains plasmid pCF218, which bears DNA that overexpresses TraR from a vector promoter (17). While WCF47(pCF372) synthesizes extremely low levels of TraR, WCF47(pCF372)(pCF218) synthesizes amounts that

TABLE 1. Abilities of *A. tumefaciens* strains to respond to autoinducer analogs

Compound	β-Galactosidase activity in[a]:			
	WCF47(pCF372) with analog at concn (nM):		WCF47(pCF372) (pCF218) with analog at concn (nM):	
	10	10^4	10	10^4
3-Oxobutanoyl-HSL (A)	1.8	0.4	1.6	24
3-Oxopentanoyl-HSL (B)	2.1	1.6	1.2	710
3-Oxohexanoyl-HSL (C)	1.6	2.5	180	1,200
3-Oxoheptanoyl-HSL (D)	10	470	1,200	1,400
3-Oxooctanoyl-HSL (E)	70	700	1,300	1,500
3-Oxoundecanoyl-HSL (F)	4.9	45	440	1,100
3-Oxododecanoyl-HSL (G)	4.3	73	91	1,300
4-Oxa-3-oxohexanoyl-HSL (H)	2.2	0.3	1.9	850
Butanoyl-HSL (I)	1.2	0.8	1.8	1.7
Pentanoyl-HSL (J)	1.6	1.2	2.2	210
Hexanoyl-HSL (K)	2.5	2.1	0.9	1,500
Heptanoyl-HSL (L)	1.4	2.5	300	680
Octanoyl-HSL (M)	3.2	6.1	650	1,100
Decanoyl-HSL (N)	2.6	1.9	1.7	1,000
Dodecanoyl-HSL (O)	2.2	1.6	2.3	72
3-Hydroxynonanoyl-HSL (P)	2.7	0.7	290	1,400
3-Hydroxydodecanoyl-HSL (Q)	1.8	2.0	1.1	1,000
2-Butenoyl-HSL (R)	2.1	2.3	2.0	2.4
2-Pentenoyl-HSL (S)	1.9	1.1	1.7	640
2-Hexenoyl-HSL (T)	2.5	1.8	1.1	280
2-Octenoyl-HSL (U)	1.3	2.2	3.1	940
2-Nonenoyl-HSL (V)	1.8	1.8	1.7	800
2-Decenoyl-HSL (W)	2.0	1.3	0.8	300
2-Butynoyl-HSL (X)	3.0	1.0	1.3	1.6
2-Hexynoyl-HSL (Y)	2.3	0.7	1.3	120
5-Hexynoyl-HSL (Z)	1.9	0.5	2.1	380
2-Octynoyl-HSL (AA)	1.9	1.1	1.3	710
3-Oxo-7-octynoyl-HSL (AB)	1.3	45	710	1,500
3-Oxo-11-octadecenoyl-HSL (AC)	2.8	1.7	1.3	27
diHSL decandioate (AD)	1.7	1.3	1.8	17
diHSL-3,12-dioxotetradecandioate (AE)	1.6	1.0	19	460
p-Propylbenzoyl-HSL (AF)	ND	1.5	ND	1.8
O-Hexyl-*N*-HSL carbamate (AG)	2.9	1.2	7.1	1,000

[a] The indicated analogs were provided at the noted concentrations to cultures of WCF47(pCF372) containing or lacking pCF218 in AT broth supplemented with 2 mg of opine per ml. Bacteria were cultured for 12 h and assayed for β-galactosidase specific activity (29). β-Galactosidase expression in the absence of autoinducers was less than 2 Miller units. ND, not determined.

TABLE 2. Measure of competitive inhibition in *A. tumefaciens* strains with 100 nM 3-oxo-C_8-HSL

Inhibitor	β-Galactosidase activity in[a]:					
	WCF47(pCF372) with analog at concn (nM):			WCF47(pCF372)(pCF218) with analog at concn (nM):		
	10^2	10^3	10^4	10^2	10^3	10^4
None	340	NA	NA	1,400	NA	NA
A	360	250	150	1,600	1,300	1,200
B	350	240	110	1,500	1,500	1,300
C	340	11	0.9	1,600	1,500	1,200
D	210	120	470	1,500	1,500	1,100
F	280	38	74	1,300	1,200	1,000
G	250	200	180	1,400	1,400	1,000
H	360	320	17	1,800	1,500	850
I	400	330	380	1,400	840	1,300
J	350	350	97	1,600	1,600	1,200
K	380	340	46	1,500	1,600	940
L	180	19	2.4	1,400	1,200	950
M	95	6.0	5.7	1,600	1,500	1,200
N	370	93	2.6	1,400	1,300	1,100
O	410	330	17	1,600	1,400	1,500
P	220	9.5	3.6	1,500	1,500	1,300
Q	350	99	1.9	1,400	1,400	1,000
R	320	330	330	1,600	1,600	1,500
S	380	320	260	1,600	1,600	1,400
T	330	320	76	1,400	1,600	890
U	340	170	3.9	1,500	1,300	1,100
V	370	250	8.6	1,500	1,500	780
W	360	360	140	1,400	1,300	1,100
X	340	330	310	1,500	990	1,300
Y	360	350	340	1,600	930	1,700
Z	370	390	300	1,600	1,100	1,500
AA	320	370	210	1,300	1,500	1,100
AB	230	48	33	1,300	1,400	940
AC	380	370	300	1,400	1,500	950
AD	340	310	270	1,600	1,200	1,500
AE	340	220	13	1,600	1,500	970
AF	360	350	320	1,600	1,500	970
AG	370	350	64	1,400	1,100	1,000

[a] The indicated analogs were provided at the noted concentrations to cultures of WCF47(pCF372) containing or lacking pCF218 in AT broth supplemented with 2 mg of opine per ml. Bacteria were cultured for 12 h and assayed for β-galactosidase specific activity (29). β-Galactosidase expression in the absence of autoinducers was less than 2 Miller units. NA, not applicable.

are readily detectable by Western immunoblotting (Fig. 3). WCF47(pCF372)(pCF218) was far more sensitive to low concentrations of 3-oxo-C_8-HSL than its parent, since it was detectably induced at concentrations as low as 0.1 nM and the response was saturated by an approximately 10 nM concentration of this autoinducer (Fig. 4A). The reporter fusion in this strain, when fully induced, expressed about twofold more β-galactosidase than the same reporter fusion in the strain that expresses wild-type levels of TraR.

We also tested the ability of the 32 autoinducer analogs to induce the reporter fusion of WCF47(pCF372)(pCF218). The responses to two concentrations of these compounds are shown in Table 1, and dose-response curves for most compounds are shown in Fig. 4. Surprisingly, this strain was detectably stimulated by no fewer than 29 of the 33 tested compounds. Among the most active compounds were 3-oxo-C_7-HSL (compound D), 3-oxo-7-octynoyl-HSL (AB), and C_8-HSL (M), all of which can half-maximally induce the fusion when they are provided at 3 to 10 nM. All three compounds are closely related to 3-oxo-C_8-HSL. Slightly less active compounds include 3-oxo-C_6-HSL (C), C_7-HSL (L), 3-oxo-C_{11}-HSL (F), and 3-hydroxy-C_9-HSL (P). Almost all other compounds were stimulatory when they were supplied at higher concentrations. The four inactive compounds were C_4-HSL (I), 2-butenoyl-HSL (R), 2-butynoyl-HSL (X), and *p*-propylbenzoyl-HSL (AF). 3-Oxo-C_4-HSL (A) was only weakly active, indicating that TraR is stimulated very poorly or not at all by autoinducers having four-carbon acyl moieties. Two other compounds, 3-oxo-11-octadecenoyl-HSL

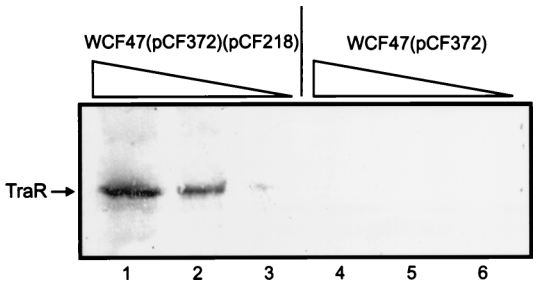

FIG. 3. Western immunoblot of serially diluted extracts of WCF47(pCF372)(pCF218) (left) and WCF47(pCF372) (right). Lanes 1 and 4, 10 μl of lysate; lanes 2 and 5, 2.5 μl of lysate; lanes 3 and 6, 0.625 μl of lysate.

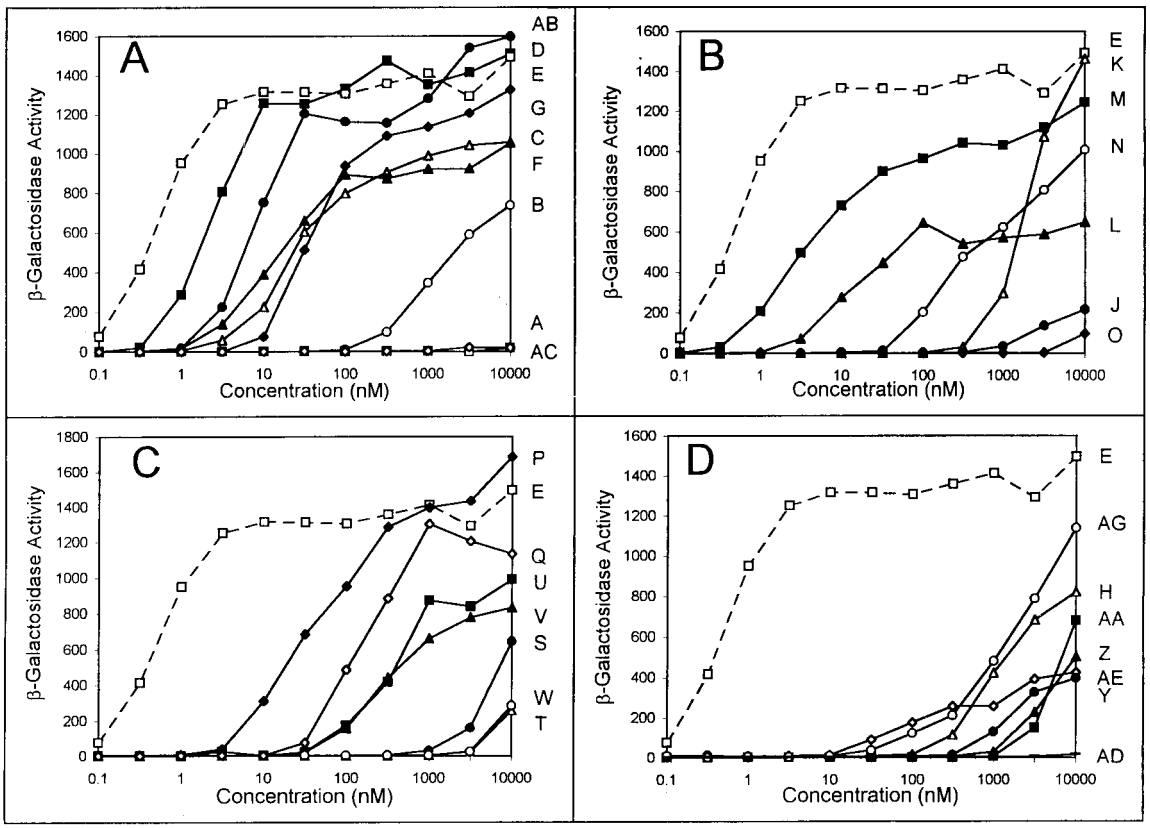

FIG. 4. Dose-response curves with an *A. tumefaciens* strain that overexpresses TraR. (A) Results with compounds A (□), B (○), C (△), D (■), E (□), F (▲), G (◆), AB (●), and AC (◇); (B) results with compounds E (□), J (●), K (△), L (▲), M (■), N (○), and O (◆); (C) results with compounds E (□), P (◆), Q (◇), S (●), T (△), U (■), V (▲), and W (○); (D) results with compounds E (□), H (△), Y (●), Z (▲), AA (■), AD (–), AE (◇), and AG (○).

(AC) and diHSL-decandioate (AD), were also very weak inducers.

We tested these 32 compounds for the ability to antagonize 3-oxo-C_8-HSL. Strikingly, none of these compounds was able to inhibit induction more than twofold (Table 2). Several compounds appeared to cause very modest inhibition, although these differences may lie within experimental error.

Production of multiple autoinducers by TraI. We have shown that several naturally occurring autoinducers are potent antagonists of 3-oxo-C_8-HSL in a strain that expresses wild-type levels of TraR. In an earlier report, Zhang and colleagues reported that an *A. tumefaciens* strain containing an octopine-type Ti plasmid synthesized compounds having molecular masses identical to those of 3-oxo-C_8-HSL, 3-oxo-C_6-HSL, and C_6-HSL (46). We have found that the latter two compounds can antagonize 3-oxo-C_8-HSL. To determine whether *A. tumefaciens* synthesizes inhibitory concentrations of these compounds, we used TLC (40) to visualize all bioactive autoinducers made by *A. tumefaciens* and to estimate the concentrations of some of these compounds.

Strain R10(pCF372) was cultured in broth (AT salts supplemented with octopine) to stationary phase and assayed for β-galactosidase activity. The culture expressed only 36 U of activity, indicating that its *tra* regulon was only weakly induced and suggesting that the culture medium contained only limited amounts of autoinducers. These data agree with earlier observations that the high cell densities required for induction of this system are more readily achieved on semisolid medium than in broth culture (17). We therefore diluted this culture fivefold into fresh broth (AT salts and octopine) and incubated it once again to stationary phase. This second culture expressed 282 U of β-galactosidase activity, indicating that its *tra* regulon was far more strongly induced than that of the first culture, presumably due to the autoinducers released during the first growth interval. Bacteria were removed by centrifugation from 20 ml of this culture, and the cell-free supernatant was extracted and resuspended in 200 μl of ethyl acetate as described in Materials and Methods. The concentrated extract was serially diluted in fivefold steps, and 1 μl of each dilution was applied to a reversed-phase TLC plate.

In the most concentrated sample, no fewer than six bioactive compounds were detected (Fig. 5A). The two most prominent spots had R_{fs} identical to those of 3-oxo-C_8-HSL and 3-oxo-C_6-HSL, and smaller amounts of a compound with an R_f identical to that of C_6-HSL was also detected, consistent with the data of Zhang and colleagues (46). In addition, trace amounts of several more hydrophobic compounds were detected. Two very polar compounds were also detected, as described more fully below. WCF47(pCF372), which lacks *traI*, did not synthesize any compounds active in this assay (data not shown), suggesting that all detected compounds were synthesized by TraI.

The concentrations of 3-oxo-C_8-HSL and 3-oxo-C_6-HSL were estimated by serially diluting each concentrated extract and identifying the greatest dilution that contained detectable amounts of each compound. These spots were compared to spots made by serially diluted synthetic autoinducers (Fig. 5B). From these data, we estimated that the culture contained 16 μM 3-oxo-C_8-HSL and 0.6 μM 3-oxo-C_6-HSL.

We also tested the production of autoinducers by strain R10

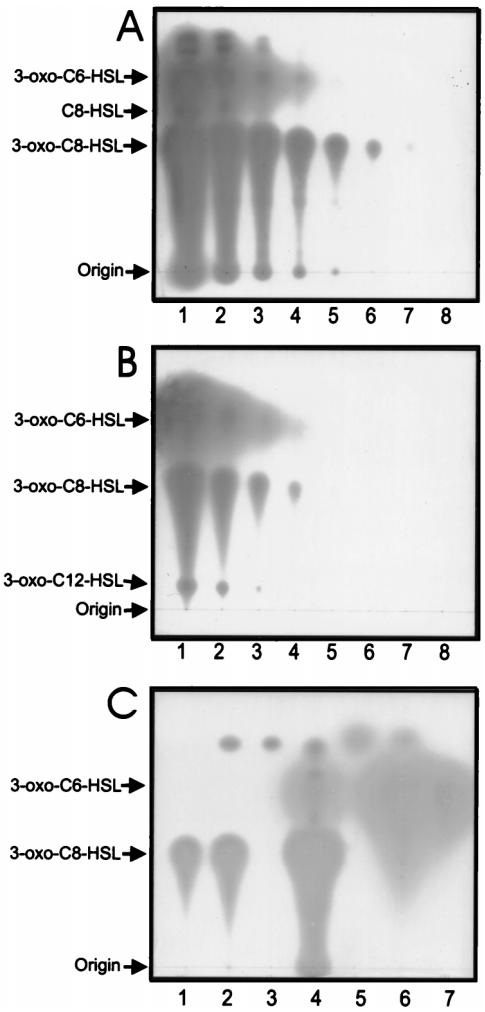

FIG. 5. TLC of autoinducers synthesized by a wild-type *A. tumefaciens* strain. (A) Lane 1 represents the activities of autoinducers obtained from 100 μl of culture supernatant. Lanes 2 to 8 show fivefold serial dilutions of the sample chromatographed in lane 1. (B) Synthetic racemic autoinducers. Lane 1 contains 50 pmol of 3-oxo-C_6-HSL, 50 pmol of 3-oxo-C_8-HSL, and 1,500 pmol of 3-oxo-C_{12}-HSL. Lanes 2 to 8 show fivefold serial dilutions of the sample chromatographed in lane 1. (C) Conversion of 3-oxo-C_8-HSL and 3-oxo-C_6-HSL to a more polar active form by treatment with alkali. Lane 1, 3-oxo-C_8-HSL incubated at pH 7.0; lane 2, 3-oxo-C_8-HSL incubated at pH 12 for 1 h; lane 3, 3-oxo-C_8-HSL incubated at pH 13 for 1 h; lane 4, autoinducers produced by strain R10 (pCF372); lane 5, 3-oxo-C_6-HSL incubated at pH 13 for 1 h; lane 6, 3-oxo-C_6-HSL incubated at pH 12 for 1 h; lane 7, 3-oxo-C_6-HSL incubated at pH 7.

(pCF372)(pCF218), which overexpresses TraR and therefore constitutively overexpresses TraI (17), as well as strain KYC6, which contains a null mutation in *traM* and therefore expresses the *tra* regulon at elevated levels (15). The culture supernatants of these strains contained all of the bioactive compounds detected above (data not shown). Furthermore, these compounds were synthesized in ratios similar to those in R10 (pCF372). The culture supernatant of strain R10(pCF372)(pCF218) contained approximately 1,100 μM 3-oxo-C_8-HSL and 25 μM 3-oxo-C_6-HSL, while that of KYC6 contained 800 μM 3-oxo-C_8-HSL and 50 μM 3-oxo-C_6-HSL. Therefore, artificial overexpression of the *tra* regulon increased the production of all detectable autoinducers but did not greatly alter the ratios of their concentrations. Since 3-oxo-C_6-HSL and C_6-HSL inhibit *tra* gene expression only when they are present at concentrations higher than that of 3-oxo-C_8-HSL (Table 2), the concentrations found in *A. tumefaciens* supernatants are unlikely to interfere with expression of the *tra* regulon.

As described above, two very polar bioactive compounds present in culture supernatants were detected in these TLC assays. Neither of these compounds is likely to be 3-oxo-C_4-HSL, because synthetic 3-oxo-C_4-HSL was poorly detected in this assay and formed an extremely diffuse spot (data not shown). We hypothesized that the two polar compounds could be a consequence of spontaneous hydrolysis of the HSL ring of two of the autoinducers described above. To test this, we treated synthetic 3-oxo-C_8-HSL and 3-oxo-C_6-HSL with alkali at pH 12 or 13 for 1 h at room temperature and chromatographed and bioassayed the resulting compounds. Incubation at pH 13 destroyed both compounds (Fig. 5C, lane 3 and 5). However, hydrolysis of 3-oxo-C_8-HSL caused the appearance of a bioactive compound having an R_f identical to the that of most polar compound found in *A. tumefaciens* culture supernatants (Fig. 5C, lanes 2, 3, and 4). Hydrolysis of 3-oxo-C_6-HSL caused the appearance of an even more polar compound (Fig. 5C, lanes 5 and 6) that did not comigrate with any bioactive compound in lane 4. Since alkali opens the HSL ring, these polar compounds are probably 3-oxo-C_8-homoserine and 3-oxo-C_6-homoserine. These compounds are either detectably active in this bioassay or else undergo lactonization during the drying of the TLC plates, forming bioactive acyl-HSLs. These data are reminiscent of the data of Zhang and colleagues, who reported that an acyclic methyl ester of 3-oxo-C_8-HSL is bioactive (46).

DISCUSSION

We have tested the ability of two strains of *A. tumefaciens* to discriminate between the autoinducer 3-oxo-C_8-HSL and a variety of related compounds. The strain expressing wild-type levels of TraR was far more strongly induced by its cognate autoinducer than by any other tested compound and was completely nonresponsive to all but four analogs. In contrast, an isogenic strain that constitutively overexpressed TraR was far more sensitive to these compounds and seemed to be less discriminatory, since 29 of the 33 compounds tested were stimulatory and several of these showed a half-maximal responses at only 3 to 30-fold-higher concentrations than that of the native autoinducer. Furthermore, in the strain expressing wild-type levels of TraR, many analogs were potent inhibitors of induction, while in a strain that overexpressed TraR, inhibition was not detected.

The narrow substrate specificities of wild-type strains might be interpreted to mean that only 3-oxo-C_8-HSL and a few closely related compounds can bind TraR at the concentrations used. However, this possibility is inconsistent with our finding that many of the same compounds are potent antagonists, since this antagonism is presumably a consequence of competition for autoinducer binding sites. We therefore prefer a model in which TraR can bind a wide variety of autoinducer analogs but in which only a small subset can cause a conformational change in TraR necessary to convert it to an active form.

As described above, the strain expressing wild-type levels of TraR was stimulated only by compounds that closely resemble the cognate autoinducer (compounds D, F, G, and AB). Furthermore, the most effective antagonists (compounds C, L, M, N, P, Q, U, and V) also closely resemble the cognate autoinducer. However, there was little if any overlap in performance between effective agonists and the most effective antagonists. All agonists had acyl chains of seven carbon residues or more, and all had 3-oxo substituents. Therefore, TraR tolerated acyl groups one carbon shorter or up to four carbons longer than

the cognate autoinducer and tolerated a triple bond at the 7-8 position but did not tolerate other alterations. In contrast, with one exception, the strongest antagonists lacked the 3-oxo substituent and had methylene residues, hydroxyl residues, or 2-3 unsaturated bonds at this position. The single best antagonist (compound M) is identical to the best autoinducer (compound E) except for the fact that compound M lacks the 3-oxo group. The exception to this pattern is compound C, which has a 3-oxo group but has a six-carbon fatty acyl group. Since antagonism is probably a consequence of competitive binding, we conclude that the 3-oxo group is completely dispensable for TraR binding but that it plays an important role in converting TraR into an active conformation.

Overproduction of TraR did not lead to constitutive expression of a target promoter. Rather, promoter activity still required an autoinducer, but the number of active compounds was drastically increased. Overexpression of TraR therefore potentiated its ability to activate transcription. Perhaps these data can best be explained by postulating that autoinducers increase the affinity of TraR either for other TraR monomers or for *tra* box DNA (or conceivably for some other macromolecule such as RNA polymerase). If so, autoinducer analogs may cause similar but smaller increases in affinity. Small increases in affinity would be insufficient to drive activation of wild-type TraR pools but would be sufficient to cause activation when TraR is overproduced. To help illustrate this point, we propose that 3-oxo-C_8-HSL may act by decreasing the K_d for TraR dimerization from, perhaps, 10^{-4} to 10^{-7} M, while 3-oxo-C_6-HSL may decrease the K_d to 10^{-6} M. According to this hypothetical example, a low concentration of TraR (for example, 10^{-7} M TraR monomers) would permit significant dimerization by 3-oxo-C_8-HSL but not by 3-oxo-C_6-HSL while a higher concentration of TraR (for example, 10^{-5} M TraR monomers) would permit significant dimerization by both autoinducers. If autoinducers decrease the K_d for TraR-DNA or TraR-RNA polymerase interactions, very similar models can be proposed.

There are at least two indications that active TraR is di- or oligomeric, as was previously suggested for LuxR (13). First, a truncated TraR-like protein lacking a DNA binding domain exerts dominant negative effects on *tra* gene expression, suggesting that it forms inactive heteromultimers (31, 47). Similar data have been reported for LuxR (7). Second, several putative TraR-binding sites show a strong dyad symmetry (16), suggesting that one monomer of a dimer contacts one arm of the dyad and that a second monomer makes identical contacts on the opposite arm.

Our observations are reminiscent of the data of Sitnikov and coworkers (41), who found that 3-hydroxy-C_4-HSL failed to activate *lux* genes in *V. fischeri* but did so when the *lux* operon was cloned in *E. coli*, where LuxR may have been overexpressed. In the same study, C_{10}-HSL was an antagonist of *lux* genes in *V. fischeri* but was an agonist in *E. coli*. Similarly, Schaefer and colleagues reported that 15 of 17 autoinducer analogs inhibited bioluminescence in *V. fischeri* (in the weakly bioluminescent strain B-61; see reference 13), while in a separate study, many of the same compounds did not inhibit bioluminescence in a recombinant strain of *E. coli* carrying a reconstituted *lux* regulon (37). Kuo and colleagues also demonstrated that C_8-HSL is an antagonist in *V. fischeri* (28). LuxR was overexpressed in these *E. coli* strains, and this may have increased autoinducer sensitivity in a fashion similar to that observed for TraR in our study.

Similar studies have been carried out with an *E. coli* strain expressing LasR (32). In this study, LasR showed a broad substrate specificity, comparable to the response we observed using the TraR-overexpressing strain. Many of these compounds competed against radiolabeled 3-oxo-C_{12}-HSL in a binding assay, but none interfered with induction of a target promoter. It is possible that LasR would have shown a narrower autoinducer specificity and would have been more effectively antagonized had it been expressed at wild-type levels. This may have important clinical implications, where autoinducer antagonists may be useful in treating *Pseudomonas* infections.

The ability of wild-type *A. tumefaciens* to discriminate between 3-oxo-C_8-HSL and all other tested compounds should prevent induction by noncognate autoinducers released by other bacterial species. In fact, the only noncognate autoinducer that efficiently induced the *tra* regulon at low concentrations (3-oxo-C_7-HSL) has an odd number of carbon atoms and is therefore unlikely to be found in nature. On the other hand, the finding that many compounds actively inhibited induction suggests that other bacterial species in the rhizosphere may interfere with autoinduction and thereby inhibit Ti plasmid conjugation. Whether this has important ecological consequences is not known. By analogy, one can imagine that 3-oxo-C_6-HSL synthesized by *A. tumefaciens* may well perturb autoinduction by other bacterial species.

TraI can synthesize detectable amounts of several autoinducers in addition to 3-C_8-HSL (Fig. 5). It is noteworthy that at least two of these compounds can inhibit induction of wild-type strains (Table 1). However, these compounds are synthesized at levels lower than that of 3-oxo-C_8-HSL and are therefore unlikely to interfere with induction.

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