# Synthesis and validation of a probe to identify quorum sensing receptors†

**Luba Dubinsky,‡[a] Lucja M. Jarosz,‡[b] Neri Amara,[a] Pnina Krief,[a] Vladimir V. Kravchenko,[c] Bastiaan P. Krom*[b] and Michael M. Meijler*[a]**

***Received (in Cambridge, UK) 25th August 2009, Accepted 28th September 2009***
***First published as an Advance Article on the web 28th October 2009***
**DOI: 10.1039/b917507e**



**The synthesis and evaluation of a 'tag-free' probe to isolate and identify receptors for *N*-acyl homoserine lactones is described.**

The term "quorum sensing" (QS) is used to describe the mechanism that bacteria use to coordinate their behavior in a cell-density dependent manner.[1] Using this mechanism, which is based on the exchange of small diffusible signaling molecules between cells, bacterial cultures can essentially function as multicellular organisms. Examples of QS-controlled behaviors are production of light (bioluminescence), virulence factor expression and biofilm formation. These processes are advantageous to a bacterial population only when they are carried out simultaneously by all its members.[2]

During the past two decades a wealth of information regarding intercellular communication among bacteria have been uncovered. QS is mediated by secretion and recognition of small diffusible molecules, of which an example is the class of *N*-acyl homoserine lactones (AHLs), used by many known Gram-negative bacteria, such as the opportunistic pathogen *Pseudomonas aeruginosa*. The QS molecule *N*-(3-oxododecanoyl) homoserine lactone (3-oxo-$C_{12}$-HSL, C12, Fig. 1, **1**) has been identified as the primary signal to enable QS in *P. aeruginosa* and this system involves the LasI (synthase) and LasR (receptor) proteins. This is part of a sophisticated strategy to survive under conditions that do not favor bacteria living solely as individual cells.

A growing number of reports indicate that bacterial AHL autoinducers can influence gene expression in eukaryotic cells. Most of these studies focus on the effects of the *P. aeruginosa* autoinducer C12 on the production of a number of cytokines, such as IFN-γ, by immune cells *in vitro* and *in vivo*.[3–11] A recent study by Kravchenko *et al.* has uncovered a mechanism in which C12 selectively disrupts NF-κB signaling in activated mammalian cells.[9,10] These findings indicate that *P. aeruginosa* has developed means to detect host immune activation and to institute countermeasures.[12] The resulting response in eukaryotes seems to point to a conserved downregulation of the immune response, which is beneficial to the bacteria and may or may not be beneficial to the host. In another example of eukaryotic response to bacterial crosstalk, studies have shown that the nematode *Caenorhabditis elegans* senses the presence and possibly also the population size of *P. aeruginosa* by recognizing and responding to C12.[13,14]

Recently, it has been shown that in many cases of *Candida albicans* infection, biofilm formation is involved. For both the infection process and biofilm formation, a morphological transition from yeast cells to filamentous cells (hyphae) is essential.[15] Because of its importance, this morphological switch is tightly regulated. Recently, C12 was shown to repress hyphal formation.[16] The mechanisms underlying the interactions between C12 and putative receptors in the mentioned eukaryotes are completely unknown. Jahoor *et al.* recently proposed that PPARγ is a mammalian C12 receptor, although no binding studies were reported;[11] their observed effects of C12 on PPARγ activity could be the result of C12 binding to a different protein, ultimately resulting in downstream activity. We set out to isolate, identify and characterize eukaryotic receptors for C12, through a 'tag-free' activity-based protein profiling approach based on a highly selective copper(I) mediated azide–alkyne cycloaddition reaction.[17–19] Our design scheme was based on the following findings: (1) it was shown previously that any modification in the lactone ring leads to diminished activity in mammalian cells;[4,10] (2) small modifications in the alkyl chain did not cause a significant loss of activity.

We therefore chose to design AHL analogs with only minimal structural deviations in the alkyl chain so as to increase the chances of recognition. In this approach, the probe is first conjugated to its receptor through irradiation of a functional moiety, such as diazirine. This smallest possible photoactive group, that can be activated by light irradiation to generate a highly active carbene, has been used successfully by others to label proteins.[20–22] Then, following the methodology

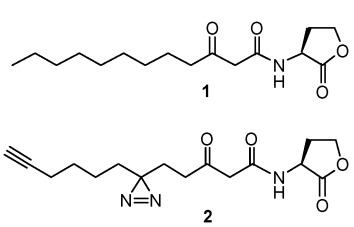


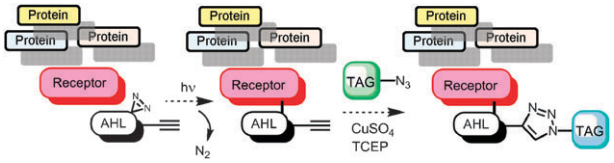

**Fig. 1** Structures of 3-oxo-$C_{12}$-HSL (C12, **1**) and diazirine alkynyl probe **2**.

[a] *Department of Chemistry, Ben-Gurion University of the Negev, Be'er-Sheva 84105, Israel. E-mail: meijler@bgu.ac.il; Fax: +972-8-6472751; Tel: +972-8-6472751*
[b] *Department of Biomedical Engineering, The Kolff Institute, University Medical Center Groningen and the University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands. E-mail: b.p.krom@med.umcg.nl; Fax: +31-50-3633159; Tel: +31-50-3633160*
[c] *Department of Immunology and Microbial Sciences, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA*

† Electronic supplementary information (ESI) available: Synthetic procedures and characterization of compounds. Experimental details of biological evaluations and analysis by mass spectrometry. See DOI: 10.1039/b917507e

‡ These authors contributed equally to the project.




**Scheme 1** Labeling of unknown receptors with use of activatable bifunctional AHL-based probes. Addition of two minimally perturbing moieties to the homoserine lactone will allow for specific binding to an AHL receptor amidst a wide variety of proteins. Irradiation with UV light (365 nm) will result in a covalent bond between the probe and the receptor. A highly specific 1,3-dipolar cycloaddition reaction ('click') with a tag (or fluorophore) will then allow for isolation and identification of the receptor.

developed by Cravatt and co-workers for the identification of unannotated enzymes,[17] the probes, while attached to their target proteins, will undergo reaction with their cycloaddition partner, which itself is attached to a tag/reporter group such as biotin or rhodamine (Scheme 1). This will then enable isolation of the probe-bound protein, followed by identification by mass spectrometry. By applying this general scheme, we aim to develop a method to characterize the interaction between AHLs and unknown proteins that bind the AHLs.

Probe **2** was prepared from 6-chlorohexyne as shown in Scheme 2. Following procedures described by Hodgson *et al.*,[23] 4-oxononynoic acid **5** was prepared, and upon treatment with liquid ammonia the aziridine intermediate was obtained. Oxidation with iodine resulted in the formation of diazirine **6**, followed by homologation with mono *tert*-butyl malonate. Deprotection of **7** and coupling with homoserine lactone yielded diazirine alkynyl probe **2**.

Probe **2** was first tested for its ability to mimic C12 using *Escherichia coli* pSB1075 as the reporter strain. Induction of luminescence, indicative of activation of the LasI–LasR QS system was observed (Fig. 2A). In addition, the induction of elastase, a virulence factor under control of the Las system, was analyzed in a *P. aeruginosa* LasI mutant unable to synthesize C12. $IC_{50}$ values ranged from 1 μM for C12 to 0.03 μM for probe **2** (ESI†, Fig. S1). We then verified whether **2** would mimic the activity of C12 in two eukaryotic systems. Phosphorylation of the initiator factor eIF2α in macrophages

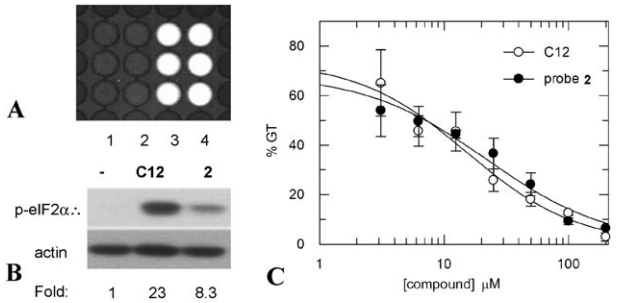
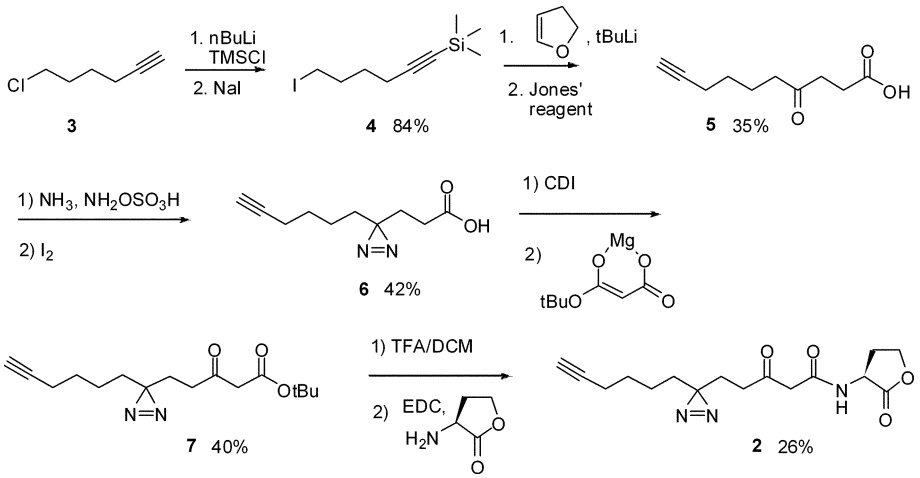



**Fig. 2** (A) Induction of bioluminescence by 5 μM C12 (lane 4, triplicates) and 5 μM probe **2** (lane 3) in a lasR-based AHL biosensor (*E. coli* pSB1075) strain. Luminescence was measured after 3 h growth. Lane 1 contains 0.5% DMSO in LB medium, lane 2 only LB medium. All wells were inoculated with a 1/100 diluted overnight culture; (B) biological activity of C12 and its diazirine alkynyl analog **2** in bone marrow-derived macrophages (BMDM). BMDM were treated with C12 or **2** for 30 min, and the cellular extracts were analyzed by Western blot for the phosphorylated form of eIF2α (p-eIF2α), as a biochemical marker of C12-mediated activation of mammalian cells. Western blot for actin was used as a control. Relative fold of p-eIF2α induction was estimated by densitometry. See ref. 9 for experimental details; (C) effects of C12 and probe **2** on germ tube formation in *C. albicans*. Calculated $IC_{50}$: 15.3 μM (C12), 21.8 μM (**2**).

was induced by **2**, albeit slightly less than by C12 (Fig. 2B). Inhibition of germ tube (GT) formation in *C. albicans* was inhibited by C12 and probe **2** similarly (Fig. 2C). These experiments indicate that probe **2** is capable of mimicking C12 in diverse microbial and eukaryotic systems.

In order to determine accurate and optimized conditions for protein labeling by this probe and other diazirine-based probes, we focused on labeling of LasR. Bottomley *et al.* published the first crystal structure of the ligand binding domain (LBD) of LasR,[24] bound to C12, and following their protocol we overexpressed this protein in *E. coli*.

During expression of the protein in the presence of **2**, cells were irradiated for 15 min with a UV-lamp (365 nm), after which cell membranes were lysed and LasR-LBD was isolated through affinity purification. Proteins were analyzed by LC/MS as described by us previously,[25] and while extracted protein that was expressed in the presence of C12 alone




**Scheme 2** Synthesis of 'tag-free' diazirine alkynyl AHL probe **2**.

(20 μM) yielded a single peak (22 427 Da, Fig. 3A) corresponding to the expected mass of LasR-LBD (22 430 Da), the experiment in which LasR was expressed in the presence of probe **2** (20 μM) resulted in an additional peak with a mass difference of 292 Da (Fig. 3B, expected difference: 290 Da). In a third experiment in which LasR was expressed in the presence of both C12 and **2** (20 μM each) the additional peak had disappeared (Fig. 3C), showing that **2** competes with C12 for the same binding site, albeit with lower affinity. From these experiments it follows that under the crosslinking conditions a significant fraction of LasR is labeled covalently and specifically.

As the interaction between C12 and LasR is highly specific and small changes in the structure of C12 have been reported to lead to a significant loss in activity of C12 analogs, the specific labeling of LasR by probe **2** as observed in our experiments is encouraging in our attempts to isolate and identify putative receptors in eukaryotes that bind C12. We are currently using probe **2** in pursuing this target. As the exact location of the diazirine moiety may influence specific binding to C12-binding proteins in different organisms we are also currently expanding our toolset with C12-based probes with diazirine moieties at various locations in the alkyl chain.

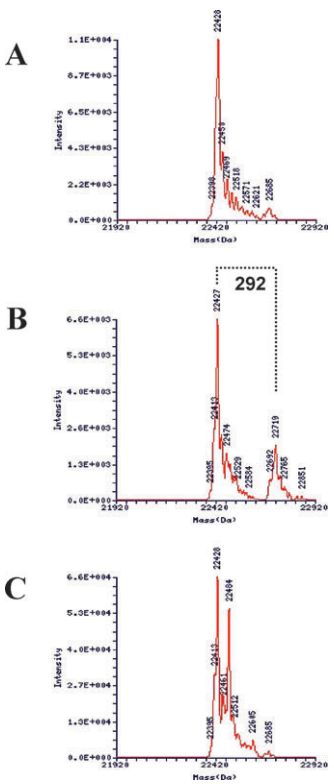



**Fig. 3** Deconvoluted ESI mass spectra of crude LasR-LBD: (A) overexpressed in *E. coli* in the presence of C12 (20 μM). Calculated mass: 22 430 Da. Observed: 22 428 Da, and several adducts—possibly the result of phosphorylation and/or methionine oxidation; (B) overexpressed in the presence of diazirine probe **2** (20 μM). Calculated mass increase upon labeling with **2**: 290 Da. Observed increase: 292 Da; (C) overexpressed in the presence of both probe **2** (20 μM) and C12 (20 μM). 22 484 Da is an unknown (possibly oxidized) adduct. All three cultures were irradiated with a UV lamp (365 nm) for 15 min to effect crosslinking of the probe, before lysis and affinity purification.

This work was supported by the Human Frontier Science Program (Young Investigator Grant RGY0072/2007, BPK and MMM), the National Institutes of Health (AI079436, VVK), and by the US–Israel Binational Science Foundation (Startup Grant 2006287, MMM). We thank Dr R. A. Ulevitch for valuable insights and support, Dr K. D. Janda and Dr G. F. Kaufmann for helpful discussions, and Dr M. J. Bottomley, Dr D. A. Hogan and Dr W. J. Quax for bacterial strains and plasmids.

## Notes and references

1 J. Engebrecht, K. Nealson and M. Silverman, *Cell*, 1983, **32**, 773–781.
2 J. W. Hastings and K. H. Nealson, *Annu. Rev. Microbiol.*, 1977, **31**, 549–595.
3 G. Telford, D. Wheeler, P. Williams, P. T. Tomkins, P. Appleby, H. Sewell, G. S. Stewart, B. W. Bycroft and D. I. Pritchard, *Infect. Immun.*, 1998, **66**, 36–42.
4 S. R. Chhabra, C. Harty, D. S. Hooi, M. Daykin, P. Williams, G. Telford, D. I. Pritchard and B. W. Bycroft, *J. Med. Chem.*, 2003, **46**, 97–104.
5 A. J. Ritchie, A. O. Yam, K. M. Tanabe, S. A. Rice and M. A. Cooley, *Infect. Immun.*, 2003, **71**, 4421–4431.
6 K. Tateda, Y. Ishii, M. Horikawa, T. Matsumoto, S. Miyairi, J. C. Pechere, T. J. Standiford, M. Ishiguro and K. Yamaguchi, *Infect. Immun.*, 2003, **71**, 5785–5793.
7 C. Wagner, S. Zimmermann, G. Brenner-Weiss, F. Hug, B. Prior, U. Obst and G. M. Hansch, *Anal. Bioanal. Chem.*, 2006, **387**, 481–487.
8 G. L. Thomas, C. M. Bohner, H. E. Williams, C. M. Walsh, M. Ladlow, M. Welch, C. E. Bryant and D. R. Spring, *Mol. BioSyst.*, 2006, **2**, 132–137.
9 V. V. Kravchenko, G. F. Kaufmann, J. C. Mathison, D. A. Scott, A. Z. Katz, D. C. Grauer, M. Lehmann, M. M. Meijler, K. D. Janda and R. J. Ulevitch, *Science*, 2008, **321**, 259–263.
10 V. V. Kravchenko, G. F. Kaufmann, J. C. Mathison, D. A. Scott, A. Z. Katz, M. R. Wood, A. P. Brogan, M. Lehmann, J. M. Mee, K. Iwata, Q. Pan, C. Fearns, U. G. Knaus, M. M. Meijler, K. D. Janda and R. J. Ulevitch, *J. Biol. Chem.*, 2006, **281**, 28822–28830.
11 A. Jahoor, R. Patel, A. Bryan, C. Do, J. Krier, C. Watters, W. Wahli, G. Li, S. C. Williams and K. P. Rumbaugh, *J. Bacteriol.*, 2008, **190**, 4408–4415.
12 L. Wu, O. Estrada, O. Zaborina, M. Bains, L. Shen, J. E. Kohler, N. Patel, M. W. Musch, E. B. Chang, Y. X. Fu, M. A. Jacobs, M. I. Nishimura, R. E. Hancock, J. R. Turner and J. C. Alverdy, *Science*, 2005, **309**, 774–777.
13 E. Beale, G. Li, M. W. Tan and K. P. Rumbaugh, *Appl. Environ. Microbiol.*, 2006, **72**, 5135–5137.
14 Y. Zhang, H. Lu and C. I. Bargmann, *Nature*, 2005, **438**, 179–184.
15 K. E. Krueger, A. K. Ghosh, B. P. Krom and R. L. Cihlar, *Microbiology*, 2004, **150**, 229–240.
16 D. A. Hogan, A. Vik and R. Kolter, *Mol. Microbiol.*, 2004, **54**, 1212–1223.
17 A. E. Speers, G. C. Adam and B. F. Cravatt, *J. Am. Chem. Soc.*, 2003, **125**, 4686–4687.
18 M. Sawa, T. L. Hsu, T. Itoh, M. Sugiyama, S. R. Hanson, P. K. Vogt and C. H. Wong, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 12371–12376.
19 Q. Wang, T. R. Chan, R. Hilgraf, V. V. Fokin, K. B. Sharpless and M. G. Finn, *J. Am. Chem. Soc.*, 2003, **125**, 3192–3193.
20 A. Blencowe and W. Hayes, *Soft Matter*, 2005, **1**, 178–205.
21 S. J. Dilly, M. J. Bell, A. J. Clark, A. Marsh, R. M. Napier, M. J. Sergeant, A. J. Thompson and P. C. Taylor, *Chem. Commun.*, 2007, 2808–2810.
22 D. P. Smith, J. Anderson, J. Plante, A. E. Ashcroft, S. E. Radford, A. J. Wilson and M. J. Parker, *Chem. Commun.*, 2008, 5728–5730.
23 D. M. Hodgson, A. H. Labande, F. Y. Pierard and M. A. Exposito Castro, *J. Org. Chem.*, 2003, **68**, 6153–6159.
24 M. J. Bottomley, E. Muraglia, R. Bazzo and A. Carfi, *J. Biol. Chem.*, 2007, **282**, 13592–13600.
25 N. Amara, R. Mashiach, D. Amar, P. Krief, S. A. Spieser, M. J. Bottomley, A. Aharoni and M. M. Meijler, *J. Am. Chem. Soc.*, 2009, **131**, 10610–10619.