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(54) Title: 4-(4-CHLOROPHENYL)-5-METHYLENE-PYRROL-2-ONE AND 5-METHYLENE-4-(P-TOLYL)PYRROL-2-ONE FOR USE IN THE TREATMENT OF GRAM NEGATIVE BACTERIAL INFECTIONS

(57) Abstract: 4-(4-Chlorophenyl)-5-methylene-pyrrol-2-one and 5-methylene-4-(p-tolyl)pyrrol-2-one for use in the treatment of gram-negative bacterial infections, in particular infections in which Pseudomonas is implicated.

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4-(4-CHLOROPHENYL)-5-METHYLENE-PYRROL-2-ONE AND 5-METHYLENE-4-(P-TOLYL)PYRROL-2-ONE FOR USE IN THE TREATMENT OF GRAM NEGATIVE BACTERIAL INFECTIONS

This application claims priority from EP16180590.8 filed 21 July 2016, the contents of which are incorporated herein in their entirety for all purposes.

FIELD OF THE INVENTION

The invention relates to certain lactam compounds for use in a method of treatment of gram negative bacterial infections.

BACKGROUND

The emergence of drug-resistant bacteria and fungi presents a significant medical and public health problem. Consequently, there is an urgent need for the development of antimicrobial agents that can overcome drug resistance problems. Bacteria and fungi generally develop drug resistance in four ways: producing metabolizing enzymes for the degradation of the drugs, modifying their targets to render the drugs ineffective, expressing a high level of efflux proteins that "pump" the drug out in order to lower its concentration, and inducing biofilm formation to prevent permeation of drugs into the bacteria.

WO2007/085042 (Biosignal Limited) describes certain lactam structures and their use in the treatment of bacterial infections.

WO2014/118240 (Unilever) describes antimicrobial compositions comprising a lactam and a hydrotrope.

WO2014/183164 (Kumar, Perry and Kit) describes certain *N*-functionalised dihydropyrrolone compounds and methods for preparing surfaces to which the dihydropyrrolone compounds are attached.

SUMMARY OF THE INVENTION

The invention is based on the inventors' understanding and insight into the unusual biological properties of certain lactam compounds, and the utility of these compounds in methods of treatment owing to their biological profile.

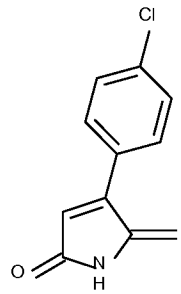
The lactams described herein are antibacterial. Their particular biological profile makes them surprisingly suitable for the treatment of gram-negative bacterial infections, in particular in long term treatment.

In a first aspect, the invention therefore relates to a lactam for use in a method of treatment of an infection caused by gram-negative bacteria.

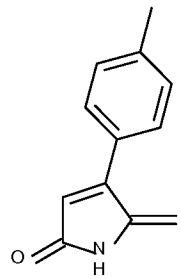
Suitably, the infection is chronic (not cleared within 12 weeks) or an infection that is considered at risk of becoming chronic.

Suitably, the infection is a bacterial infection in which *Pseudomonas*, (usually but not necessarily *P. aeruginosa*) is implicated.

Accordingly, in a first aspect, the invention may provide a lactam selected from



4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488); and



5-methylene-4-(p-tolyl)pyrrol-2-one (Ref. 491);

for use in a method of treatment of an infection caused by a gram-negative bacteria.

The lactams of Formula I and II act to prevent the formation of, retard or prevent the growth and development of, and/or reduce the extent of a bacterial biofilm.

Pseudomonas, typically *P.aeruginosa,* may be implicated in the bacterial biofilm.

In the present invention, the subject being treated is preferably a mammalian subject and most preferably a human.

The lactams of the invention may be used as the first line of treatment of an infection, or may be used in subjects already treated with other antibiotics for the same infection, for example those who have not shown a satisfactory response to those other antibiotics.

If appropriate, the lactams of the invention may be administered together with other treatments, including other antibiotic treatments.

In a second aspect, the invention provides a method of treatment of an infection caused by a gram-negative bacteria, the method comprising administering to a subject a therapeutically effective amount of 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) or 5-methylene-4-(p-tolyl)pyrrol-2-one (Ref. 491).

In a third aspect, the invention provides the use of 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) or 5-methylene-4-(p-tolyl)pyrrol-2-one (Ref. 491) in the manufacture of a medicament for the treatment of bacterial infections of skin lesion.

It will be appreciated that all preferences described with respect to the first aspect apply similarly to the second and third aspects, as appropriate.

DETAILED DESCRIPTION

The invention will now be described with reference to the following drawings in which:

LB medium = Lysogeny broth ex. Sigma Aldrich UK.
Lactam 488 = 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one
Lactam 491 = 5-methylene-4-(p-tolyl)pyrrol-2-one

and

Figure 1 shows a comparison of the expression of *P. aeruginosa lasI::lux* in the presence and absence of lactams 488 and 491 at 100 µM. Control – LB medium + 1% methanol.

Figure 2 shows a comparison of the expression of *P. aeruginosa rhlI::lux* in the presence and absence of lactams 488 and 491 at 100 µM. Control – LB medium + 1% methanol.

Figure 3 shows a comparison of the expression of *P. aeruginosa pqsA::lux* in the presence and absence of Unilever lactams 488 and 491 at 100 µM. Control – LB medium + 1% methanol.

Figure 4 shows a comparison of the expression of *P. aeruginosa tac::lux* in the presence and absence of Unilever lactams 488 and 491 at 100 µM. Control – LB medium + 1% methanol.

Figure 5 shows vials demonstrating pyocyanin production by PAO1-L grown in the presence of lactam 488 in 0 (1), 100 (2) and 10 µM (3). (4) is an LB only control.

Figure 6 shows the effect of lactam 488 on the expression of *pqsA::lux* in *P. aeruginosa* PAO1-L.

Figure 7 shows the effect of lactam 491 on expression of *pqsA::lux* in *P. aeruginosa* PAO1-L.

Figure 8 shows the effect of PQS on the expression of *pqsA::lux* in PAO1-L Δ*pqsA* in presence of lactam 488 EC_{50} for PQS increased from 2 µM to 31 µM in the presence of 488 (215 µM).

Figure 9 shows the effect of 488 at 0, 50, 100, 200 µM on the PQS-dependent expression of *pqsA'::lux* in *P. aeruginosa* PAO1-L Δ*pqsA.* The data show that PQS competes with 488 for PqsR-dependent activation of *pqsA::lux* fully restoring light output in the presence of 50 µM but not 100 µM or 200 µM of 488.

Figure 10 shows the effect lactam 488 on the expression of *pqsA'::lux* in PAO1Δ*pqsA* in the presence of PQS at 0 µM, 10 µM or 40 µM.

Figure 11 shows the PqsR-independent activity of 488 on the expression of *pqsA'::lux* in PAO1-N Δ*pqsAHR*.

Figure 12 shows the inhibitory effect of 488 on the expression of *pqsA'::lux* in *P.aeruginosa*.

Figure 13 shows TLC analysis of HHQ production. (**Panel A**) TLC plate under UV light at 312 nm, showing PQS (lane 1 upper spot) and HHQ (lane 1 lower spot) standards together, 488 standard (lane 2), organic solvent supernatant extracts of PAO1-N Δ*pqsAHR* grown without (lane 3) and with (lane 4) the compound 488 and organic solvent supernatant extracts of PAO1-N Δ*pqsAHR pqsABCD* grown without (lane 5) and with 488

(lane 6). Pyocyanin production (**Panel B**) and light output (**Panel C**) occurs via PAO1-L Δ*pqsA* CTX::*pqsA'-luxCDABE* present in the agar overlay. Both pyocyanin and light output are dependent on the presence of AQs. Bioluminescence was captured using a luminograph photon camera. The uppermost bright spot on the UV illuminated plate (Panel A, lanes 2, 4 and 6) is 488.

Gram-negative bacteria

The proteobacteria are a major group of gram-negative bacteria, including *Escherichia coli* (*E. coli*), *Salmonella*, *Shigella*, and other Enterobacteriaceae, *Pseudomonas*, *Moraxella*, *Helicobacter*, *Stenotrophomonas*, *Bdellovibrio*, acetic acid bacteria, *Legionella* etc. Other notable groups of gram-negative bacteria include the cyanobacteria, spirochaetes, green sulfur, and green non-sulfur bacteria.

Medically relevant gram-negative cocci include the four organisms that cause a sexually transmitted disease (*Neisseria gonorrhoeae*), a meningitis (*Neisseria meningitidis*), and respiratory symptoms (*Moraxella catarrhalis*, *Haemophilus influenzae*).
Medically relevant gram-negative bacilli include a multitude of species. Some of them cause primarily respiratory problems (*Klebsiella pneumoniae*, *Legionella pneumophila*, *Pseudomonas aeruginosa*), primarily urinary problems (*Escherichia coli*, *Proteus mirabilis*, *Enterobacter cloacae*, *Serratia marcescens*), and primarily gastrointestinal problems (*Helicobacter pylori*, *Salmonella enteritidis*, *Salmonella typhi*).
Gram-negative bacteria associated with hospital-acquired infections include *Acinetobacter baumannii*, which cause bacteremia, secondary meningitis, and ventilator-associated pneumonia in hospital intensive-care units.

Accordingly, the gram-negative bacteria may be selected from *Escherichia coli* (*E. coli*), *Salmonella*, *Shigella*, and other Enterobacteriaceae, *Pseudomonas*, *Moraxella*, *Helicobacter*, *Stenotrophomonas*, *Bdellovibrio*, acetic acid bacteria, *Legionella,* cyanobacteria, spirochaetes, green sulfur, and green non-sulfur bacteria, *Neisseria gonorrhoeae*, *Neisseria meningitidis Moraxella catarrhalis*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis*, *Enterobacter cloacae*, *Serratia marcescens, Helicobacter pylori*, *Salmonella enteritidis*, *Salmonella typhi, Acinetobacter baumannii*.

Pseudomonas aeruginosa

Preferably, the gram-negative bacteria is a *P.aeruginosa*.

There are a number of *P.aeruginosa* strains, including PA01, PA7, USBPP-PA14 and strain 2192. Except where indicated otherwise, a reference to *P.aeruginosa* is intended to refer to any and all strains.

The methods described herein may be directed to treatment of infections in which *P.aeruginosa* is implicated. The *P.aeruginosa* may be a strain that produces AQs (alkylquinoline compounds). The *P.aeruginosa* may be a strain that produces one or both of PQS (*Pseudomonas* quinolone signal; 2-heptyl-3-hydroxy-4-(1H)-quinolone) and HHQ (4-hydroxy-2-heptylquinoline). The *P.aeruginosa* may be a strain belonging to one of the two major *P. aeruginosa* genomic groups (PAO1 and PA14).

Quorum sensing

Quorum sensing (QS) is a mechanism whereby microorganisms, and in particular bacteria, communicate with each other and exhibit community-wide behaviour coordination through the secretion and detection of chemical signals called autoinducers (AIs). Quorum sensing has been demonstrated in a large number of bacteria species/strains and important in regulating bacterial virulence, drug resistance, expression of efflux transporters, and biofilm formation, and therefore is attracting attention in the antimicrobial field. (*Frontiers in microbiology 6 (1036) September 2015*).

Quorum sensing is a cell-density based intercellular communication system to regulate collective behaviour, which plays a key role in regulation of bacterial virulence and biofilm formation. The process relies on the production, release and group-wide detection of signal molecules called autoinducing peptides (AIPs), which in gram-negative bacteria are typically homoserine lactones, (HSLs), especially *N*-acyl-homoserine lactones (AHLs). Other quorum sensing molecules are known, including epinephrine/ norepinephrine.

Biofilm formation enables the bacteria to resist antibiotics because once the bacteria sense that the outer layer of the biofilm is being destroyed, the inner layers will grow stronger to re-establish the community. The present invention is based on the inventors'

investigation into the properties of certain lactams as described herein and their insight into the way in which said lactams influence QS in gram negative bacteria such as *P.aeruginosa*.

The QS network of *P.aeruginosa* is organised in a multi-layered hierarchy consisting of at least four interconnected signalling mechanisms and demonstrates plasticity, in that it can respond to bacterial population changes and possibly also environmental stress cues. *P.aeruginosa* orchestrates biofilm formation – and production of virulence factors – by reliance on two QS systems, both part of the LuxI/R signalling cascade, the Las and Rh1 systems.

In *Pseudomonas aeruginosa*, the acyl-homoserine lactone (AHL) and alkyl quinolone (AQ) QS systems are important for virulence and biofilm formation. One acyl-HSL QS regulator is LasR. A non-AHL signalling molecule produced by *P.aeruginosa* is known as PQS (*Pseudomonas* quinolone signal), which is 2-heptyl-3-hydroxy-4-(1H)-quinolone.

Biofilm

The term "biofilm" as used herein refers to biological films that develop and persist at interfaces in aqueous environments. These biological films are composed of microorganisms embedded in organic gelatinous matrices composed of one or more matrix polymers that are secreted by the resident microorganisms. Biofilms can develop into macroscopic structures and are also capable of trapping nutrients and particulates that can contribute to their enhanced development and stability. Biofilms can also prevent penetration of antimicrobial agents, which may lead to persistent infections. Formation of biofilms provides bacteria with a protected environment can withstand various stresses, including many antibiotic treatments.

Methods of Treatment

It will be appreciated that the term “methods of treatment” as used herein includes prophylaxis, treatment that hamper bacterial colony population growth, treatments that keep a bacterial colony population stable, and treatments that reduce or eradicate a bacterial population.

Owing to their unusual biological profile, the lactams of the invention may be useful in the long term treatment of infections. This is because, owing to their selective interactions with the various bio-pathways of the bacteria, and in particular *P.aeruginosa,* the lactams may permit population control and reduction without triggering the mechanisms that are thought to lead to evolutionary resistance.

In some cases, the bacterial infection is an infection in which *Pseudomonas*, (usually but not necessarily *P. aeruginosa*) is implicated.

The methods described herein may be suitable for long term use. Accordingly, the methods may include regular administration of the lactam to a subject for a period of at least several weeks, several months, at least one year, at least two years, at least three years, at least 5 years, at least 8 years, or at least 10 years.

EXAMPLES

To elucidate the mechanism of action of compounds described herein, the impact of each lactam on both *N*-acylhomoserine lactone (AHL) and 2-alkyl-4-quinolone-(AQ)-dependent quorum sensing (QS) in *P. aeruginosa* was explored.

The expression of *rhl* and *las* AHL- and the *pqsA* AQ- synthase genes was evaluated using *lux*-based *lasI* (**Figure 1**), *rhlI* (**Figure 2**) and *pqsA* (**Figure 3**) chromosomally integrated promoter fusions. Luminescence was quantified as a function of bacterial growth. The applicant further determined that none of the lactams is growth inhibitory at 100 µM using a large number of strains (figure not shown).

The *P. aeruginosa* PAO1-N *tac*::*lux strain* was used as a positive control biosensor as it constitutively expresses *luxCDABE* and hence light (**Figure 4**). Any reduction in light output in this strain in the presence of a lactam will show whether or not the compound has an adverse effect on luminescence *per se*. The *tac::lux* expression profile shows that there is no interference between the compounds and the enzymes involved in light generation. Any effects observed on incubation of the QS reporter strains with the lactams will therefore be due to alterations in promoter expression.

The qualitative effect of compound 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) on pyocyanin production by PAO1-L was then investigated.

Pyocyanin is a blue redox-active secondary metabolite and a putative signalling molecule in *P. aeruginosa* (Jayaseelan *et al*, 2014). The pyocyanin biosynthetic (*phz*) genes are regulated by QS and in part via the transcriptional regulator PqsR acting through PqsE (Rampioni *et al* 2011). Pyocyanin production was visually assessed after 8 h of incubation at 37°C in *P. aeruginosa* strain PAO1-L in the presence and absence of compound 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488), which **Figure 3** shows was the most potent inhibitor of the PqsR-dependent *pqsA::lux* promoter fusion. **Figure 5** shows the culture supernatants of PAO1-L grown in the absence or presence of lactam 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488).

The inhibition of pyocyanin production by lactam 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) was observed as the absence of green pigmentation in culture supernatant in vial 2 (**Figure 5**). Although qualitative, this observation offers further confirmation of the inhibitory effects of lactam 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) on AQ-dependent QS.

The inhibitory properties of compounds of the invention on AQ-dependent QS in *P. aeruginosa* were then investigated.

In *P. aeruginosa,* the *pqsABCDE* genes code for the biosynthetic pathway required for the synthesis and action of 2-alkyl-4(1*H*)-quinolones (AQs) (Heeb *et al* 2010). The transcriptional activator PqsR regulates the expression of the *pqs* biosynthetic pathway that in turn controls secondary metabolites such as elastase, pyocyanin and phospholipase as well as biofilm maturation and swarming motility. In this QS system, the primary AQ signal molecules are 4-hydroxy-2-heptylquinoline (HHQ) and 2-heptyl-3-hydroxy-4-quinolone (PQS) both of which act as co-inducers of PqsR (Heeb *et al* 2010; Ilangovan *et al* 2013).

The results from the first screening experiments (**Figure 1-3**) using AHL and AQ biosensor strains clearly show that inhibition of the *pqs*-system is the main effect of lactams 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) and 5-methylene-4-(p-

tolyl)pyrrol-2-one (Ref. 491) on QS in *P.* aeruginosa. To determine the relative inhibitory activities (IC_{50s}) of 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) and 5-methylene-4-(p-tolyl)pyrrol-2-one (Ref. 491), dose response curves were constructed using the same biosensor strain (*P. aeruginosa* PAO1 *pqsA::lux;* Fletcher *et al*, 2007; Ilangovan *et al* 2013). This strain produces light in response to the endogenous production of AQs such as PQS and HHQ. The data obtained are presented in **Figure 6** and **Figure 7** from which IC_{50s} were calculated as 22 μM for 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) and 44 μM for 5-methylene-4-(p-tolyl)pyrrol-2-one (Ref. 491) respectively. While 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) is the more potent of the two compounds, 5-methylene-4-(p-tolyl)pyrrol-2-one (Ref. 491) showed the desired effect. It can also be seen that 5-methylene-4-(p-tolyl)pyrrol-2-one (Ref. 491) did not inhibit *pqsA* expression as strongly as 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488); at high concentrations while 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) completely inhibited light output and hence *pqsA* expression.

Figures 6 and **7** demonstrate the binding and inhibition of the PQS quorum sensing in pseudomonas, with compound 488 showing the ability to fully knock out the PQS. The 491 compound in figure 7 whist proving inhibition did not give complete knock out at higher concentration.

The mode of action on AQ-dependent QS was investigated.

The major QS molecules in the *pqs* system are the AQs, PQS and its precursor HHQ (Williams & Camara 2009). Their biosynthesis requires the regulator PqsR and the biosynthetic proteins, PqsABCD and the mono-oxygenase, PqsH (Heeb *et al* 2010).

The inhibitory action of 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) may involve inhibition of AQ biosynthesis or 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) may behave like an antagonist and block the PQS receptor, PqsR. These experiments do not discriminate between inhibition of PqsR activation and inhibition of AQ biosynthesis because the biosensor strain used maintains an intact AQ-dependent QS system. Experiments were therefore designed to determine the nature and degree of 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) inhibitory activity. Firstly, the possibility that 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) is an inhibitor of

the response regulator protein, PqsR that is essential for expression of the *pqsABCDE* genes and hence AQ production was explored.

By using the AQ-non producing *P. aeruginosa* strain PAO1-L Δ*pqsA*, *pqsA::lux,* the EC_{50} value for the native PqsR agonist, PQS was calculated as ~2 μM (Ilangovan *et al*. 2013). In the presence of a fixed concentration of lactam 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) and a range of PQS concentrations, the EC_{50} value increased ~15 fold to 31 μM (**Figure 8**). This suggests that 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) acts as a competitive antagonist of AQ signalling potentially inhibiting the interaction between PqsR and its co-inducer, PQS.

The experiment shown in **Figure 8** was then repeated using 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) concentrations of 0, 50, 100 and 200 μM. The results obtained are shown in **Figure 9** which shows that as 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) concentrations are increased, maximal light output is substantially reduced. The shape of the dose response curves indicates that 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) is a competitive antagonist as increasing the PQS concentration, restores *pqsA::lux* expression.

These data demonstrate the selective binding and competitive antagonist activity of 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488).

A PAO1-L Δ*pqsA pqsA'::lux* biosensor-based assay was performed to determine whether lactam 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) exhibits any partial agonist activity at higher concentrations. A partial agonist is a molecule that can bind to and activate a receptor resulting in a non-complete response compared with a full agonist. Graphically, an antagonist that is also a partial agonist exhibits agonist activity at high concentrations. **Figure 10** shows that 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) has no partial agonist activity as it was unable to activate *pqsA* expression at concentrations up to 800 μM. This contrasts with its activity as an antagonist in the presence of 10 or 40 μM PQS. This demonstrates that at higher concentrations there is no activation of receptor binding, offering low or even no toxicity at higher doses.

PQS is capable of weakly activating *pqsA* in the absence of PqsR via a mechanism that is not fully understood but appears to depend on the iron chelating properties of PQS. The aim of this experiment was to clarify whether lactam 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) is also inhibitory for *pqsA* expression via the PqsR independent pathway. As previously described, the *pqsA'::lux* biosensor based on the triple mutant PAO1-N Δ*pqsAHR* cannot produce AQs and lacks *pqsR* but still responds to exogenous PQS. This response is however much weaker that the PqsR-dependent response. **Figure 11** shows the impact of 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) on the PqsR-independent expression of *pqsA* in the presence or absence of 40 µM PQS. 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) has a small inhibitory effect on the PqsR-independent pathway. This suggests that while the compounds of the invention may have some effect on *P. aeruginosa* that do not express AQs, they show most promise for in the treatment of disorders in which AQ-producing strains are implicated.

Indeed for 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) the PQS pathway is the dominant inhibitory route. Taking into account the role of quorum sensing in toxicity and virulence of pseudomonas, then this technology is most effective against those strains capable of quorum sensing.

To investigate the inhibitory effect of 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) on *P. aeruginosa* PA14, a *pqsA'::lux* fusion was introduced onto the chromosome. Comparable inhibitory effects with those previously described for strain PAO1 were observed. The IC_{50} extrapolated from the inhibition curve in **Figure 12** was calculated to be 17.9 µM.

To determine whether 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) is capable of inhibiting the enzymes (PqsABCD) involved in AQ biosynthesis, the *P. aeruginosa* PAO1-N Δ*pqsAHR* was exploited. This mutant cannot produce AQs because the genes coding for the key biosynthetic enzymes (PqsA and PqsH) and regulation (PqsR) have been deleted. To restore AQ production in a PqsR-independent manner in PAO1-N Δ*pqsA,* the *pqsABCD* genes were introduced on a plasmid (pBBR *pqsABCD*; Niewerth *et al.* 2011). *P. aeruginosa* PAO1-N Δ*pqsAHR* and *P. aeruginosa* PAO1-N Δ*pqsAHR* pBBR *pqsABCD* were both incubated overnight in LB with and without 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) (200 µM), resuspended to an OD_{600} 1.0. The cultures

were extracted with acidified ethyl acetate, the organic phase removed, dried and resuspended in methanol subjected to thin layer chromatography (TLC).

1	PQS, 10 mM + HHQ, 10 mM, 2 µl
2	488 10 mM, 5 µl
3	PAO1-N Δ*pqsAHR* organic extract, 10 µl
4	PAO1-N Δ*pqsAHR* + 488 200 µM organic extract, 10 µl
5	PAO1-N Δ*pqsAHR pBBR-pqsABCD* organic extract, 10 µl
6	PAO1-N Δ*pqsAHR pBBR-pqsABCD* + 488 200 µM organic extract, 10 µl

TABLE 1

After chromatography using a dichloromethane-methanol mobile phase, the TLC plates were overlaid with a thin agar layer containing the AQ biosensor strain PAO1-L Δ*pqsA* CTX::*pqsA'-luxCDABE* (Fletcher *et al.* 2007) incubated and examined for reporter output (**Figure 13**). **Figure 13 panel A** shows the TLC plate under UV illumination after chromatography. The biosensor strain produces light (**Figure 13 panel C**) and pyocyanin (**Figure 13 panel B**) in response to AQs that bind to PqsR and activate the *pqsA* promoter.

The biosensor revealed the presence of HHQ both in the samples of PAO1-N Δ*pqsAHR pBBR-pqsABCD* without (**Figure 13 panels B and C; lane 5**) and with (**Figure 13 panels B and C lane 6**) compound 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488) indicating that the lactam did not inhibit HHQ biosynthesis.

Sources of bacterial strains

The bacterial strains were obtained or made as follows:

Strain	**Antibiotic**	**Source or reference**
Escherichia Coli:		
S17-1 pMiniCTX-*pqsA*::*lux*	Gentamycin 25µg/ml	Diggle *et al.*, 2007
DH5 pBBRMCS-5::*pqsABCD*	Gentamycin 25µg/ml	Niewerth *et al.*, 2011
Pseudomonas aeruginosa:		
PAO1-L	-	Halloway collection
PAO1-N	-	Halloway collection

PAO1-L Δ*pqsA*	-	This study
PAO1-L Δ*pqsA*, *pqsA::lux*	Gentamycin 25µg/ml	This study
PAO1-N Δ*pqsA*	-	Aendekerk *et al.*, 2005
PAO1-N Δ*pqsA*, *pqsA::lux*	Tetracyclin 125µg/ml	Diggle *et al.*, 2007
PAO1-N Δ*pqsAH*		Diggle *et al.*, 2007
PAO1-N Δ*pqsAH*, *pqsA::lux*	Gentamycin 25µg/ml	Diggle *et al.*, 2007
PAO1-N Δ*pqsAHR*		Ilangovan *at al.,* 2013
PAO1-N Δ*pqsAHR*, *pqsA::lux*	Gentamycin 25µg/ml	By applicant
PAO1-L Δ*pqsAHR, pqsABCD*	Gentamycin 25µg/ml	By applicant
PAO1-L Δ*rhlI*	-	Dr. Matthew Fletcher
PAO1-L Δ*lasI*	-	Dr. Matthew Fletcher
PAO1-L *pqsA::lux*	Gentamycin 25µg/ml	By applicant
PAO1-L *las::lux*	Gentamycin 25µg/ml	James Lazenby. Unpublished'
PAO1-L *rhlI::lux*	Gentamycin 25µg/ml	James Lazenby. Unpublished'
PAO1-N *tac::lux*	Tetracyclin 125µg/ml	By applicant
PA ATCC 15442	-	Unilever*
PA ATCC 15442, *pqsA::lux*	Gentamycin 25µg/ml	By applicant
PA14		Rahme *et al.,* 1995
PA14, *pqsA::lux*	Gentamycin 25µg/ml	By applicant

*accession no. AYUC00000000 on DDBJ/EMBL/GenBank

TABLE 2

Summary of biological activity

The examples demonstrate that compounds of the invention have been shown to:

- inhibit alkylquinolone (AQ) dependent quorum sensing (QS) in *P. aeruginosa*.
- inhibit PqsR in representative strains belonging to the major *P. aeruginosa* genomic groups (PAO1 and PA14 respectively).
- interact antagonistically with the LysR-type regulator PqsR (in a competitive manner without partial-agonist activity).
- do not appear to directly inhibit AQ biosynthesis but block AQ synthesis by acting as a PqsR antagonist.
- potentially interact with the co-inducer binding domain of PqsR ($PqsR^{CBD}$) acting an allosteric inhibitors.

This provides an unusual biological profile that may enable compounds of the invention to be useful in the treatment of chronic bacterial infections. This is because the compounds of the invention have a biological activity profile that controls multiplication and biofilm formation, but does not trigger the bio-pathways that are associated with developing antibacterial resistance.

References

The references cited herein are incorporated by reference in their entirety for all purposes:

Aendekerk S, Diggle SP, Song Z, Høiby N, Cornelis P, Williams P, Cámara M. The MexGHI-OpmD multidrug efflux pump controls growth, antibiotic susceptibility and virulence in *Pseudomonas aeruginosa* via 4-quinolone-dependent cell-to-cell communication. (2005) *Microbiology* 151(4) 1113–25.

Fletcher MP, Diggle SP, Cámara M & Williams, P. (2007) Biosensor-based assays for PQS, HHQ and related 2-alkyl-4-quinolone quorum sensing signal molecules. *Nature Protocols*, 2, 1254 - 1262 doi:10.1038/nprot.2007.158.

Fletcher MP, Diggle SP, Cámara M. and Williams P. (2007) Biosensor-based assays for PQS, HHQ and related 2-alkyl-4-quinolone quorum sensing signal molecules. *Nature protocols*, 2, 1254–62.

Freschi *et al*. (2015) Clinical utilization of genomics data produced by the international *Pseudomonas aeruginosa* consortium. *Frontiers in microbiology* 6, Article 1036.

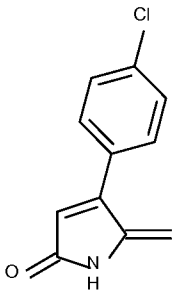
Ilangovan A, Fletcher M, Ramioni G, Pustelny C, Rumbaugh K, Heeb S, Camara M, Truman A, Chhabra SR, Emsley J & Williams P. (2013) Structural basis for native agonist and synthetic inhibitor recognition by the *Pseudomonas aeruginosa* quorum sensing regulator PqsR (MvfR). *PLOS Pathogens* 9(7):e1003508.

Niewerth H, Bergander K, Chhabra S, Williams P, and Fetzner S. (2011) Synthesis and biotransformation of 2-alkyl-4(1H)-quinolones by recombinant Pseudomonas putida KT2440, *Applied Microbiology and Biotechnology*, 91, 1399–1408.

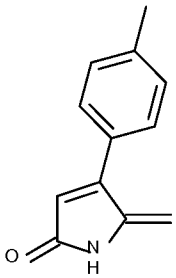
Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, et al. (1995) Common virulence factors for bacterial pathogenicity in plants and animals. *Science*, 268: 1899–1902.

CLAIMS

1. A lactam selected from



4-(4-chlorophenyl)-5-methylene-pyrrol-2-one (Ref. 488); and



5-methylene-4-(p-tolyl)pyrrol-2-one (Ref. 491);

for use in a method of treatment of an infection caused by a gram-negative bacteria.

2. The lactam for use in a method of treatment of claim 1, wherein the lactam is 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one.

3. The lactam for use in a method of treatment of claim 1, wherein the lactam is 5-methylene-4-(p-tolyl)pyrrol-2-one.

4. The lactam for use in a method of treatment of any one of claims 1 to 3, wherein the infection is a bacterial infection in which *Pseudomonas* is implicated.

5. The lactam for use in a method of treatment of claim 4, wherein the *Pseudomonas* is *P. aeruginosa*.

6. The lactam for use in a method of treatment of any preceding claim, wherein the infection is characterised by biofilm formation.

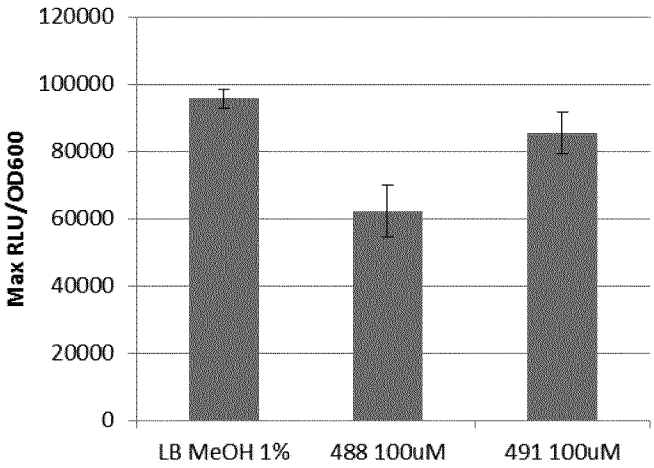


Figure 1

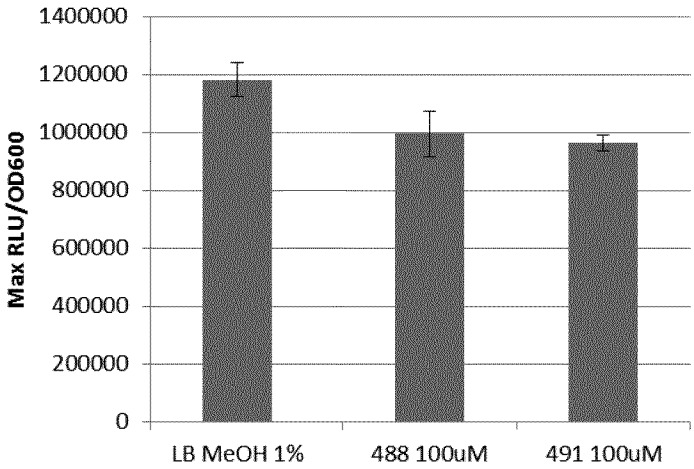


Figure 2

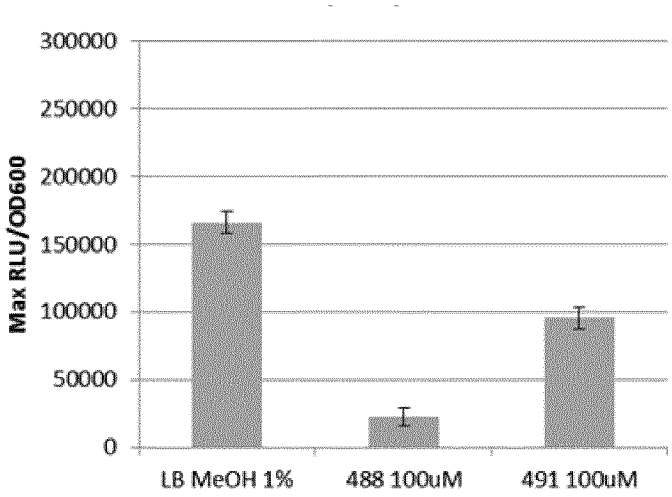


Figure 3

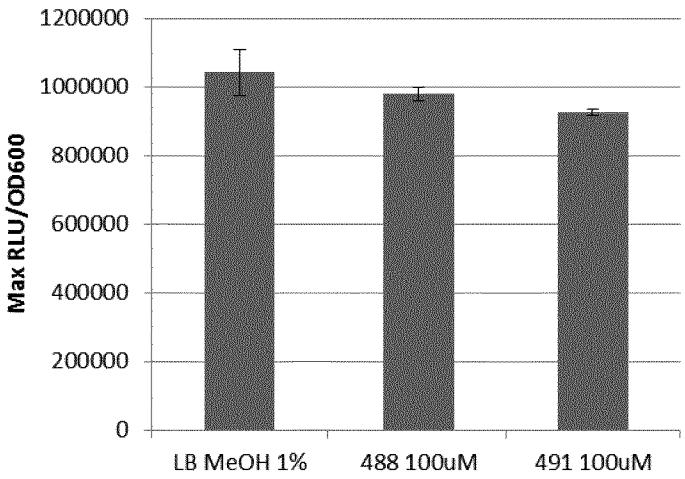


Figure 4

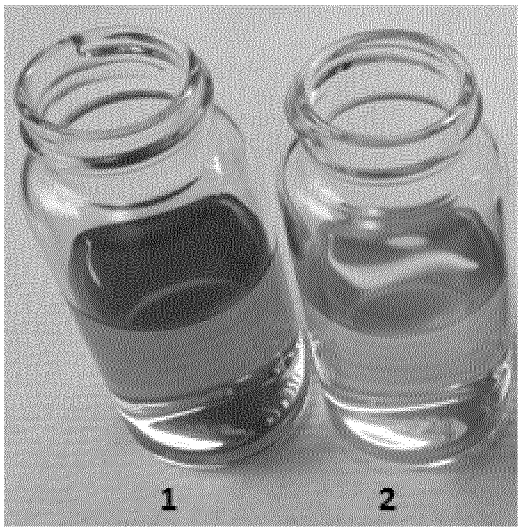
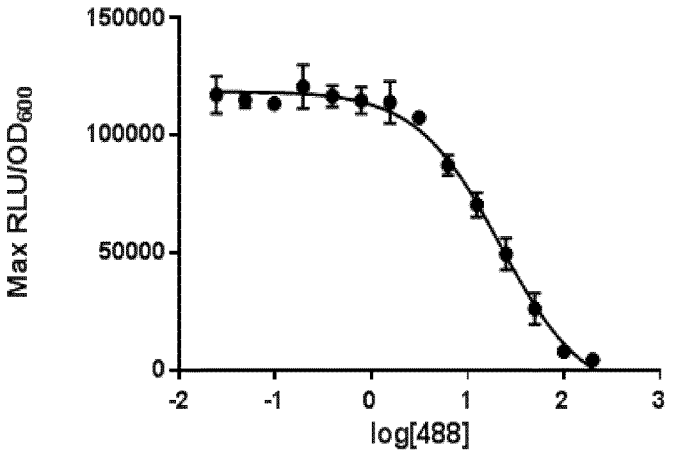


Figure 5



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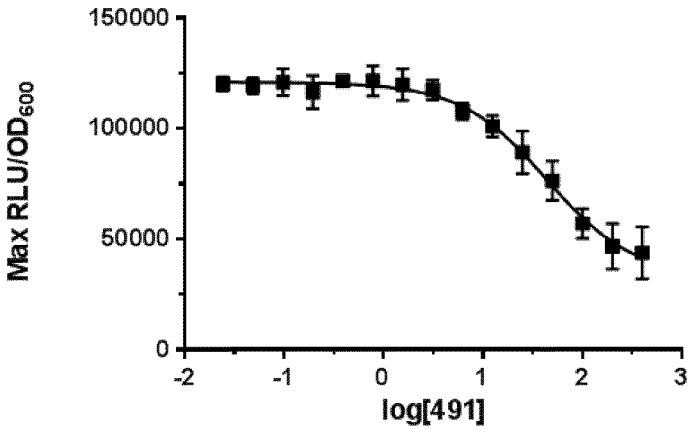

Figure 7

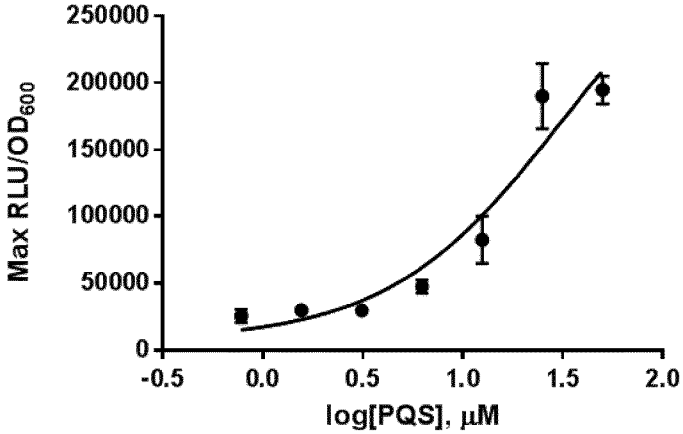

Figure 8

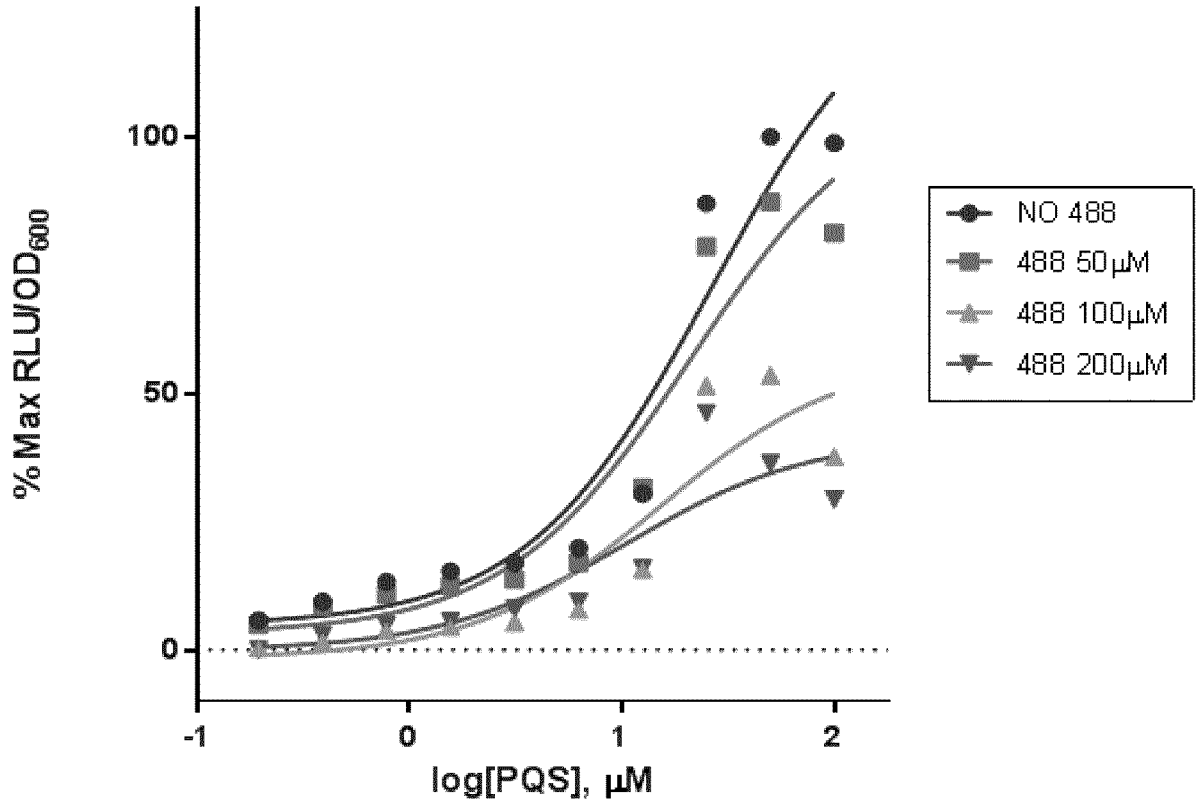

Figure 9

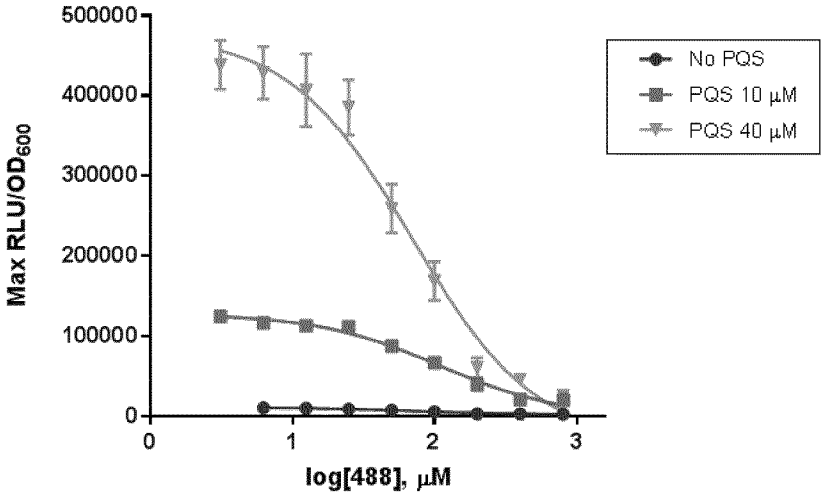

Figure 10

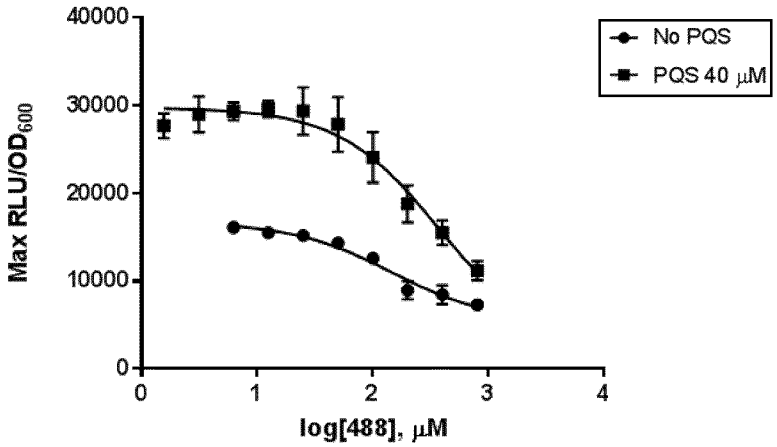

Figure 11

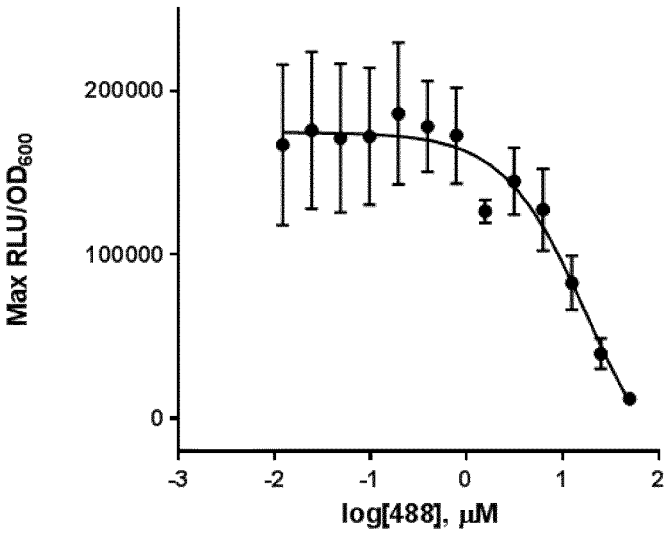

Figure 12

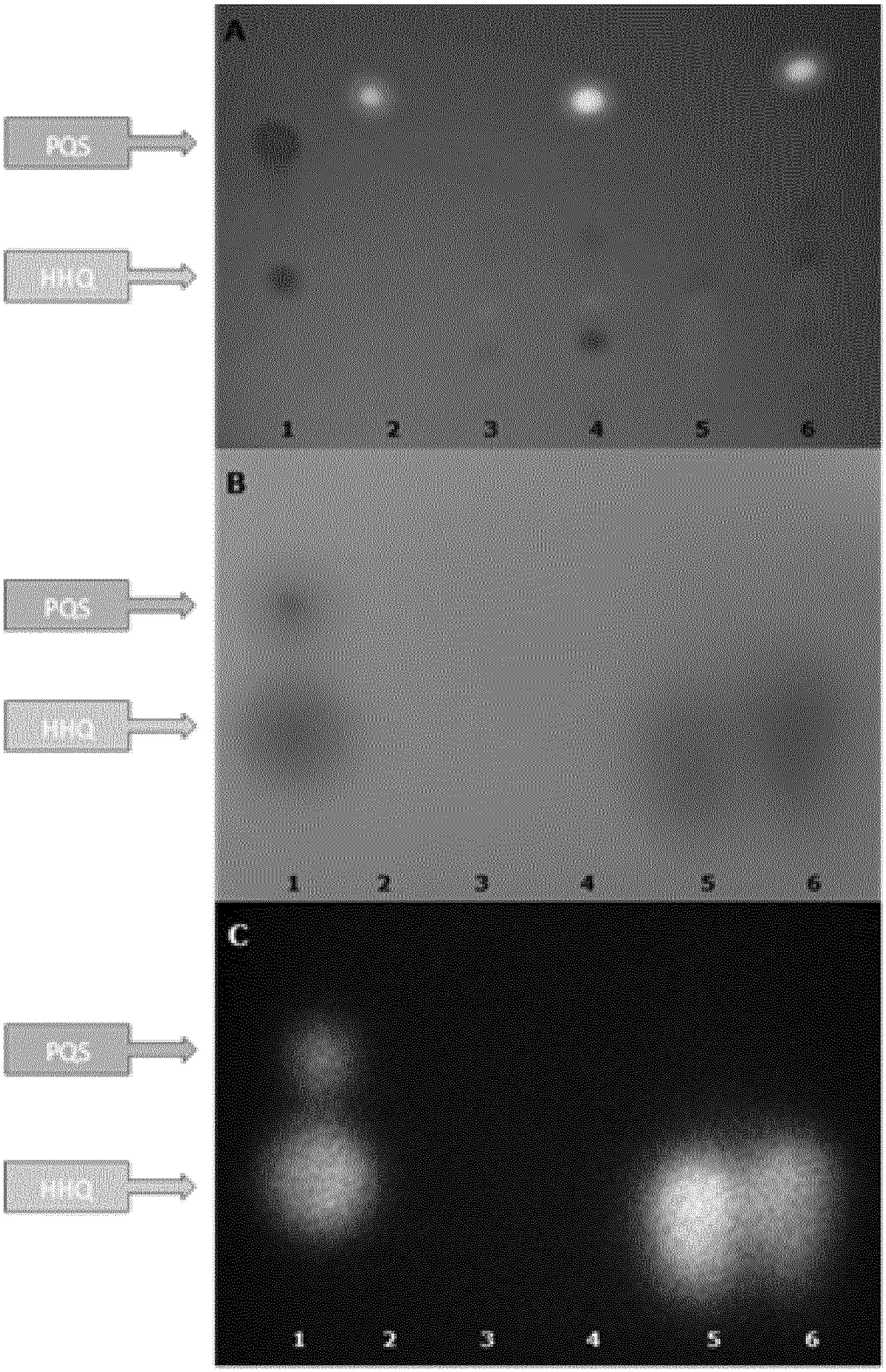

Figure 13

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/067783

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/4015 A61P31/00 A61K31/402
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/085042 A1 (BIOSIGNAL LTD [AU]; KUMAR NARESH [AU]; ISKANDER GEORGE [AU]) 2 August 2007 (2007-08-02) page 33 - page 34; table 2 page 8, line 5 - line 11 page 22 compound 219; page 40 claims 7,8 page 7, line 23 - page 8, line 2 page 8, line 16 - page 9, line 4	1-6
A	WO 2014/118240 A1 (UNILEVER PLC [GB]; UNILEVER NV [NL]; CONOPCO INC DBA UNILEVER [US]) 7 August 2014 (2014-08-07) page 7 claims	1-6

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 October 2017	20/10/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Werner, Doris

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2014/183164 A1 (KUMAR NARESH [AU]; WILLCOX MARK DUNCAN PERRY [AU]; HO KA KIT [AU]) 20 November 2014 (2014-11-20) claim 7 page 25 - page 26 page 5	1-6
A	KRENK O ET AL: "Methodology for Synthesis of Enantiopure 3,5-Disubstituted Pyrrol-2-ones", EUROPEAN JOURNAL OF ORGANIC CHEMISTRY, WILEY - V C H VERLAG GMBH & CO. KGAA, DE, 17 July 2015 (2015-07-17), pages 5414-5423, XP002752111, ISSN: 1434-193X, DOI: 10.1002/EJOC.201500620 the whole document	1-6

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/067783

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 2014183164 A1	20-11-2014	NONE	