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Full title: Quantitative ^{1}H NMR spectroscopy (qNMR) in the early process development of a new quorum sensing inhibitor.

Short title: ^{1}H qNMR to ensure quality of a new quorum sensing inhibitor.

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Abstract

2-methyl-5,6,7,8-tetrahydro-2*H*-chromen-4(3*H*)-one (called 6-oxo) is presented as a new AI-1 quorum sensing inhibitor for *Vibrio harveyi*. The development of a chemical process to afford traceable materials for new biological assays demands the development of analytical methods to ensure their purity and quality. This work describes the use of quantitative ^{1}H NMR spectroscopy (qNMR) to assess the purity of a sample of 6-oxo (99.88%) and a sample of its major process impurity (*E*)-1-(2-hydroxycyclohex-2-en-1-yl)but-2-en-1-one (called HCB; 98.28%). To explore the scope of the use of qNMR to quantify the amount of low-content components in samples related to the chemical process for 6-oxo synthesis, this work also determined the amount of 6-oxo in two HCB samples: (1) the high-purity HCB sample described above and (2) a crude HCB sample collected during the chemical process. Despite the complexity of the crude sample, the amount of 6-oxo was readily assessed and could help to estimate the extent to which 6-oxo was already formed during the HCB synthesis. This information can help the understanding of how the process parameters can be modified to improve the performance of the whole process, by controlling the reaction mechanisms working at each step of this chemical process. In this context, our results reinforce qNMR as a complementary analytical tool for the quantification of the main component found in a sample, contributing to the standardization of reference materials, and thus allowing the development of analytical methods for process control and traceability of the samples used for biological assays.

Keywords: purity; quantitative nuclear magnetic resonance; process development; related compound; process control.

1. Introduction

In recent decades, health authorities have reported an increase in bacterial antibiotic resistance and the emergence of multi-resistant pathogenic strains. These problems have driven the pharmaceutical industry to discover new therapeutic alternatives for conventional drugs [1][2][3]. Recent studies have showed the relationship between bacterial quorum sensing (QS), a chemical signaling system, and virulence expression factors [4][5]. Greater knowledge concerning QS mechanisms and the role of their signaling molecules, called autoinducers (AIs), can lead to the discovery of new strategies to help control bacterial infections [3]. In this context, the prospection of molecules to work as quorum sensing inhibitors (QSIs) has been introduced as promising anti-virulence drugs for treating bacterial infections. [1][5][6][7]

Preliminary studies in our workgroup have shown 2-methyl-5,6,7,8-tetrahydro-2*H*-chromen-4(3*H*)-one, called 6-oxo (**1**), as a new bacterial QS inhibitor. In order to determine the effect of 6-oxo on luminescence in *Vibrio harveyi* and to dismiss artifacts possibly generated by inhibitory effects on bacterial growth, the luminescence expression was measured simultaneously at different points of the bacterial growth curve (Fig. 1). The assays showed that 6-oxo reduced the *V. harveyi* luminescence at 60 %, without interfering with bacterial population growth (luminometry assay and statistical analyses are described in the supplementary information). Moreover, the inhibitory effect produced by 6-oxo occurred in a dose-dependent manner and was seen to be more effective in the AI-1 QS system (Fig. 2) [8]. Driven by this biological response, the synthesis of batches of this chromenone with known quality is indispensable, as good as that required for active pharmaceutical ingredients (APIs), aiming to obtain this product with high purity for further biological assays [9].

To assess the purity requirements of organic molecules to ensure a desired quality, the development of analytical methods addressing quality is mandatory. However, during the early development of a new drug substance, no commercially available certified reference material is expected to be found on the market. For this reason, most common chromatographic methods applied to purity determination cannot be used for this proposal (e.g. HPLC and GC)[10].

Quantitative Nuclear Magnetic Resonance (qNMR) is an attractive analytical tool to assess the purity of organic molecules, due to its easy procedure for sample preparation with a simple method development [9][10][11][12]. The qNMR analysis can be considered as a primary ratio method of measurement, especially because it allows the direct comparative measurement of the signal area ratio between an analyte and a standard of the same quantity. This method can be described completely by mathematical equations, from which a full uncertainty budget may be derived. For this reason, calibrations or reference standards analogous to analytes are not needed [13][14]. Other positive features include: the assignment of the chemical structure of the analyte and its impurities, the absence of intensity calibration requirements for determination of ratios (the signal area is proportional to the number of resonant nuclei), relatively short measuring times (this parameter depends on the relaxation times, of the analyte concentration and the spectrometer used), easy handling and sample preparation and the simultaneous analysis of different analytes in the same sample, particularly important for the analysis of impurities [11][13][14]. This technique has already been used in the analysis of drugs and made official by some pharmacopoeias, such as in the U.S. Pharmacopoeia (USP) monographs on orphenadrine citrate and isoamyl nitrite [13][15]. Recently, the methodology (based on an internal standard approach) was extended and validated for quantification of MDMA in ecstasy tablets.[16]

Given that 6-oxo is a potential pharmaceutical candidate during early stages of development (scheme 1), it is indispensable to define an assay method for its purity determination in terms of mass fractions [17][18]. However, current regulatory requirements for the pharmaceutical industry demands not only the synthesis of 6-oxo samples as a highly pure active pharmacological substance, but also knowledge about the potential impurities present in these samples, and the definition of process parameters that are directly responsible for impurities formation [19]. Therefore, our contribution describes the characterization of 6-oxo and its main impurity (*E*)-1-(2-hydroxycyclohex-1-en-1-yl)but-2-en-1-one, called HCB (**2**) by ^{1}H NMR. It also describes the application of ^{1}H qNMR using two different certificated internal reference standards, for the determination of the purity of 6-oxo and HCB samples. In addition, qNMR methodology was performed in two different approaches to quantify the amount of 6-oxo as minor chemical component. The first one was focused on assessing the individual amount of 6-oxo in a high-purity HCB sample prepared to be used as a reference material in our analytical developments. The other application of the qNMR method was focused on measuring the amount of 6-oxo already produced after the enamine acylation step of the 6-oxo synthesis, highlighting the potential of the use of qNMR for the optimization of this chemical process.

2. Experimental section

2.1. Chemicals and reagents

Chemical compounds and reagents used were purchased and supplied by Sigma-Aldrich Chemicals. All chemicals were of analytical grade and used without additional purification procedures. Benzoic acid (Sigma-Aldrich, Buchs, CH, TraceCERT quality - 99.97 %) and maleic acid (Sigma-Aldrich, Buchs, CH, TraceCERT quality - 99.94 %) were used as certified reference material (internal standard).

2.2. Synthesis of 6-oxo (2-methyl-5,6,7,8-tetrahydro-2*H*-chromen-4(3*H*)-one)

To a three-neck round-bottomed flask (250 mL) equipped with condenser, addition funnel and anhydrous $CaCl_2$ tube, 1-morpholinocylohexene (4.8 mL, 30 mmol), triethylamine (5.4 mL, 38 mmol) and anhydrous dichloromethane (30 mL) were added. To this solution, magnetically stirred and kept in an ice bath (10-12 °C), a solution of crotonoyl chloride (3.2 mL) in anhydrous dichloromethane (25 mL) was added over 30 minutes. The reddish-brown mixture was kept in a warm water bath (38-40 °C) for 18 h. A solution prepared with 30 mL of HCl (10 % w/w) and 15 mL of ethanol (95 %) was sequentially added, and the resulting mixture was refluxed for a further 10 h. After cooling, the organic layer was separated, and the aqueous portion was extracted with chloroform (3 x 20 mL). The combined organic extract was washed successively with water (3 x 50 mL), saturated aqueous solution of sodium bicarbonate (30 mL) and saturated aqueous solution of sodium chloride (30 mL). Drying over anhydrous Na_2SO_4 and evaporation of solvent gave a reddish-brown liquid. Without further purification, a solution prepared with Acetic acid (99.85%): HCl (37%): H_2O (1:1:1, 1 mL of each component for 1 g of reddish-brown liquid) was added to the round-bottomed flask containing the reddish-brown liquid. A condenser was connected to the round-bottomed flask and the reaction mixture was refluxed (72-75 ºC), under stirring for 4h. The cooled reaction mixture was diluted with water (20 mL) and extracted with dichloromethane (3 x 20 mL). The combined extract was washed with water (3 x 20 mL), saturated aqueous solution of sodium bicarbonate (30 mL) and brine (30 mL). Drying over anhydrous Na_2SO_4 and rotoevaporation of the solvent gave the crude product (brown solid). The crude was dissolved in hot hexane/ethyl acetate solution (95:5 v/v, 20 mL, 38-40 °C) and kept under stirring over activated charcoal for 6 h. This solution was vacuum filtered through a diatomaceous earth layer, and the solvent was removed by rotoevaporation to give a light

yellow solid. This product was solubilized in hot hexane (25 mL). After cooling, the crystallization yielded the pure 6-oxo as white needles (3.3 g, 65 %).

2-methyl-5,6,7,8-tetrahydro-2*H*-chromen-4(3*H*)-one *6-oxo* (1)

White needles. mp 47- 48 °C (literature 47-48 °C [20]).

^{1}H NMR (600 M*Hz*, DMSO-d_6) δ (ppm) 4.45 (dqd, *J* = 12.7, 6.3, 3.8 *Hz*, 1H), 2.41 (dd, *J* = 16.7, 13.3 *Hz*, 1H), 2.34 (dd, *J* = 16.7, 3.8 *Hz*, 1H), 2.25 – 1.98 (m, 4H), 1.75 – 1.37 (m, 4H), 1.34 (d, *J* = 6.3 *Hz*, 3H).

^{13}C NMR (151 M*Hz*, $CDCl_3$) δ (ppm) 192.5, 171.4, 112.0, 74.6, 43.3, 28.7 22.2, 22.0, 20.8 20.7.

IR (KBr, υ_{max}/ cm^{-1}) 2973, 2929, 2865, 1664, 1612.

EI/MS (70 eV) *m/z* 166 (M^+), 151, 138, 124, 110, 96, 83, 68 (100), 55.

2.3. Synthesis of HCB ((*E*)-1-(2-hydroxycyclohex-1-en-1-yl)but-2-en-1-one)

The pure sample of HCB was obtained by the chromatographic purification (silica-gel and dichloromethane) of the crude HCB (reddish-brown liquid collected just after the first extraction step).

Yellow solid. mp 47-48 ºC (literature 46-48 °C [21]).

^{1}H NMR (600 M*Hz*, DMSO-d_6) δ (ppm) 6.88 (dq, *J* = 15.2, 6.9 *Hz*, 1H), 6.46 (dq, *J* = 15.2, 1.7 *Hz*, 1H), 2.38 (t, *J* = 6.2 *Hz*, 2H), 2.34 (t, *J* = 6.2 *Hz*, 2H), 1.92 (dd, *J* = 6.9, 1.7 *Hz*, 3H), 1.68 – 1.60 (m, 4H).

^{13}C NMR (151 M*Hz*, $CDCl_3$) δ (ppm) 191.7, 182.2, 141.6, 124.5, 106.1, 33.7, 23.7, 23.0, 21.8, 18.7.

EI/MS (70 eV) *m/z* 166 (M^+), 151(100), 138, 123, 109, 95, 79, 69, 55.

2.4. ^{1}H NMR and qNMR analysis.

The NMR solutions were prepared with Sigma-Aldrich deuterated chloroform ($CDCl_3$) for ^{13}C NMR analyses (Figs. S3 and S4, supplementary information) and deuterated dimethyl sulfoxide (DMSO-d_6) for ^{1}H qNMR analyses. Five qNMR samples were prepared weighing 12 to 16 mg of analyte and internal standard, both dissolved in 600 µL of deuterated solvent.

The qNMR experiments were carried out on a Bruker Avance III HD 600 M*Hz* spectrometer equipped with a 5 mm broadband observe (BBFO) probe. Locking and shimming adjustments were performed automatically. Tuning and matching adjustment were done manually for each sample. The ^{1}H NMR spectra were acquired without sample spinning and with ^{13}C decoupling during acquisition (zgig30 pulse sequence). T_1 measures of all analytes were previously performed in qNMR experiments. To ensure complete relaxation, all acquisitions were made with a relaxation delay of 7 times the largest T_1 value. Acquisition parameters (Table 1) were adjusted to ensure a signal-to-noise ratio at least 300 for the relevant peaks. The integration of the internal standard signal and sample signal were done in the same way. Aiming to integrate completely each quantified signal, the integration range was extended from the centre of the signal at least eighty times of the full width at half height (FWHM) on either side of the measured signal. The limits of the integration range to multiplets were based on the outermost signals. Spectrum data were processed with the TopSpin 3.2 software. Manual adjustments in phase and baseline were performed for each sample, and all spectra were referenced using TMS signal (0 ppm). The spectra showed were edited with MestReNova software (Mestrelab Research S.L., Santiago de Compostela, Spain, version: 11.0.4, 2017).

Equation S1 was used to calculate the purity (% w/w) of each analyte in the samples (supplementary information) [12][13][14].

The uncertainty budget was based on main uncertainty sources to our qNMR measurements and these are illustrated in a cause-effect diagram in the supplementary information (fig. S1). The combined uncertainty $u_c(P_x)$ was calculated by equation S2, (supplementary information). The expanded uncertainty (U) was calculated to a confidence level of 95%, by multiplying of the combined uncertainty to a confidence level factor of $k = 2$ [14][22][23].

3. Results and discussion

3.1. Chemical synthesis of the samples

The synthesis of 6-oxo (**1**) was based on the acylation of 1-morpholinocyclohexene (**3**) by crotonoyl chloride (**4**) in the presence of triethylamine, followed by acid hydrolysis to afford the crude HCB (**2**). After that, the final tandem isomerization / cyclization step was performed under acidic conditions to yield the pure 6-oxo after crystallization in hexane (Scheme 1). The whole procedure is based on one previously described by Mahajan and Resck [20].

The intermediate HCB (**2**) is the last chemical intermediate of the 6-oxo synthesis and is its main process impurity. A high-purity sample of HCB was prepared by chromatographic purification (silica gel and dichloromethane) of the crude HCB, a process sample collected just after the work-up of the enamine acylation step.

The measurement of the mass fraction of a chemical substance present in a complex sample is a recognized qNMR limitation [24]. To address this issue, we also proposed the measurement of the 6-oxo amount present in a crude sample of HCB collected just before quenching the first reaction step, the acylation of the enamine.

3.2. NMR analyses

The 6-oxo ^{1}H NMR spectrum (Fig. 3a) showed chemical shifts and multiplicities that endorse its molecular structure. A double quartet of doublets (dqd), observed at 4.45 ppm,

was assigned to H13, due to its coupling with H12, H9´ and H9´´. Two doublets doubles of doublets (dd), observed at 2.41 and 2.34 ppm, were assigned to the diastereotopic hydrogens H9´ and H9´´, due to their couplings with H13. A multiplet, 2.25 – 1.98 ppm, integrating for 4 hydrogens, was assigned to the allylic methylenes H3 and H6. From 1.75 to 1.37, a multiplet could be observed corresponding to the methylenes H1 and H2. Finally, at 1.42 ppm, a doublet could be observed, assigned to the methyl H12, due to its coupling with H13.

The HCB ^{1}H NMR spectrum also matched the proposed chemical structure (Fig. 4a). At 6.88 ppm, a double of quartets (dq) was attributed to H9, due to its coupling with H8 and H10. At 6.46 ppm another dq was attributed to H8, due to its coupling with H9 and H10. At 2.38 and 2.34 ppm, two triplets, which were respectively assigned to the methylene hydrogens H1 and H4, due to their couplings with H2 and H3, could also be detected. At 1.92 ppm a double doublet (dd) assigned to the methyl H10, due to its coupling with H8 and H9. A multiplet corresponding to the resonance of the methylenes H2 and H3 was detected, ranging from 1.68 to 1.60 ppm. A doublet observed at 1.34 ppm was assigned to the methyl group of 6-oxo, which was detected as an impurity in this HCB sample, confirming the information previously obtained by the GC-MS and HPLC analysis.

3.3. Purity determination and 6-oxo quantification in samples of HCB

The 6-oxo ^{1}H NMR spectrum has a signal at 4.45 ppm (dqd) that was resolved enough to allow its integration free of the contribution of another hydrogen nuclei (T_1: 4 s $CDCl_3$ and 3 s to $DMSO_4$-d). For this reason, this signal was chosen to perform the purity assay of this 6-oxo sample based on the integral value of the maleic acid signal (6.27 ppm, Fig. 3a) and benzoic acid signal (8.13 ppm, Fig. 3b), chosen to be separately used as the internal standards. The results of the ^{1}H qNMR analysis are presented in Table 2 for both internal

standards, where the purity calculation was performed by means of eqn. 1. The analyzed samples showed a high purity for both standards, (P_{MA}= 99.88%, U_{MA}=0.33%) and (P_{BA}= 99.87%, U_{BA}=0.31%), having the acceptable purity required for its use in our future biological assay related to QS.

The purity analysis of the pure HCB sample by ^{1}H NMR spectroscopy was also performed with maleic acid (Fig. 4a) and benzoic acid (Fig. 4b) as internal standards. The HCB characteristic resonance signals for all its hydrogen nuclei could be readily assessed. In addition, a doublet at 1.34 ppm (T_1: 2 s $CDCl_3$ and 1 s to $DMSO_4$-d_6), assigned to 6-oxo methyl group, was detected (Fig. 4a). The presence of this impurity had already been predicted by GC-MS and HPLC analysis.

For maleic acid as the internal standard, the purity analysis of HCB was performed with the same parameters used to assess 6-oxo purity (table 1). The signal at 6.88 ppm (T_1: 6 s $CDCl_3$ and 4 s to $DMSO_4$-d_6) had enough resolution to be used as the reference resonance signal to acquire the qNMR data of this sample of HCB (Fig. 4a) and yielded a purity of 98,28 % (U_{MA}=0.33%) (Table 3). The percentage of 6-oxo in this sample could also be calculated through its doublet in 1.34 ppm (Fig. 4a) and showed an amount of 1.58 % .

Benzoic acid was also used as an internal standard to assess the purity of the HCB by ^{1}H qNMR analysis. In addition to the expected resonance signals for HCB and 6-oxo, some extra signals were detected (highlighted by squares in Fig. 4b). These signals were credited to unexpected chemical interactions between the analyte and internal standard. Not necessarily due to the acid promoted decomposition of the analyte, but most probably due to the acid/base equilibrium between HCB and benzoic acid. The afforded salt could have its ionic

pair close enough to allow differential anisotropic shield/deshield promoted by the benzoic acid aromatic ring for some nuclei. This hypothesis was supported by the lower HCB purity (97.56%, U_{BA}=0.40%) assessed when benzoic acid was the internal standard in contrast to the previous purity (98,28 %, U_{MA}=0.33%) observed when maleic acid was the internal standard. These findings highlight the need to scan different internal standards to assess purity by ^{1}H qNMR analysis.

The possibility to measure the amount of 6-oxo when it is an impurity in a sample of HCB prompted us to answer an opened question in respect to our process development. How much 6-oxo is formed just after the hydrolysis step that affords HCB? This hydrolysis is performed under acidic conditions (aqueous HCl solution) and, in the same way as that promoted by AcOH/HCl/H_2O for the cyclization of HCB, it could promote 6-oxo formation (Scheme 1). The confirmation of this hypothesis could open the opportunity to redesign the process to a one pot hydrolysis/cyclization step to yields 6-oxo, reducing the number of the unit operations and, consequently, lowering the process timeline and the exposure of the desired product to acidic conditions responsible to degrade it.

To evaluate the ^{1}H qNMR methodology with this different approach, ^{1}H NMR spectrum of the crude HCB was acquired (Figure 5a) and revealed new resonance signals ranging especially from 6.0 to 6.5 ppm, when compared to the pure HCB sample (Figure 4). For this reason, only benzoic acid was used as an internal standard. Despite the high complexity of the acquired spectrum, the 6-oxo signal at 4.45 ppm showed enough resolution, even after the addition of the internal standard, and was used to perform qNMR analysis (Fig. 5b). The results showed a 6.58 % mass fraction of 6-oxo on average and a minor data variation (Table S1, supplementary information). Despite the success on the detection and

quantification of 6-oxo at this process step, the low amount of the final product discouraged us from attempting the redesign 6-oxo chemical process.

4. Conclusion.

The measurement of the mass fraction of the components of a sample, especially the assay of its main compound, is important to properly evaluate the data obtained by the biological activity studies. This scientific contribution detailed the use of quantitative ^{1}H NMR spectroscopy to determine the purity of a new quorum sensing inhibitor called 6-oxo. The ^{1}H qNMR results, assessed by two different internal standards, provided high accuracy and demonstrated the achievement of a high-purity product.

The same ^{1}H qNMR methodology was used to assess the purity of an HCB sample, a synthetic intermediate of the 6-oxo chemical process, usually observed as the main impurity in final samples of 6-oxo. The data showed good results when maleic acid was used as the internal standard. Attempts to perform the analysis when benzoic acid was the internal standard led to additional resonance signals in the ^{1}H NMR spectrum, yielding a lower percentage of HCB when compared to the result returned using maleic acid as internal standard. This was credited to unwanted chemical interaction between the analyte and the internal standard, highlighting the need for the screening of the proper internal standard to the desired purity determination.

The ^{1}H qNMR methodology was also used successfully to assess the mass fraction of the 6-oxo in a crude sample of HCB, despite the presence of other impurities. This can allow 6-oxo formation to be followed at each step of the chemical process and potentially helps its optimization toward the improvement of 6-oxo yield. This technique can be incorporated into the daily workflow of 6-oxo quantification by NMR, even if it is applied to the analysis of a complex matrix, as usually observed for in-process control steps.

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References

[1] T. Defoirdt, *Trends Microbiol.* **2018**, *26*, 313.

[2] A.K. Bhardwaj, K. Vinothkumar, N. Rajpara, *Recent Pat. Antiinfect. Drug Discov.* **2013**, *8*, 68.

[3] L. Cegelski, G.R. Marshall, G.R. Eldridge, S.J. Hultgren, *Nat Rev Microbiol*. **2008**, *6*, 17.

[4] K. Reuter, A. Steinbach, V. Helms, *Perspect. Medicin. Chem.* **2016**, *8*, 1.

[5] R. Salini, S. Santhakumari, R.A. Veera, S.K. Pandian, *Aquaculture* **2019**, *498*, 162.

[6] M. Cámara, P. Williams, A. Hardman, *Lancet Infect. Dis*. **2002**, *2*, 667.

[7] D. A. Rasko, V. Sperandio, *Nat. Rev. Drug Discov.*, **2010**, *9*, 117.

[8] F.L. Silva, A.H.L. Machado, I.S. Resck, L.G. Giugliano, A.L. Pereira, "Prospecting synthetic lactones for quorum sensing interference", 26th Brazilian Congress of Microbiology, Foz do Iguaçu, Brazil, **2011**.

[9] M.R. Siddiqui, Z.A. Othman, N. Rahman, *Arab. J. Chem.* **2017**, *10*, 1409.

[10] M. Shivani, I.P. Singh, *Magn. Reson. Chem.* **2013**, *51*, 676.

[11] U. Holzgrabe, R. Deubner, C. Schollmayer, B. Waibel, *J. Pharm. Biomed. Anal.* **2005**, *38*, 806.

[12] Q. Yang, H. Qiu, W. Guo, D. Wang, X. Zhou, D. Xue, J. Zhang, S. Wu, Y. Wang, *Molecules* **2015**, *20*, 12114.

[13] M.S. Santos, L.A. Colnago, *Quim. Nova* **2013**, *36*, 324.

[14] F. Malz, H. Jancke, *J. Pharm. Biomed. Anal.* **2005**, *38*, 813.

[15] U. Holzgrabe, B. Wawer, B. Diehl, *NMR spectroscopy in pharmaceutical analysis*, 1st ed., Elsevier, Oxford, **2008**, p.135.

[16] N. S. Almeida, L. E. C., Benedito, A.O. Maldaner, A.L. Oliveira, *J. Braz. Chem. Soc.* **2018**, *29*, 1944.

[17] G.F. Pauli, S. Chen, C. Simmler, D.C. Lankin, T. Gödecke, B.U. Jaki, J.B. Friesen, J.B. McAlpine, J.G. Napolitatno, *J. Med. Chem.* **2014**, 57, 9220.

[18] Y. Huang, B. Su, Q. Ye, V.A. Palaniswamy, M.S. Bolgar, T.V. Raglione, J *Pharm. Biomed. Anal*. **2014**, *88*, 1.

[19] R.M. Maggio, N.L. Calvo, S.E. Vignaduzzo, *J. Pharm. Biomed. Anal.* **2014**, *101*, 102.

[20] J.R. Mahajan, I.S. Resck, *J. Braz. Chem. Soc.* **1997**, *8*, 603.

[21] M. Casey, J.A. Donnelly, J.C. Ryan, S. Ushioda, *ARKIVOC* **2003**, (*vii*), 310.

[22] M.Weber, C. Hellriegel, A. Rueck, J. Wuethrich, P. Jenks, M. Obkircher, *Anal. Bioanal. Chem.* **2015**, *407*, 3115.

[23] M.Weber, C. Hellriegel, A. Rueck, R. Rück, R. Sauermoser, J. Wüthrich, *Accred. Qual. Assur*. **2013**, *18*, 91

[24] M.Weber, C. Hellriegel, A. Rueck, J. Wuethrich, P. Jenks, *J. Pharm. Biomed. Anal.* **2014**, *93*, 102.

Table 1 - qNMR acquisition parameters

Parameter (Bruker code)	Value
Digitization mode (DIGMOD)	Baseopt
Filter correction (FILCOR)	1.5 µs
Number of dummy scans	4
Pre-scan delay (DE)	10 µs
Receiver gain (RG)	automatic
Relaxation delay (D1)	15 s
Sweep width (SW)	24 ppm
Time domain size (TD)	64k
Total number of scans (NS)	32

Table 2 - Data of 6-oxo ^{1}H qNMR analysis with maleic acid (MA) or benzoic acid (BA) as internal standard.

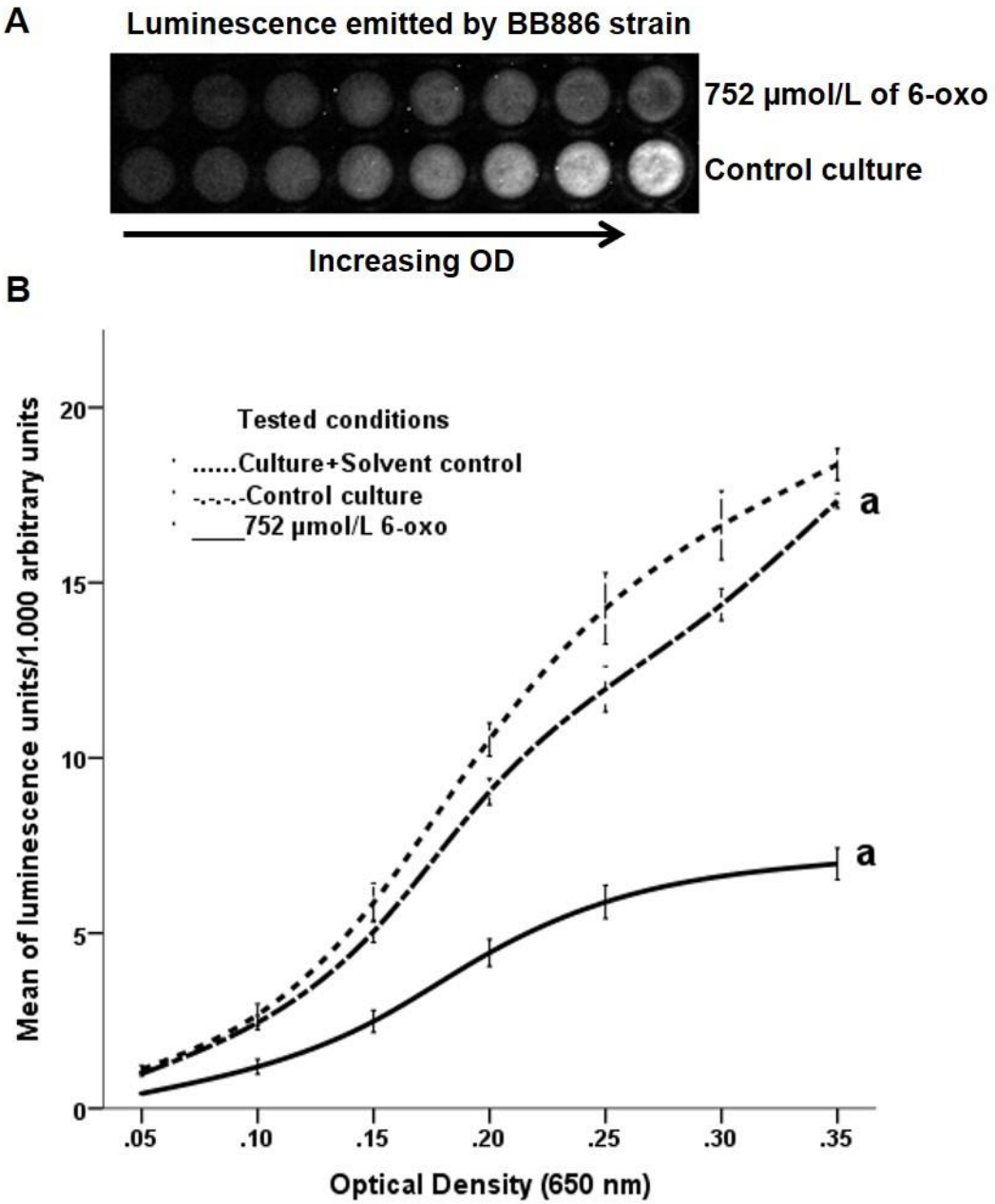
Sample	Std	I_x /10	IS_{td} /10	N_{Std}	N_x	M_x (g/mol)	M_{Std} (g/mol)	m_{Std} (mg)	m (mg)	P_{Std} (%)	$\boldsymbol{P_x}$ (%)
1		3.19	10.00	2	1	166.22	116.07	14.0	12.8	99.94	99.90
2		3.86	10.00	2	1	166.22	116.07	12.4	13.7	99.94	99.87
3	MA	3.99	10.00	2	1	166.22	116.07	12.0	13.7	99.94	99.94
4		3.88	10.00	2	1	166.22	116.07	12.5	13.9	99.94	99.95
5		3.90	10.00	2	1	166.22	116.07	12.5	14.0	99.94	99.73
1		2.87	10.00	2	1	166.22	122.12	15.9	12.4	99.97	99.99
2		2.93	10.00	2	1	166.22	122.12	15.3	12.2	99.97	99.89
3	BA	2.85	10.00	2	1	166.22	122.12	15.6	12.1	99.97	99.84
4		3.08	10.00	2	1	166.22	122.12	14.9	12.5	99.97	99.91
5		3.08	10.00	2	1	166.22	122.12	15.0	12.6	99.97	99.72

Table 3 - Data from HCB ^{1}H qNMR analysis with maleic acid as internal standard

Sample	Analyte	I_x/10	IS_{td}/10	N_{Std}	N_x	M_x (g/mol)	M_{Std} (g/mol)	m_{Std} (mg)	m (mg)	P_{Std} (%)	$\boldsymbol{P_x}$ (%)
1		3.72	10.00	2	1	166.22	116.07	14.2	15.4	99.94	98.25
2		3.82	10.00	2	1	166.22	116.07	13.4	14.9	99.94	98.37
3	HCB	3.69	10.00	2	1	166.22	116.07	13.0	14.0	99.94	98.17
4		3.81	10.00	2	1	166.22	116.07	12.6	14.0	99.94	98.21
5		3.79	10.00	2	1	166.22	116.07	12.7	14.0	99.94	98.40
1		0.18	10.00	2	3	166.22	116.07	14.2	15.4	99.94	1.54
2		0.17	10.00	2	3	166.22	116.07	13.4	14.9	99.94	1.45
3	6-oxo	0.19	10.00	2	3	166.22	116.07	13.0	14.0	99.94	1.67
4		0.19	10.00	2	3	166.22	116.07	12.6	14.0	99.94	1.65
5		0.19	10.00	2	3	166.22	116.07	12.7	14.0	99.94	1.60

Table 4 - Data from HCB ^{1}H qNMR analysis with benzoic acid as internal standard.

Sample	I_x/10	I_{Std}/10	N_{Std}	N_x	M_x (g/mol)	M_{Std} (g/mol)	m_{Std} (mg)	m (mg)	P_{Std} (%)	$\boldsymbol{P_x}$ (%)
1	3.79	10.00	2	1	166.22	122.12	15.1	16.0	99.97	97.37
2	3.50	10.00	2	1	166.22	122.12	15.7	15.3	99.97	97.77
3	3.74	10.00	2	1	166.22	122.12	13.4	14.0	99.97	97.41
4	3.72	10.00	2	1	166.22	122.12	13.4	13.9	99.97	97.69
5	3.90	10.00	2	1	166.22	122.12	12.4	13.5	99.97	97.54



Lines represent spline interpolations
Error bars indicate standard errors of the means
"a" indicates $p < 0.001$ for the comparison between two indicated groups

Figure 1 - Effect of 6-oxo at concentration of 752 µmol/L on QS-induced luminescence produced by *V. harveyi* strain BB886. a) Representative image showing the inhibitory effect of 6-oxo on the luminescence produced by *V. harveyi* strain BB886. b) 6-oxo reduced the luminescence produced by *V. harveyi* luminescence at 60.7% without interfering with bacterial population growth.

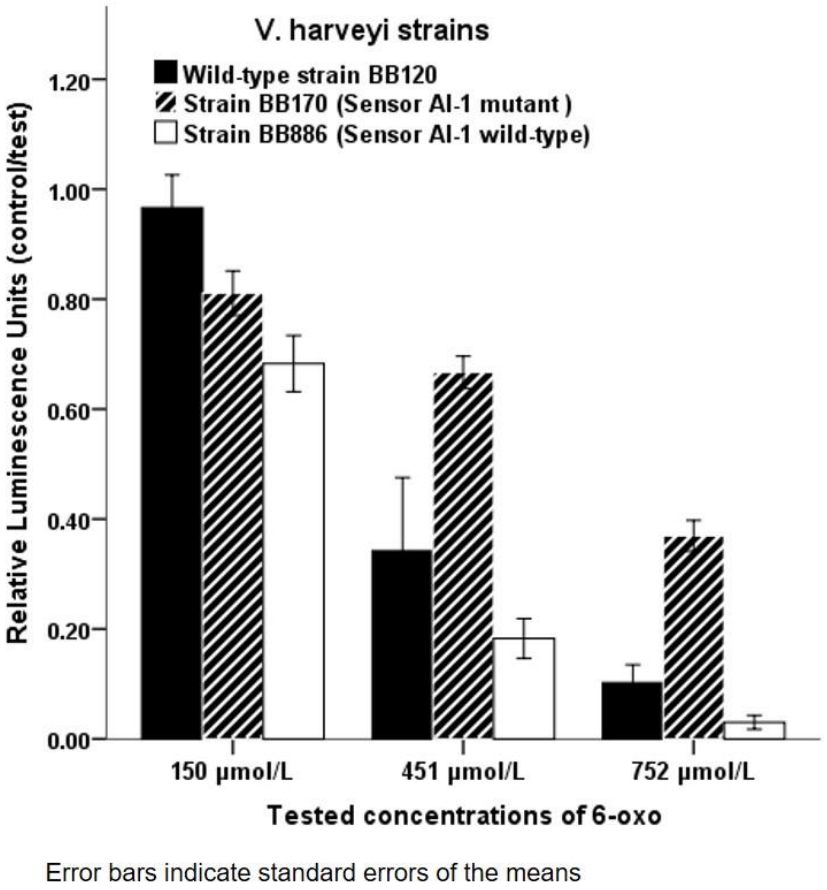


Figure 2 - 6-oxo inhibits the QS system AI-1 more efficiently in *V. harveyi*. 6-oxo at concentration of 752 µmol/L reduced the luminescence of the strains BB120 (wild-type strain: sensor AI-1^{+}, sensor AI-2^{+}), BB170 (mutant strain: sensor AI-1 mutant, sensor AI-2 wild-type) and BB886 (mutant strain: sensor AI-1 wild-type, sensor AI-2 mutant), respectively at 89.7% ± 6.6; 63.1% ± 5.6; and 97.0% ± 2.5. When compared to the controls the reductions were statistically significant ($P < 0.05$). [1]

[1] Further comments about the quorum sensing system in the *Vibrio harveyi* bacteria can be found in the supplementary information

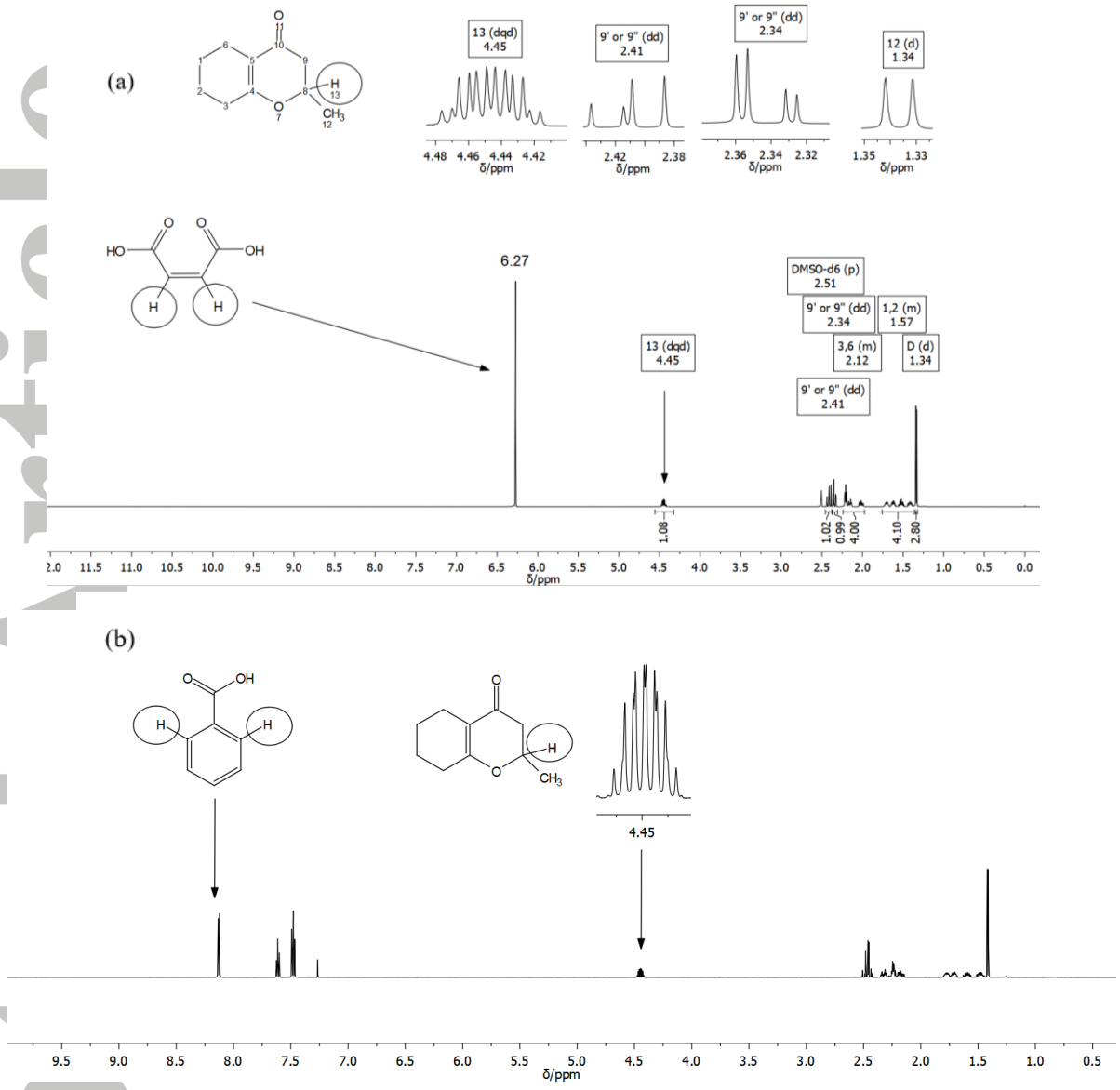


Figure 3 - ^{1}H NMR spectrum (600 MHz) of the pure 6-oxo with maleic acid (a) or benzoic acid (b) as internal standard.

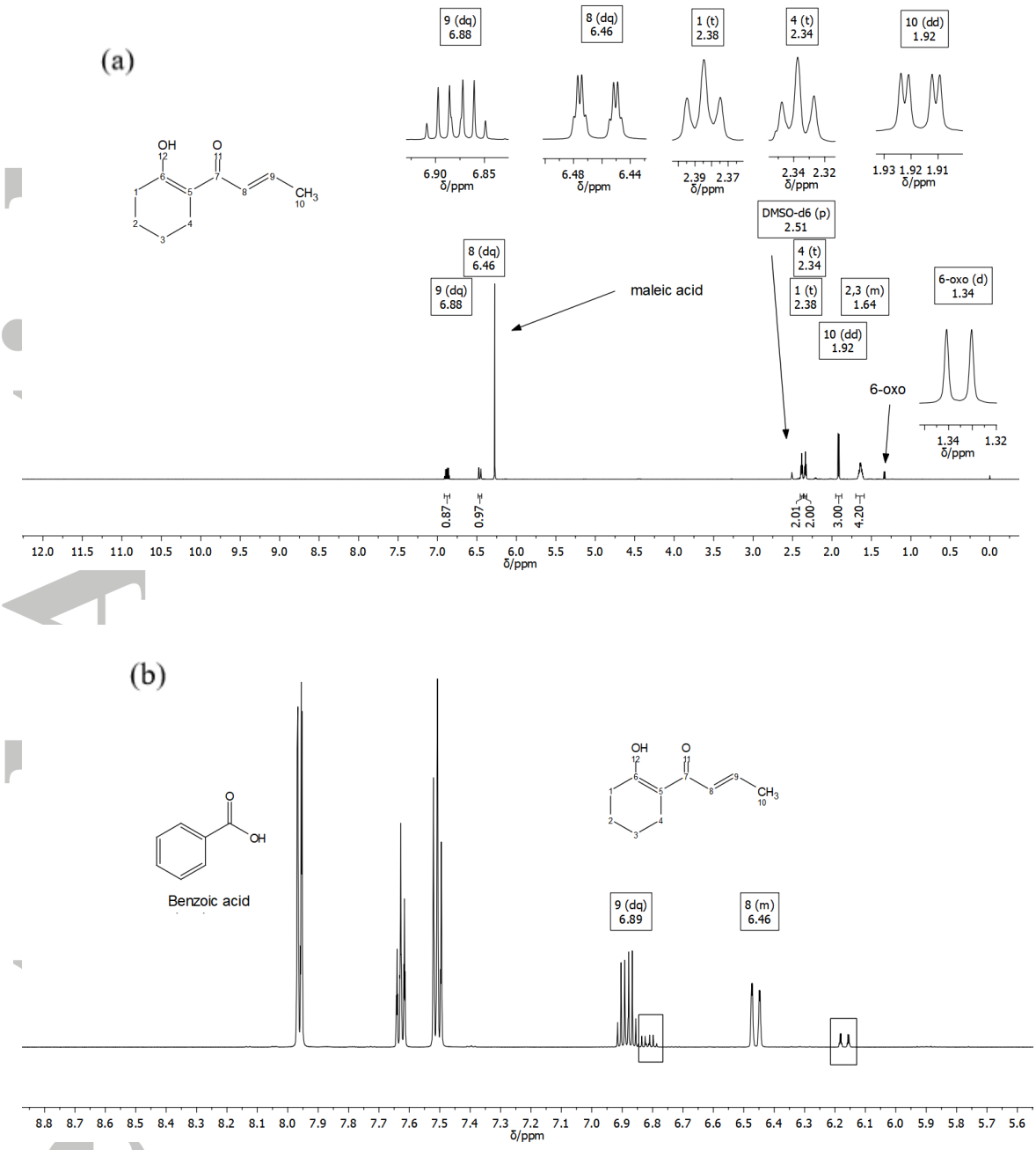


Figure 4 - ^{1}H NMR spectra (600 MHz, DMSO-d_6) of the pure HCB with maleic acid (a) or benzoic acid (b) as internal standard (Resonance signals highlighted by the squares were attributed to chemical interactions between HCB and benzoic acid).

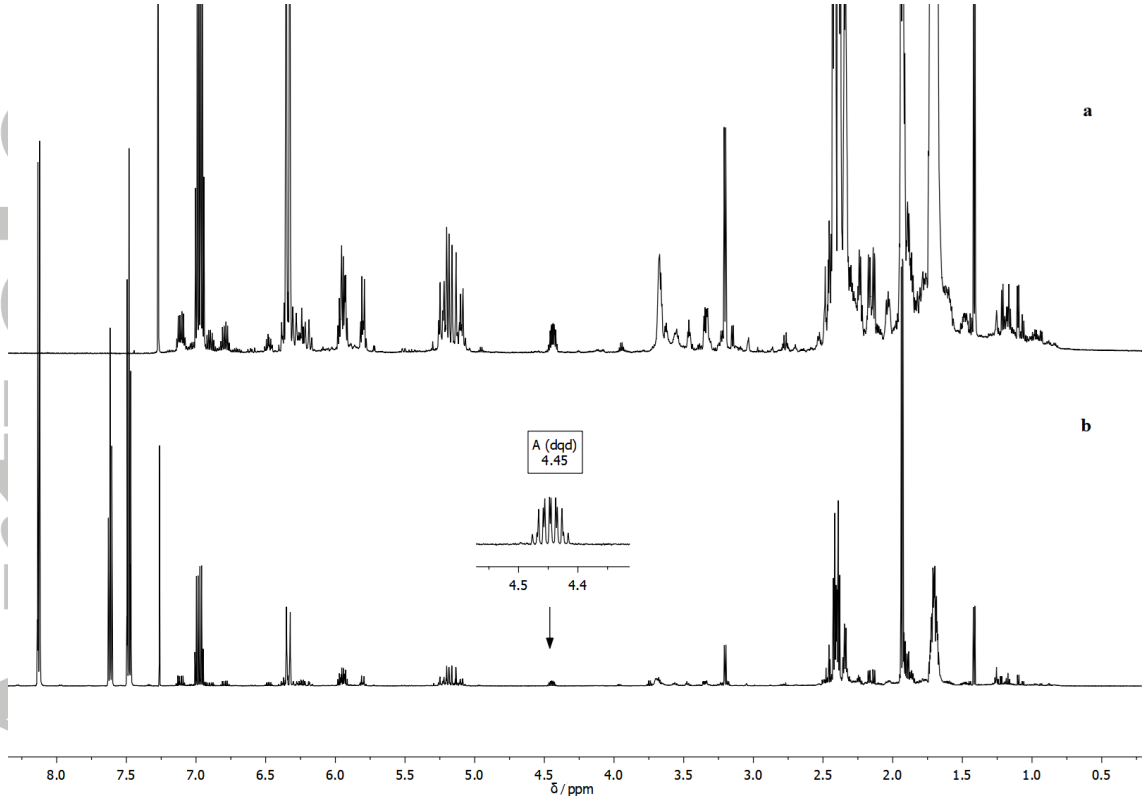
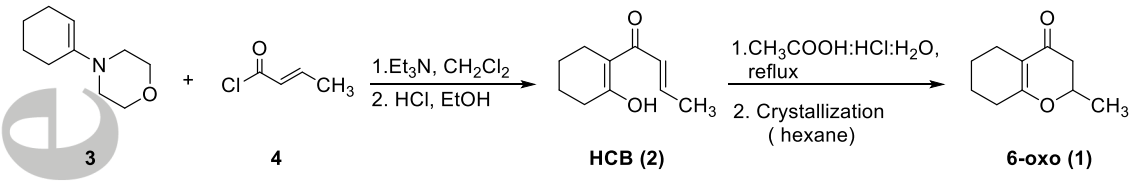


Figure 5 - ^{1}H NMR spectra (600 MHz, DMSO-d_6) of the crude HCB (a) and the crude HCB with benzoic acid (b)



Scheme 1 - Chemical steps for the synthesis of 6-oxo (1)

Graphical Abstract