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NOVEL SYNTHETIC ANALOGS OF THE *PSEUDOMONAS* AUTOINDUCER

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Abstract: Release of virulence factors in *Pseudomonas aeruginosa* is regulated by two N-acylhomoserine lactones, PAI-1 and PAI-2, that activate the respective transcription factors LasR and RhlR. With the goal of developing novel therapeutic agents, we synthesized constrained analogs of PAI-1 and evaluated them in *P. aeruginosa*. Two of the novel analogs bound to LasR and showed agonist activity in LasR stimulation of a *lasI-lacZ* reporter construct.

Pseudomonas aeruginosa persists as a serious pathogen for patients with compromised immune systems, notably patients with cystic fibrosis, burns, and deep wounds.[1] Many of the highly necrotic and toxic aspects of *P. aeruginosa* infections, including the formation of drug-resistant biofilm colonies, are regulated by a quorum-sensitive mechanism that activates the appropriate genes only when a sufficient concentration of bacteria can make a concerted attack on the host.[2] A master regulatory transcription factor, LasR, controls a number of the individual genes; LasR is activated by a small molecule autoinducer, N-(3-oxododecanoyl)-L-homoserine lactone (PAI-1). A second regulator-autoinducer complex, RhlR and its cognate agonist N-butyryl-L-homoserine lactone (PAI-2), are also part of the *P. aeruginosa* virulence factor cascade; the Las and Rhl systems function in a hierarchical manner.[3]

Several observations lay the foundation for developing small-molecule antagonists to autoinducers as potential therapeutics. Mutants lacking one or more components of these regulatory systems have demonstrated pronounced avirulence in mouse model studies[4, 5a] and decreased adherence to human bronchial epithelial cells in vitro.[3b] Antagonists have been described for the N-acylhomoserine lactone (NAHL) quorum sensing systems in other Gram-negative organisms including *V. fischeri*,[6] *S. liquefaciens*,[7] *A. tumefaciens*,[8] and *C. violaceum*.[9] Perhaps significantly, these bacteria use the relatively shorter-chain NAHLs. The longer chain NAHLs seem more capable of being antagonists of the shorter chain NAHLs than the reverse. PAI-1, despite promotion of RhlR expression, is an antagonist of PAI-2-RhlR binding;[10] PAI-1 is also an antagonist of the *Vibrio* autoinducer (3-oxohexanoyl-L-homoserine lactone) at LuxR.[11] This latter observation is encouraging since it demonstrates the potential of cross-antagonism among species, and sets the stage for using PAI-1 as a lead for PAI-2 antagonists. Despite the successes in antagonizing some of the shorter-chain NAHLs, no antagonist of PAI-1 has been described. Previous attempts at developing autoinducer antagonists for any species have focused largely on modifying the length of the acyl tail, and all of the modified acylating moieties remained highly flexible.[7,8,11,12,13] In contrast to these earlier efforts, our strategy was to retain the full atom content of PAI-1 while limiting and locking the tautomeric possibilities (E

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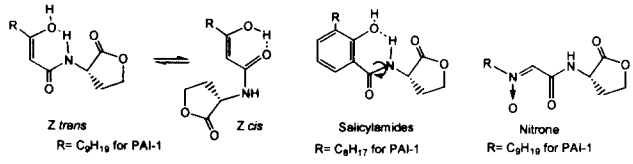
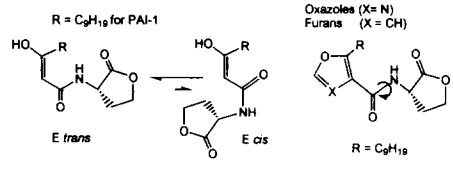
enol, Z enol, fully extended) of the β-keto amide system. Thus our compounds were a departure from previous compounds by virtue of being constrained analogs in which a rigid organic functional group would be used to probe the bioactive tautomer of the β–ketoamide.

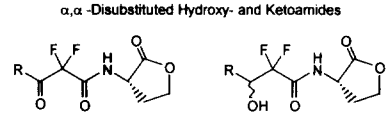
Salicylamides were selected as a scaffold to lock in the Z enol while allowing rotation around the amide



bond.[14] The β-nitrone, a more open structure, can also be considered a Z enol mimetic with increased polarization and lacking the hydrogen bond potential. Simple heterocycles such as furans and oxazoles can enforce the E relationship of the enolic oxygen with respect to the amide carbonyl.

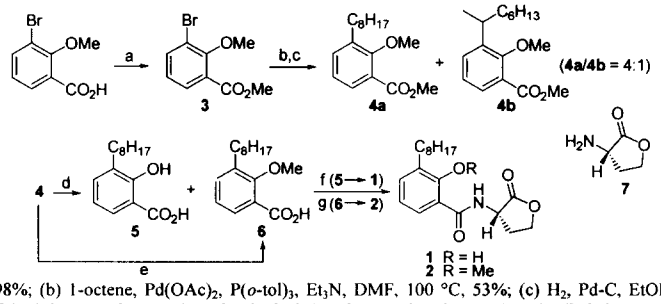


For the extended structures we selected the *gem* difluorosubstituted analogs that would restrict tautomerization but introduce minimal steric perturbation.[15]



Chemistry

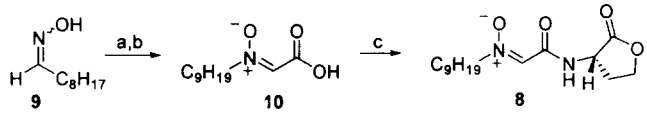
Z-mimetics **1** and **2** were synthesized as shown in Scheme 1. Conversion of 3-bromoanisic acid to methyl ester **3** and subsequent Heck reaction with 1-octene gave a mixture of E and Z styrene products.[16] Hydrogenation provided 3-*n*-octyl methyl salicyate **4a** and minor regioisomer **4b** in 98% yield. Treatment of **4** with BBr_3-SMe_2 in refluxing dichloroethane resulted in partial demethylation, leading to a 1.2:1 ratio of carboxylic acids **5** and **6** respectively.[17] Benzoic acid **5** could be obtained alternatively by direct saponification of ester **4**. Carbodiimide-mediated coupling of **5** and **6** to (S)-α-amino-γ-butyrolactone **7** afforded salicylamides **1** and **2** respectively.



(a) HCl (g), MeOH, 98%; (b) 1-octene, $Pd(OAc)_2$, P(*o*-tol)$_3$, Et_3N, DMF, 100 °C, 53%; (c) H_2, Pd-C, EtOH, 98%; (d) BBr_3-SMe_2 (40 equiv), DCE, reflux, 72 h, 67%; (e) KOH, THF-MeOH-H_2O, 97%; (f) 7, EDC, HOAt, DIEA, DMF, 81%; (g) 7, DCC, HOAt, DIEA, DMF, 64%.

Scheme 1

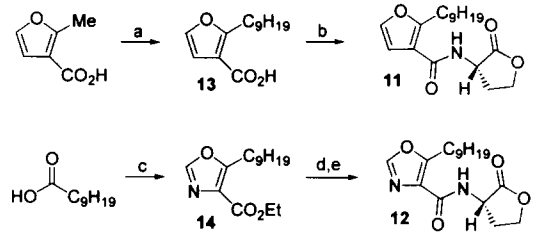
Nitrone **8** was synthesized (Scheme 2) in two steps from known oxime **9**.[18] Sodium cyanoborohydride reduction of **9** resulted in formation of the unstable N-nonylhydroxylamine that decomposed upon standing,[19] but could be rapidly condensed with glyoxylic acid to give nitrone **10** as a single isomer in 85% yield over two steps.[20] Amide formation using a water-soluble carbodiimide provided the nitrone **8**.



(a) $NaCNBH_3$, MeOH-HCl; (b) glyoxylic acid, $CaCl_2$, $NaHCO_3$, Et_2O, 85% (two steps); (c) **7**, EDC, HOAt, DIEA, DMF, 10%.

Scheme 2

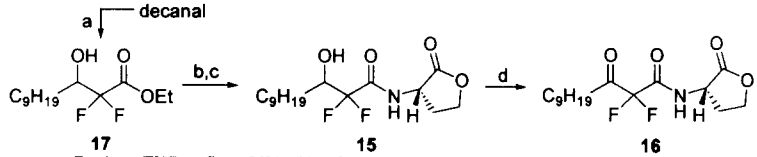
Scheme 3 illustrates the syntheses of E-mimetics furan **11** and oxazole **12**. Alkylation of 2-methyl-3-furoic acid[21] and conversion of the resulting acid **13** to the amide gave furan **11** in 36% yield over two steps. The structurally related oxazole **12** was prepared by initial cyclization of decanoic acid with ethyl isocyanoacetate to give the heterocycle **14** in 76% yield.[22] Saponification and coupling to amine **7** afforded oxazole **12** in 82% yield.



(a) *n*-BuLi (2 equiv), THF, -20 °C, iodooctane, 45%; (b) **7**, EDC, HOAt, DIEA, DMF, 81%; (c) diethyl cyanophosphonate, ethyl isocyanoacetate, Et_3N, DMF, 76%; (d) KOH, THF-EtOH-H_2O, 98%; (e) **7**, EDC, HOAt, DIEA, CH_2Cl_2, 82%.

Scheme 3

To incorporate the α,α-difluoro functionality into the side chain of PAI-1 analogs **15** and **16** (Scheme 4), decyl aldehyde was subjected to a Reformatsky reaction with ethyl bromodifluoroacetate to give the β-hydroxy ester **17** in 56% yield.[23] Amide formation gave **15** as a mixture of diastereomers in 83% yield. The β-ketoamide **16** was readily obtained by oxidation with Dess–Martin periodinane.[24]



(a) ethyl bromodifluoroacetate, Zn dust, THF, reflux, 56%; (b) KOH, THF-EtOH-H_2O, 98%; (c) **7**, EDC, HOAt, DIEA, DMF, 85%; (d) Dess–Martin periodinane, CH_2Cl_2, 92%

Scheme 4

Results

With **1**, **2**, **8**, **11**, **12**, **15**,and **16** in hand we determined the ability of the analogs to stimulate or diminish the autoinducer effects in both the PAI-1-LasR and PAI-2-RhlR systems. For this we used PAO-JP2, a defined

mutant deficient in both *lasI* (for PAI-1 production) and *rhlI* (for PAI-2 production), but with fully functional LasR and RhlR.

The *P. aeruginosa lasI* gene is induced by the transcriptional activator LasR in the presence of an autoinducer (e.g. PAI-1) capable of activating LasR. Therefore PAO-JP2(*lasI-lacZ*) can serve as a reporter to monitor LasR activation. If a synthetic autoinducer capable of activating LasR is present, the activated LasR induces *lasI-lacZ* expression that can be measured as β-galactosidase activity.[25] The results for the PAI-1-LasR interaction are shown in Figure 1. None of the constrained enolic analogs showed agonist or antagonist activity at LasR. In contrast, both extended analogs **15** and **16** were agonists, with the β-diketo analog **16** stimulating the *lasI-lacZ* response at 10 μM to a degree achieved by PAI-1 between 0.1-1μM.

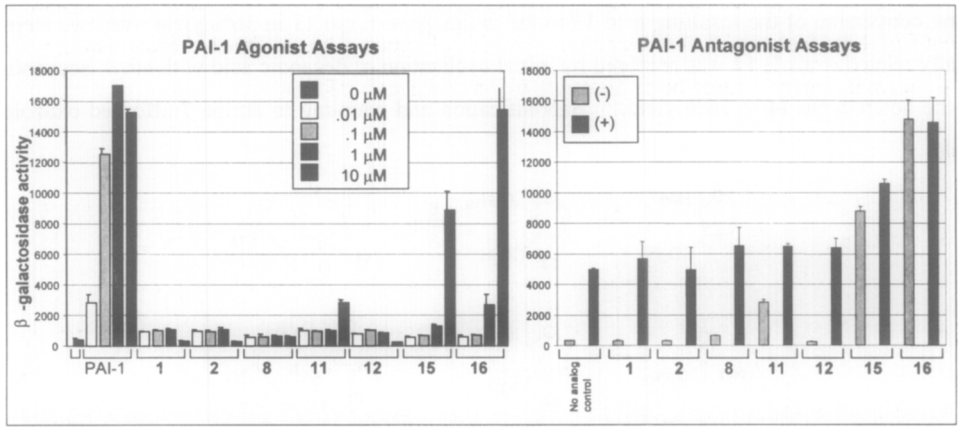


Figure 1. Agonist (left) or antagonist (right) effects of N-acylhomoserine lactone analogs of the PAI-1-LasR interaction, measured as Miller units of β-galactosidase activity in a *lasI-lacZ* fusion in PAO-JP2 as described note 25. The antagonist data were acquired with 10 μM analog in the absence (-) or presence (+) of 40 nM PAI-1, a concentration selected to give half-maximal response.

To determine any activity with RhlR, stimulation of a *rhlA-lacZ* reporter construct in PDO-100 (*rhlI*) was measured. For all analogs there was very weak, although dose-dependent PAI-2 activity as an agonist, and no activity as an antagonist, of the PAI-2- RhlR interaction (data not shown).

To confirm that the PAI-1 agonist effects were a direct result of analog-LasR interactions, we measured competition of selected analogs **8, 15** and **16** with [^{3}H]PAI-1.[26] As shown in Figure 2, incubation of *E. coli* MG4 (pKDT37), a strain that has no intrinsic LasR but harbors a plasmid that overexpresses the *P. aeruginosa* LasR, with 2 μM of our analogs discerns compounds that compete with the binding of 200 nM [^{3}H]PAI-1. The bar at the far left showed that pKDT33, an *E. coli* strain that carries no *lasR* on a plasmid, bound only the amount of [^{3}H] PAI-1 that can be considered as background, whereas the pKDT37 strain bound [^{3}H] PAI-1 robustly.

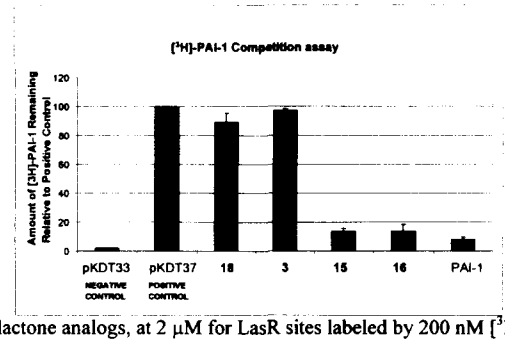


Figure 2. Competition of lactone analogs, at 2 μM for LasR sites labeled by 200 nM [^{3}H] PAI-1. Details of the assay are given in note 26.

Neither compound **18** (Sedanolide, an inactive lactone) nor compound **8** displaced the labeled ligand, whereas **15**, **16**, and PAI-1 reduced the level of binding to 10–15% of control levels. This indicated that the activity of **15** and **16** shown in Figure 1 could be attributed to direct action at LasR.

Discussion

N-acylhomoserine lactone analogs representative of Z enol, E enol, and extended structures have been assayed as agonists or antagonists of both PAI-1-LasR and PAI-2-RhlR. Only the non-enolizable analogs were active, with β-diketo analog **16**, and to a somewhat lesser extent the racemic β-hydroxy analog **15**, showing agonist activity at LasR. We have further shown that this activity is a direct result of binding at [^{3}H] PAI-1 sites on Las R. Previous attempts to develop antagonists of the quorum sensing systems, particularly those of *P. aeruginosa*, have emphasized the need to retain close adherence to the native chain length of the acylating sidechain.[13] Through the use of our constrained analogs, we can add to that requirement the necessity of retaining extended chain geometry in order for a compound to be recognized at LasR.

The recent patent on biofilm regulators reported that when *P. aeruginosa* PAO1 was grown in the continuous presence of 20 μM **16** for 10 days, the resultant biofilm was rendered sensitive to 0.2% SDS, whereas the biofilm from a control PAO1 culture was insensitive to SDS.[15] Despite the apparent discrepancy , this may not necessarily be at variance with our data that show that **16** is an agonist, not an antagonist, of PAI-1. Biofilm formation, and the coordinate regulation of *Pseudomonas* virulence factors, are complex processes that will require detailed investigations. Our continuing work on *P. aeruginosa* autoinducer antagonists, guided in part by the SAR developed in this study, will provide the tools to pursue these questions

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25. For the PAI-1 agonist assay, an overnight culture of PAO-JP2(*lasI-lacZ*) was diluted to an $OD_{660} = 0.1$ in PTSB medium (peptone trypticase soy broth). The culture was allowed to grow to $OD_{660} = 0.3$ (approximately 2 h). 1 mL aliquots were added to tubes containing either various concentrations of the analogs being tested or control synthetic PAI-1. After allowing the cultures to grow for 1 h, β-galactosidase activity was measured. For the antagonist assay, 1 mL aliquots of an overnight culture of a PAO-JP2(*lasI-lacZ)* culture, described above, were added to tubes containing either the analogs being tested (at a concentration of 1 μM) or control synthetic PAI-1 (40 nM – previously determined to be the concentration required for half-maximal activation of *lasI-lacZ*). The cultures were then allowed to grow for 15 min. After this time, 1 mL aliquots of each culture were added to 2 tubes: the first tube contained no PAI-1; the second tube contained 40 nM PAI-1. The cultures were allowed to grow for an additional hour before being assayed for β-galactosidase activity.
The PAI-2 agonist and antagonist assays were essentially the same as that described for PAI-1 except that PDO-100(*rhlA-lacZ*) was used as the reporter strain. Furthermore, in the PAI-2 antagonist assay 10 μM PAI-2 was added to the tubes after the fifteen minute incubation with test analog. This concentration was used to maximize the assay sensitivity, and the PAI-2 assays are less sensitive than those of PAI-1.
26. An overnight culture of *E.coli* MG4 (pKDT33) [control vector] or of *E.coli* (pKDT37)[*lasR* under *tac* promoter] grown at 37 °C in LB broth (+ ampicillin 100 μg/mL) was used to inoculate (1:100) fresh medium. Cultures were grown at 37 °C with shaking for three hours. Each culture was induced by the addition of IPTG to a final concentration of 1 mM and allowed to grow for one additional hour.
One mL of the appropriate culture was added to a microfuge tube containing either buffer alone or 2 μM of the compound of interest. The tubes were thoroughly mixed and held at 25 °C for 15 min to allow binding to occur. After the incubation the contents of the tubes were placed into fresh microfuge tubes containing 200nM (5μCi) of [^{3}H]-PAI-1 (125 Ci/mmol) and returned to the 25 °C incubation for an additional 15 min. Following the second incubation, the tubes were spun at 16,000 x *g* for 10 min and the pellets resuspended in cold phosphate buffered saline (PBS, pH 7.2). The cells were pelleted twice more and the final pellet was resuspended in 30–50 μL of PBS. The resuspended pellets were placed into a scintillation vial containing scintillation fluid (Ecoscint A) and counted.