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Research Article

Synthesis of carbon-14 labelled (5*Z*)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone: a potent quorum sensing inhibitor

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Summary

The potent quorum sensing inhibitor (5*Z*)-4-bromo-5-(bromomethylene)-2(5*H*)-[2-^{14}C]furanone has been prepared in five steps in 7.7% overall yield starting from bromo[1-^{14}C]acetic acid. Condensation of ethyl bromo[1-^{14}C]acetate with ethyl acetoacetate followed by decarboxylation was accelerated by microwave heating to afford [1-^{14}C]levulinic acid. Subsequently, bromination and oxidation gave the targeted furan-2-one with a radiochemical purity of $>97\%$ and a specific activity of 57 mCi/mmol. Copyright © 2004 John Wiley & Sons, Ltd.

Key Words: carbon-14; quorum sensing inhibitor; levulinic acid; microwave; decarboxylation; condensation

Introduction

Several gram-negative bacteria control expression of virulence factors by a phenomenon termed quorum sensing.[1] The bacteria produces extracellular signal molecules, which accumulate during growth, and at a certain threshold level, interaction with the receptor (LuxR homologue) leads to a burst in

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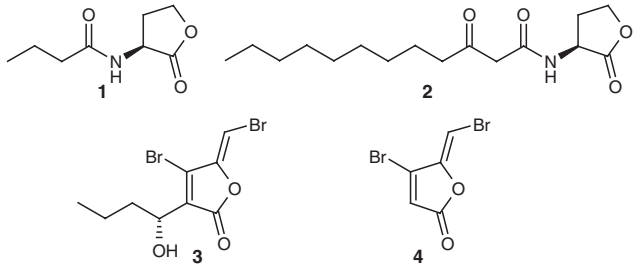


Figure 1. Signal molecules *N*-butanoyl-L-homoserine lactone (1) and *N*-(3-oxododecanoyl)-L-homoserine lactone (2) and quorum sensing inhibitors (*1′R*, 5*Z*)-4-bromo-3-(1′-hydroxybutyl)-5-(bromomethylene)-2(5*H*)-furanone (3) and (5*Z*)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone (4)

expression of the target genes. In *Pseudomonas aeruginosa*, the major signal molecules are *N*-butanoyl-L-homoserine lactone (**1**) and *N*-(3-oxododecanoyl)-L-homoserine lactone (**2**) (Figure 1) with the components of the dual quorum sensing apparatus encoded by *rhlI*/*rhlR* and *lasI*/*lasR*, respectively.[1] The lactone moiety of the signal molecule (e.g. homoserine lactone in **1** and **2**) is a generic feature of ligands bound to the transcriptional regulator, and interestingly, several other five-membered lactones have been shown to antagonise quorum sensing.[2,3] One example is fimbrolide **3**, discovered as a secondary metabolite of red seaweed *Delisea pulchra*, expressing antimicrobial and antifungal activity.[4–6] More recent findings demonstrate that fimbrolides are potent inhibitors of quorum sensing in *P. aeruginosa*.[7]

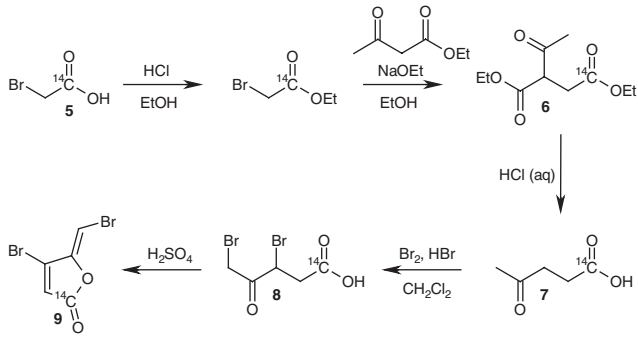
In order to investigate the biological activity by means of reactivity and mode of action, a suitable labelled inhibitor was needed. One of the most convenient tools for labelling of biological compounds is the use of radioactive isotopes such as tritium or carbon-14. Compared to other labelling techniques, this methodology has the advantage of not altering the biological activity, as the labelled compound is identical to the native compound except that one or more atoms has been replaced by their radioactive counterparts. Furthermore, radioactivity can be measured and quantified in trace amounts by simple methods. While labelling with tritium offers the advantage of high specific activity and short synthetic routes in general, it also offers considerably less radiochemical and metabolic stability compared to carbon-14. For this reason, a carbon-14 labelled tracer is normally used in more complex studies, even though the synthetic route often proves longer and more complex. Attempts to use tritium-labelled fimbrolide **3** have been reported as unsuccessful,[8] but since then, another more efficient inhibitor has been discovered. The five-membered cyclic product obtained by Wolff and Rüdel[9] in 1896 by bromination and oxidation of levulinic acid was reassigned as (5*Z*)-4-bromo-5-(bromomethy-

lene)-2(5*H*)furanone (**4**) in 1963.[10] Substance **4**, a non-natural representative of the fimbrolides, proved to have superior *in vitro* biological activity compared to the natural fimbrolides,[8] and recently, this was extended to an *in vivo* mouse model showing attenuation of quorum sensing.[7] Here, we describe the synthesis of carbon-14 labelled **4** to be used in future binding-assays and animal tests.

Results and Discussion

A number of different strategies for a small-scale radiochemical synthesis of (5*Z*)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone (**4**) were considered. The last two steps of the procedure described by Wolff and Rüdel[9] (bromination followed by simultaneous dehydration and oxidation of levulinic acid) were, as it turned out, easily transferred to a radiolabelling strategy. With this protocol in hand, a retrosynthetic C–C disconnection of levulinic acid was needed in order to bring the strategy to a level at which carbon-14 labelled starting materials were available. Carbon-14 labelled ethyl bromoacetate could be envisioned as the synthon together with an enol provided either as the corresponding stannane or masked in ethylacetoacetate. The latter route has been described by Joubert *et al.* for the synthesis of carbon-13 labelled levulinic acid.[11] Carbon-14 labelled ethyl bromoacetate, in turn, is easily prepared from commercially available carbon-14 labelled bromoacetic acid by esterification in acidic ethanol. The synthetic route employing the stannane methodology was one step shorter and offered a faster and more selective bromination procedure.[12] However, it turned out that even though the coupling between ethyl bromoacetate and acetonyltributylstannane worked well in preliminary unlabelled experiments run in THF, the stannane was unstable to the esterification mixture. As ethyl bromoacetate is highly volatile, removal of the solvent after the esterification proved very difficult, and therefore, this approach was abandoned. Instead, we turned to the ethyl acetoacetate approach (Scheme 1), by which the condensation reaction could be made in the same solvent as the esterification. Even though this was a longer route, it was in part negated by careful optimization of the reaction conditions for all three steps leading to levulinic acid. As part of the optimization procedure we included microwave-assisted chemistry, which has gained significant attention lately with numerous examples from a wide range of application areas.[13] Starting from bromo[1-^{14}C]acetic acid (**5**) (74% purity[†]), less than an hour of microwave irradiation in all was needed for the three steps leading to [1-^{14}C]levulinic acid (**7**), which was obtained in 73%

[†]The acid/ester mixture of bromo[1-^{14}C]acetic acid used as starting-material was 74% pure according to HPLC-analysis. Although the extra impurities obviously could interfere in the synthesis, no simple procedure for purifying bromoacetic acid or the corresponding ethyl ester exists. Instead, we were confident that the impurities could be separated at the final purification stage.



Scheme 1. Synthesis of (5*Z*)-4-bromo-5-(bromomethylene)-2(5*H*)-[2-^{14}C]furanone (9)

yield and 53% radiochemical purity. The purity at this stage, although seemingly on the low side, was quite acceptable considering the purity of the starting material. The [1-^{14}C]levulinic acid obtained was used without further purification. However, this direct approach made the subsequent bromination difficult to control due to the impurities present. The reaction had to be carefully monitored by HPLC analysis to ensure an optimum distribution of 3,5-dibromo[1-^{14}C]levulinic acid (**8**) in preference to mono- and tri-substituted analogues as well as the 5,5-disubstituted analogue. To facilitate the final reaction step, some of the polar impurities were separated at this stage by solid-phase extraction of the reaction mixture. This provided **8** (64%) with a radiochemical purity of 37%, which is satisfying considering the distribution of formed products in the reaction.

The lactonization and oxidation step was performed in concentrated sulfuric acid. We found that most of the impurities did not propagate through this step, the reason most likely being that the brominated analogues are not lactonized or oxidized in a similar manner. In preliminary experiments, we observed that a bromide shift was seen under certain reaction conditions, so despite the lack of 5,5-dibromolevulinic acid in the mixture, the final product rearranged to the undesired 5-(dibromomethylene)-2(5*H*)-[2-^{14}C]furanone. This has been described in the literature, when 20% oleum was used as oxidizing agent.[10] On the other hand, we observed this bromide shift during early attempts to promote the efficiency in the reaction by microwave heating at higher temperatures. The time of heating together with the temperature was found to be critical to the regioselectivity, and therefore, termination of the reaction was made by simply pouring the reaction mixture onto ice to impede any bromide shifts. Using this approach, a clean reaction was observed making it straightforward to purify the product by HPLC. It was very surprising though to discover that the product disappeared when evaporating to dryness *in vacuo*. Instead, **9** was concentrated by solid-phase extraction and

obtained in 13% yield with a radiochemical purity of >97%, the only impurity seemingly being the 5-(dibromomethylene)-2(5*H*)-[2-^{14}C]furanone. The overall yield of **9** starting from **5** was 5.9% (7.7% corrected for the purity of the starting material) and the specific activity was 57 mCi/mmol as determined by mass spectroscopy, which also confirmed the identity of the compound. All other identifications were made by HPLC based on characterisation performed during unlabelled optimization experiments using NMR and MS. No assignment of stereochemistry concerning the exocyclic bond in **9** was made, but characterisation on similar compounds and **4** implies it is the *Z*-isomer.[14]

Conclusion

Quorum sensing inhibitor (5*Z*)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone (**4**), labelled with carbon-14 in the carboxylic position, has been synthesised from bromo[1-^{14}C]acetic acid (**5**) in 7.7% corrected overall yield with a radiochemical purity of >97% and a specific activity of 57 mCi/mmol. The initial three steps of the synthesis were accelerated by microwave heating offering a fast route to carbon-14 labelled levulinic acid from simple building blocks. Levulinic acid, a well-used synthetic intermediate, was converted in two steps into (5*Z*)-4-bromo-5-(bromomethylene)-2(5*H*)-[2-^{14}C]furanone (**9**). The successful implementation of microwave heating in this and other projects underlines the value of this technology in the radiochemistry laboratory. Currently, compound **9** is being applied in bioassays to elucidate the biological activity and mechanism-of-action by this potent quorum sensing inhibitor.

Experimental

General

Bromo[1-^{14}C]acetic acid (specific activity: 58 mCi/mmol) was supplied by Amersham Biosciences, UK and had a 74% radiochemical purity (as a free acid/ethyl ester mixture) at the time of use. All other reagents and solvents were of analytical grade and used without further purification. HPLC was performed using a Merck Hitachi Intelligent Pump L6200A equipped with a Supertherm Column Thermostat (Mikrolab Aarhus) (set at 40°C), a Merck LC Organizer with a Rheodyne injector, and a Merck Hitachi UV Detector L4000 (detection at 220 nm). Detection of carbon-14 was performed on a Canbarra Packard flow detector 500TR.

Analytical HPLC was performed on a RP C18 column (4.6 × 250 mm, 5 μm, OdDMeSi 120 Å, Novo Nordisk) with a flow of 1.0 ml/min using one of the two following systems. System 1: 100% → 40% A 0-30 min and system 2: 80% A 0-30 min followed by 0% A for 10 min (A: 10% acetonitrile in 0.1% aq.

TFA; B: 90% acetonitrile in 0.1% aq. TFA). Purifications were performed using system 2 employing a 10×250 mm column (5.0 ml/min).

Radioactivity measurements were performed on a Packard Tri-Carb 1000 liquid scintillation analyzer using Ultima Flo^{TM} M (PerkinElmer) as liquid scintillation cocktail. Specific activity was determined on a ThermoFinnigan LCQ mass spectrometer operating in the APCI mode. Microwave heating was performed on an Emrys^{TM} Creator from Personal Chemistry, Sweden. Concentrations were made on a rotary evaporator at a temperature below 40°C.

*1,4-Diethyl-acetyl[1-^{14}C]succinate (**6**)*

A solution of bromo[1-^{14}C]acetic acid (**5**) (9.88 mCi, 74% radiochemical purity, specific activity: 58 mCi/mmol) in abs. EtOH (2 ml) was added to a microwave vessel containing abs. EtOH (0.5 ml) acidified with AcCl (2 µl) and the mixture was heated by microwaves at 110°C for 0.5 h. Then, ethylacetoacetate (0.18 mmol, 23 µl) dissolved in an EtOH/NaOEt-solution (0.20 mmol, 0.5 ml, 2.4 M) was added in one portion, and the mixture was heated by microwaves at 60–65°C for a total of 16 min (4 + 4 + 8 min). The solvent was removed using a gentle stream of nitrogen giving **6** as an oily residue with a radiochemical purity of 39% (System 1).

*[1-^{14}C]Levulinic acid (**7**)*

1,4-Diethyl-acetyl[1-^{14}C]succinate (**6**) (9.20 mCi, 62 µmol) was emulsified with 2 M HCl (3 ml) carefully providing for the oil being covered by the solvent. The mixture was heated by microwaves at 130°C for 10 min. Evaporation to dryness afforded **7** (7.17 mCi, 73% from **5**) as a semi-solid with a radiochemical purity of 53% (System 1).

*3,5-Dibromo[1-^{14}C]levulinic acid (**8**)*

A solution of [1-^{14}C]levulinic acid (**7**) (7.17 mCi, 66 µmol) in dichloromethane (1 ml) was cooled to 0°C. Then, HBr (10 µl, 33% in AcOH) was added followed by dropwise addition of Br_2 (10 µl) dissolved in dichloromethane (0.5 ml). The reaction mixture was heated to room temperature and then to reflux while following the progress of the reaction by HPLC analysis (System 1). Additional equivalents of HBr and Br_2 were added to the reaction mixture, whenever the reaction seemed to slow down. After 10 h of reflux, the loss of product seemed to be increasing faster than the formation and the reaction was stopped. In total, 72 µl Br_2 (1.4 mmol) and 130 µl HBr (33% in AcOH) were added. The reaction mixture was eluted through a Sep-Pak® cartridge (Waters, SiO_2, 2 g) and concentrated to give **8** (4.59 mCi, 64%) as a colorless oil with a radiochemical purity of 37% (System 1).

*(5Z)-4-Bromo-5-(bromomethylene)-2(5H)-[2-^{14}C]furanone (**9**)*

3,5-Dibromo [1-^{14}C]levulinic acid (**8**) (4.59 mCi, 29 μmol) was dissolved in sulfuric acid (95–97%, 1 ml) and the reaction mixture heated at 100°C for precisely 10 min in an oil bath. Then, the mixture was poured onto crushed ice followed by extraction with dichloromethane (4 × 5 ml). The combined organic phases were eluted through a Sep-Pak® cartridge (Waters, SiO_2, 0.5 g) and concentrated giving crude **9** (1.77 mCi) with a radiochemical purity of 60% (System 1). The crude product was purified by HPLC (System 2). As the product was found to disappear when concentrated *in vacuo*, the combined fractions containing **9** were diluted with water to an acetonitrile content of 5% and then applied‡ to a pre-activated Sep-Pak® cartridge (Waters, RP-C18, 2 g) followed by washing with water (20 ml). Eluting with acetonitrile (20 ml) afforded **9** (0.58 mCi, 13%) in 5.9% overall yield (7.7% corrected for the purity of the starting material and the final product). The radiochemical purity was greater than 97% as determined by HPLC (System 2) and the specific activity 57 mCi/mmol as determined by mass spectrometry.

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‡Since the product slowly elutes from the Sep-Pak cartridge when it is applied in 5% aq. acetonitrile, the cartridge was changed to a new one, whenever the product could be detected in the effluent.

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