The *lux* autoinducer–receptor interaction in *Vibrio harveyi*: binding parameters and structural requirements for the autoinducer

Jie-Gang CAO,* Zhong-Yong WEI* and Edward A. MEIGHEN†

Department of Biochemistry, McGill University, Montreal, Quebec, Canada H3G 1Y6

To assess the binding parameters and the structure–function relationship of the *Vibrio harveyi lux* autoinducer, *N*-(*D*-3-hydroxybutanoyl)homoserine lactone (*D*-HBHL), to light emission, a series of acylhomoserine lactone analogues were synthesized and their effects on the stimulation of luminescence of an autoinducer-deficient mutant of *V. harveyi*, D1, examined. Of the analogues with 3-hydroxyacyl chains, only *N*-(3-hydroxyvaleryl)homoserine lactone (HVHL) could act as an inducer, with about 85% of the potency of *D*-HBHL in stimulation of luminescence; the apparent K_d of the putative receptor for HVHL was 3.8 μ M, close to that for the natural autoinducer (1.4 μ M). Analogues with longer 3-hydroxyacyl chains, *N*-(3-hydroxyhexanoyl)homoserine lactone and *N*-(3-hydroxyheptanoyl)homoserine lactone, acted as competitive

inhibitors of HBHL with apparent K_i values of 77 and 53 μ M respectively. Replacement of the 3-hydroxybutanoyl moiety with a 3-methylbutanoyl or 3-methoxybutanoyl group created weak competitive inhibitors, *N*-(isovaleryl)- and *N*-(3-methoxybutanoyl)-homoserine lactones, with apparent K_i values of 150 and 360 μ M respectively. Two other analogues, *N*-(2-hydroxybutanoyl)- and *N*-(4-hydroxybutanoyl)-homoserine lactone, could neither stimulate nor inhibit luminescence. The approach used in these studies to demonstrate binding of autoinducer analogues at the same site, as well as measurement of the relative dissociation constant, may be of value in analysing analogues activating or inhibiting luminescence and other processes that are under control of acylhomoserine lactone autoregulators.

INTRODUCTION

In the marine bacteria *Vibrio fischeri* and *Vibrio harveyi*, the development of luminescence is under the control of species-specific autoinducers [1–3], which could be considered as bacterial pheromones [4–6]. The structures of the *lux* autoinducers of *V. fischeri* and *V. harveyi* are closely related, being *N*-(3-oxohexanoyl)homoserine lactone (OHHL) and *N*-(*D*-3-hydroxybutanoyl)homoserine lactone (*D*-HBHL) respectively.

In earlier work, it was shown that a number of non-luminescent bacteria of marine and terrestrial origins, including the important human pathogen *Vibrio cholera*, appeared to excrete a substance into the media which can stimulate light emission in *V. harveyi* [7,8]. This result suggested that *D*-HBHL or related analogues may be involved in the regulation of other pathways in these bacteria. Although *D*-HBHL synthesis has not been demonstrated in other bacteria, it has recently been shown that this compound controls polyhydroxybutyrate biosynthesis as well as luminescence in *V. harveyi* [9].

The first demonstration of the synthesis of acylhomoserine lactone inducers by non-luminescent bacteria was accomplished by Bainton et al. [10,11]. 3-Oxoacylhomoserine lactone autoregulators, which were identical to or closely related in structure with the *V. fischeri lux* autoinducer, have now been identified in a wide range of bacteria [6,10–12], including plant and animal pathogens. A set of 3-oxoacylhomoserine lactones has been found in *Pseudomonas aeruginosa*, with *N*-(3-oxododecanoyl)-homoserine lactone being the most effective in stimulation of the expression of elastase, an enzyme implicated in pathogenesis during infection [13,14]. In the plant pathogen *Erwinia carotovora*, OHHL controls both the synthesis of the virulence

factors and the antibiotic, carbapenem [11,15]. Similarly, *N*-(3-oxo-octanoyl)homoserine lactone has been identified as the conjugation factor of *Agrobacterium tumefaciens* [16]. Moreover, recent evidence indicates that acylhomoserine lactones are produced by an even wider range of bacteria including species of *Citrobacter*, *Enterobacter*, *Hafnia*, *Proteus*, *Rahnella* and *Serratia* [11,12]. Genetic data have demonstrated that genes in *V. fischeri* responsible for synthesis and response to autoinducer, *luxI* and *luxR* respectively, have their counterparts in other bacteria [6,14,17–21]. These discoveries have indicated the importance of acylhomoserine lactones as general autoregulators and generated considerable interest in the regulation of luminescence by the *lux* autoinducers.

In *V. harveyi*, a protein receptor for the *lux* autoinducer, *D*-HBHL, has not yet been clearly identified. However, a new gene product (LuxN), which contains a potential membrane-spanning domain in the N-terminal region, has been proposed as a potential binding site for the *V. harveyi lux* autoinducer, *D*-HBHL [22]. Transposon insertions in *luxN* blocked the stimulation of luminescence of *V. harveyi* by HBHL, consistent with a receptor function.

The structure–function relationships of the 3-oxoacylhomoserine lactone autoinducers of *V. fischeri* and *E. carotovora* have been studied using analogues in which the fatty acyl chain or homoserine lactone ring have been modified. In all cases, agonist activity was reduced [23,24]. Removal of the 3-carbonyl group of the acyl moiety of the *V. fischeri* autoinducer resulted in strong antagonists [23], while the abilities of the *E. carotovora* autoinducer analogues to act as antagonists were not examined [24]. Although these studies measured the relative efficiency of the analogues, they did not address other aspects of the

Abbreviations used: *D*-HBHL or HBHL, *N*-(*D*-3-hydroxybutanoyl)homoserine lactone; HVHL, *N*-(3-hydroxyvaleryl)homoserine lactone; HXHL, *N*-(3-hydroxyhexanoyl)homoserine lactone; HPHL, *N*-(3-hydroxyheptanoyl)homoserine lactone; IVHL, *N*-(isovaleryl)homoserine lactone; MBHL, *N*-(3-methoxybutanoyl)homoserine lactone; α HBHL, *N*-(2-hydroxybutanoyl)homoserine lactone; γ HBHL, *N*-(4-hydroxybutanoyl)homoserine lactone; OHHL, *N*-(3-oxohexanoyl)homoserine lactone.

* Present address: Astra Pain Research Unit, 275 bis, boul. Armand-Frappier, Edifice 3000, Laval, Quebec, Canada H7V 4A7

† To whom correspondence should be addressed.

autoinducer–receptor interaction such as the binding affinity and whether the analogues bound to the same site as the inducer in a competitive fashion.

To more accurately assess the parameters of the autoinducer–receptor binding, we adopted Clark's method [25,26] based on the law of mass action to establish a quantitative relationship between the ligand concentrations and levels of stimulation of luminescence in an autoinducer-deficient mutant, D1, which responds to a low level of HBHL [3]. We also investigated the structural requirements for the *V. harveyi* lux autoinducer by synthesizing a number of analogues of D-HBHL and examining their biological impacts on stimulation of luminescence, including the measurement of their apparent dissociation constants. The results indicate that the autoinducer and its analogues interact at the same site to confer stimulation or inhibition of luminescence in *V. harveyi*, consistent with a specific receptor for the acylhomoserine lactone autoregulator.

MATERIALS AND METHODS

Strains and growth conditions

The bacterial strains used in this study were *V. harveyi* (B392) and an autoinducer-responsive mutant of *V. harveyi*, D1 [3]. The cells were grown in complex medium [2 g of Na_2HPO_4 , 1 g of KH_2PO_4 , 0.5 g of $(\text{NH}_4)_2\text{HPO}_4$, 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0 g of tryptone (Difco), 0.5 g of yeast extract (Difco), 10 g of NaCl, and 2 g of glycerol per litre] at 27 °C in rotary shakers at 200 rev./min.

Preparation of 3-hydroxy acids

The 3-hydroxy acids, which were not commercially available, were synthesized by the procedure of Gawronski [27]. Trimethylchlorosilane (2.4 mmol) was added to a suspension of zinc powder (32 mmol) in 50 ml of anhydrous ether and stirred for 15 min at room temperature. The suspension was heated to reflux to activate the zinc. After heating was stopped, a mixture of methyl bromoacetate (24 mmol) and an aldehyde (propanal, butanal or pentanal, 24 mmol) was added slowly so that a gentle reflux was achieved. Heating was then resumed so that the solution continued to reflux for an additional hour. The mixture was cooled, 200 ml of 2 M HCl added and stirred for 15 min. The aqueous phase was extracted twice with 50 ml of ether. The extracts were combined and washed twice with 50 ml of 5% NaHCO_3 solution. After drying over MgSO_4 and then evaporating the ether, a crude 3-hydroxy ester preparation was obtained. Each 3-hydroxy ester was hydrolysed overnight in 120 ml of a methanol/water solution (3:1, v/v) containing 120 mmol of LiOH according to the method of Corey et al. [28].

Synthesis and purification of the autoinducer and its analogues

D-HBHL, *N*-(2-hydroxybutanoyl)-, *N*-(4-hydroxybutanoyl)- and *N*-(isovaleryl)-homoserine lactone (α HBHL, γ HBHL and IVHL respectively) were synthesized by condensing 0.5 g of the corresponding fatty acid (Aldrich) with a racemic sample of 1 g of α -amino- γ -butyrolactone hydrobromide (Aldrich) using 1 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma) as a carbonyl group activator as described previously [2].

To synthesize *N*-(3-hydroxyvaleryl)-, *N*-(3-hydroxyhexanoyl)- or *N*-(3-hydroxyheptanoyl)-homoserine lactone (HVHL, HXHL or HPHL respectively), the pH of each of the preparations of the 3-hydroxy acids (3-hydroxyvaleric, 3-hydroxyhexanoic and 3-hydroxyheptanoic acid) obtained above was first adjusted with 12 M HCl to 7.5 and 1 g each of α -amino- γ -butyrolactone hydrobromide and 1-ethyl-3-(3-dimethylaminopropyl)-

carbodiimide were added. *N*-(3-Methoxybutanoyl)homoserine lactone (MBHL) was synthesized by oxidation of 3-methoxybutanol with KMnO_4 to 3-methoxybutyric acid and then condensed with α -amino- γ -butyrolactone. The reaction mixtures were incubated overnight and the compounds extracted three times with 2 vol. of chloroform. The autoinducer and each analogue were purified on a silica gel (Bio-Rad) column (13 cm $\times$ 0.7 cm) by elution with the specific solvent given in Table 1. The purities of these compounds as judged by NMR spectroscopy were found to be higher than 99% for D-HBHL, HVHL, γ HBHL, MBHL and IVHL, 95% for HPHL and 90% for α HBHL and HXHL.

The purified autoinducer and analogues were dried under nitrogen gas, weighed, redissolved in chloroform, dried again and stored in aliquots at -20°C . Diluted stock solutions prepared in 1% NaCl of the autoinducer and its analogues were made just before use so that the same volume of 1% NaCl (10 μl), containing the appropriate amount of autoinducer and/or an analogue, was added to 1.0 ml of culture. To control cultures the same volume of 1% NaCl was also added.

Bioassay for the autoinducer and its analogues

Cells were inoculated into fresh complex medium ($D_{660} < 0.01$) until an attenuation of 0.25–0.3 at 660 nm was reached. An aliquot (1 ml) of culture was added to a 20-ml scintillation vial containing the autoinducer and/or one of its analogues. The cultures were then shaken for an additional 2 h and *in vivo* luminescence in light units/ml (LU/ml; 1 LU = 10^{10} quanta/s) was followed. As the absolute level of luminescence at a specific time could vary 3–5-fold, depending on the history and inoculum of the cells, control (no addition) and reference cultures (with different amounts of D-HBHL) were grown for each set of analogues.

^1H -NMR spectrometry

The ^1H -NMR spectra were taken on Varian XL-200 instruments. The data are reported in p.p.m. relative to the CHCl_3 reference line with the multiplicity and number of protons given in parentheses.

D-HBHL ^1H NMR (C^2HCl_3 , p.p.m.): 1.24 (d, 3 H), 2.08–2.52 (m, 3 H), 2.68–2.85 (m, 1 H), 3.30 (broad s, 1 H), 4.12–4.38 (m, 2 H), 4.40–4.68 (m, 2 H), 6.78 (m, 1 H).

HVHL ^1H NMR (C^2HCl_3 , p.p.m.): 0.95 (t, 3 H), 1.50 (m, 2 H), 2.08–2.50 (m, 3 H), 2.65–2.85 (m, 1 H), 3.38 (broad s, 1 H), 3.90 (m, 1 H), 4.26 (m, 1 H), 4.38–4.68 (m, 2 H), 6.88 (m, 1 H).

HXHL ^1H NMR (C^2HCl_3 , p.p.m.): 0.92 (m, 3 H), 1.48 (m, 4 H), 2.05–2.55 (m, 3 H), 2.80 (m, 1 H), 3.20 (broad s, 1 H), 4.03 (m, 1 H), 4.29 (m, 1 H), 4.42–4.85 (m, 2 H), 6.78 (m, 1 H).

HPHL ^1H NMR (C^2HCl_3 , p.p.m.): 0.88 (m, 3 H), 1.30 (m, 6 H), 2.20–2.55 (m, 3 H), 2.75 (m, 1 H), 3.30 (broad s, 1 H), 3.98 (m, 1 H), 4.25 (m, 1 H), 4.40–4.75 (m, 2 H), 7.00 (m, 1 H).

α HBHL ^1H NMR (C^2HCl_3 , p.p.m.): 0.97 (t, 3 H), 1.57–1.78 (m, 2 H), 2.15–2.31 (m, 1 H), 2.67–2.79 (m, 1 H), 4.06–4.18 (m, 1 H), 4.20–4.33 (m, 1 H), 4.42–4.77 (m, 2 H).

γ HBHL ^1H NMR (C^2HCl_3 , p.p.m.): 1.62 (s, 3 H), 1.82–1.94 (q, 2 H), 2.02–2.24 (m, 1 H), 2.36–2.43 (t, 2 H), 2.73–2.87 (m, 1 H), 4.19–4.32 (m, 1 H), 4.42–4.61 (m, 2 H), 6.39 (m, 1 H).

IVHL ^1H NMR (C^2HCl_3 , p.p.m.): 0.92 (m, 6 H), 1.17–1.35 (m, 1 H), 1.98–2.18 (m, 3 H), 2.69–2.83 (m, 1 H), 4.17–4.32 (m, 1 H), 4.38–4.66 (m, 2 H), 6.51 (m, 1 H).

MBHL ^1H NMR (C^2HCl_3 , p.p.m.): 1.14 (dd, 3 H), 2.02–2.24 (m, 1 H), 2.33–2.37 (m, 2 H), 2.61–2.74 (m, 1 H), 3.28 (s, 3 H), 3.60–3.74 (m, 1 H), 4.13–4.27 (m, 1 H), 4.30–4.62 (m, 2 H), 7.10 (m, 1 H).

Table 1 Purification and effects of autoinducer analogues on stimulation of luminescence

Agonist activities were measured by adding 1 ml cultures of the D1 mutant (D_{660} 0.27) to a vial containing 20 $\mu\text{g/ml}$ (90–110 μM) of the compound and the luminescence compared after 2 h to cultures with no additions. Apparent dissociation constants for α -HBHL and HVHL were determined as described in Figure 3. Antagonist activities were measured by incubation of 1 ml of D1 cultures with 0.5 $\mu\text{g/ml}$ (2.7 μM) of the autoinducer (α -HBHL) plus 20 $\mu\text{g/ml}$ (90–110 μM) of the analogue and luminescence after 2 h compared with the control culture containing 0.5 $\mu\text{g/ml}$ of α -HBHL. Dissociation constants were determined as described in Figure 5. Abbreviation: N, no effect on stimulation or inhibition of luminescence.

Analogue	Acyl moiety	Agonist activity (K_d , μM)	Antagonist activity (K_i , μM)	Purification ^a
HBHL	$\text{CH}_3\text{CH}(\text{OH})\text{CH}_2\text{CO}-$	1.4	—	2% Methanol
HVHL	$\text{CH}_3\text{CH}_2\text{CH}(\text{OH})\text{CH}_2\text{CO}-$	3.8	—	1% Methanol
HXHL	$\text{CH}_3(\text{CH}_2)_2\text{CH}(\text{OH})\text{CH}_2\text{CO}-$	N	77	0.5% Methanol
HPHL	$\text{CH}_3(\text{CH}_2)_3\text{CH}(\text{OH})\text{CH}_2\text{CO}-$	N	53	0.2% Methanol
α HBHL	$\text{CH}_3\text{CH}_2\text{CH}(\text{OH})\text{CO}-$	N	N	2% Methanol
γ HBHL	$\text{CH}_2(\text{OH})\text{CH}_2\text{CH}_2\text{CO}-$	N	N	2% Methanol
IVHL	$(\text{CH}_3)_2\text{CHCH}_2\text{CO}-$	N	151	5% Hexane
MBHL	$\text{CH}_3\text{CH}(\text{OCH}_3)\text{CH}_2\text{CO}-$	N	362	1% Methanol

^a Solvent used for elution of synthetic compound from silica gel.

RESULTS

Bioassays for the autoinducer and its analogues

The effects of autoinducer α -HBHL and analogues with longer 3-hydroxyacyl chains (HVHL, HXHL and HPHL) on stimulation of luminescence of the autoinducer-deficient D1 mutant of *V. harveyi* are given in Figure 1. α -HBHL, as well as HVHL, is a strong inducer of the *V. harveyi* luminescence system, whereas neither HXHL nor HPHL stimulates luminescence even at 40-fold higher concentrations. Mixing of either of these latter compounds with α -HBHL or HVHL caused a decrease in stimulation of luminescence compared with the addition of α -HBHL or HVHL alone (Figure 1), showing that HXHL and HPHL can function as inhibitors.

Figure 1 Bioassay with the D1 mutant to study the effects of the autoinducer and its analogues on stimulation of luminescence in *V. harveyi*

An aliquot (1 ml) of a D1 culture (D_{660} = 0.28) was added to a vial containing α -HBHL or its analogues (HVHL, HXHL and HPHL) or a combination of α -HBHL or HVHL with either HXHL or HPHL. The final concentration of α -HBHL or HVHL was 0.5 $\mu\text{g/ml}$; HXHL or HPHL was 20 $\mu\text{g/ml}$. The control culture contained no additions (NA). The cultures were incubated for 2 h and the luminescence recorded. All cultures reached the same cell density (i.e. attenuation at 660 nm).

Figure 2 Dose-response of the D1 mutant to the autoinducer and its analogues

Cultures (1 ml; D_{660} = 0.25) of D1 were added to vials containing the indicated amounts of the autoinducer (α -HBHL) or an analogue (HVHL, HXHL or HPHL) and luminescence recorded after 2 h of growth.

Using the same assay system, α HBHL, γ HBHL, IVHL and MBHL were not able to stimulate luminescence. However, IVHL and MBHL, but not α HBHL or γ HBHL, were found to be inhibitors of the stimulation of luminescence by HBHL.

Figure 2 shows the luminescence response of the D1 mutant to different amounts of the autoinducer and some of its analogues. Only about 1 $\mu\text{g/ml}$ of autoinducer is needed to cause the maximal induction of luminescence of D1. Higher concentrations of autoinducer do not cause inhibition, which is in agreement with the results previously obtained with another autoinducer-stimulatable mutant [2,29]. HVHL exhibits an inducing capacity similar to the autoinducer, α -HBHL. However, the degree of luminescence stimulation is always less than that for the autoinducer over a wide range of concentrations, indicating that it is not quite as effective. Neither HXHL nor HPHL stimulates

Figure 3 Lineweaver-Burk analysis of stimulation of luminescence of D1 by D-HBHL and HVHL

Cultures (1 ml; $D_{660} = 0.26$) of D1 were added to vials containing different concentrations (1–11 μM) of either D-HBHL or HVHL and the luminescence recorded after 2 h of growth. The results are presented in a double-reciprocal plot as the inverse of the luminescence ($1/L$) versus the inverse of the concentrations of the inducers ($1/[A]$). The lines were drawn by the least-squares regression method with a correlation coefficient for D-HBHL of 0.954 and for HVHL of 0.987. The dissociation constants are reported in Table 1.

luminescence at any concentration tested up to 100 $\mu\text{g}/\text{ml}$, in agreement with the results in Figure 1.

Interaction of HBHL or HVHL with a receptor

The mechanism by which the autoinducer stimulates luminescence is still unknown. However, if we adopt the approach pioneered by Clark [25,26] assuming that the autoinducer (A) binds reversibly to a single site on a receptor (R) to form a complex (AR) as shown below:

where luminescence (L) is proportional to the concentration of the complex, then we can write eqn. (1).

$$L/L_m = A/(K_d + A) \quad (1)$$

A is the free autoinducer concentration and L_m is the maximum luminescence when all the receptor molecules are bound by HBHL (or the agonist, HVHL) at a given time during cell growth.

Figure 3 shows a double-reciprocal plot from which the apparent K_d and L_m can be determined for the autoinducer (D-HBHL) or HVHL by eqn. (2).

$$1/L = 1/L_m + (K_d/L_m)(1/A) \quad (2)$$

It should be recognized that the value for K_d is based on the assumption that light emission is proportional to the amount of receptor–autoinducer complex and therefore should only be considered as an apparent K_d for the dissociation constant. The results indicate that there is a 1:1 stoichiometry between the homoserine lactone autoregulator and a putative receptor and show that this approach can be used to provide a measurement of the interaction of the autoinducer and its analogues. The apparent K_d of the receptor for the autoinducer in this experiment is 1.4 μM and that for HVHL is 3.8 μM (Table 1). The ratio of

Figure 4 Competition of HVHL and D-HBHL

Cultures (1 ml; $D_{660} = 0.25$) each containing different concentrations (0.0054–54 μM) of autoinducer (D-HBHL) were mixed with HVHL (0, 2, or 4 μM). Luminescence was recorded after 2 h of growth and is plotted as the percentage of maximal light ($100 \times L/L_m$) versus the concentration of D-HBHL.

the L_m s of HVHL to HBHL is 0.87 (Figure 3), which is simply the potency (P) of HVHL relative to the autoinducer. Since the amount of receptor, and hence L_m , is dependent on the time during the bioassay, P can be determined by analysis of the data at different stages of cell growth. K_d values of 1–1.8 μM for D-HBHL and 3.8–8 μM for HVHL, with a range of 0.87–0.91 for the relative potency (P) of HVHL, were found between 1 and 2.5 h after the addition of autoinducer.

HVHL binds to the same site as D-HBHL

To examine whether HVHL interacts at the same site as the autoinducer (D-HBHL), both compounds were added together and the effects on stimulation of luminescence tested. Under the same assumptions as above, the stimulation of luminescence by the mixture of D-HBHL and HVHL can then be described by the following equation:

$$L/L_m = (A/K_d + P \times V/K_{dv}) / (A/K_d + V/K_{dv} + 1) \quad (3)$$

where A and K_d are the concentration and dissociation constant, respectively, for HBHL, V and K_{dv} are the concentration and dissociation constant for HVHL, and P is the relative potency of HVHL.

In Figure 4, each curve is obtained by plotting the percentage of maximal luminescence ($L/L_m \times 100$) induced by a fixed level of HVHL (0, 2 or 4 μM) and varied amounts of the autoinducer (0.053–53 μM) as a function of the concentration of autoinducer (A). According to eqn. (3) all the curves intersect when the autoinducer concentration A equals $K_d \times P/(1 - P)$. At this point, the stimulation of luminescence is independent of the concentration of HVHL (V) and it can be easily verified from eqn. (3) that the value of L/L_m is equal to P . In Figure 4, this value ($L/L_m = 0.83$) is in close agreement with that of the relative potency for HVHL calculated from the data in Figure 3. When L/L_m is less than P , D-HBHL concentrations are low and consequently HVHL stimulates luminescence as most receptor sites will be unoccupied, whereas luminescence is inhibited at

Figure 5 Shild plot analyses showing competitive inhibition of the HBHL luminescence stimulation of D1 by HPHL, HXHL, IVHL and MBHL

Sets of 1-ml D1 cultures ($0.25\text{--}0.3 D_{660}$) containing 0.54 , 1.1 or $2.2 \mu\text{M}$ of autoinducer (D-HBHL) were mixed with different concentrations ($9\text{--}110 \mu\text{M}$ for HPHL, HXHL and IVHL; $50\text{--}250 \mu\text{M}$ for MBHL) of each antagonist. The cultures were incubated for 2 h and luminescence recorded. For analysis of each analogue, a set of reference cultures with identical amounts of D1 cells containing different concentrations ($0.54\text{--}5.4 \mu\text{M}$) of D-HBHL were included to determine the specific L_m at the identical stage of growth and thus allow calculation of the autoinducer concentration (A') that gave the same level of luminescence as that observed (A) in the presence of the different concentrations of each antagonist. As three different concentrations of autoinducer (0.54 , 1.1 , $2.2 \mu\text{M}$) were each mixed with a given concentration of an antagonist, the three values of $\log(A'/A-1)$ were averaged for each inhibitor concentration and used to construct Shild plots. The data were analysed by the least-squares regression method. HPHL: slope = -1.01 , $r = 0.989$; HXHL: slope = -1.01 , $r = 0.988$; IVHL: slope = -1.03 , $r = 0.987$; MBHL: slope = -0.987 , $r = 0.947$. The dissociation constant for each inhibitor was calculated as the concentration of an inhibitor corresponding to $\log(A'/A-1) = 0$ and is presented in Table 1.

$L/L_m > P$ since HVHL blocks binding of D-HBHL and its potency in stimulation of luminescence is lower than that of the autoinducer. These results provide strong evidence that HVHL interacts at the same site as the autoinducer.

HPHL, HXHL, IVHL and MBHL are competitive inhibitors of the autoinducer

To determine whether the nature of inhibition of autoinducer activity by HPHL, HXHL, IVHL or MBHL is competitive, the data were analysed using a Shild plot [30]. In the absence of inhibitor, the stimulation of luminescence by the autoinducer, A , is given by eqn. (1). When a competitive inhibitor, I , is present then the relative luminescence (L/L_m) is given by the following equation:

$$L/L_m = A/[K_d + A + (K_d \times I/K_i)] \quad (4)$$

where K_i and I are the dissociation constant and concentration of the inhibitor respectively. Consequently, a higher concentration of autoinducer (A') is needed to give the same level of luminescence (L/L_m) as in the absence of inhibitor. Under these circumstances where the stimulation of luminescence is equal

$$A/(K_d + A) = A'/[K_d + A' + (K_d \times I/K_i)] \quad (5)$$

or

$$\log(A'/A-1) = -(-\log I) - \log K_i \quad (6)$$

For competitive inhibition caused by an antagonist which does not have agonist activity, a plot of $\log(A'/A-1)$ versus $-\log I$

Figure 6 Inhibition of luminescence of wild-type *V. harveyi* cells by HBHL analogues

V. harveyi cells were inoculated at a D_{660} of 0.05 into complex medium containing $100 \mu\text{g/ml}$ ($450\text{--}550 \mu\text{M}$) each of HXHL, HPHL, IVHL and MBHL. The cultures were incubated for 4 h and luminescence measured at 0.5 h intervals.

would be linear with a slope of -1 . Figure 5 shows Shild plots demonstrating that HPHL, HXHL, IVHL and MBHL are competitive inhibitors of the autoinducer. The slopes are very close to -1 , also confirming that these compounds are not agonists. The apparent dissociation constants (K_i s), which correspond to the inhibitor concentrations where $\log(A'/A-1) = 0$ (i.e. $A'/A = 2$), for HPHL, HXHL, IVHL and MBHL, are 53 , 77 , 150 and $360 \mu\text{M}$ respectively (Table 1).

When these competitive inhibitors were added to the cultures of wild-type cells of *V. harveyi*, inhibition of luminescence was observed (Figure 6). Their relative efficacy in inhibition of luminescence of wild-type cells is in the same order as inhibition of D-HBHL stimulation of light emission of the D1 mutant, i.e. HPHL > HXHL > IVHL > MBHL. The relative inhibition of luminescence by these compounds decreases with cell growth, consistent with the further synthesis and accumulation of autoinducer in the medium with time which can reach levels as high as $20 \mu\text{M}$ [3]. Using the K_d for D-HBHL and the K_i s for HPHL, HXHL, IVHL and MBHL measured with the autoinducer-deficient D1 mutant (Table 1) as well as the relative inhibition of luminescence of wild-type cells (Figure 6), the concentration of the autoinducer at later stages of growth of *V. harveyi* can be estimated to be $15\text{--}20 \mu\text{M}$, in excellent agreement with the value given above.

DISCUSSION

The present work has demonstrated that *in vivo* luminescence was dependent on reversible binding between the autoinducer and a receptor in a $1:1$ stoichiometry. Therefore, an analysis based on the law of mass action [25,26] could be applied to quantitatively study, in terms of the autoinducer-receptor interaction, the stimulation and/or inhibition of luminescence of *V. harveyi* and an autoinducer-deficient mutant, D1, by the *lux* autoinducer and its analogues. D1 was an important strain for this study since it has a high sensitivity to the autoinducer. Previous studies have not detected autoinducer ($\leq 0.1 \mu\text{M}$) in medium conditioned by this mutant [3].

Seven analogues of the autoinducer were synthesized, purified (Table 1) and their effects on the stimulation or inhibition of luminescence of an autoinducer-deficient mutant (D1) of *V. harveyi* investigated. The apparent dissociation constants for the autoinducer and its analogues (Table 1), as well as the relative potency of HVHL to the autoinducer in stimulation of luminescence (Figure 1), have been determined. As the 3-hydroxyacyl chain increased in length, only HVHL retained autoinducer activity; HXHL and HPHL acted as antagonists. Longer 3-hydroxyacyl chains resulted in a decrease in affinity and the inability to trigger an inducer-receptor complex to activate transcription of the *lux* genes. IVHL and MBHL had no autoinducer activity but had the ability to act as weak competitive inhibitors, indicating the 3-hydroxy group is not essential for binding but is required for autoinducer-mediated receptor function. The results are consistent with molecular modelling studies showing that the conformations of the fatty acid moieties of these analogues were similar to the autoinducer (results not shown). In contrast, α HBHL and γ HBHL lost the ability to bind to the same site as D-HBHL and could cause neither stimulation nor inhibition of luminescence, consistent with major differences in conformation of their fatty acid moieties as predicted by molecular modelling analyses (results not shown). It seems that the structural requirements for the function of the autoinducer are very stringent, in agreement with other cases in which minor modifications of the structures of signal molecules reduced or abolished their biological effects [23,24].

The stereoisomer, D-3-hydroxybutyrate, was used for the synthesis of D-HBHL as the L-isomer (L-HBHL) is inactive [3]. Since racemic samples of the acyl chains were used to synthesize the analogues, except IVHL which does not have a chiral centre in its acyl moiety, the apparent dissociation constants for these analogues may be reduced by one-half assuming only one stereoisomer can bind to the putative receptor. Similarly, a racemic sample of α -amino- γ -butyrolactone hydrobromide was used to generate the homoserine lactone ring in all cases. Assuming only one of the two diastereoisomers is functional, the apparent dissociation constants of the autoinducer and all its analogues could be further reduced.

The mechanism by which the autoinducer is transported into the *V. harveyi* cell has not yet been studied. Although we have used the word 'receptor' or 'putative receptor' throughout this manuscript, its meaning should not be restricted to the LuxR type of receptor of *V. fischeri* which has been proposed to bind to the autoinducer and activate the transcription of the *luxICDABEG* operon [17]. The data obtained in this study also fit a model of regulation at the level of transport of the autoinducer. Since the autoinducer of *V. harveyi* is more polar than that of *V. fischeri*, the energy barrier for D-HBHL to cross the cell membrane should be much higher and thus may require binding (and saturation) to a transport protein. It is of interest in this regard that the *V. harveyi* protein implicated in reception of the autoinducer (LuxN) contains a potential membrane-spanning domain [22].

At present, we do not yet know the specific mechanism by which the autoinducer (D-HBHL) stimulates the *lux* system in *V. harveyi*. A set of regulatory genes, including *luxN*, have been implicated with D-HBHL as well as possibly another small signal molecule in relieving inhibition by a repressor, LuxO [22,31]. The signal transduction has been suggested by Bassler et al. [22,31] to occur via a mechanism similar to the 'transmitter/receiver two-component system' [32,33]: the signal binds to the receptor which then relays information to a second site which in turn

results in activation of transcription (e.g. by phosphorylation). Alternatively, the possibility that the signal simply binds to a receptor (on the cell surface or inside the cell) acting directly as a transcription activator [17,34], cannot yet be excluded.

The synthesis of the *V. harveyi* autoinducer and its analogues and the measurement of their ability to act as agonists and/or antagonists in this study are important for defining the structure-function relationship between the *V. harveyi lux* autoinducer. Moreover, the approach used to demonstrate that the analogues and inducer bind to the same site in a competitive fashion may prove useful to characterize analogues of homoserine auto-regulators which serve as inhibitors or activators of other processes in bacteria.

This work was supported by a grant (MT-7672) from the Medical Research Council of Canada.

REFERENCES

- 1 Eberhard, A., Burlingame, A. L., Eberhard, C., Kenyon, G. L., Nealson, K. H. and Oppenheimer, N. J. (1981) *Biochemistry* **20**, 2444-2449
- 2 Cao, J.-G. and Meighen, E. A. (1989) *J. Chem. Biol.* **264**, 21670-21676
- 3 Cao, J.-G. and Meighen, E. A. (1993) *J. Bacteriol.* **175**, 3856-3862
- 4 Meighen, E. A. (1991) *Microbiol. Rev.* **55**, 123-142
- 5 Meighen, E. A. (1994) *Annu. Rev. Genet.* **28**, 117-139
- 6 Fugua, W. C., Winans, S. C. and Greenberg, E. P. (1994) *J. Bacteriol.* **176**, 269-275
- 7 Greenberg, E. P., Ulitzur, S. and Hastings, J. W. (1979) *Arch. Microbiol.* **120**, 87-91
- 8 Hada, H. S., Stemmler, J., Grossbard, M. L., West, P. A., Potrikus, C. J., Hastings, J. W. and Colwell, R. R. (1985) *System. Appl. Microbiol.* **6**, 203-209
- 9 Sun, W., Cao, J.-G., Teng, K. and Meighen, E. A. (1994) *J. Biol. Chem.* **269**, 20785-20790
- 10 Bainton, N. J., Bycroft, B. W., Chhabra, S. R. et al. (1992) *Gene* **116**, 87-91
- 11 Bainton, N. J., Stead, P., Chhabra, S. R., Bycroft, B. W., Salmond, G. P. C., Stewart, G. S. and Williams, P. (1992) *Biochem. J.* **288**, 997-1004
- 12 Swift, S., Winson, M. K., Chan, P. F. et al. (1993) *Mol. Microbiol.* **10**, 511-520
- 13 Jones, S., Yu, B., Bainton, N. J. et al. (1993) *EMBO J.* **12**, 2477-2482
- 14 Pearson, J. P., Gray, K. M., Passador, L., Tucker, K. D., Eberhard, A., Iglewski, B. H. and Greenberg, E. P. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 197-201
- 15 Pirhonen, M., Flego, D., Heikiheimo, R. and Palva, E. T. (1993) *EMBO J.* **12**, 2467-2476
- 16 Zhang, L., Murphy, P. J., Kerr, A. and Tate, M. E. (1993) *Nature (London)* **362**, 446-448
- 17 Engbrecht, J., Nealson, K. and Silverman, M. (1983) *Cell* **32**, 773-781
- 18 Hwang, I., Li, P. L., Zhang, L., Piper, K. R., Cook, D. M., Tate, M. E. and Farrand, S. K. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 4639-4643
- 19 Piper, K. R., Beck von Bodman, S. and Farrand, S. K. (1993) *Nature (London)* **362**, 448-450
- 20 Gray, K. M., Passador, L., Iglewski, B. H. and Greenberg, E. P. (1994) *J. Bacteriol.* **176**, 3076-3080
- 21 Pierson, L. S., Keppenne, V. D. and Wood, D. W. (1994) *J. Bacteriol.* **176**, 3966-3974
- 22 Bassler, B. L., Wright, M., Showalter, R. E. and Silverman, M. R. (1993) *Mol. Microbiol.* **9**, 773-786
- 23 Eberhard, A., Widrig, C. A., McBath, P. and Schineller, J. B. (1986) *Arch. Microbiol.* **146**, 35-40
- 24 Chhabra, S. R., Stead, P., Bainton, N. J., Salmond, G. P., Stewart, G. S., Williams, P. and Bycroft, B. W. (1993) *J. Antibiotics* **46**, 441-454
- 25 Clark, A. J. (1933) *The Mode of Action of Drugs on Cells*, E. Arnold, London
- 26 Clark, A. J. (1937) *General Pharmacology*, Springer Verlag, Berlin
- 27 Gawronski, J. K. (1984) *Tetrahedron Lett.* **25**, 2605-2608
- 28 Corey, E. J., Szekely, I. and Shiner, C. S. (1977) *Tetrahedron Lett.* **40**, 2529-2532
- 29 Cao, J.-G., Swartzman, E., Miyamoto, C. and Meighen, E. A. (1989) *J. Biol. Chem.* **264**, 207-212
- 30 Shild, H. O. (1947) *Br. J. Pharmacol.* **2**, 189
- 31 Bassler, B. L., Wright, M. and Silverman, M. R. (1994) *Mol. Microbiol.* **12**, 403-412
- 32 Kofoid, E. C. and Parkinson, J. S. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3939-3943
- 33 Nixon, B. T., Ronson, C. W. and Ausubel, F. M. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7850-7854
- 34 Miller, V. L., Taylor, R. K. and Mekalanos, J. J. (1987) *Cell* **48**, 271-279