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Biosynthesis and biological role of 2-alkyl-4-quinolones in *Pseudomonas aeruginosa* and *Burkholderia thailandensis*

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Abstract

Pseudomonas aeruginosa and *Burkholderia* species particularly contribute to chronic pulmonary infections in cystic fibrosis patients, leading to considerable mortality. Both of these bacteria uniquely produce a range of 2-alkyl-4-quinolones and finely tune the chemical structures of these natural products, optimizing their roles in quorum sensing, antimicrobial activity, and modulation of the host immune response.

The goal of this thesis was to broaden our knowledge of bacterial quinolone biosynthesis, a process specific to only three bacterial families, making these metabolites rather unique. In our study, we aimed to predict a new class of bacterial quinolones, based on substrates formed within fatty acid biosynthesis in *P. aeruginosa*. We were also interested in the biological activity of discovered quinolones, including their role in bacteria-bacteria and bacteria-host interactions. Another aspect of our research aims to enhance our understanding of the functionalities associated with PQS, the main quorum sensing quinolone in *P. aeruginosa*. Finally, in collaboration with the Fetzner group, we aimed to fill the gaps in the methylated quinolone biosynthesis in *Burkholderia* spp. and obtain more information about the enzymes' kinetics.

Here we report a new class of hydroxylated 2-alkyl-4-quinolones, produced by *P. aeruginosa*. We could also clearly show that this hydroxy moiety comes from the fatty acid precursor, formed within fatty acid biosynthesis/ β -oxidation. Additionally, these new quinolones triggered a strong pro-inflammatory immune response even at nanomolar concentrations, indicating their potential involvement in *P. aeruginosa*-induced lung inflammation. Our second work reveals PQS's capacity to trap other metal ions, in addition to being a known iron trap, highlighting the crucial role of PQS in *P. aeruginosa*'s metal ion regulation. Our third project could finally demonstrate at which stage the methylation occurs in 3-methyl-2-alkyl-4-quinolone biosynthesis in *B. thailandensis*, and why quinolone-producing *Burkholderia* spp. preferably form quinolones with Δ^2 -unsaturation in fatty side chains.

Zusammenfassung

Insbesondere *Pseudomonas aeruginosa* und *Burkholderia*-Arten tragen zu chronischen Lungeninfektionen bei Mukoviszidose-Patienten bei und führen zu einer erheblichen Mortalität. Beide Bakterien produzieren eine Reihe von seltenen 2-Alkyl-4-chinolonen mit fein abgestimmten chemischen Strukturen dieser Naturstoffe, wodurch ihre Rolle beim Quorum Sensing, der antimikrobiellen Aktivität und der Modulation der Immunantwort des Wirts optimiert wird.

Das Ziel dieser Arbeit war es, unser Wissen über die Biosynthese von bakteriellen Chinolonen zu erweitern, einem Prozess, der nur für drei Bakterienfamilien spezifisch ist und daher diese Metaboliten einzigartig macht. Ein Ziel unserer Studie war es, eine neue Klasse bakterieller Chinolone vorherzusagen, die aus Substraten der Fettsäurebiosynthese in *P. aeruginosa* gebildet werden. Wir waren auch an der biologischen Aktivität der entdeckten Chinolone interessiert, einschließlich ihrer Rolle bei Bakterien-Bakterien- und Bakterien-Wirt-Interaktionen. Ein weiterer Aspekt unserer Forschung zielt darauf ab, unser Verständnis der Funktionalitäten zu verbessern, die mit PQS, dem wichtigsten Quorum-Sensing-Chinolon in *P. aeruginosa*, verbunden sind. Schließlich wollten wir in Zusammenarbeit mit der Fetzner-Gruppe die Lücken in der Biosynthese methylierter Chinolone in *Burkholderia* spp. schließen und weitere Informationen über die Kinetik der beteiligten Enzyme erhalten.

Hier berichten wir über eine bisher unbekannte Klasse hydroxylierter 2-Alkyl-4-chinolone, produziert von *P. aeruginosa*. Wir konnten auch klar zeigen, dass diese Hydroxylgruppe von der Fettsäurevorstufe stammt, die im Rahmen der Fettsäurebiosynthese/ β -Oxidation gebildet wird. Darüber hinaus lösten diese neuen Chinolone selbst bei nanomolaren Konzentrationen eine starke entzündungsfördernde Immunantwort aus, was auf ihre mögliche Beteiligung an der durch *P. aeruginosa* verursachten Lungenentzündung hinweist. In unserer zweiten Arbeit konnten wir zum ersten Mal zeigen, dass PQS die Fähigkeit besitzt, zusätzlich zu der bekannten Funktion als Eisenfalle auch andere Metallionen einzufangen, was die entscheidende Rolle von PQS bei der Metallionenregulation von *P. aeruginosa* unterstreicht. Unser drittes Projekt konnte letztendlich zeigen, auf welcher Stufe der Methylierungsschritt in der 3-Methyl-2-alkyl-4-chinolon-Biosynthese von *B. thailandensis* stattfindet und warum Chinolon produzierende *Burkholderia* spp. vorzugsweise Chinolone mit Δ^2 -ungesättigten Fettsäureresten bilden.

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List of Abbreviations

2'-OH-NQ - 2'-Hydroxy-2-nonyl-4-quinolone

2'-OH-NQNO - 2'-Hydroxy-2-nonyl-4-quinolone *N*-oxide

3-oxo-C12-HSL - *N*-(3-Oxo-dodecanoyl)-L-homoserine lactone

ACP - Acyl Carrier Protein

AHLs - Acylated Homoserine Lactones

AI - Autoinducers

AQ – 2-Alkyl-4-quinolone

AQNO - 2-Alkyl-4-quinolone *N*-oxide

BGCs - Biosynthetic Gene Clusters

C4-HSL - *N*-Butanoyl-L-homoserine lactone

C8-HSL - *N*-Octanoyl-L-homoserine lactone

°C - Degrees Celsius

DCM – Dichloromethane

Δ^1 -NQ - *trans*- Δ^1 -Unsaturated 2-nonyl-4-quinolone

Δ^1 -NQNO - *trans*- Δ^1 -Unsaturated 2-nonyl-4-quinolone *N*-oxide

Δ^2 -MAQ - *trans*- Δ^2 -Unsaturated 3-methyl-2-alkyl-4-quinolone

Δ^2 -MAQNO - *trans*- Δ^2 -Unsaturated 3-methyl-2-alkyl-4-quinolone *N*-oxide

Δ^2 -MHQ - *trans*- Δ^2 -Unsaturated 3-methyl-2-heptyl-4-quinolone

Δ^2 -MNQ - *trans*- Δ^2 -Unsaturated 3-methyl-2-nonyl-4-quinolone

DHQ - 2,4-Dihydroxyquinolone

EtOH - Ethanol

h - Hours

HAQ - 3-Hydroxy-2-alkyl-4-quinolone

HHQ - 2-Heptyl-4-quinolone

HQNO - 2-Heptyl-4-quinolone *N*-oxide

H₂O₂ - Hydrogen peroxide

KOH - Potassium hydroxide

LPS - Lipopolysaccharides

MAQ - 3-Methyl-2-alkyl-4-quinolone

MAQNO - 3-Methyl-2-alkyl-4-quinolone *N*-oxide

MHQ - 3-Methyl-2-heptyl-4-quinolone

***m*-CPBA** - *m*-Chloroperbenzoic acid

NQ - 2-nonyl-4-quinolone

OMVs - Outer Membrane Vesicles

o/n - Overnight

Ph₂O - Diphenyl ether

PQS - *Pseudomonas* quinolone signal

QS - Quorum Sensing

***t*-BuOK** - Potassium *tert*-butoxide

THF - Tetrahydrofuran

TsOH - *p*-Toluenesulfonic acid

1 Introduction

1.1 Quorum sensing (QS)

For many years, the dominant belief was that bacterium exists as an autonomous organism, living without the need for communication among the population. However, during the 1960s-1970s, pioneering studies from Tomasz and Hastings unveiled the concept of intercellular communication in a bacterial population.^[1-2] It was found that previously unknown "hormone-like activators" at specific concentrations altered gene expression, consequently coordinating the collective behavior of *Streptococcus pneumoniae* (*Pneumococcus*) at a population level.^[1] In another example, bioluminescent marine bacteria *Vibrio fischeri* and *Vibrio harveyi* demonstrated a unique pattern of light production. Interestingly, only when the bacterial cells were in high density and not in diluted cultures the luminescence was observed.^[2] In the 1990s, Bassler and Schaefer significantly contributed to our understanding of cell-to-cell communication, when the LuxI/LuxR system, a crucial component of microbial intercellular communication, was identified in *Vibrio fischeri*.^[3-4] These revolutionary studies opened the door for many other significant discoveries in the field of microbiology, ultimately leading to the characterization of the mechanism, now known as quorum sensing (QS).^[5]

Quorum Sensing (QS) is a mechanism built on a cell-density-based regulation of gene expression in bacterial communities. A cell density can be recognized by a bacterial population through the use of small chemical signals that it synthesizes. These small molecules are released by bacteria and accumulated in the environment while the population density increases. As these QS signals can induce their own biosynthesis, they are often called autoinducers (AIs). The accumulation of AIs to a critical concentration triggers the activation of various genes that regulate behaviors and activities at a population level.

In general, Gram-negative bacteria employ acylated homoserine lactones (AHLs) as their AIs, while Gram-positive bacteria typically rely on processed oligopeptides. AHLs consist of a lactone ring attached to an acyl side chain. Some AHLs are specific to a particular bacterial species and can vary in the length and structure of the acyl side chain. Unlike AHLs, which are small organic molecules, processed oligopeptides are produced from peptide precursors. The signaling peptide is initially

produced as an immature pro-peptide, which becomes functional only after the processing. This differentiation in AIs types is an important aspect of bacterial QS, highlighting the diversity and complexity of communication mechanisms in the microbial world. The specific AHL or oligopeptide used by a bacterium, as well as the genes and processes it regulates, can vary widely between different bacterial species and even strains, reflecting the adaptability of QS systems.^[6]

QS is crucial for the coordination of microbial behaviors, including virulence, overcoming the host's defense mechanisms, regulating the formation of biofilms, stress adaptation, and bacteria-bacteria interaction.^[7-9] An understanding of microbial QS is essential for developing strategies to combat bacterial infections by disrupting their communication — a potential target for novel anti-infectives.

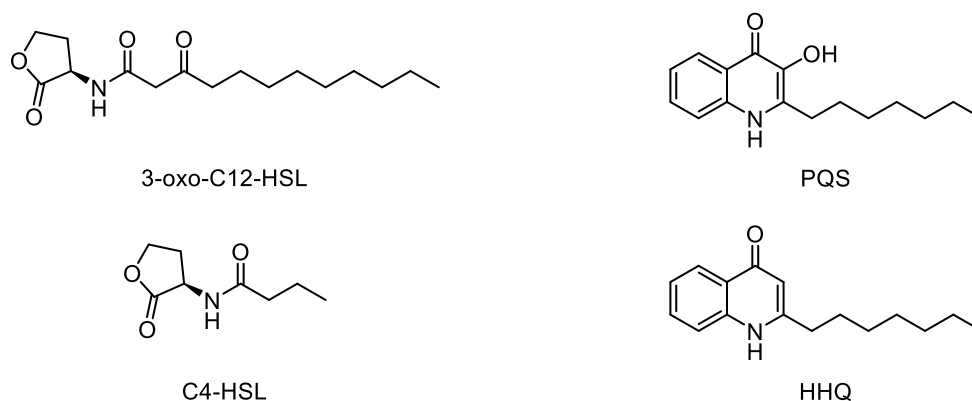
1.2 QS systems in *Pseudomonas aeruginosa* and *Burkholderia thailandensis*

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium known for its remarkable adaptability and virulence. It can be found in diverse environments, including soil, water, plants, animals, and humans. This pathogenic bacterium is commonly associated with a wide spectrum of serious human illnesses, for instance, conditions such as sepsis, skin infections, respiratory tract infections, as well as joint and bone infections.^[10-11] One of *P. aeruginosa*'s defining features is the QS systems it employs. This bacterium utilizes three QS systems: Las, Rhl, and Pqs. The first two are the most extensively studied QS systems in *P. aeruginosa* and use AHLs as QS signals (Figure 1a). The third one, Pqs, is rather unique, as it utilizes 2-alkyl-4-quinolones (AQs) as AIs (Figure 1a).

The Las QS system is controlled by the LasR transcriptional regulator. LasI, the AHL synthetase, is responsible for generating *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), which can bind to the cytoplasmic transcription activator LasR. Consequently, the expression of several genes gets activated, including those associated with the production of virulence factors, such as exotoxin A and elastase.^[12] The Rhl's signal molecule is *N*-butanoyl-L-homoserine lactone (C4-HSL), which binds to the RhlR transcriptional regulator, thus driving the expression of genes associated with other virulence factors, such as pyocyanin and hydrogen cyanide.^[13] The Pqs QS system relies on the production and detection of AQs, such as *Pseudo*-

monas quinolone signal (PQS) and its direct precursor 2-heptyl-4-quinolone (HHQ). PqsR receptor protein reacts to these QS signals, which results in the production of more AQS and the upregulation of several virulence factors. All three QS systems of *P. aeruginosa* are interconnected and work together to finely regulate the bacterial population behavior and expression of various secondary metabolites. The Las circuit primarily controls the initiation of infections, while Rhl comes into play during later stages, contributing to chronicity and biofilm formation. Remarkably, the Las system is the highest in the QS hierarchy, as it induces both Rhl and Pqs circuits, [14-17] whereas Rhl is able to control the Pqs QS system.[18]

a



b

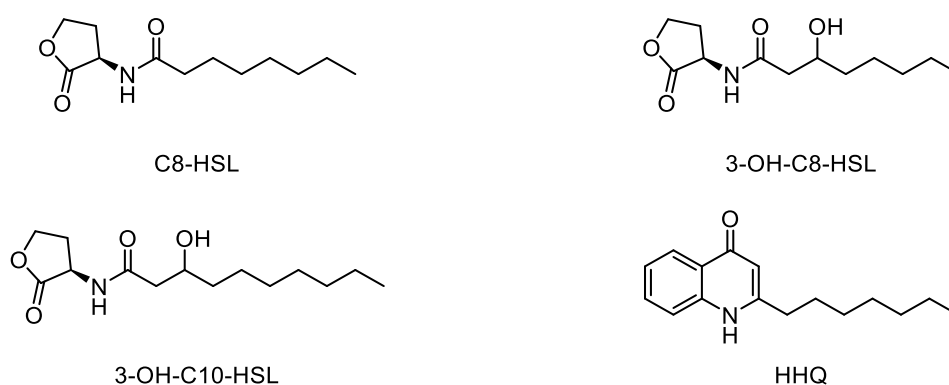


Figure 1. Quorum sensing (QS) signals of **a)** *P. aeruginosa* and **b)** *Burkholderia thailandensis*.

Burkholderia thailandensis is a Gram-negative bacterium, that is mostly found in soil. Due to its non-virulent nature, *B. thailandensis* is often employed as an alternative research model to the closely related human pathogen *Burkholderia pseudomallei*. *B. thailandensis* employs multiple QS systems, including three AHL-based (Bta1, Bta2, and Bta3) and one AQ-based (Hmq). The first three are canonical Lux-

type QS systems, that utilize *N*-octanoyl-L-homoserine lactone (C8-HSL), *N*-3-hydroxy-decanoyl-L-homoserine lactone (3-OH-C10-HSL), and *N*-3-hydroxy-octanoyl-L-homoserine lactone (3-OH-C8-HSL) signals, respectively (Figure 1b).^[19-21] *B. thailandensis* carries genes for the respective signals' synthases (btaI1, btaI2, and btaI3) as well as their transcriptional regulators BtaR1, BtaR2, and BtaR3. Notably, these QS circuits are also present in *B. pseudomallei*.^[22] The interplay between these three QS systems is very complex and is surprisingly growth-phase-dependent, as described in detail in the study by Guillouzer et al. from 2017.^[23] In contrast to *P. aeruginosa*, the AQ-based QS circuit of *B. thailandensis* and related species uses only the HHQ molecule as its AI (Figure 1b).^[24] This is due to the fact, that quinolone-producing species of the genus *Burkholderia* lack PqsH monooxygenase homolog, which would be able to oxidize HHQ into PQS.

1.3 2-Alkyl-4-quinolone (AQ) biosynthesis

The main signaling molecules of *P. aeruginosa*, PQS and HHQ, belong to the AQ family. The *pqsABCDE* operon, as well as *pqsH* and *pqsL* genes, are responsible for the AQ and 2-alkyl-4-quinolone *N*-oxide (AQNO) biosynthesis in this species (Figure 2b). This bacterium produces over 50 various quinolones, including 16 AQNOs.^[25] These secondary metabolites mostly vary in their side chains, including the length and degree of saturation at the C-2. While several AQ(NO)s exhibit anti-bacterial or immunomodulatory activity,^[26-29] the biological role of the majority of these quinolones remains unstudied.

The first step in the AQ biosynthesis is the anthranilate conversion into an activated anthraniloyl-Coenzyme A (anthraniloyl-CoA) by PqsA (anthranilate-CoA ligase) (Figure 2a).^[30-31] Anthranilate is initially activated with ATP/Mg²⁺, resulting in anthranilyl-AMP, which then undergoes thioesterification with CoA to yield anthraniloyl-CoA.^[32] Thus, the generation of anthranilate is crucial for the biosynthesis of all quinolones in *P. aeruginosa*. Interestingly, there are three known independent and non-redundant pathways for anthranilate biosynthesis in *P. aeruginosa*: *phnAB*, *trpEG*, and kynurenine pathway.^[30, 33-35] The *phnAB* operon is located directly downstream of the *pqsABCDE* and is positively regulated by PqsR (Figure 2b).^[16, 36] Notably, *phnAB* is predominantly expressed when a bacterial population reaches high density, indicating its regulation by QS.^[37] In the *trpEG* pathway, anthranilate synthase TrpEG con-

verts chorismate into anthranilate in the presence of L-glutamine, which is also the initial step in tryptophan biosynthesis in *P. aeruginosa*. In the kynurenine pathway of the anthranilate production, KynA and KynB enzymes convert tryptophan into kynurenine, which is subsequently converted into anthranilate by KynU via L-alanine cleavage.^[35]

Formed in the first step anthraniloyl-CoA is further converted with a condensing PqsD enzyme into 2-aminobenzoylacetate-CoA (2-ABA-CoA), in the reaction with malonyl-CoA (Figure 2a).^[38-39] Next, the PqsE thioesterase hydrolyzes 2-ABA-CoA into free 2-ABA.^[40] Interestingly, before the work by Drees and Fetzner,^[40] it was believed that PqsE does not participate in AQ(NO) biosynthesis in *P. aeruginosa*, as it was shown to act as a transcription factor for many virulence targets. Notably, a formation of 2,4-dihydroxyquinolone (DHQ) as a side product of quinolone biosynthesis is also observed, which can be non-enzymatically formed from 2-ABA-CoA or 2-ABA via spontaneous cyclization (Figure 2a). Moreover, $\Delta pqsE$ mutants of *P. aeruginosa* are shown to produce DHQ at higher concentrations compared to wild-type strains. 2-Aminoacetophenone (2-AA) can also be generated as a side product, by spontaneous decarboxylation of 2-ABA (Figure 2a). 2-AA is known to regulate PqsR through the negative feedback loop, promoting *P. aeruginosa* phenotypes favoring chronic lung infections.^[41]

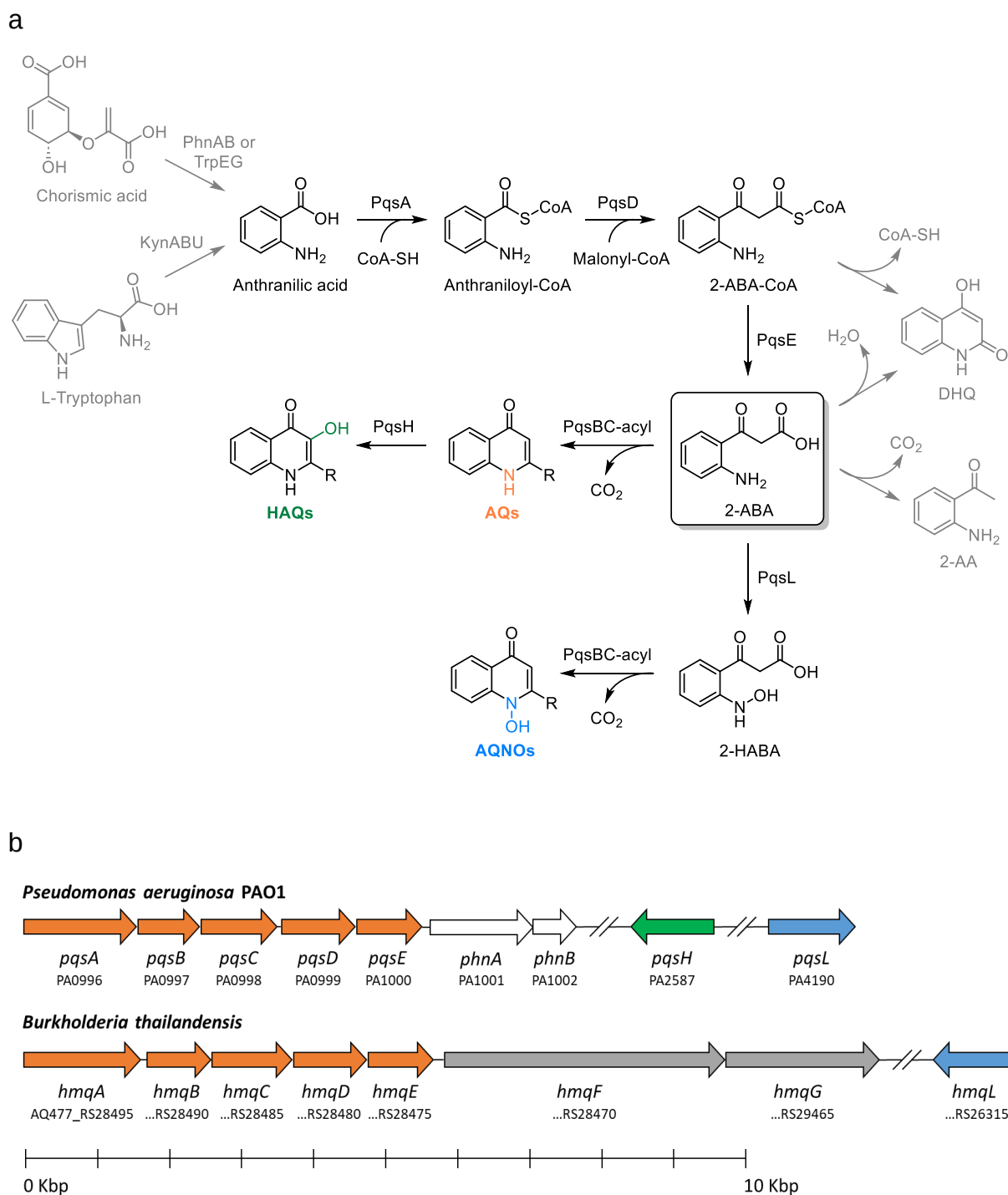


Figure 2. a) 2-Alkyl-4-quinolone (AQ) (orange), 2-alkyl-4-quinolone *N*-oxide (AQNO) (blue), and 3-hydroxy-2-alkyl-4-quinolone (HAQ) (green) biosynthesis in *P. aeruginosa*. **b)** Biosynthetic gene clusters (BGCs) responsible for enzymes involved in the production of quinolones and their *N*-oxides in *P. aeruginosa* and *B. thailandensis*.

2-ABA formation creates a branching point in AQ(NO) biosynthesis, leading to either AQs or AQNOs, additionally to 2-AA and DHQ side products, as discussed earlier. PqsBC is a heterodimeric protein complex that catalyzes a condensation reaction

between 2-ABA and CoA-activated fatty acids, resulting in the AQs formation.^[42-43] The PqsL enzyme, which shares similarities with a flavoprotein monooxygenase, catalyzes the conversion of 2-ABA to 2-HABA through oxidation. 2-HABA is then accepted as a substrate by PqsBC heterodimer and cyclized into AQNOs.^[44] Such PqsBC's ability to use diverse acyl-CoA substrates was explained due to the flexible regions around the acyl-CoA binding channel near active-site residues His₂₆₉-Cys₁₂₉ in PqsC.^[45]

This biosynthetic pathway produces AQs and AQNOs, but for 3-hydroxy-2-alkyl-4-quinolones (HAQs), such as the PQS signal, an extra oxidation step at the C-3 of the quinolone core is necessary (Figure 2a). PqsH monooxygenase serves as the catalyst for this final step, using NADH and oxygen.^[46] Exploration of substrate preferences for PqsH indicates that alkyl side chains of AQs play a key role, with the best activity noted for alkyl side chains with 7-9 carbons. Notably, because PqsH relies on oxygen, anaerobic *P. aeruginosa* is not able to produce PQS and virulence factors regulated by PQS.^[46]

Various biological functions of AQ(NO)s may be linked to the diversity of their alkyl side chains. This variation depends on fatty acids, available to be introduced into quinolone biosynthesis by PqsBC. These necessary fatty acids can originate from bacterial fatty acid biosynthesis, β -oxidation, or be consumed from a medium.^[42, 47-49]

Fatty acid biosynthesis is a fundamental metabolic pathway that plays a key role in both bacterial and eukaryotic organisms. Membrane phospholipids, lipoproteins, and lipoglycans depend on fatty acids, as well as QS molecules, including discussed quinolones. Fatty acids are generated in the cytoplasm, by adding two carbons at one cycle, a process that involves repetitive rounds of Claisen-type condensation reactions between the malonyl-ACP donor and an acyl acceptor (Figure 3a).^[50] The final product is usually a saturated fatty acid with 16 carbons (palmitic acid).

β -Oxidation is a metabolic process in bacteria that breaks down fatty acids to generate energy. It occurs in the cell's cytoplasm and is essential for maintaining an energy balance. During β -oxidation, fatty acids are enzymatically cleaved two carbons at once from the acyl-CoA molecules, producing acetyl-CoA (Figure 3b).^[51-52] These acetyl-CoA molecules are further processed through the citric acid cycle to yield ATP.

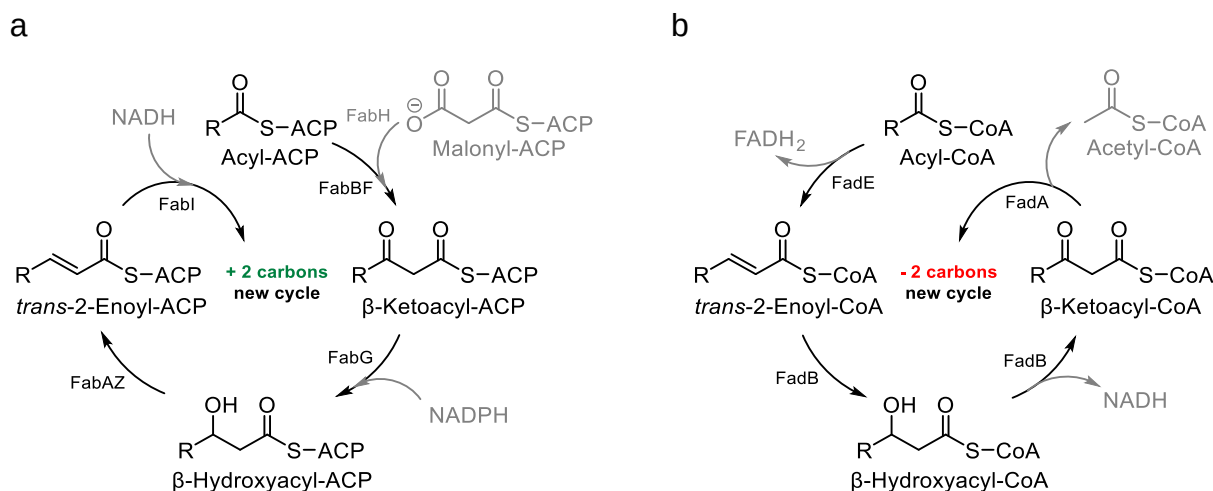


Figure 3. a) Fatty acid biosynthesis and **b)** fatty acid degradation (β -oxidation) in *Escherichia coli*.

AQ(NO)s with odd-numbered carbon atoms in their alkyl side chains are more common than even-numbered ones and, despite the range of fatty acid options, AQ(NO)s with 7-9 carbons are especially frequent, which gives a hint into the PqsBC fatty acid's preference.

The quinolone biosynthetic pathway in *B. thailandensis* closely resembles that of *P. aeruginosa*, with minor variations. For instance, compared to *P. aeruginosa*, *B. thailandensis* and related species preferably produce quinolones methylated at the position C-3: 3-methyl-2-alkyl-4-quinolones (MAQs) and their *N*-oxides (MAQNOs) (Figure 4). Moreover, while *P. aeruginosa* produces quinolones with saturated and *trans*- Δ^1 -unsaturated side chains, *Burkholderia* spp. prefers *trans*- Δ^2 -unsaturated MAQ(NO)s (Δ^2 -MAQ(NO)s), additionally to their saturated counterparts. This is due to the fact, that quinolone BGCs in *Burkholderia* spp. have two additional genes, *hmqF* and *hmqG*, immediately downstream of the *pqsABCDE* homolog, called *hmqABCDE* (Figure 2b). HmqF, consisting of three domains, plays a key role in de-saturating fatty acids at the C-3 position, leading to the generation of Δ^2 -MAQs and Δ^2 -MAQNOs.^[53] The HmqG enzyme, functioning as a methyltransferase, facilitates the synthesis of 3-methylated quinolones and their corresponding *N*-oxides within quinolone-producing *Burkholderia*. Interestingly, *B. thailandensis* and related species don't carry genes encoding for PhnA and PhnB homologs. Instead, it relies on the *trpEG* and kynurenine pathways to produce the required anthranilate.^[54]

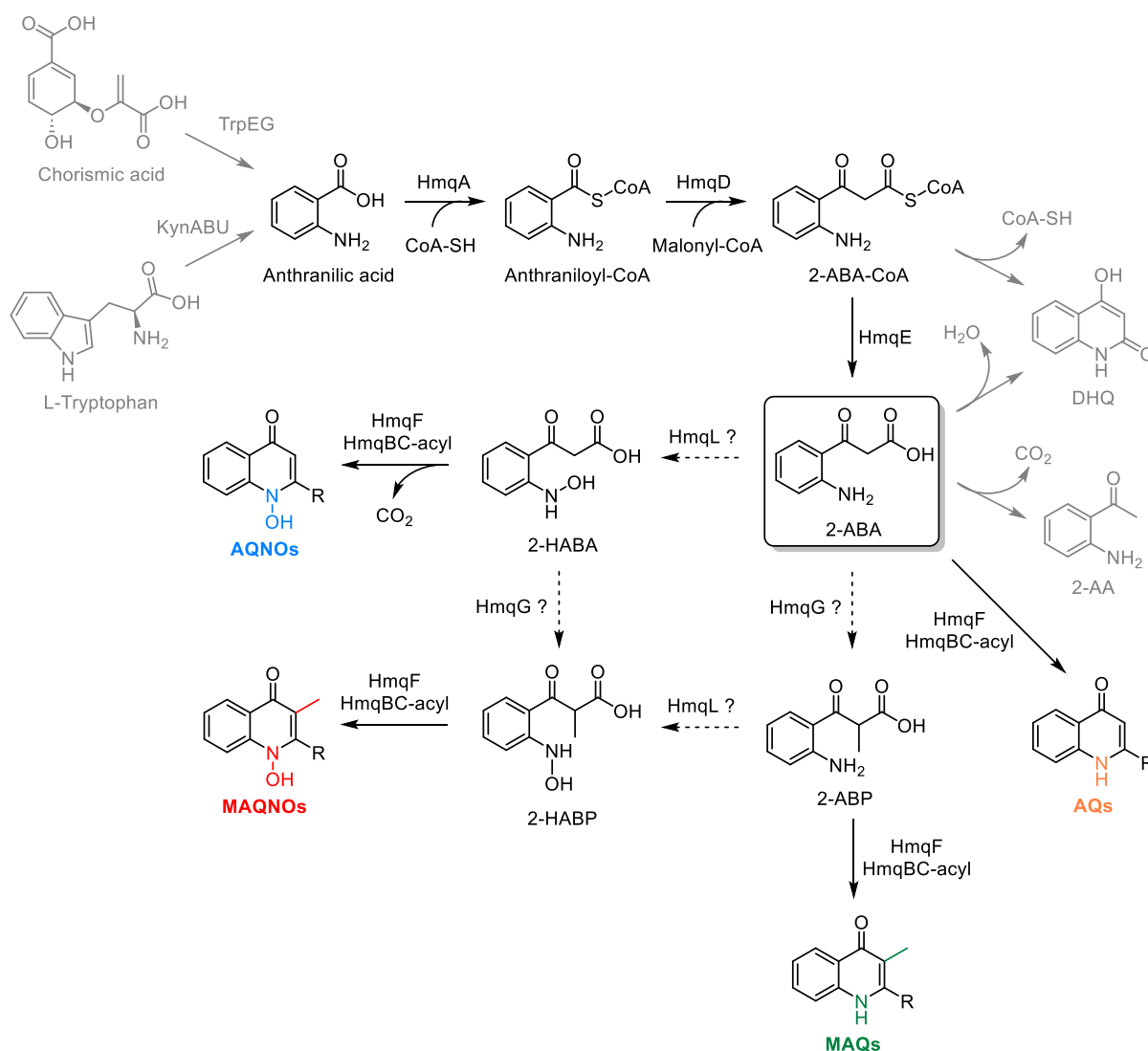


Figure 4. 2-Alkyl-4-quinolone (AQ) (orange), 2-alkyl-4-quinolone *N*-oxide (AQNO) (blue), 3-methyl-2-alkyl-4-quinolone (MAQ) (green), and 3-methyl-2-alkyl-4-quinolone *N*-oxide (MAQNO) (red) biosynthesis in *B. thailandensis*.

An inhibition of even one of the enzymes involved in quinolone biosynthesis in *P. aeruginosa* and *Burkholderia* spp. would lead to disturbed QS, thus decreasing bacterial virulence. A thorough examination of AQ(NO) biosynthesis and the associated enzymes opens the way for innovative treatment strategies against infectious diseases caused by *P. aeruginosa* and *Burkholderia* spp..

1.4 Quinolones' activity beyond QS

PQS and HHQ signals were shown to have multiple other biological functions in *P. aeruginosa* beyond QS. For example, Bredenbruch et al. found that adding PQS to *P. aeruginosa* PAO1 cultures upregulated genes related to oxidative stress response as well as made the bacteria more sensitive to H₂O₂-induced oxidative stress.^[55] Interestingly, a similar tendency was observed during ciprofloxacin antibiotic supplementation, when the PQS overproducing strain was more sensitive to this antibiotic compared to the PQS non-producing mutant.^[56] In the same study, it was demonstrated that the exogenous PQS led to a dose-dependent decrease in intracellular levels of reactive oxygen species. Consequently, it was determined that additionally to QS function, PQS serves as a primary antioxidant, exhibiting activity similar to ascorbate. Remarkably, HHQ does not show such antioxidant properties, highlighting the functional importance of the hydroxy group at the C-3 position of the quinolone core of PQS.

Respective HHQ's *N*-oxide, HQNO, was observed to induce autolysis in *P. aeruginosa*.^[57] HQNO-driven programmed cell death was linked to respiratory chain poisoning as a result of the generation of reactive oxygen species and damage to the cell membrane. This autolytic response was also found to promote biofilm formation and enhance antibiotic resistance in the remaining *P. aeruginosa* population.

The iron-trapping role of PQS has been extensively investigated in numerous studies. It was shown that PQS has strong affinity for iron, forming PQS-iron complexes in 2:1 and 3:1 ratios, as reported in studies by Bredenbruch and Diggle.^[55, 58] Notably, the exogenous addition of PQS into *P. aeruginosa* cultures was shown to deplete iron in the growth medium, proving its iron-trapping capabilities.^[55] Moreover, the supply of PQS triggers an iron starvation response in *P. aeruginosa*, promoting the production of siderophores and influencing the Rhl QS system.^[55, 58-59] Experiments indicate that PQS primarily acts as an iron trap, concentrating free iron ions at the cell envelope, rather than functioning as a classical siderophore.^[58, 60] As the hydroxy group of the PQS molecule is crucial for the iron trapping function, no such activity was observed for AQs and AQNOs, including the HHQ signal.

PQS molecule was also shown to play a key role in outer membrane vesicles (OMVs) formation. Nano-sized bilayer spherical vesicles, known as OMVs, are released from the Gram-negative bacteria's outer membrane. These OMVs are capa-

ble of transporting small molecules, proteins, lipids, lipopolysaccharides (LPS), and nucleic acids. As a QS signal, PQS must be released into the extracellular space. However, this process is challenging for hydrophobic QS signals like PQS. *P. aeruginosa* has overcome this challenge by packing PQS into OMVs.^[61-62] Interestingly, the majority of PQS (~86%) produced by *P. aeruginosa* is associated with OMVs.^[61] The same study demonstrates that OMVs from *P. aeruginosa* as well contain various other quinolones, such as the HHQ and NQ (2-nonyl-4-quinolone). OMVs production in *P. aeruginosa* is specifically linked to the presence of PQS. External supplementation of PQS at physiological concentrations can restore OMVs formation in a $\Delta pqsH$ mutant (PQS non-producing), indicating the direct role of PQS in OMVs biogenesis.^[61] Moreover, it was discovered that PQS exhibits a strong interaction with acyl chains and the 4'-phosphate of bacterial LPS.^[62] This leads to the initiation of a membrane curvature. The mechanism of OMVs biogenesis via PQS is proposed to involve an asymmetric outer leaflet's expansion of the outer membrane, leading to its curvature, until an OMV can be secreted from the surface.^[63]

P. aeruginosa's quinolones are also studied for their role in bacteria-bacteria interaction. Numerous studies explore QS signals and related quinolones as powerful tools used by *P. aeruginosa* in its competition against other bacteria. For example, 10 μ M PQS inhibits motility in gram-positive bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes* without affecting their growth rates.^[64] They also found that HHQ, at the same concentration, specifically targets *Algoriphagus* sp., *Vibrio cholerae*, and *Vibrio fischeri* without impacting the growth of other gram-negative bacteria, like *E. coli*, *Serratia* sp., and *Vibrio parahaemolyticus*. In-depth investigation expanded the analysis of HHQ's species-specific antibacterial effects by subjecting multiple strains of *Aliivibrio* and *Vibrio* spp. to 10 μ M HHQ on agar plates.^[65] This revealed four distinct outcomes, including complete growth inhibition (observed in *Vibrio gallaecicus* and various *Aliivibrio fischeri* strains) and examples of HHQ resistance (evident in *V. parahaemolyticus*, *Verbena litoralis*, and *Vibrio* sp. 1.19).

Since the 1950s, the strong antibacterial properties of AQNOs have been consistently drawing attention.^[66] Lightbown and Jackson were the first to report that HQNO effectively inhibits bacterial cytochrome systems at micromolar concentrations.^[66] Later, the activity of HQNO against the standard strain of *S. aureus* was demonstrated.^[67] In a subsequent investigation, Hoffman et al. explored the antistaphylococcal effects of HQNO. They achieved this by co-culturing *S. aureus* with various strains of *P. aeru-*

ginosa.^[68] Their findings revealed that long exposure of *S. aureus* strains to physiologically relevant concentrations of HQNO led to the dominance of small-colony variants within the *S. aureus* population. Furthermore, our research group demonstrated that Δ^1 -NQNO (*trans*- Δ^1 -unsaturated 2-nonyl-4-quinolone *N*-oxide), an abundant quinolone *N*-oxide synthesized by *P. aeruginosa*, displays exceptional activity against *S. aureus*. Additionally, it prompts the formation of small-colony variants, suppresses respiration and the production of virulence factors in *S. aureus*.^[28]

Studies indicate that *P. aeruginosa*'s quinolones can interact in diverse ways with the human immune system. These secondary metabolites can either trigger or suppress immune responses when the pathogen invades. PQS is frequently identified in sputum samples from cystic fibrosis patients infected with *P. aeruginosa*, showing a strong link to a lung damage severity. This might be explained by PQS's ability to induce reactive oxygen species in lung epithelial and macrophage cell lines, prompting early apoptosis in A549 epithelial cells.^[69] Moreover, PQS has been known to reduce the production of HIF-1, a key transcription factor involved in activating the innate pro-inflammatory response.^[70]

Methylated quinolones as well exhibit various biological activities beyond QS. For instance, Δ^2 -MHQ (*trans*- Δ^2 -unsaturated 3-methyl-2-heptyl-4-quinolone) and Δ^2 -MNQ (*trans*- Δ^2 -unsaturated 3-methyl-2-nonyl-4-quinolone) show antifungal activity against fungal plant pathogens, like *Phytophthora capsica*.^[71] Another study demonstrated that Δ^2 -MHQ, Δ^2 -MNQ, and saturated MHQ exhibited an inhibition effect on the growth of *Tenacibaculum maritimum*, the bacterium responsible for skin ulcers in marine fish.^[72]

1.5 Known strategies of AQ and AQNO synthesis

1.5.1 AQ synthesis

4-Quinolones, originating from quinolines, are organic compounds that coexist in equilibrium with 4-hydroxyquinolines, which are their minor tautomeric forms (Figure 5).^[73] The pH usually dictates the dominance of one or another form.

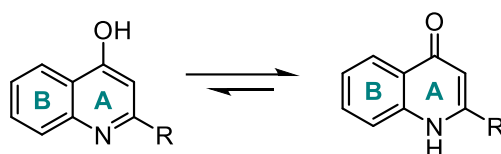


Figure 5. The tautomeric equilibrium between 4-hydroxyquinoline (left) and 4-quinolone (right), with labeled rings (green).

Several techniques have been established to synthesize AQs, typically involving the formation of an activated substrate followed by the cyclization of the A-ring. Such cyclizations often require high temperatures or the use of potent bases.

Wells in 1952 reported the first synthesis of HHQ, NQ (2-nonyl-4-quinolone), and Δ^1 -NQ (*trans*- Δ^1 -unsaturated 2-nonyl-4-quinolone),^[74] which back then were called Pyo Ib/c and Pyo III and were isolated from *P. aeruginosa*. The method employed, known as the Conrad-Limpach reaction using β -ketoesters and aniline, remains the most widely used approach for synthesizing AQs.^[28, 75]

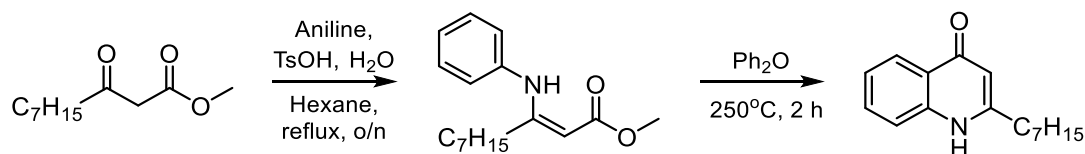


Figure 6. Conrad-Limpach reaction optimized for the synthesis of HHQ.

Conrad-Limpach reaction is known since 1887.^[76] This reaction is based on the thermally triggered shift between keto- and enol-forms within a carboxylate ester originating from a Schiff base. It proceeds with a cyclization of the resulting enamine

tautomer, typically at temperatures nearing 250°C, within solvents like diphenyl ether or biphenyl. Notably, the accessibility of starting materials — anilines and β -ketoesters — coupled with the lack of strong bases or acids in the synthesis, enables the reaction's applicability across a wide spectrum of derivatives. These final compounds are obtained in high yields via precipitation.

Another similar and widely used technique in producing 4-quinolones is the Gould–Jacobs reaction.^[77] This method utilizes anilinomethylene malonates that, when subjected to harsh conditions, typically using diphenyl ether or Dowtherm A under elevated temperatures, undergo cyclization to yield the 4-quinolones (Figure 7).

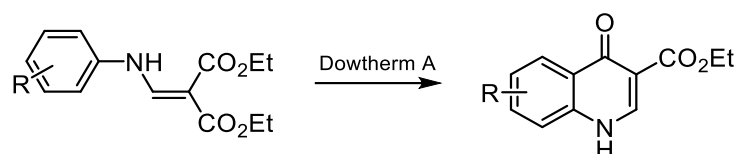


Figure 7. Gould–Jacobs quinolone synthesis.

The next method utilizes 2'-amino-chalcones as starting material, easily synthesized through an aldol condensation from 2'-amino-acetophenones and aldehydes. Various methodologies are available for this cyclization, often employing an acid-catalyzed intramolecular aza-Michael reaction, resulting in 2-alkyl-2,3-dihydro-4-quinolones. These intermediates can be further transformed into 2-alkyl-4-quinolones through oxidation using iodine or diacetoxyiodobenzene (Figure 8).^[78-79]

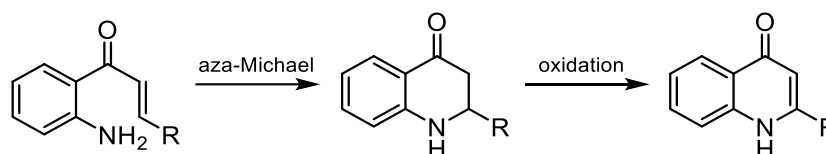


Figure 8. Simplified scheme for the 4-quinolone synthesis from 2'-amino-chalcones, using aza-Michael reaction, with subsequent oxidation.

Another important strategy is known as the Niementowski reaction.^[80-81] This process utilizes anthranilic acid and either ketones or aldehydes at elevated temperatures (120-130°C). This reaction generates 4-quinolones through the formation of a

Schiff base, which subsequently undergoes an intramolecular condensation reaction to yield the desired compounds (Figure 9).

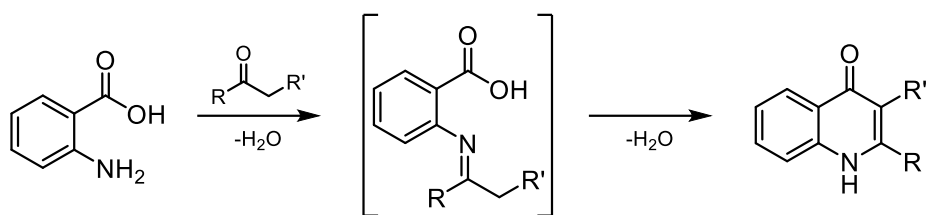


Figure 9. Niementowski reaction for the 4-quinolone synthesis from anthranilic acid.

The Camps quinolone synthesis, alternatively referred to as the Camps cyclization, is one of the most famous reactions for quinolone formation.^[82-83] This chemical process requires harsh reaction conditions that include strong bases and high temperatures, and it converts *o*-acylaminoacetophenones into two types of quinolones (4-quinolones and 2-quinolones) (Figure 10). The ratio of these quinolones relies on both the reaction conditions and the nature of R and R' substituents.

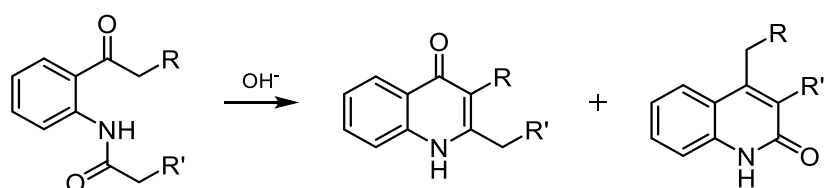
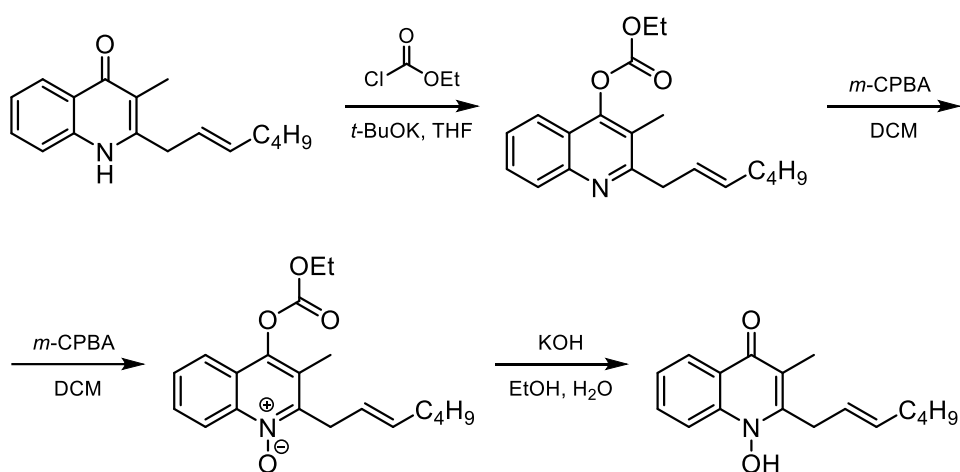


Figure 10. The Camps quinolone synthesis for either 4-quinolones or 2-quinolones.

1.5.2 AQNO synthesis

The synthesis of AQNOs can be achieved through the oxidation of AQs to form the corresponding *N*-oxides. This oxidation process can be carried out using peroxy reagents, such as *m*-chloroperbenzoic acid (*m*-CPBA),^[84] perbenzoic acid,^[85] and bis(*N,N*-dimethylformamide- κ O)oxodiperoxymolybdenumand (Figure 11).^[86] Notably, the first synthesis of natural AQNOs from *P. aeruginosa* was described already in 1956, using perbenzoic acid as an oxidant.^[85]

a



b

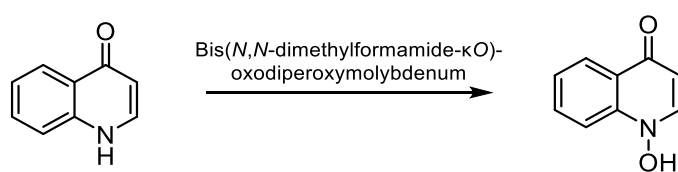


Figure 11. 4-Quinolone *N*-oxide synthesis using **a)** *m*-CPBA and **b)** bis(*N,N*-dimethylformamide- κ O)oxodiperoxymolybdenumand as oxidants.

2 Publications

- **Publication 1.** Savchenko, V., Szamosvári, D., Bao, Y., Pignitter, M., Böttcher, T. Biosynthetic flexibility of *Pseudomonas aeruginosa* leads to hydroxylated 2-alkylquinolones with proinflammatory host response. Commun. Chem. 6, 138 (2023). <https://doi.org/10.1038/s42004-023-00937-y> (**Published**).

V.S. contributed in designing the project and experiments, synthesized all standard AQNO compounds, established LC–MS/MS methodologies, and conducted all experimental measurements with subsequent data analysis. V.S. was involved in the manuscript writing.

- **Publication 2.** Szamosvári, D., Savchenko, V., Badouin, N., Böttcher, T. Beyond iron: metal-binding activity of the *Pseudomonas* quinolone signal-motif. Org. Biomol. Chem. 21, 5158-5163 (2023). <https://doi.org/10.1039/D3OB00710C> (**Published**).

V.S. was involved in designing the experiments and performed the identification of PQS-metal ion complexes via HRMS.

- **Publication 3.** Savchenko, V., Jaegers, M., Herrmann, E., König, S., Kümmel, D., Böttcher, T., Fetzner, S., Ernst, S. Unraveling key steps in the biosynthesis of antimicrobial methylated unsaturated 2-alkyl-4-quinolones of *Burkholderia thailandensis*. Cell Reports Physical Science. 5, 102100 (2024). <https://doi.org/10.1016/j.xcrp.2024.102100> (**Published**).

V.S. designed the synthetic methodology and conducted chemical synthesis, NMR, small-molecule HR-MS, and MS/MS analysis with subsequent data analysis. V.S. was involved in the manuscript writing.

2.1 Publication 1. Manuscript

communications chemistry

ARTICLE



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OPEN

Biosynthetic flexibility of *Pseudomonas aeruginosa* leads to hydroxylated 2-alkylquinolones with proinflammatory host response

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The human pathogen *Pseudomonas aeruginosa* produces various 4(1H)-quinolones with diverse functions. Among these, 2-nonyl-4(1H)-quinolone (NQ) and its N-oxide (NQNO) belong to the main metabolites. Their biosynthesis involves substrates from the fatty acid metabolism and we hypothesized that oxidized fatty acids could be responsible for a so far undetected class of metabolites. We developed a divergent synthesis strategy for 2'-hydroxy (2'-OH) and 2'-oxo- substituted quinolones and N-oxides and demonstrated for the first time that 2'-OH-NQ and 2'-OH-NQNO but not the corresponding 2'-oxo compounds are naturally produced by PAO1 and PA14 strains of *P. aeruginosa*. The main metabolite 2'-OH-NQ is produced even in concentrations comparable to NQ. Exogenous availability of β -hydroxydecanoic acid can further increase the production of 2'-OH-NQ. In contrast to NQ, 2'-OH-NQ potently induced the cytokine IL-8 in a human cell line at 100 nM, suggesting a potential role in host immune modulation.

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The compound classes of 2-alkyl-4(1H)-quinolones (AQs) and 2-alkyl-4(1H)-quinolone *N*-oxides (AQNOs) are produced most notably by the opportunistic human pathogen *Pseudomonas aeruginosa*, which is one of the major pathogens involved in the infection of the lungs of cystic fibrosis patients and other body sites^{1–3}. While AQs, like 2-heptyl-4-hydroxyquinoline (HHQ), are known to act as quorum sensing molecules in *P. aeruginosa*^{4,5}, AQNOs were found to exhibit a profound antibiotic activity against Gram-positive bacteria like *Staphylococcus aureus* including MRSA with the *trans*-unsaturated 2-nonenyl-4(1H)-quinolone *N*-oxide (Δ^1 -NQNO) as its most potent member⁶. AQs and AQNOs are characterized by a great diversity in length and saturation of their alkyl side chains, which may correlate with functional specialization in their biological activities. In *P. aeruginosa* alone, over 50 different 2-alkyl-4(1H)-quinolone compounds were already identified⁷. This variety is likely a result of the rather broad substrate specificity of the β -ketoacyl-(acyl-carrier-protein) synthase III (FabH)-like enzymes PqsB and PqsC. The heterodimeric PqsBC complex uses CoA-activated fatty acids to catalyze a decarboxylative Claisen-condensation of acyl-ACP and 2-aminobenzoylacetate (2-ABA) or 2-hydroxy-aminobenzoylacetate (2-HABA) to form AQs or AQNOs, respectively^{8,9}. The fatty acids used in the biosynthesis of AQs and AQNOs may be provided by the bacterial fatty acid synthesis, β -oxidation, or be utilized from the medium (Fig. 1). Surprisingly, only saturated and unsaturated AQs and AQNOs have been unambiguously identified so far^{6,7}. We here aim to investigate if β -hydroxy fatty acids and β -oxo fatty acids of the fatty acid metabolism could also be utilized as substrates for the AQ and AQNO biosynthesis and potentially yield 2'-hydroxy-2-alkyl-4(1H)-quinolones (2'-OH-AQs) and 2'-oxo-2-alkyl-4(1H)-quinolones (2'-oxo-AQs) as well as their corresponding *N*-oxides (Fig. 1c, d). The discovery of these molecules would expand our understanding of the molecular arsenal of *P. aeruginosa* and allow to investigate their function in microbial competition and microbe host interactions.

In this work, we have generated synthetic standards confirming that 2'-OH-NQ and 2'-OH-NQNO but not the 2'-oxo compounds are produced by *P. aeruginosa*. 2'-OH-NQ was among the major 4(1H)-quinolones of *P. aeruginosa* and, in contrast to NQ, caused a potent stimulation of cytokine IL-8 in an epithelial cell line.

Results and discussion

Synthesis of quinolone standards. In order to investigate if 2'-OH-NQ and 2'-oxo-NQ and their corresponding *N*-oxides are produced by *P. aeruginosa*, we first aimed to prepare synthetic standards of these compounds. Several well established methods are known for the synthesis of saturated and unsaturated 2-alkyl

and 2-alkenyl-4(1H)-quinolones and their *N*-oxides¹⁰. However, the key step of the cyclization requires relatively harsh conditions, which are not compatible with more sensitive functional groups. We thus aimed for a strategy to directly generate functionalized AQs and AQNOs. Sicker et al. reported 1987 the cyclization of 2'-nitrocinnamoyl derivatives to 2-methyl-4(1H)-quinolone *N*-oxides through a Pt(OH)₂ catalyzed hydrogenation reaction under acidic conditions at room temperature¹¹. In 2001, Jung et al. used a similar nitro-substrate for the reductive cyclization with Pd/C under neutral conditions to obtain 2-substituted 3-carboxyethyl-4(1H)-quinolone *N*-oxides¹². Inspired by these earlier works, we developed a divergent reductive cyclization that allows the controlled synthesis of AQs or AQNOs, depending on the choice of catalyst and reaction time. Its mild reaction conditions appeared highly suitable for the synthesis of our more functionalized target compounds 1g, 1h, 2g and 2h.

We started our synthesis by using methyl 3-oxodecanoate as common precursor whose keto group was either reduced with NaBH₄ and subsequently protected with TBS (1a) or directly protected as ketal (2a) (Fig. 2). After reduction of the methyl ester group with DIBAL-H, the protected decanal derivatives 1b and 2b were used in a Lewis-acid catalyzed Mukaiyama aldol-type reaction¹³ with 2'-nitroacetophenone to obtain the key intermediates 1c and 2c in good yields. To allow later cyclization, compounds 1c and 2c were oxidized to their respective β -ketoenol compounds 1d and 2d via Dess-Martin oxidation. Other oxidation reagents like Jones reagent or IBX failed to provide the desired compounds. With 1d and 2d in hand, we performed the cyclization to their respective 4(1H)-quinolones and 4(1H)-quinolone *N*-oxides under reductive conditions at neutral pH. Using Pd/C as catalyst, we observed the cyclization of 1d and 2d to the protected 4(1H)-quinolones 1e and 2e after 24 h. Shorter reaction times gave significantly less 4(1H)-quinolones with equal amounts of 4(1H)-quinolone *N*-oxides. Surprisingly, when using Pt/C for 2–3 h, exclusively 4(1H)-quinolone *N*-oxides 1f and 2f were obtained without simultaneous production of the respective 4(1H)-quinolones. Longer reaction times led to complex mixtures of products with 4(1H)-quinolones as side products complicating later separation. Finally, deprotection with TBAF (for 1e and 1f) or HCl (for 2e and 2f) gave the desired 2'-OH-NQ (1g), 2'-OH-NQNO (1h), 2'-oxo-NQ (2g) and 2'-oxo-NQNO (2h) in high purity after column chromatography or HPLC.

Qualitative and quantitative analysis of metabolites produced by *Pseudomonas aeruginosa*. We subsequently used the synthesized quinolones as standards for the identification and quantification of 2'-hydroxy and 2'-oxo-4(1H)-quinolones and their *N*-oxides using LC-MS/MS (Fig. 3 and Table S3). Remarkably, the MS² fragmentation pattern of the quasi-molecular ions of 2'-OH-

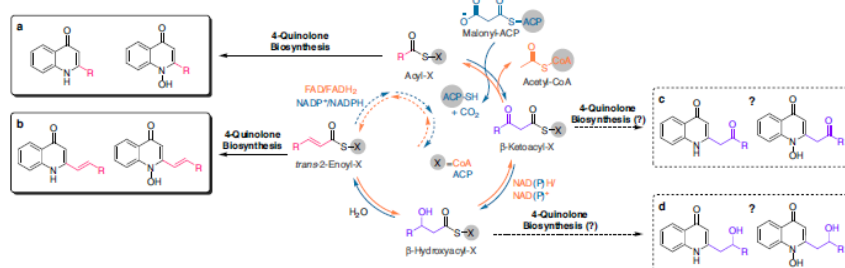


Fig. 1 Fatty acid biosynthesis (cyan arrows) and fatty acid β -oxidation (orange arrows) in *P. aeruginosa* with known (black arrows) and hypothetical (dashed black arrows) substrate incorporation in 4-quinolone biosynthesis. **a** 2-alkyl-4(1H)-quinolones (AQs) and 2-alkyl-4(1H)-quinolone *N*-oxides (AQNOs); **b** Δ^1 -AQs and Δ^1 -AQNOs; **c** 2'-oxo-AQs and 2'-oxo-AQNOs; **d** 2'-OH-AQs and 2'-OH-AQNOs.

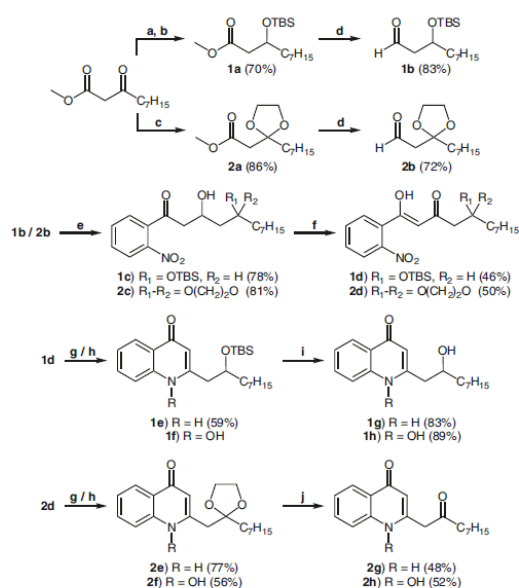


Fig. 2 Synthesis of 2'-OH-NQ (1g), 2'-oxo-NQ (2g), and their

corresponding *N*-oxides (1h and 2h). Reaction conditions: **a** NaBH₄, MeOH, rt, 1 h; **b** TBS-Cl, imidazole, DMF, 0 °C, o/n; **c** trimethylorthoformate, pTsOH, ethylene glycol, rt, 5 h; **d** DIBAL-H, DCM/toluene, -78 °C, 2 h; **e** 2-nitroacetophenone, Bu₂BOTf, DIPEA, DCM, 40 min at 0 °C, 30 min at rt, 30 min at -78 °C, 2.5 h at 0 °C; **f** DMP, DCM, 1 h at 0 °C, 4 h at rt; **g** H₂, 10% Pd/C, EtOH, rt, 24 h; **h** H₂, 10% Pt/C, EtOH, rt, 3 h; **i** TBAF, THF, rt, 30 min; **j** 6 M HCl aq./THF (1:1), rt, 24 h.

NQ and 2'-OH-NQNO showed a unique fragment $[M + H - 18]^+$, which is most likely due to the elimination of water from the 2'-hydroxyalkyl side chain (Fig. 3a, b). Interestingly, all other detected fragments are typical for Δ^1 -unsaturated AQs and their *N*-oxides^{7,14}, which supports the proposed structure of the unique $[M + H - 18]^+$ fragment.

While 2'-OH-NQ has the same parental mass as previously detected NQNO, our LC-MS/MS method allows us to confidently distinguish between these two isomeric quinolones via significantly different MS² fragmentation and retention time (RT) (Table S3a, b). Moreover, we could exclude the possibility of the detection of isomeric quinolones with the different position of the hydroxylation in the side chain, as not only RT of natively produced quinolones fit our synthetic standards, but also the fragmentation pattern precisely matches (including fragment ions distribution) (Fig. 3a, b and Fig. 3e, f, see Supplementary Information MS² Assignments).

In order to investigate the native production of the new quinolones via their retention times and unique fragmentation fingerprints, we selected *P. aeruginosa* strains PAO1 and PA14 and analyzed extracted culture supernatants. Additionally, we investigated culture supernatants of *Burkholderia thailandensis* and *Burkholderia ambifaria* AMMD, which are known to produce a large variety of 2-alkyl-4(1*H*)-quinolones^{14,15}. Indeed, both strains of *P. aeruginosa* produced 2'-OH-NQ and 2'-OH-NQNO (Fig. 3e, f). In contrast, 2'-oxo-NQ and corresponding *N*-oxide were not detected at any time point during 24 h of incubation (Tables S8 and S9). Interestingly, none of the new quinolones were found in the two *Burkholderia* strains at any time or growth condition tested (Tables S10-S13). These results indicate that at least *P. aeruginosa* is able to produce the compound classes of 2'-hydroxy-4(1*H*)-quinolones and their *N*-

oxides but not the corresponding 2'-oxo classes. In order to quantify the detected quinolones, mass transitions of the most intense or unique ions of the MS² spectra were selected. External calibration was performed using concentration series of standard compounds (Table S2). *P. aeruginosa* strains produced 2'-OH-NQ and 2'-OH-NQNO while grown for 3-24 h, peaking at 12 h of incubation (Figs. 3g, h, S1, S2, and Tables S4, S5). NQ and NQNO were also quantified in this experiment for direct comparison with production levels of new quinolones (Tables S6 and S7). In both *P. aeruginosa* PAO1 and PA14 strains, 2'-OH-NQ was produced in higher concentrations (max. 0.55 mg L⁻¹ or 1.9 μ M) than the corresponding *N*-oxide (max. 0.08 mg L⁻¹ or 0.3 μ M), while maximum NQ and NQNO levels were 0.63 mg L⁻¹ and 3.1 mg L⁻¹, respectively.

Insights into substrate utilization and biosynthesis of hydroxylated quinolones. To test if exogenous addition of hydroxylated fatty acid can boost the production of hydroxylated quinolones, we performed a feeding experiment with synthetic β -hydroxydecanoic acid, the proposed biosynthetic precursor of 2'-OH-NQ and 2'-OH-NQNO. Native levels of 2'-OH-NQ and 2'-OH-NQNO production in *P. aeruginosa* PAO1 were quantified by LC-MS/MS and compared to those after the fatty acid feeding. Changes in NQNO levels were analyzed as well for comparison (Fig. 4).

Remarkably, the external addition of β -hydroxydecanoic acid to *P. aeruginosa* PAO1 cultures led to a significant boost in 2'-OH-NQ and 2'-OH-NQNO production (Fig. 4b, c, S5a-S6a, and Table S15). 2'-OH-NQ concentrations increased 5.8-fold from native 0.26 mg L⁻¹ (0.9 μ M) up to 1.51 mg L⁻¹ (5.3 μ M) after feeding 50 μ M of the fatty acid, and for 2'-OH-NQNO 5.5-fold from 0.17 mg L⁻¹ (0.6 μ M) up to 0.93 mg L⁻¹ (3.2 μ M). These results demonstrate that the production of 2'-OH-NQ and 2'-OH-NQNO can be strongly increased by exogenic supplementation of β -hydroxydecanoic acid. Feeding higher concentrations of β -hydroxydecanoic acid, however, did not further increase production levels of 2'-OH-NQ and 2'-OH-NQNO. In contrast, NQNO production considerably decreased by β -hydroxydecanoic acid supplementation (Fig. S7a). This may be explained by substrate competition of added β -hydroxydecanoic acid with native decanoic acid for utilization in quinolone biosynthesis by the enzyme complex PqsBC. Strikingly, feeding of synthetic β -oxodecanoic acid to *P. aeruginosa* PAO1 did not lead to a detectable production of 2'-oxo-NQ and 2'-oxo-NQNO, which suggests that direct incorporation of β -oxo fatty acids in 4-quinolone biosynthesis is not possible. However, as β -oxodecanoic acid is a precursor of β -hydroxydecanoic acid in the fatty acid biosynthesis, we still observed a β -oxodecanoic acid dependent increase in the concentrations of 2'-OH-NQ and 2'-OH-NQNO (Figs. S5b and S6b). Surprisingly, NQNO concentration was nearly two times decreased after β -oxodecanoic acid addition and remained unaffected with the increase of fatty acid concentration (Fig. S7b). To investigate the possibility of a direct incorporation of β -hydroxydecanoic acid, we performed an additional feeding experiment in *P. aeruginosa* PAO1 with 100 μ M of the deuterated analog β -hydroxydecanoic acid-d (3d). LC-MS/MS analysis of the $[M + 1 \text{ Da} + H]^+$ signal for 2'-OH-NQ and 2'-OH-NQNO revealed a 4.2-fold and 4.6-fold increase in production of the parent mass + 1 Da, respectively, compared to the DMSO control (Figs. 4d, e, S12, and Table S14) suggesting that hydroxylated fatty acids can be utilized as direct substrates of the quinolone biosynthesis machinery.

Investigation of biological activities. Some quinolone *N*-oxides exhibit strong antimicrobial effects^{6,14} and we were thus

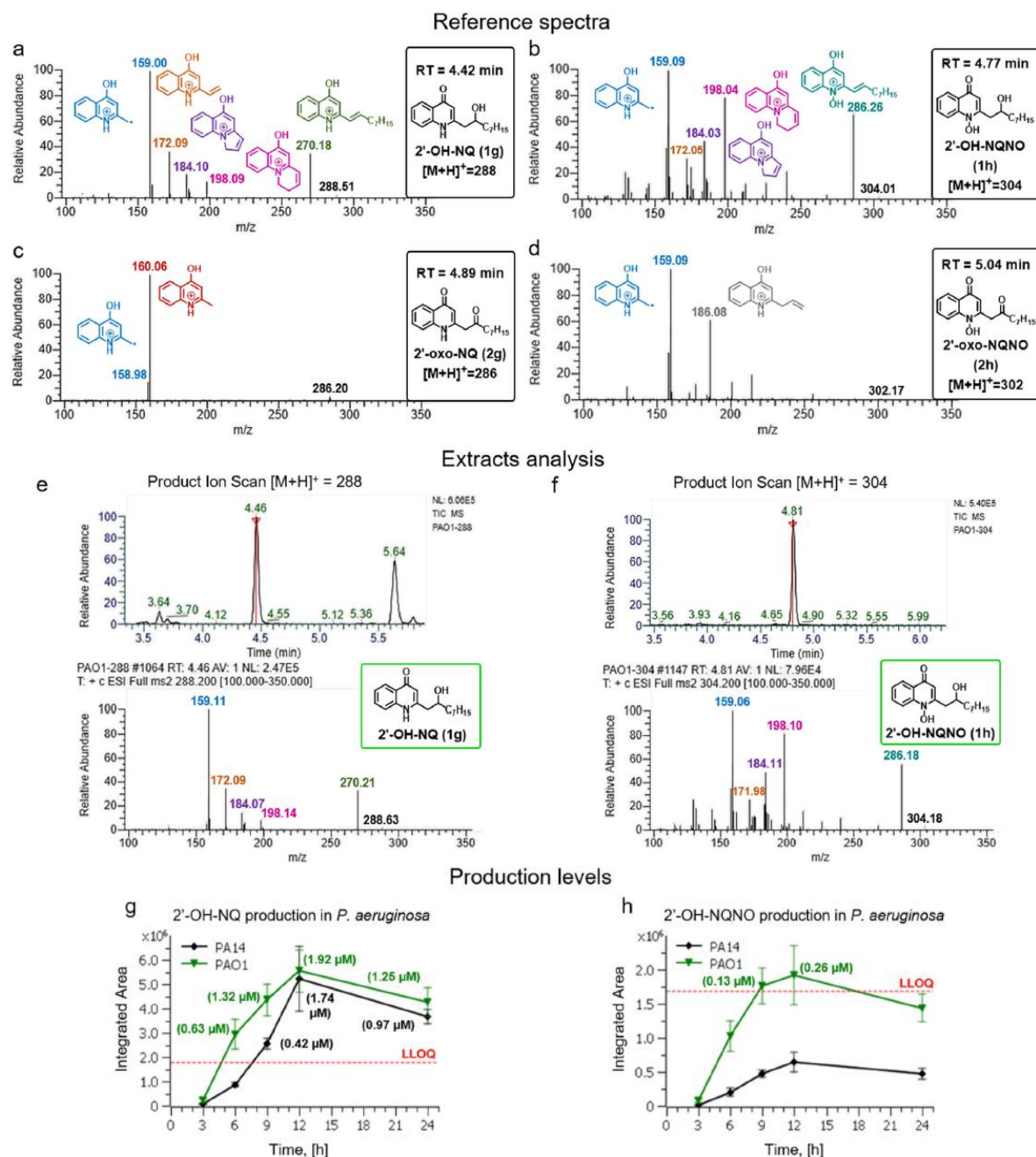


Fig. 3 MS² reference spectra with fragmentation of parent ions (product ion scan) of synthesized quinolones and analysis of extracted culture supernatants of *P. aeruginosa*. **a–d** MS² fragmentation pattern and proposed structures of fragment ions of synthetic standards. **e, f** MS² spectra of detected 2'-OH-NQ and 2'-OH-NQNO in extracts of *P. aeruginosa* culture supernatants. **g, h** Integrated area of monitored mass transitions of 2'-OH-NQ and 2'-OH-NQNO produced by *P. aeruginosa* PA14 (black) and *P. aeruginosa* PAO1 (green) during 24 h of incubation. Quantified concentrations are shown in brackets. LLOQ lower limit of quantification. Mean values are given for biological triplicates with the error bars representing the population standard deviation.

interested to examine the antibiotic activity of the new quinolones 1g, 1h, 2g, and 2h against the methicillin-resistant *S. aureus* (MRSA) strain USA300. Since an endpoint assay testing metabolic activity did not indicate inhibition after 8 h at up to 200 μ M (Fig. S3), we performed growth curve measurements for a more detailed assessment (Supplementary Data 2). Hereby, NQ

derivatives were always less active than the corresponding NQNOs (Figs. 5a and S4). Interestingly, 2'-OH-NQ and 2'-OH-NQNO were both considerably less active than their unsubstituted counterparts, which suggests that the overall antimicrobial effect of this new class of quinolones against *S. aureus* is even lower compared to 2-alkyl-4(1H)-quinolones and their

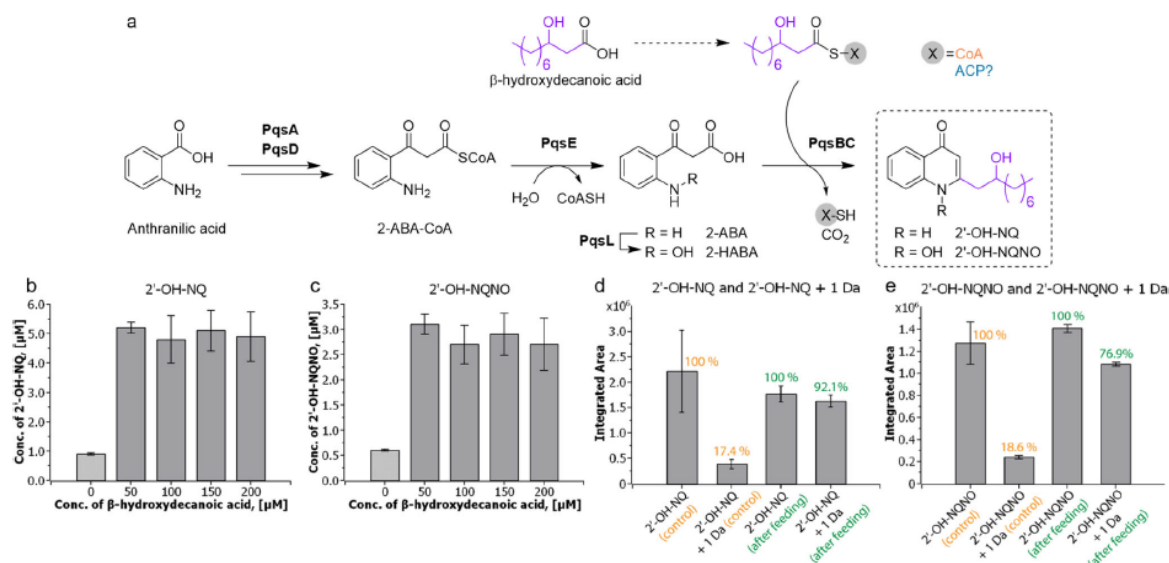


Fig. 4 Quinolone production in dependence of fatty acid availability. **a** β -Hydroxydecanoic acid incorporation in 4(1H)-quinolone biosynthesis in *P. aeruginosa*. Production levels of 2'-OH-NQ (**b**) and 2'-OH-NQNO (**c**) in *P. aeruginosa* PAO1 after feeding β -hydroxydecanoic acid. Production levels of **d** 2'-OH-NQ and 2'-OH-NQ + 1 Da; **e** 2'-OH-NQNO and 2'-OH-NQNO + 1 Da measured in *P. aeruginosa* PAO1 in control sample (orange labels) and after feeding 100 μM deuterated β -hydroxydecanoic acid (**3d**) to bacterial cultures (green labels). Predicted m/z distribution for 2'-OH-NQ [$M + H$] $^+$: 288.1959 (100.0%), 289.1992 (19.5%), 290.2026 (1.8%). Predicted m/z distribution for 2'-OH-NQNO [$M + H$] $^+$: 304.1908 (100.0%), 305.1941 (19.5%), 306.1975 (1.8%). Mean values are given for biological triplicates with the error bars representing the population standard deviation.

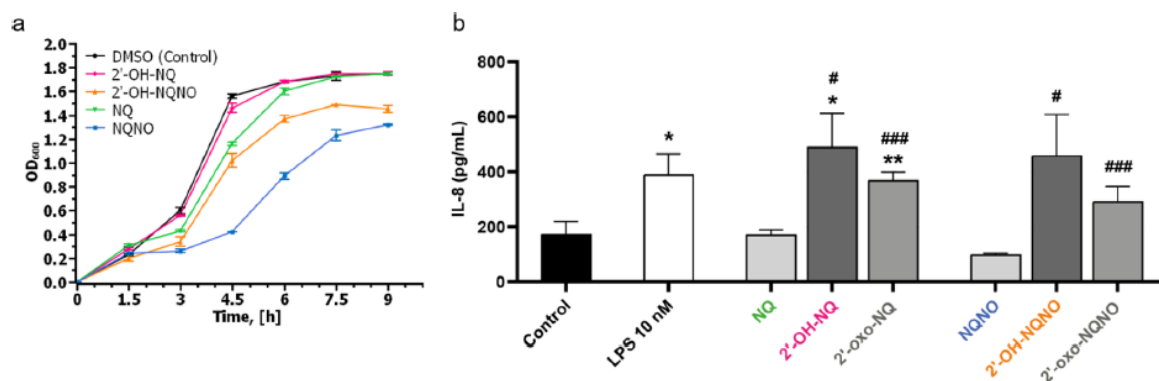


Fig. 5 Influence of quinolones on *S. aureus* growth and host immune response. **a** Growth curves of *S. aureus* USA300 in the presence of 50 μM 2'-OH-NQ (pink), 2'-OH-NQNO (orange), NQ (green), NQNO (blue), and DMSO as a control (black). Mean values are given for biological triplicates with the error bars representing the population standard deviation. **b** IL-8 release from differentiated Caco-2 cells after 24 h of treatment with medium (Control), 10 nM LPS, 100 nM NQ, 100 nM 2'-OH-NQ, 100 nM 2'-oxo-NQ, 100 nM NQNO, 100 nM 2'-OH-NQNO, and 100 nM 2'-oxo-NQNO. Data are presented as Mean $\pm$ SEM of five independent biological repetitions in triplicates. * ($p < 0.05$) or ** ($p < 0.01$) indicates statistical significance compared with the control group. # ($p < 0.05$) or ### ($p < 0.001$) indicates statistical significance compared with NQ or NQNO.

N-oxides. Quinolones produced by *P. aeruginosa* exhibit great functional diversity ranging from quorum sensing signals, antibiotics, and iron traps to modulating the innate immune response of their hosts¹⁶. We thus compared the effects of our new quinolones on cytokine response with that of NQ and NQNO. While none of the compounds (1–500 nM) exhibited cytotoxicity (Figs. S8 and S10), we observed a very potent effect on the production of pro-inflammatory interleukine-8 (IL-8) by Caco-2 cells already at 100 nM with all 2'-hydroxy and 2'-oxo substituted NQs and NQNOs (Fig. 5b, S9, S11, and Supplementary Data 3). At 100 nM and 500 nM, 2'-OH-NQ showed hereby the strongest effect, comparable with the stimulation by 10 nM LPS. In contrast, neither NQ nor NQNO showed any stimulation of IL-8

even at 500 nM (Fig. 5b and S11). Chronic lung inflammation mediated by the neutrophil recruiting chemokine IL-8 plays an important role in cystic fibrosis and is stimulated by infections with *P. aeruginosa*^{17,18}. Stimulation of proinflammatory IL-8 by *P. aeruginosa* is also observed during infection of intestinal epithelial cells¹⁹. Surprisingly, the effect of AQs and AQNOs on IL-8 production has not been investigated before. However, it has been shown that the quorum sensing signal 3-oxo-dodecanoyl-L-homoserine lactone of *P. aeruginosa* can induce IL-8 production, although its activity was reported in the upper micromolar range, i.e. at 300- to 1000-fold higher concentrations compared to 2'-OH-NQ^{20,21}. Regarding 2-alkyl-4(1H)-quinolones, immune modulating effects have only been studied on the example of

various other cytokines with the quorum sensing signals HHQ and the *Pseudomonas* quinolone signal (PQS). These studies show that HHQ and PQS at high concentrations exhibit activities suppressing the host innate immune response by down-regulating the NF- κ B pathway²², inhibiting mitogen-stimulated IL-2 release²³, or decreasing the LPS-stimulated production of IL-12²⁴. These effects were observed in the micromolar concentration range and are hence significantly less potent than the nanomolar immune modulatory effects by 2'-OH-NQ and 2'-OH-NQNO described in the present work. Interestingly, 2'-OH-NQ also has been recently reported as a metabolite of a *Pseudomonas* strain isolated from a Chinese soil sample²⁵. Additionally, this metabolite class was putatively identified using feature-based molecular networking analysis for secondary metabolites of *P. aeruginosa*, although the positioning of the hydroxylation in the side chain could not be resolved²⁶.

Our results suggest that with native concentrations of up to 0.55 mg L⁻¹ (1.9 μ M), 2'-OH-NQ is one of the main 4(1H)-quinolones of *P. aeruginosa* strains. Already at 20-fold lower concentrations, we have shown potent stimulation of cytokine IL-8 by 2'-OH-NQ, indicating that host immune modulation could be achieved under physiologically relevant concentrations. While the biosynthesis of 2-alkyl-4(1H)-quinolones via the PqsBC complex is well established, it remained unclear if and how side-chain oxidized compounds could be produced. Our results with isotope labeled β -hydroxydecanoic acid suggest that oxidized fatty acids can be used even directly as substrates of this promiscuous biosynthetic pathway.

Conclusions

We have reported the first synthesis of 2'-hydroxy and 2'-oxo substituted 2-alkyl-4(1H)-quinolones and demonstrated that 2'-OH-NQ and 2'-OH-NQNO but not the corresponding 2'-oxo-NQ and 2'-oxo-NQNO are naturally produced by *P. aeruginosa* strains PAO1 and PA14. We have shown that 2'-OH-NQ occurs in remarkably high concentrations and is together with NQ one of the major 4(1H)-quinolones of *P. aeruginosa*. Importantly, our results indicate that exogenous fatty acids may greatly boost product concentration and that 2'-OH-NQ has strong immunomodulatory activity with nanomolar potency.

Methods

Materials. See Supplementary Information (page 2).

Preparation of overnight cultures. A small amount of a bacterial cryo-stock (20% glycerol, stored at -80 °C) was inoculated in 3 mL LB (lysogenic broth)-Lennox medium (LB medium) in sterile 13 mL polypropylene tubes (Sarstedt, ref 62.515.028), and allowed to grow for 16–18 h at indicated growth conditions (Table S1).

Growth curves. An overnight culture was diluted 1:1000 in 3 mL of LB medium in a sterile 13 mL polypropylene tube. Quinolones were added from DMSO stocks, to reach their final concentration of 50 μ M. The cultures were incubated at 37 °C and 180 rpm. The growth was monitored by every 1.5 h measurement of the OD₆₀₀ value (Fisher Scientific cell density meter model 40) of 100 μ L culture in plastic cuvettes (Sarstedt UV-transparent cuvettes for use >220 nm, 12.5 $\times$ 12.5 $\times$ 45 mm). The experiment was performed in triplicates.

Feeding experiments. An overnight culture (45 μ L) of *P. aeruginosa* PAO1 was inoculated into 3 mL of LB medium in sterile 15 mL polypropylene centrifugal tubes with screw caps (VWR). A respective DMSO stock solution of fatty acid (or DMSO as a control) (3 μ L) was added to reach its final concentration of 50, 100, 150, and 200 μ M. Caps of the tubes were loosely opened by a 180 degree turn and fixed in this position to ensure equal oxygen supply. Cultures were incubated for 9 h at 37 °C in a shaking incubator at 180 rpm. After incubation, samples were centrifuged at 4500 rpm for 5 min and supernatants were sterile filtrated. Three hundred microliters of culture supernatant were added in 1.5 mL glass vials (LABSOLUTE, Art. Nr. 7612960) with caps containing a PTFE membrane (LABSOLUTE, Art. Nr. 7623097). Three hundred microliters of EtOAc was added

and immediately vortexed for 5 s. After the separation of organic and water phases, 100 μ L of the EtOAc layer was transferred via pipetting into mass spec vials containing a glass insert (MACHEREY-NAGEL, Art. Nr. 702007). EtOAc was evaporated by a gentle stream of nitrogen. For LC-MS/MS analysis, 100 μ L of sample solvent (MeOH/H₂O 1:1) was added into glass inserts and the residue redissolved. The experiment was performed in triplicates.

Cell culture and treatment. Caco-2 cells were maintained at 37 °C and 5% CO₂ in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2% L-glutamine and 1% penicillin/streptomycin under a humidified atmosphere. For differentiation into an enterocyte cell model, cells were seeded in 96-well plates with the density of 3 $\times$ 10⁴ cells/well, and the medium was replaced with fresh culture medium every 2–3 days until differentiation was complete (after 21 days). For treatment, the differentiated Caco-2 cells were incubated with fresh medium alone (control) and fresh medium containing 10 nM LPS (lipopolysaccharide from *Escherichia coli*), 1, 10, 50, 100, 200 and 500 nM quinolones. After 24 h of incubation, the supernatants were collected for cytokine analysis, and cells were used for cell viability estimation based on MTT assay.

Cell viability. Caco-2 cells in each well were incubated with 100 μ L MTT working solution (0.83 mg mL⁻¹) diluted in serum-free medium for 10 min. By replacing the MTT working solution with 150 μ L DMSO, the formazan product, formed during the incubation, was dissolved. The absorbance was measured at 570 nm with 650 nm as a reference wavelength.

Measurement of Interleukin-8 (IL-8) level. The concentration of IL-8 was evaluated by ELISA kits (BD Biosciences) based on the manufacturer's instructions. Data are expressed in pg mL⁻¹.

Quantification of quinolones in bacterial cultures. An overnight culture (60 μ L) was inoculated into 4 mL of LB medium in a sterile 15 mL polypropylene centrifugal tubes with screw caps (VWR). Caps of the tubes were loosely opened by a 180° turn and fixed in this position to ensure equal oxygen supply. Cultures were incubated for 3, 6, 9, 12, and 24 h at 37 °C in a shaking incubator at 180 rpm. After incubation, samples were centrifuged at 4500 rpm for 5 min and supernatants were sterile filtrated. Three hundred microliters of culture supernatant were added in 1.5 mL glass vials (LABSOLUTE, Art. Nr. 7612960) with caps containing a PTFE membrane (LABSOLUTE, Art. Nr. 7623097). Three hundred microliters of EtOAc was added and immediately vortexed for 5 s. After the separation of organic and water phases, 100 μ L of the EtOAc layer was transferred via pipetting into mass spec vials containing a glass insert (MACHEREY-NAGEL, Art. Nr. 702007). The EtOAc was evaporated by a gentle stream of nitrogen. For LC-MS/MS analysis, 100 μ L of sample solvent (MeOH/H₂O 1:1) was added into glass inserts and the residue redissolved. The experiment was performed in triplicates.

LC-MS/MS analysis. Ultra-high performance liquid chromatography was performed on a Dionex Ultimate 3000 UHPLC (Thermo Fisher Scientific) and Vanquish™ UHPLC system (Thermo Fisher Scientific) using a Nucleodur C18 Gravity-SB 100 $\times$ 2 mm, 3 μ m column (Macherey-Nagel). The flow rate was 0.5 mL min⁻¹ and the column temperature was kept at 40 °C. The injection volume was 10 μ L. Eluent A was 0.1% formic acid in water and eluent B was 0.1% formic acid in acetonitrile. The gradient was set to 20–100% B in 10 min, 100% B for 2 min, 100–20% B in 1 min, and 20% B for 2 min. MS/MS analysis was performed by Finnigan™ TSQ Quantum (Thermo Scientific) and TSQ Series II Quantum (Thermo Fisher Scientific) mass spectrometers. A heated-electrospray ionization (HESI-II probe, Thermo Scientific) was used as an ion source. In the optimized conditions the ion spray voltage was 3500 V, vaporizer temperature 300 °C, capillary temperature 380 °C, sheath gas pressure 60 psi, ion sweep gas pressure 2 psi, and aux gas 10 psi. The fragmentation pattern of quinolone standards was acquired in a Product Ion Scan mode using a fixed collision energy of 30 eV to fragment the corresponding precursor ion before recording the fragments in a mass range of m/z 130–350. Quinolones were quantified in Selected Reaction Monitoring scan mode. MS/MS spectra were acquired in a positive mode. The software Quan Browser Thermo Xcalibur was used for quantitative analysis. The peak area of the respective product ion was fitted by linear regression versus the known concentrations to generate a standard curve. Note: Analysis of extracts after feeding of deuterated β -hydroxydecanoic acid (3d) were performed on Vanquish™ UHPLC system (Thermo Fisher Scientific) in combination with TSQ Series II Quantum (Thermo Fisher Scientific), while all other LC-MS/MS experiments on Dionex Ultimate 3000 UHPLC (Thermo Fisher Scientific) in combination with Finnigan™ TSQ Quantum (Thermo Fisher Scientific).

MS² assignments. See Supplementary Information (page 6).

Synthesis. See Supplementary Information (page 15).

Reporting summary. Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

LC-MS/MS spectra are deposited in the GNPS database under the spectra identifiers given in the Supplementary Information. NMR data are provided in Supplementary Data 1 file. Optical density values from growth curve experiment are provided in Supplementary Data 2. IL-8 release data are provided in Supplementary Data 3. All relevant data are also available from the authors.

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Author contributions

V.S., D.S., and T.B. designed the project and experiments. V.S. synthesized all standard AQNO compounds, established LC-MS/MS methodologies and conducted all experimental measurements with subsequent data analysis. D.S. designed the synthetic methodology and contributed to the synthesis of the standard AQNO compounds. Y.B. and M.P. performed the cytokine and cell viability tests. T.B. directed the project. V.S., D.S., and T.B. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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2.1 Publication 1. Supporting Information

Supporting Information

Biosynthetic flexibility of *Pseudomonas aeruginosa* leads to hydroxylated
2-Alkylquinolones with proinflammatory host response

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1. Supplementary Methods

1. Materials and Methods

Chemicals and solvents for the synthesis were purchased from Sigma-Aldrich, Carl Roth, Acros Organics or VWR Chemicals and were used without further purification. Distilled technical grade solvents and silica gel 60 A (Carl Roth) were used for silica gel chromatography. Thin layer chromatography (TLC) was performed using aluminium sheets "TLC Silica gel 60 F254" from Merck Millipore® and analysed with short/long wave UV-light or by permanganate staining. NMR spectra were obtained on Bruker Avance-III 400 and Bruker Avance-III 600 NMR spectrometers at ambient temperature. Multiplicities are given as follows: s - singlet, d - doublet, t - triplet, q - quartet, quint. - quintet, m - multiplet. Chemical shifts (δ) are given in parts per million (ppm) relative to the solvent residual signal with CDCl_3 $\delta_{\text{H}} = 7.26$ ppm and $\delta_{\text{C}} = 77.16$ ppm, DMSO-d_6 $\delta_{\text{H}} = 2.50$ ppm and $\delta_{\text{C}} = 39.52$ ppm, CD_3OD $\delta_{\text{H}} = 3.31$ ppm and $\delta_{\text{C}} = 49.00$ ppm.^[1] The obtained data were processed and analysed with MestReNova 14.2.0-26256 software. High-resolution mass spectrometry data were obtained on a Hybrid FT Mass Spectrometer LTQ Orbitrap Velos (Thermo Scientific). LC-MS/MS analysis was performed on Dionex Ultimate 3000 UHPLC (Thermo Fisher Scientific) in combination with Finnigan™ TSQ® Quantum (Thermo Fisher Scientific) and on Vanquish™ Horizon/Flex UHPLC system (Thermo Fisher Scientific) in combination with TSQ® Series II Quantum (Thermo Fisher Scientific).

2. Biological experiments

Preparation of overnight cultures

A small amount of a bacterial cryo-stock (20% glycerol, stored at -80°C) was inoculated in 3 mL LB-(lysogenic broth)-Lennox medium (LB medium) in sterile 13 mL polypropylene tubes (Sarstedt, ref 62.515.028), and allowed to grow for 16-18 h at indicated growth conditions (Table S1).

Growth curves

An overnight culture was diluted 1:1000 in 3 mL of LB medium in a sterile 13 mL polypropylene tube. Quinolones were added from DMSO stocks, to reach their final concentration of 50 μM . The cultures were incubated at 37°C and 180 rpm. The growth was monitored by every 1.5 h measurement of the OD_{600} value (Fisher Scientific cell density meter model 40) of 100 μL culture in plastic cuvettes (Sarstedt UV-transparent cuvettes for use >220 nm, 12.5 x 12.5 x 45mm). The experiment was performed in triplicates.

Antibiotic activity assay

An overnight culture of *S. aureus* USA300 was diluted with LB medium (1:1000) in a sterile 13 mL polypropylene tube. 99 μL of the bacteria suspension and 1 μL of a respective DMSO stock solution of quinolone (or DMSO for medium control) were added subsequently in a round bottom 96-well plate. The plate was incubated at 37°C and 180 rpm for 8 h. After incubation, 100 μL of a 0.02% resazurin solution* were added and the plate was incubated for 2 h at 37°C and 180 rpm. An inhibition was considered positive if the solution remained blue. Wells that

showed a pink or violet colour or were colourless were considered negative for inhibition. The experiment was performed in duplicates.

*20 mg of resazurin were added to 100 mL of LB media. After sonication, the solution was sterile filtered and stored for up to 2 weeks at 4°C.

Feeding experiments

An overnight culture (45 µL) of *P. aeruginosa* PAO1 was inoculated into 3 mL of LB medium in sterile 15 mL polypropylene centrifugal tubes with screw caps (VWR). A respective DMSO stock solution of fatty acid (or DMSO as a control) (3 µL) was added to reach its final concentration of 50, 100, 150, and 200 µM. Caps of the tubes were loosely opened by a 180 degree turn and fixed in this position to ensure equal oxygen supply. Cultures were incubated for 9 h at 37°C in a shaking incubator at 180 rpm. After incubation, samples were centrifuged at 4500 rpm for 5 min and supernatants were sterile filtrated. 300 µL of culture supernatant were added in 1.5 mL glass vials (LABSOLUTE, Art. Nr. 7612960) with caps containing a PTFE membrane (LABSOLUTE, Art. Nr. 7623097). 300 µL of EtOAc was added and immediately vortexed for 5 sec. After the separation of organic and water phases, 100 µL of the EtOAc layer was transferred via pipetting into mass spec vials containing a glass insert (MACHEREY-NAGEL, Art. Nr. 702007). EtOAc was evaporated by a gentle stream of nitrogen. For LC-MS/MS analysis, 100 µL of sample solvent (MeOH/H₂O 1:1) was added into glass inserts and the residue redissolved. The experiment was performed in triplicates.

Cell culture and treatment

Caco-2 cells were maintained at 37°C and 5% CO₂ in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2% L-glutamine and 1% penicillin/streptomycin under a humidified atmosphere. For differentiation into an enterocyte cell model, cells were seeded in 96-well plates with the density of 3×10⁴ cells/well, and the medium was replaced with fresh culture medium every 2 to 3 days until differentiation was complete (after 21 days).

For treatment, the differentiated Caco-2 cells were incubated with fresh medium alone (control) and fresh medium containing 10 nM LPS (lipopolysaccharide from *Escherichia coli*), 1, 10, 50, 100, 200 and 500 nM quinolones. After 24 h of incubation, the supernatants were collected for cytokine analysis, and cells were used for cell viability estimation based on MTT assay.

Cell viability

Caco-2 cells in each well were incubated with 100 µL MTT working solution (0.83 mg/mL) diluted in serum-free medium for 10 min. By replacing the MTT working solution with 150 µL DMSO, the formazan product, formed during the incubation, was dissolved. The absorbance was measured at 570 nm with 650 nm as a reference wavelength.

Measurement of Interleukin-8 (IL-8) level

The concentration of IL-8 was evaluated by ELISA kits (BD Biosciences) based on the manufacturer's instructions. Data are expressed in pg/mL.

1. Analytical methods

Quantification of quinolones in bacterial cultures

An overnight culture (60 μ L) was inoculated into 4 mL of LB medium in a sterile 15 mL polypropylene centrifugal tubes with screw caps (VWR). Caps of the tubes were loosely opened by a 180 degree turn and fixed in this position to ensure equal oxygen supply. Cultures were incubated for 3, 6, 9, 12, and 24 h at 37°C in a shaking incubator at 180 rpm. After incubation, samples were centrifuged at 4500 rpm for 5 min and supernatants were sterile filtrated. 300 μ L of culture supernatant were added in 1.5 mL glass vials (LABSOLUTE, Art. Nr. 7612960) with caps containing a PTFE membrane (LABSOLUTE, Art. Nr. 7623097). 300 μ L of EtOAc was added and immediately vortexed for 5 sec. After the separation of organic and water phases, 100 μ L of the EtOAc layer was transferred via pipetting into mass spec vials containing a glass insert (MACHEREY-NAGEL, Art. Nr. 702007). The EtOAc was evaporated by a gentle stream of nitrogen. For LC-MS/MS analysis, 100 μ L of sample solvent (MeOH/H₂O 1:1) was added into glass inserts and the residue redissolved. The experiment was performed in triplicates.

Calibration curves for quantification

MeOH stocks of all calibration standards were prepared at 1 mg/mL in glass vials and stored at -80°C for up to 3 months. Calibration standard samples for quantification were prepared in triplicates by serial dilution in sample solvent (MeOH/H₂O 1:1) (**Table S2**). Calibration equations and R-values were obtained using the Thermo Xcalibur Quan Browser.

LC-MS/MS analysis

Ultra-high performance liquid chromatography was performed on a Dionex Ultimate 3000 UHPLC (Thermo Fisher Scientific) and Vanquish™ UHPLC system (Thermo Fisher Scientific) using a Nucleodur C18 Gravity-SB 150 x 2 mm, 3 μ m column (Macherey-Nagel). The flow rate was 0.5 mL min⁻¹ and the column temperature was held at 40°C. The injection volume was 10 μ L. Eluent A was 0.1% formic acid in water and eluent B was 0.1% formic acid in acetonitrile. The gradient was 20-100 % B in 10 min, 100 % B for 2 min, 100-20 % B in 1 min, and 20 % B for 2 min. MS/MS analysis was performed by Finnigan™ TSQ® Quantum (Thermo Scientific) and TSQ® Series II Quantum (Thermo Fisher Scientific) mass spectrometers. A heated-electrospray ionization (HESI-II probe, Thermo Scientific) was used as an ion source. In the optimized conditions the ion spray voltage was 3500 V, vaporizer temperature 300°C, capillary temperature 380°C, sheath gas pressure 60 psi, ion sweep gas pressure 2 psi, and aux gas 10 psi. The fragmentation pattern of quinolone standards was acquired in a Product Ion Scan mode using a fixed collision energy of 30 eV to fragment the corresponding precursor ion before recording the fragments in a mass range of m/z 130-350. Quinolones we quantified in Selected Reaction Monitoring scan mode. MS/MS spectra were acquired in a positive mode. The software Quan Browser Thermo Xcalibur was used for quantitative analysis. The peak area of the respective product ion was fitted by linear regression versus the known concentrations to generate a standard curve.

The LC-MS/MS spectra were made available in the GNPS database under the following spectrum identifiers:

CCMSLIB00011427575 (2'-OH-NQ synthetic standard)

CCMSLIB00011427576 (2'-OH-NQNO synthetic standard)

CCMSLIB00011427581 (2'-oxo-NQ synthetic standard)
CCMSLIB00011427582 (2'-oxo-NQNO synthetic standard)
CCMSLIB00011427574 (NQ synthetic standard)
CCMSLIB00011427583 (NQNO synthetic standard)

CCMSLIB00011427580 (2'-OH-NQ in *P. aeruginosa* PAO1 supernatant)
CCMSLIB00011427579 (2'-OH-NQ in *P. aeruginosa* PA14 supernatant)
CCMSLIB00011427578 (2'-OH-NQNO in *P. aeruginosa* PAO1 supernatant)
CCMSLIB00011427577 (2'-OH-NQNO in *P. aeruginosa* PA14 supernatant)

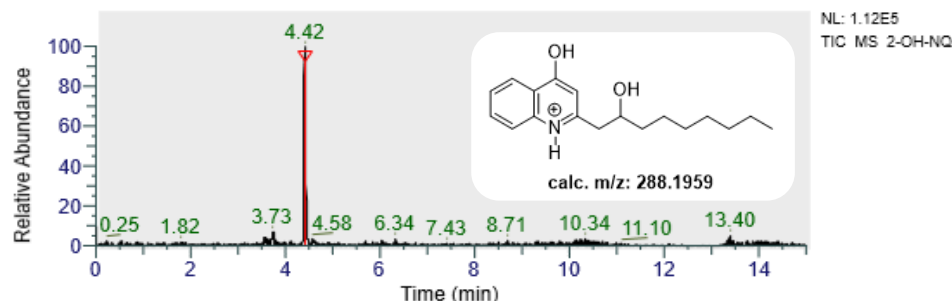
Note: extracts analysis after feeding of deuterated β -Hydroxydecanoic acid (**3d**) were performed on Vanquish™ UHPLC system (Thermo Fisher Scientific) in combination with TSQ® Series II Quantum (Thermo Fisher Scientific), while all other LC-MS/MS experiments on Dionex Ultimate 3000 UHPLC (Thermo Fisher Scientific) in combination with Finnigan™ TSQ® Quantum (Thermo Fisher Scientific).

2. MS² Assignments

Reference spectra (on Vanquish™ UHPLC + TSQ® Series II Quantum)

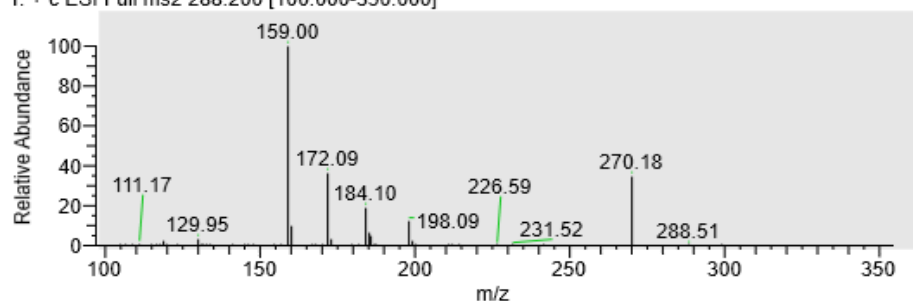
2'-OH-NQ (RT = 4.42; Parental mass = 288)

RT :0.00-15.00



~Spectrum 1 1055 - 2-OH-NQ - C1T1

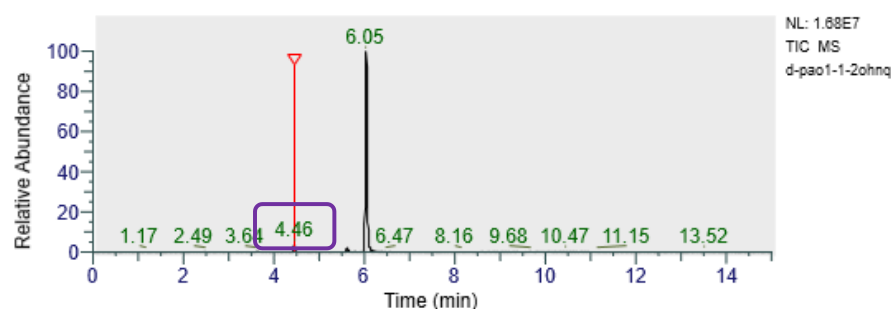
2-OH-NQ #1055 RT: 4.42 AV: 1 NL: 4.37E4
 T: + c ESI Full ms2 288.200 [100.000-350.000]



Extracts analysis (on Vanquish™ UHPLC + TSQ® Series II Quantum). Example spectra of detected 2'-OH-NQ and 2'-OH-NQNO in *P. aeruginosa* PAO1 extracts

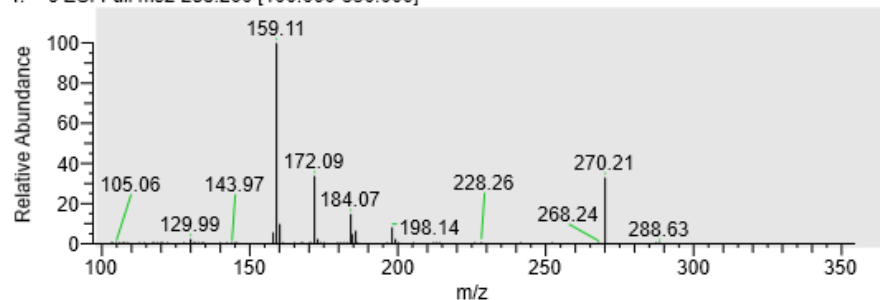
2'-OH-NQ (RT = 4.46; Parental mass = 288)

RT :0.00-15.00



~Spectrum 1 1064 - d-pao1-1-2ohnq - C1T1

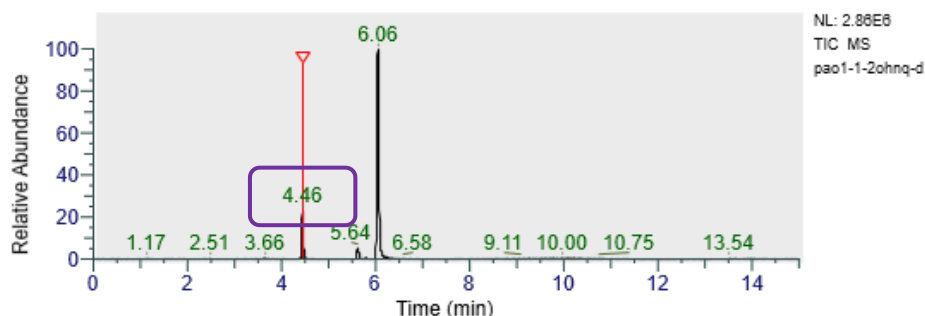
d-pao1-1-2ohnq #1064 RT: 4.46 AV: 1 NL: 2.47E5
 T: + c ESI Full ms2 288.200 [100.000-350.000]



Extracts analysis (on Vanquish™ UHPLC + TSQ® Series II Quantum). Example spectra of detected 2'-OH-NQ + 1 Da in *P. aeruginosa* PAO1 extracts

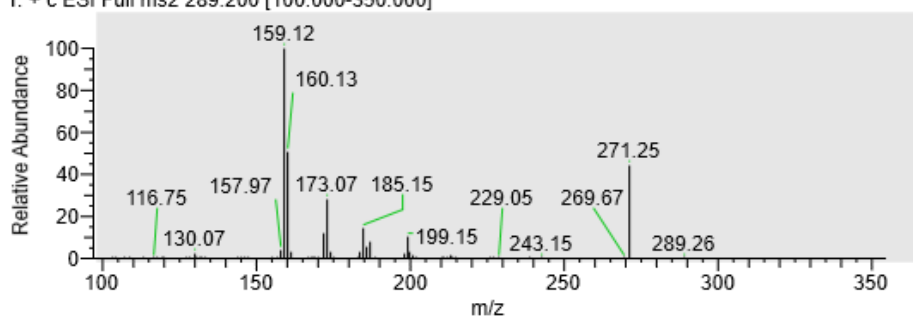
2'-OH-NQ + 1 Da (RT = 4.46; Parental mass = 289)

RT: 0.00-15.00



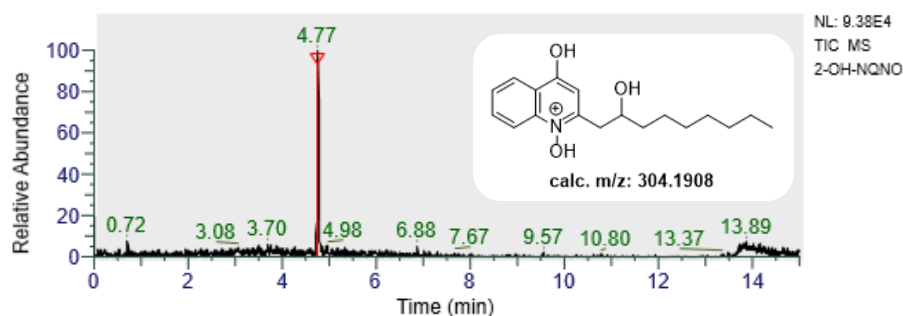
~Spectrum 1 1063 - pao1-1-2ohnq-d - C1T1

pao1-1-2ohnq-d #1063 RT: 4.46 AV: 1 NL: 2.05E5
T: + c ESI Full ms2 289.200 [100.000-350.000]



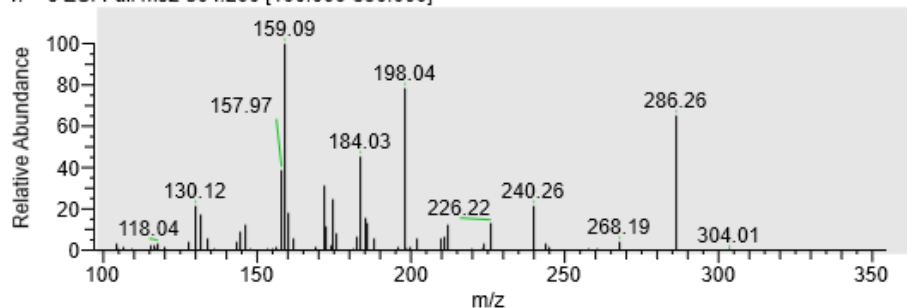
Reference spectra (on Vanquish™ UHPLC + TSQ® Series II Quantum)
2'-OH-NQNO (RT = 4.77; Parental mass = 304)

RT :0.00-15.00



~Spectrum 1 1138 - 2-OH-NQNO - C1T1

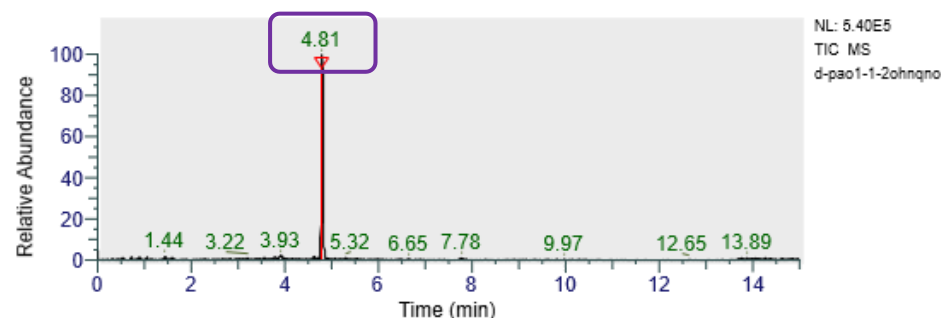
2-OH-NQNO #1138 RT: 4.77 AV: 1 NL: 1.40E4
 T: + c ESI Full ms2 304.200 [100.000-350.000]



Extracts analysis (on Vanquish™ UHPLC + TSQ® Series II Quantum). Example spectra of
 detected 2'-OH-NQ and 2'-OH-NQNO in *P. aeruginosa* PAO1 extracts

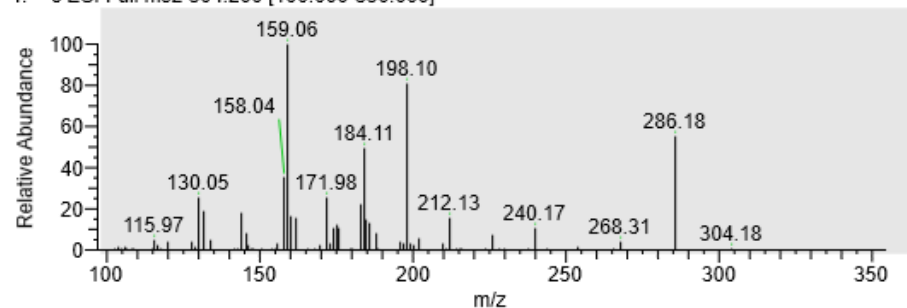
2'-OH-NQNO (RT = 4.81; Parental mass = 304)

RT :0.00-15.00



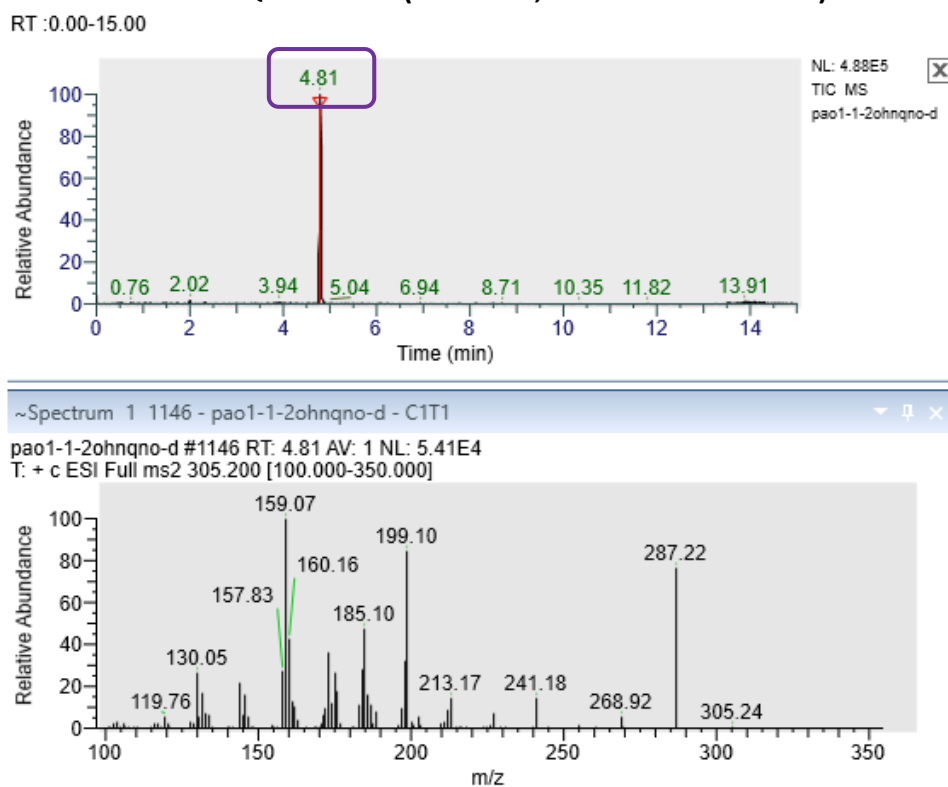
~Spectrum 1 1147 - d-pao1-1-2ohnqno - C1T1

d-pao1-1-2ohnqno #1147 RT: 4.81 AV: 1 NL: 7.96E4
 T: + c ESI Full ms2 304.200 [100.000-350.000]

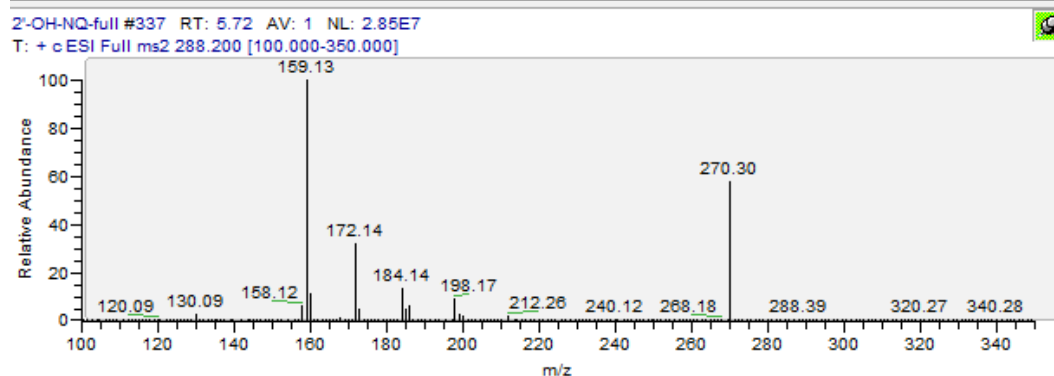
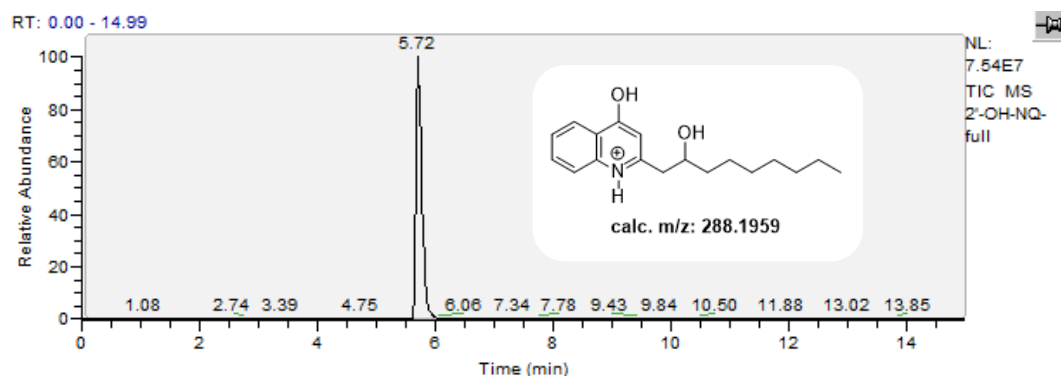


Extracts analysis (on Vanquish™ UHPLC + TSQ® Series II Quantum). Example spectra of detected 2'-OH-NQNO + 1 Da in *P. aeruginosa* PAO1 extracts

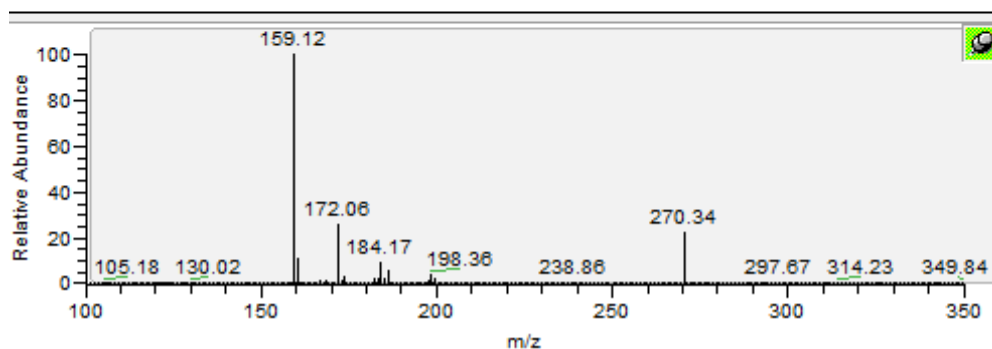
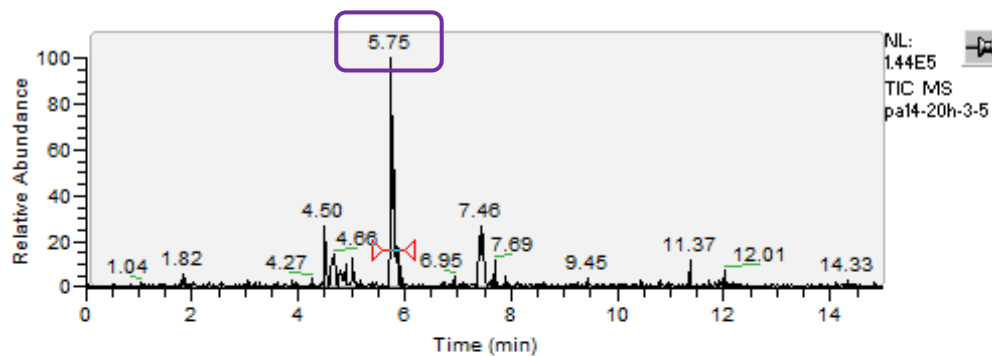
2'-OH-NQNO + 1 Da (RT = 4.81; Parental mass = 305)



Reference spectra (on Dionex Ultimate 3000 UHPLC + Finnigan™ TSQ® Quantum)
2'-OH-NQ (RT = 5.72; Parental mass = 288)

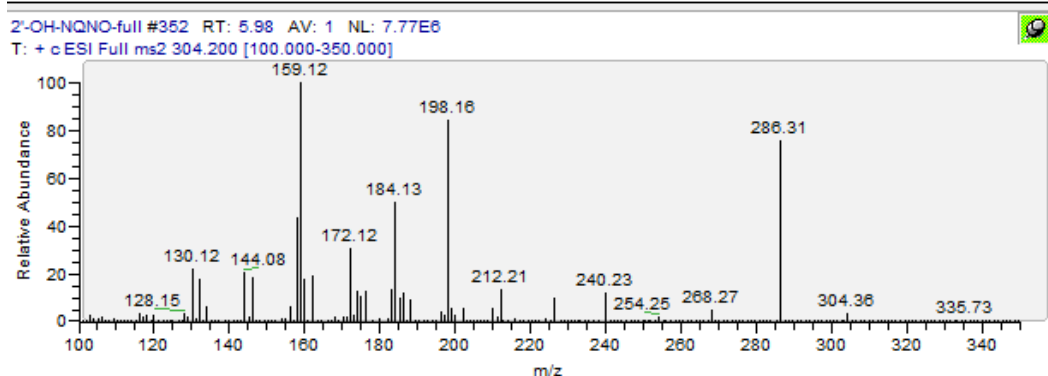
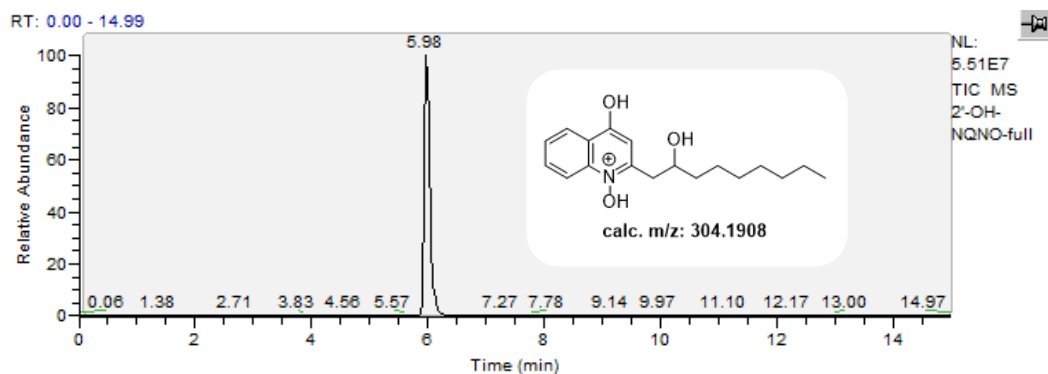


Extracts analysis (on Dionex Ultimate 3000 UHPLC + Finnigan™ TSQ® Quantum). Example
 spectra of detected 2'-OH-NQ and 2'-OH-NQNO in *P. aeruginosa* PA14 extracts
2'-OH-NQ (RT = 5.75; Parental mass = 288)



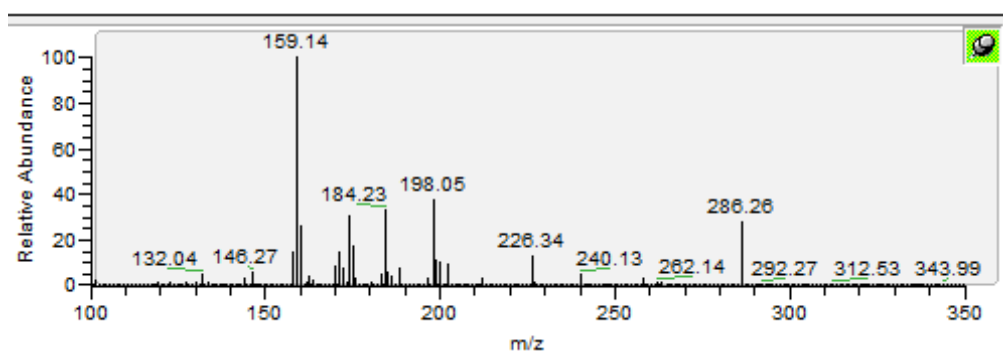
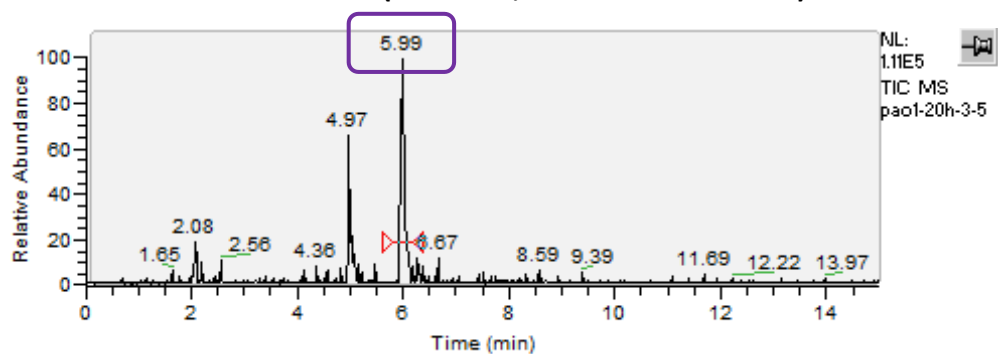
Reference spectra (on Dionex Ultimate 3000 UHPLC + Finnigan™ TSQ® Quantum)

2'-OH-NQNO (RT = 5.98; Parental mass = 304)



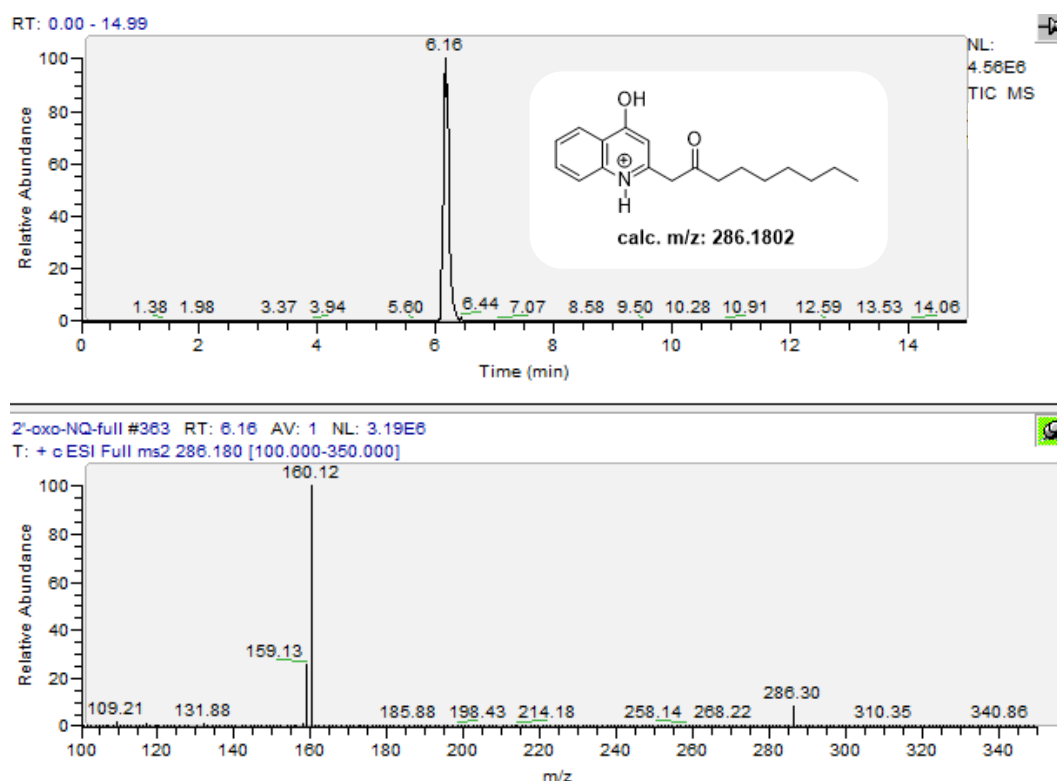
Extracts analysis (on Dionex Ultimate 3000 UHPLC + Finnigan™ TSQ® Quantum). Example spectra of detected 2'-OH-NQ and 2'-OH-NQNO in *P. aeruginosa* PAO1 extracts

2'-OH-NQNO (RT = 5.99; Parental mass = 304)

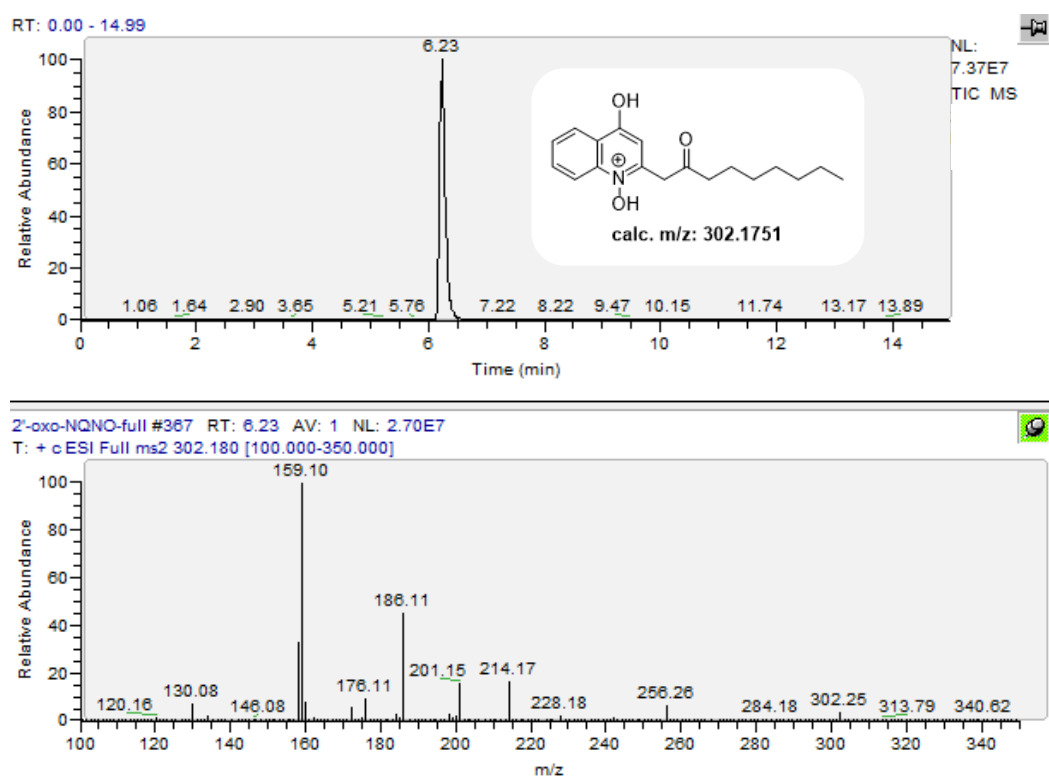


Reference spectra (on Dionex Ultimate 3000 UHPLC + Finnigan™ TSQ® Quantum)

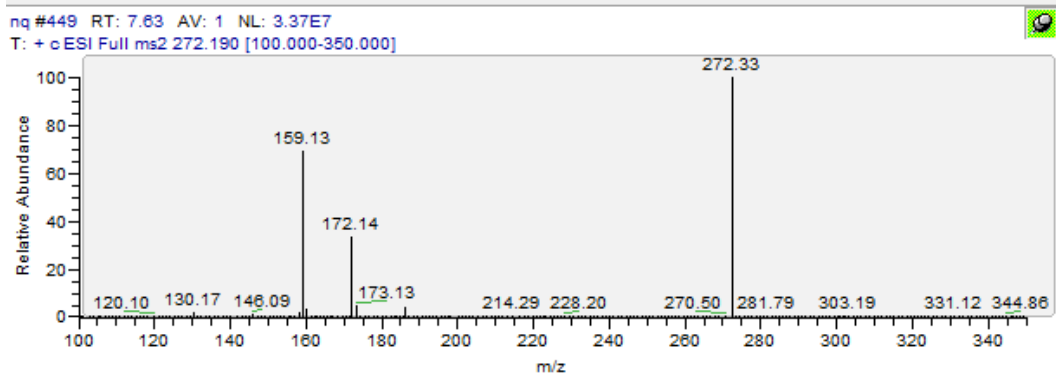
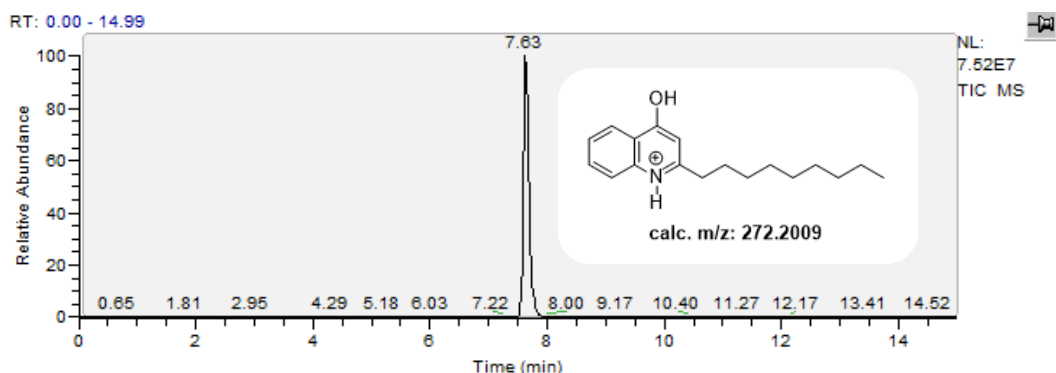
2'-oxo-NQ (RT = 6.16; Parental mass = 286)



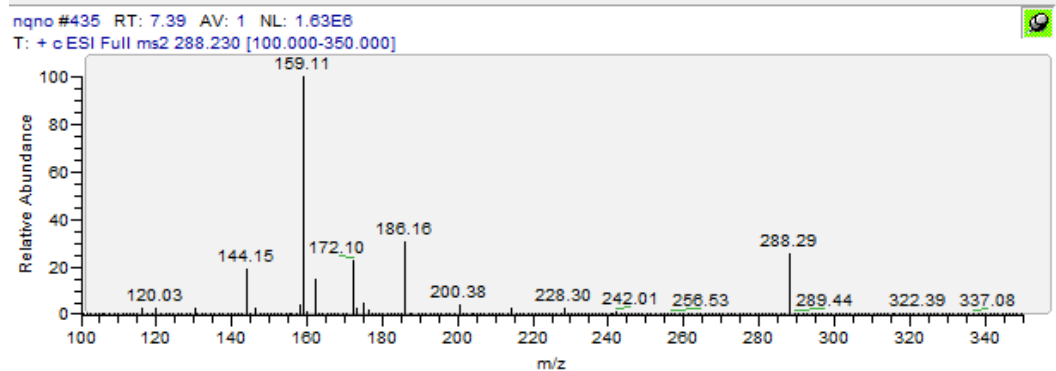
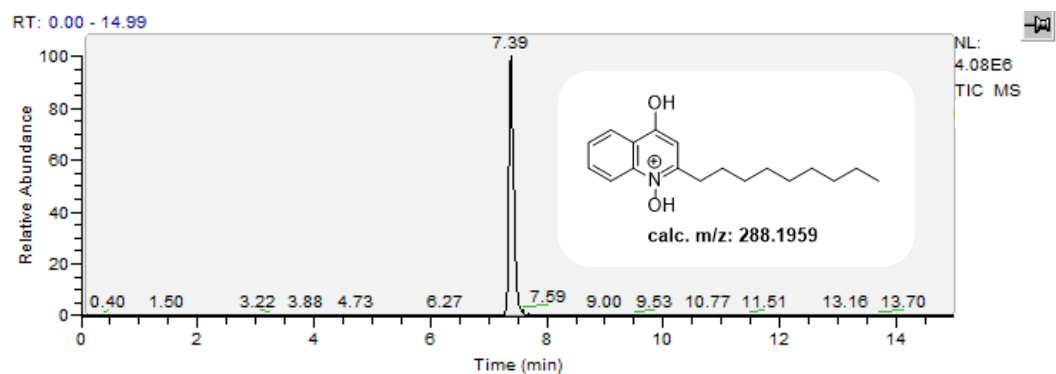
2'-oxo-NQNO (RT = 6.23; Parental mass = 302)



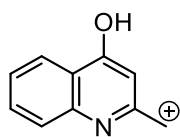
NQ (RT = 7.63; Parental mass = 272)



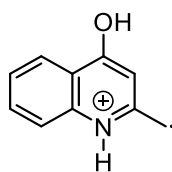
NQNO (RT = 7.39; Parental mass = 288)



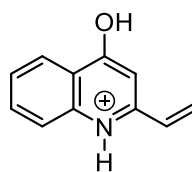
Proposed structures of fragment ions*



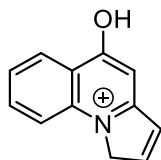
calc. m/z: 158.0601



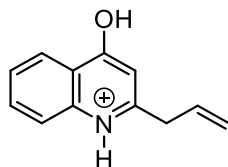
calc. m/z: 159.0679



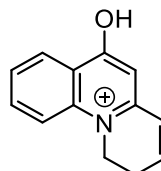
calc. m/z: 172.0757



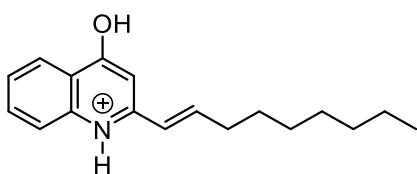
calc. m/z: 184.0757



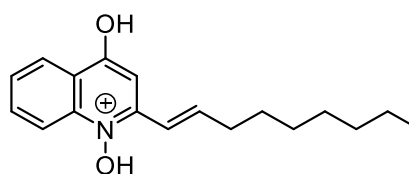
calc. m/z: 186.0914



calc. m/z: 198.0914



calc. m/z: 270.1853

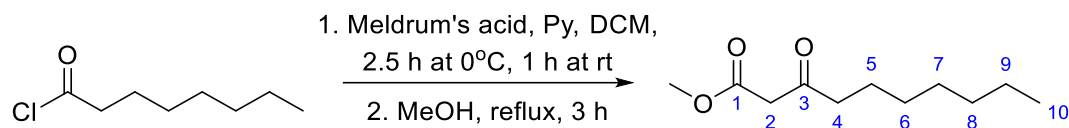


calc. m/z: 286.1802

*For relative abundance of detected fragments see **Table S3**.

3. Synthesis

Methyl 3-oxodecanoate (**a**)



Compound **a** was synthesized based on procedures described by Zheng *et. al.* [2] and Vleeschouwer *et. al.* [3].

1. 2,2-dimethyl-1,3-dioxane-4,6-dione (Meldrum's acid) (17.72 g, 122.96 mmol) was dissolved in DCM (200 mL) and cooled to 0°C. Pyridine (19.81 mL, 245.92 mmol) was added to this solution. After stirring the mixture for 40 min at 0°C, octanoyl chloride (21.00 mL, 122.96 mmol) was added dropwise. The resulting orange solution was stirred at 0°C for 2 h and at room temperature for 1 h. The obtained mixture was washed with 5% aq. HCl solution, distilled water, and Brine solution. The organic phase was dried over MgSO₄, filtered, and concentrated *in vacuo*.

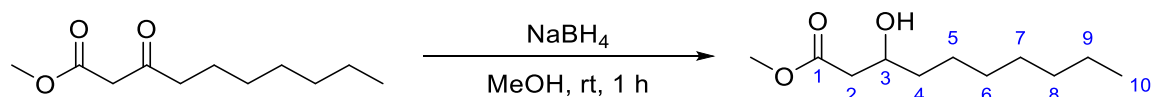
2. The resulting orange oil was dissolved in dry MeOH (150 mL) and refluxed for 3 h. The solvent was removed under reduced pressure. The residue was purified by silica column chromatography (petroleum ether/EtOAc 9:1). Product **a** was obtained as a slightly yellow oil (16.57 g, 82.73 mmol, 67 %). *R*_f = 0.53 (petrol ether/EtOAc 9:1).

¹H-NMR (CDCl₃ 400 MHz) δ (ppm): 0.85-0.88 (m, 3H, H-10), 1.22-1.31 (m, 8H, H-6 – H-9), 1.54-1.61 (m, 2H, H-5), 2.52 (t, 2H, *J* = 7.4 Hz, H-4), 3.43 (s, 2H, H-2), 3.72 (s, 3H, O-CH₃).

¹³C-NMR (CDCl₃ 101 MHz) δ (ppm): 14.16 (C-10), 22.70, 29.08, 29.12, 31.75 (C-9 – C-6), 23.59 (C-5), 43.20 (C-4), 49.13 (C-2), 52.42 (O-CH₃), 167.84 (C-1), 202.98 (C-3).

ESI-HRMS: *m/z* = 223.1305 [M+Na]⁺, calc. for C₁₁H₂₀O₃ + Na⁺ = 223.1305.

Methyl 3-hydroxydecanoate (**b**)



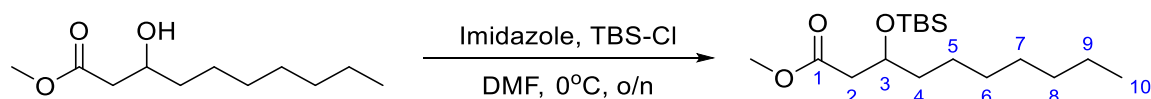
Methyl 3-oxodecanoate (**a**) (4.07 g, 20.30 mmol) was dissolved in dry MeOH (25 mL) and added slowly to a suspension of NaBH₄ (921.0 mg, 24.36 mmol) in dry MeOH (60 mL). The obtained mixture was stirred for 1 h at room temperature and quenched with 1 M aq. HCl solution. MeOH was evaporated and the remaining water mixture was extracted with diethyl ether 2 times. The combined organic phases were washed with brine solution, dried over MgSO₄, filtered, and concentrated *in vacuo*. The residue was purified by silica column chromatography (petrol ether/EtOAc 3:1) to yield product **b** as a colorless oil (*m* = 3.30 g, 16.30 mmol, 80 %). *R*_f = 0.58 (petrol ether/EtOAc 3:1).

¹H-NMR (CDCl₃ 400 MHz) δ (ppm): 0.86-0.89 (m, 3H, H-10), 1.21-1.37 (m, 10H, H-5 – H-9), 1.39-1.47 (m, 2H, H-4), 2.41 (dd, 1H, *J* = 16.3, 9.2 Hz, H-2), 2.51 (dd, 1H, *J* = 16.3, 3.2 Hz, H-2), 3.71 (s, 3H, O-CH₃), 3.97-4.03 (m, 1H, H-3).

¹³C-NMR (CDCl₃ 101 MHz) δ (ppm): 14.22 (C-10), 22.78, 25.62, 29.36, 29.61, 31.93 (C-9 – C-5), 36.67 (C-4), 41.24 (C-2), 51.87 (O-CH₃), 68.18 (C-3), 173.67 (C-1).

ESI-HRMS: *m/z* = 225.1460 [M+Na]⁺, calc. for C₁₁H₂₂O₃ + Na⁺ = 225.1462.

Methyl 3-((*tert*-butyldimethylsilyl)oxy)decanoate (**1a**)



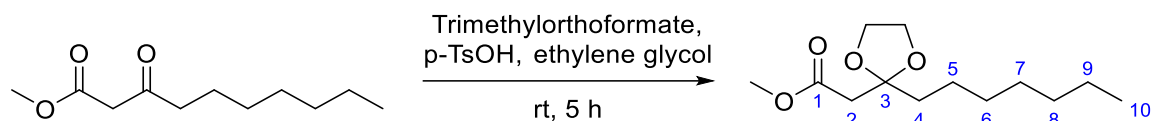
To the solution of methyl 3-hydroxydecanoate (**b**) (619.0 mg, 3.06 mmol) in DMF (2.5 mL) were added imidazole (417.0 mg, 6.12 mmol) and TBS-Cl (554.0 mg, 3.67 mmol) at room temperature. The mixture was stirred overnight at 0°C. The resulting reaction mixture was poured into sat. aq. NH_4Cl solution and extracted with diethyl ether. The combined organic layers were washed with brine solution and dried over MgSO_4 . The organic phase was evaporated to dryness to yield product **1a** as a colorless oil ($m = 851.0$ mg, 2.69 mmol, 88 %). Compound **1a** was used in the next step without further purification.

$^1\text{H-NMR}$ (CDCl_3 400 MHz) δ (ppm): 0.03 (s, 3H, Si- CH_3), 0.06 (s, 3H, Si- CH_3), 0.86 (s, 9H, $\text{C}(\text{CH}_3)_3$), 0.86-0.90 (m, 3H, H-10), 1.22-1.36 (m, 10H, H-5 – H-9), 1.45-1.51 (m, 2H, H-4), 2.43 (dd, 2H, $J = 6.3, 1.8$ Hz, H-2), 3.66 (s, 3H, O- CH_3), 4.09-4.15 (m, 1H, H-3).

$^{13}\text{C-NMR}$ (CDCl_3 101 MHz) δ (ppm): (-4.69) (Si- CH_3), (-4.36) (Si- CH_3), 14.23 (C-10), 18.14 ($\text{C}(\text{CH}_3)_3$), 22.79, 25.11, 29.39, 29.77, 31.94 (C-9 – C-5), 25.92 ($\text{C}(\text{CH}_3)_3$), 37.80 (C-4), 42.70 (C-2), 51.56 (O- CH_3), 69.68 (C-3), 172.52 (C-1).

ESI-HRMS: $m/z = 317.2508$ [$\text{M}+\text{H}$] $^+$, calc. for $\text{C}_{17}\text{H}_{36}\text{O}_3\text{Si} + \text{H}^+ = 317.2507$.

Methyl 3-(1,3-dioxolane)decanoate (**2a**)



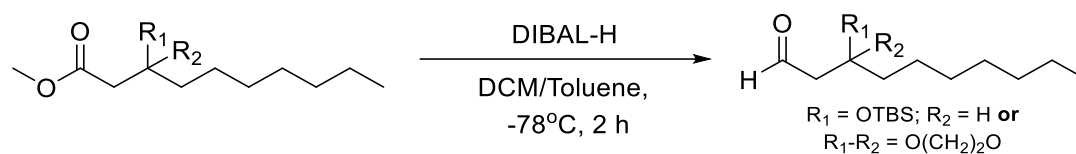
Compound **2a** was synthesized based on the procedure described by Hodgkinson *et al.* [4].

Methyl 3-oxodecanoate (**a**) (1.39 g, 6.94 mmol) was mixed with *para*-toluene sulfonic acid (132.0 mg, 694.0 μmol), trimethylorthoformate (1.37 mL, 12.49 mmol), and ethylene glycol (1.55 mL, 27.76 mmol) at room temperature under nitrogen atmosphere. The resulting mixture was stirred for 5 h at room temperature under nitrogen atmosphere. 10 mL of 5% aq. Na_2HPO_4 solution was added to the mixture and allowed to stir for 15 min. 15 mL of diethyl ether was added and the mixture was stirred vigorously for 20 min. Organic and aqueous phases were separated. The organic phase was washed with sat. aq. NaHCO_3 solution 3 times, dried over MgSO_4 , filtered, and concentrated *in vacuo*. Product **2a** was obtained as a slightly yellow oil ($m = 1.46$ g, 5.98 mmol, 85.9 %) and used in the next step without further purification.

$^1\text{H-NMR}$ (CDCl_3 400 MHz) δ (ppm): 0.87 (m, 3H, H-10), 1.20-1.33 (m, 8H, H-6 – H-9), 1.38 (m, 2H, H-5), 1.78 (m, 2H, H-4), 2.66 (s, 2H, H-2), 3.68 (s, 3H, CH_3), 3.93-4.01 (m, 4H, O- $\text{CH}_2\text{-CH}_2\text{-O}$).

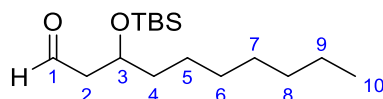
$^{13}\text{C-NMR}$ (CDCl_3 101 MHz) δ (ppm): 14.21 (C-10), 22.77, 29.36, 29.78, 31.91 (C-6 – C-9), 23.65 (C-5), 37.90 (C-4), 42.58 (C-2), 51.86 (O- CH_3), 65.25 (O- $\text{CH}_2\text{-CH}_2\text{-O}$), 109.57 (C-3), 170.18 (C-1).

General procedure 1: Generation of aldehydes



Protected methyl ester (1 eq.) was dissolved in dry DCM (4 mL/1 mmol) and cooled to -78°C . DIBAL-H solution (1 M in toluene, 1.15 eq.) was slowly added and the resulting mixture was stirred at -78°C for 2 h. The reaction was quenched by the addition of MeOH (1.5 mL/1 mmol) and sat. Rochelle salt solution (7 mL/1 mmol). The mixture was allowed to reach room temperature. Organic and aqueous layers were separated. The aqueous phase was extracted 2 times with DCM. The combined organic layers were washed with brine solution and dried over MgSO_4 . The organic phase was evaporated to dryness. The residue was purified by silica column chromatography (petrol ether/EtOAc 3:1) to yield the product.

3-((*tert*-Butyldimethylsilyl)oxy)decanal (**1b**)

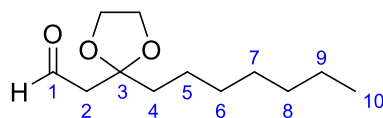


Compound **1b** was synthesized according to the general procedure 1 using 786.5 mg (2.48 mmol) of **1a** and 2.86 mL (2.86 mmol) of DIBAL-H solution. Product **1b** was obtained as a colorless oil ($m = 593.4$ mg, 2.07 mmol, 83 %) and used in the next step without further purification.

$^1\text{H-NMR}$ (CDCl_3 400 MHz) δ (ppm): 0.05 (s, 3H, Si- CH_3), 0.07 (s, 3H, Si- CH_3), 0.87 (s, 9H, $\text{C}(\text{CH}_3)_3$), 0.88 (m, 3H, H-10), 1.24-1.33 (m, 10H, H-5 – H-9), 1.46-1.52 (m, 2H, H-4), 2.50-2.52 (m, 2H, H-2), 4.17 (m, 1H, H-3), 9.81 (t, 1H, $J = 2.5$ Hz, H-1).

$^{13}\text{C-NMR}$ (CDCl_3 101 MHz) δ (ppm): (-4.55) (Si- CH_3), (-4.27) (Si- CH_3), 14.23 (C-10), 18.14 ($\text{C}(\text{CH}_3)_3$), 22.78, 25.28, 29.36, 29.71, 31.93 (C-9 – C-5), 25.91 ($\text{C}(\text{CH}_3)_3$), 38.01 (C-4), 50.98 (C-2), 68.46 (C-3), 202.69 (C-1).

3-(1,3-Dioxolane)decanal (**2b**)



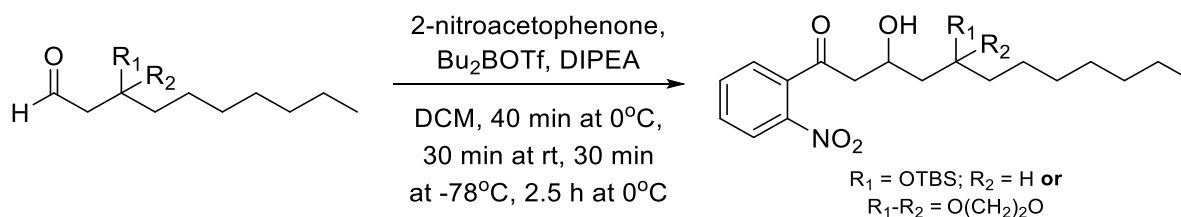
Compound **2b** was synthesized according to the general procedure 1 using 280.0 mg (1.15 mmol) of **2a** and 1.32 mL (1.32 mmol) of DIBAL-H solution. The residue was purified by silica column chromatography (petrol ether/EtOAc 3:1) to yield product **2b** as a colorless oil ($m = 502.6$ mg, 2.34 mmol, 71.5 %). $R_f = 0.54$ (petrol ether/EtOAc 3:1).

$^1\text{H-NMR}$ (CDCl_3 400 MHz) δ (ppm): 0.87 (m, 3H, H-10), 1.23-1.30 (m, 8H, H-6 – H-9), 1.33-1.39 (m, 2H, H-5), 1.67 (m, 2H, H-4), 2.67 (d, 2H, $J = 2.9$ Hz, H-2), 3.99 (m, 4H, O- CH_2 - CH_2 -O), 9.72 (t, 1H, $J = 3.0$ Hz, H-1).

$^{13}\text{C-NMR}$ (CDCl_3 101 MHz) δ (ppm): 14.19 (C-10), 22.75, 29.31, 29.75, 31.87 (C-6 – C-9), 23.67 (C-5), 38.64 (C-4), 50.76 (C-2), 65.20 (O- CH_2 - CH_2 -O), 109.68 (C-3), 200.79 (C-1).

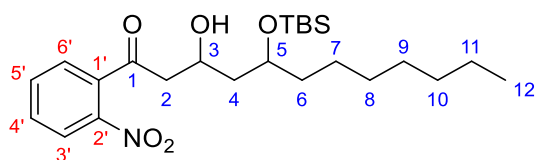
ESI-HRMS: $m/z = 451.3034$ [$2\text{M} + \text{Na}$] $^+$, calc. for $2\text{C}_{24}\text{H}_{44}\text{O}_6 + \text{Na}^+ = 451.3031$.

General procedure 2: Aldol condensation



2-Nitroacetophenone (1.15 eq.) was dissolved in dry DCM (4.9 mL/1 mmol of an aldehyde) and cooled to 0°C . Bu_2BOTf solution (1 M in DCM, 2.05 eq.) was added and the mixture stirred for 10 min at 0°C . DIPEA (2.25 eq.) was added as a solution in dry DCM (0.4 mL/1 mmol) and the resulting dark-red mixture was stirred for 30 min at 0°C . The reaction was warmed to room temperature and stirred for 30 min. The mixture was cooled to -78°C and aldehyde (1 eq.) was added slowly as a solution in dry DCM (2.33 mL/1 mmol). The mixture was stirred at -78°C for 30 min, warmed to 0°C , and stirred for 2.5 h at this temperature. The reaction was quenched at 0°C by the addition of pH 7 phosphate buffer (9.3 mL/1 mmol), MeOH (14 mL/1 mmol), and 30% H_2O_2 (9.3 mL/1 mmol). This mixture was allowed to reach room temperature and stirred vigorously for 5 min. Organic and aqueous phases were separated and the aqueous layer was extracted 2 times with DCM. The combined extracts were washed successively with sat. aq. NH_4Cl solution and brine solution. The organic phase was dried over MgSO_4 and concentrated *in vacuo*. The residue was purified by silica column chromatography (petrol ether/EtOAc 2:1) to yield the product.

5-((*tert*-Butyldimethylsilyl)oxy)-3-hydroxy-1-(2'-nitrophenyl)dodecan-1-one (**1c**)



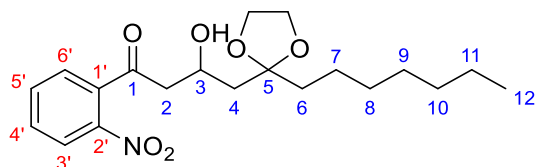
Compound **1c** was synthesized according to the general procedure 2 using 298.0 μL (2.22 mmol) of 2-nitroacetophenone, 3.96 mL (3.96 mmol) of Bu_2BOTf solution, 757.0 μL (4.35 mmol) of DIPEA, and 553.4 mg (1.93 mmol) of aldehyde **1b**. Product **1c** was obtained as an orange oil ($m = 680.8$ mg, 1.51 mmol, 78.0 %). $R_f = 0.55$ (petrol ether/EtOAc 2:1).

$^1\text{H-NMR}$ (MeOH- d_4 400 MHz) δ (mixture of 2 diastereomers (9:11), ppm): 0.06-0.09 (m, 6H, $\text{Si}(\text{CH}_3)_2$), 0.88 (s, 9H, $\text{C}(\text{CH}_3)_3$), 0.94 (m, 3H, H-12), 1.24-1.42 (m, 10H, H-7 – H-11), 1.45-1.56 (m, 2H, H-6), 1.59-1.64 (m, 1H, H-4), 1.65-1.77 (m, 1H, H-4), 2.92-3.10 (m, 2H, H-2), 3.91 (p, 0.45H, $J = 5.9$ Hz, H-5), 3.98 (p, 0.55H, $J = 5.7$ Hz, H-5), 4.29 (ddt, 1H, $J = 15.6, 12.3, 6.7$ Hz, H-3), 7.61 (dt, 1H, $J = 7.6, 1.3$ Hz, H-6'), 7.69 (td, 1H, $J = 7.9, 1.5$ Hz, H-4'), 7.81 (tt, 1H, $J = 7.6, 1.4$ Hz, H-5'), 8.12 (dd, 1H, $J = 8.2, 1.2$ Hz, H-3').

$^{13}\text{C-NMR}$ (MeOH- d_4 101 MHz) δ (mixture of 2 diastereomers (9:11), ppm): (-4.37), (-4.26), (-4.15), (-4.06) ($\text{Si}(\text{CH}_3)_2$), 14.42 (C-12), 18.89, 18.93 ($\text{C}(\text{CH}_3)_3$), 23.71, (25.92, 26.03), (30.39, 30.40), (30.84, 30.88), 32.96 (C-11 – C-7), 26.40, 26.45 ($\text{C}(\text{CH}_3)_3$), 37.96, 39.11 (C-6), 45.02, 45.51 (C-4), 51.29, 51.95 (C-2), 65.91, 66.46 (C-3), 70.66, 71.28 (C-5), 125.26, 125.38 (C-3'), 129.15, 129.21 (C-6'), 132.06, 132.10 (C-4'), 135.34 (C-5'), 138.71 (C-1'), 138.89 (C-2'), 202.87, 202.95 (C-1).

ESI-HRMS: $m/z = 474.2648$ $[\text{M}+\text{Na}]^+$, calc. for $\text{C}_{24}\text{H}_{41}\text{NO}_5\text{Si} + \text{Na}^+ = 474.2647$.

5-(1,3-Dioxolane)-3-hydroxy-1-(2'-nitrophenyl)dodecan-1-one (**2c**)



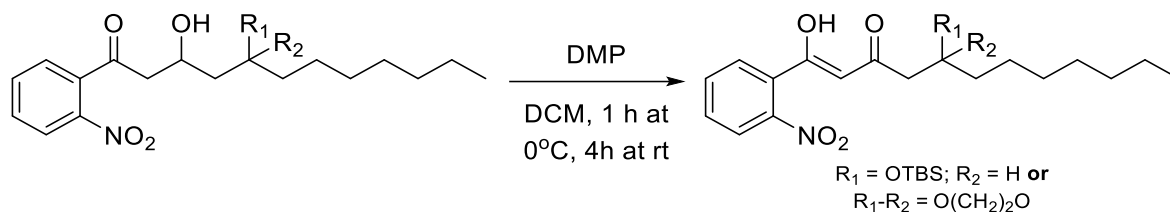
Compound **2c** was synthesized according to the general procedure 2 using 362.0 μL (2.70 mmol) of 2-nitroacetophenone, 4.81 mL (4.81 mmol) of Bu_2BOTf solution, 919.0 μL (5.28 mmol) of DIPEA, and 502.6 mg (2.35 mmol) of aldehyde **2b**. Product **2c** was obtained as a yellow oil ($m = 722.0$ mg, 1.90 mmol, 81.1 %). $R_f = 0.31$ (petrol ether/EtOAc 2:1).

$^1\text{H-NMR}$ (MeOH-d_4 400 MHz) δ (ppm): 0.88-0.92 (m, 3H, H-12), 1.27-1.34 (m, 8H, H-8 – H-11), 1.34-1.41 (m, 2H, H-7), 1.64-1.67 (m, 2H, H-6), 1.87 (dd, 1H, $J = 14.5, 7.1$ Hz, H-4), 1.93 (dd, 1H, $J = 14.5, 4.9$ Hz, H-4), 2.98 (dd, 1H, $J = 16.7, 7.9$ Hz, H-2), 3.13 (dd, 1H, $J = 16.7, 4.5$ Hz, H-2), 3.92-3.96 (m, 4H, O-CH₂-CH₂-O), 4.36 (ddt, 1H, $J = 7.9, 7.1, 4.7$ Hz, H-3), 7.62 (dd, 1H, $J = 7.6, 1.4$ Hz, H-6'), 7.69 (ddd, 1H, $J = 8.2, 7.4, 1.4$ Hz, H-4'), 7.81 (td, 1H, $J = 7.5, 1.1$ Hz, H-5'), 8.11 (dd, 1H, $J = 8.3, 1.1$ Hz, H-3').

$^{13}\text{C-NMR}$ (MeOH-d_4 101 MHz) δ (ppm): 14.42 (C-12), 23.71, 30.38, 30.92, 32.97 (C-8 – C-11), 24.84 (C-7), 38.55 (C-6), 44.07 (C-4), 51.36 (C-2), 65.62 (C-3), 65.70 (O-CH₂-CH₂-O), 112.21 (C-5), 125.33 (C-3'), 129.20 (C-6'), 132.08 (C-4'), 135.30 (C-5'), 137.39 (C-1'), 138.82 (C-2'), 202.96 (C-1).

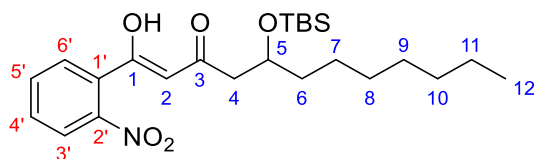
ESI-HRMS: $m/z = 402.1878$ $[\text{M}+\text{Na}]^+$, calc. for $\text{C}_{20}\text{H}_{29}\text{NO}_6 + \text{Na}^+ = 402.1888$.

General procedure 3: Alcohol oxidation



Alcohol (1 eq.) and DMP (1.5 eq.) were dissolved in dry DCM (4.5 mL/1 mmol of alcohol) under nitrogen atmosphere at 0°C. The resulting mixture was stirred under nitrogen atmosphere for 1 h at 0°C and for 4 h at room temperature. The mixture was diluted with DCM, washed with sat. aq. $\text{Na}_2\text{S}_2\text{O}_3$ solution and distilled water. The organic phase was dried over MgSO_4 and concentrated *in vacuo*. The residue was purified by prep. RP-HPLC (A: H_2O , B: MeOH ; 5% B to 100% B) (SPOT PREP Liquid Chromatography by Armen Instrument; Kinetex 5 μm C18 100 Å LC Column 250 x 21.2 mm, AXIA).

5-((*tert*-Butyldimethylsilyl)oxy)-3-oxo-1-(2'-nitrophenyl)dodecan-1-one (**1d**)



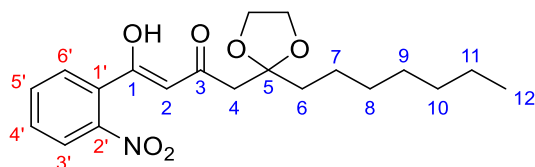
Compound **1d** was synthesized according to the general procedure 3 using 248.0 mg (549.1 μmol) of alcohol **1c** and 349.3 mg (823.6 μmol) of DMP. The product was obtained as an orange oil (m = 113.8 mg, 253.1 μmol , 46.1 %).

$^1\text{H-NMR}$ (DMSO- d_6 400 MHz) δ (mixture of enol-/keto-tautomers (4:1), ppm): (-0.03) – 0.05 (m, 6H, Si(CH $_3$) $_2$), 0.82 (s, 9H, C(CH $_3$) $_3$), 0.77-0.87 (m, 3H, H-12), 1.19-1.33 (m, 10H, H-7 – H-11), 1.46-1.51 (m, 2H, H-6), 2.44-2.70 (m, 2H, H-4), 4.10-4.17 (m, 1H, H-5), 4.21 (d, 0.4H, J = 2.7 Hz, H-2, keto-form), 6.26 (s, 0.8 H, H-2, enol-form), 7.71-7.89 (m, 3H, H-4'-H-6'), 8.00-8.15 (m, 1H, H-3').

$^{13}\text{C-NMR}$ (DMSO- d_6 101 MHz) δ (mixture of enol-/keto-tautomers (4:1), ppm): (-5.01), (-4.67) (Si(CH $_3$) $_2$), 13.90 (C-12), 17.67 (C(CH $_3$) $_3$), 22.02, 24.14, 28.54, 28.92, 31.10 (C-11 – C-7), 25.63 (C(CH $_3$) $_3$), 37.18 (C-6), 45.10 (C-4), 55.56 (C-2, keto-form, visible in HSQC-NMR), 69.53 (C-5), 100.86 (C-2, enol-form), 124.40 (C-3'), 129.27, 131.07, 133.21 (C-4'–C-6'), 147.93 (C-1'), 186.06, 189.75 (C-1, C-3).

ESI-HRMS: m/z = 450.2664 [M+H] $^+$, calc. for C $_{24}$ H $_{39}$ NO $_5$ Si + H $^+$ = 450.2671.

5-(1,3-Dioxolane)-3-oxo-1-(2'-nitrophenyl)dodecan-1-one (**2d**)



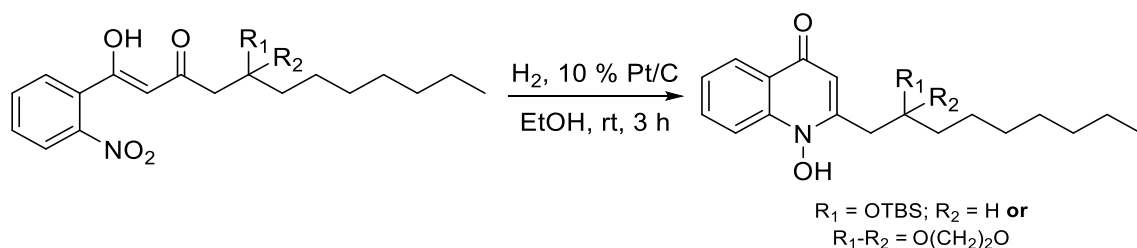
Compound **2d** was synthesized according to the general procedure 3 using 123.5 mg (325.5 μmol) of alcohol **2c** and 207.1 mg (488.2 μmol) of DMP. The product was obtained as an orange oil (m = 60.9 mg, 161.4 μmol , 49.6 %).

$^1\text{H-NMR}$ (DMSO- d_6 400 MHz) δ (mixture of enol-/keto-tautomers (7:3), ppm): 0.82-0.86 (m, 3H, H-12), 1.17-1.37 (m, 10H, H-7 – H-11), 1.54-1.69 (m, 2H, H-6), (2.71, 2.81) (s, 2H, H-4), 3.84-3.96 (m, 4H, O-CH $_2$ -CH $_2$ -O), 4.23 (s, 0.6H, H-2, keto-form, partially in exchange with solvent), 6.20 (s, 0.7H, H-2, enol-form), 7.64-7.95 (m, 3H, H-4'-H-6'), 7.95-8.13 (m, 1H, H-3').

$^{13}\text{C-NMR}$ (DMSO- d_6 101 MHz) δ (mixture of enol-/keto-tautomers (7:3), ppm): 13.92 (C-12), 22.05, 22.89, 28.59, 29.05, 31.16 (C-11 – C-7), 37.39 (C-6), 44.91 (C-4, enol form), 49.59 (C-4, keto-form), 55.70 (C-2, keto-form), 64.50 (O-CH $_2$ -CH $_2$ -O), 101.48 (C-2, enol-form), 109.38 (C-5), 124.44 (C-3'), 129.33, 132.36, 133.42 (C-4'-C-6'), 147.79 (C-1'), 186.04, 188.77 (C-1, C-3).

ESI-HRMS: m/z = 400.1725 [M+Na] $^+$, calc. for C $_{20}$ H $_{27}$ NO $_6$ + Na $^+$ = 400.1731.

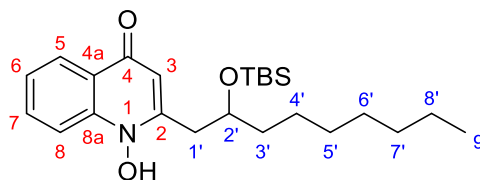
General procedure 4: Reductive cyclization to form 4-quinolone-*N*-oxide



Ketone was dissolved in EtOH (42 mL/1 mmol) and 10 % Pt/C catalyst (0.3 mg/1 mg of ketone) was added. The resulting mixture was placed under nitrogen atmosphere and hydrogen gas was supplied from a H $_2$ -filled balloon. The mixture was allowed to stir for 3 h at room

temperature. The catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was purified by silica column chromatography to yield the product.

2-(2'-((*tert*-Butyldimethylsilyl)oxy)nonyl)-1-hydroxyquinolin-4(1*H*)-one (2'-OTBS-NQNO) (**1f**)



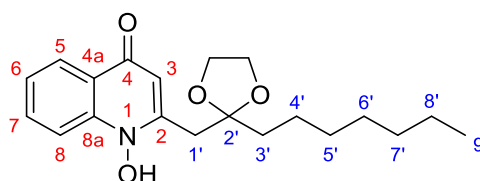
Compound **1f** was synthesized according to the general procedure 4 using 26.4 mg (58.7 μ mol) of ketone **1d** and 8.3 mg of 10 % Pt/C catalyst. The residue was purified by silica column chromatography with EtOAc to yield product **1f**, with compounds **1h** and **1e** as minor impurities (*m* = 4.5 mg). *R_f* = 0.20 (EtOAc). The product was used in the next step without further purification.

¹H-NMR (MeOH-*d*₄ 400 MHz) δ (ppm): (-0.33) (s, 3H, Si-CH₃), (-0.04) (s, 3H, Si-CH₃), 0.82 (s, 9H, C(CH₃)₃), 0.90-0.92 (m, 3H, H-9'), 1.29-1.35 (m, 8H, H-5'–H-8'), 1.42-1.52 (m, 2H, H-4'), 1.56-1.66 (m, 2H, H-3'), 2.87 (dd, 1H, *J* = 13.0, 8.5 Hz, H-1'), 3.21 (ddd, 1H, *J* = 15.4, 11.1, 4.0 Hz, H-1'), 4.34 (dq, 1H, *J* = 9.8, 5.3 Hz, H-2'), 6.40 (s, 1H, H-3), 7.53 (ddd, 1H, *J* = 8.3, 6.9, 1.2 Hz, H-6), 7.84 (ddd, 1H, *J* = 8.7, 6.9, 1.6 Hz, H-7), 8.14 (dd, 1H, *J* = 8.7, 4.2 Hz, H-8), 8.27 (dd, 1H, *J* = 8.2, 1.5 Hz, H-5).

¹³C-NMR (MeOH-*d*₄ 101 MHz) δ (ppm): (-4.90) (Si-CH₃), (-4.53) (Si-CH₃), 14.41 (C-9'), 18.76 (C(CH₃)₃), 23.70, 30.37, 30.73, 32.93 (C-5' – C-8'), 25.99 (C-4'), 26.30 (C(CH₃)₃), 39.23 (C-3'), 41.28 (C-1'), 71.45 (C-2'), 110.13 (C-3), 116.99 (C-8), 125.77 (C-5), 126.29 (C-6), 133.67 (C-7), 141.96 (C-8a), 153.34 (C-2). C-4 is not visible in ¹³C-NMR for *N*-oxides due to the electronic effects.

ESI-HRMS: *m/z* = 418.2762 [M+H]⁺, calc. for C₂₄H₃₉NO₃Si + H⁺ = 418.2772.

2-(2'-(1,3-Dioxolane)-1-hydroxyquinolin-4(1*H*)-one (2'-(1,3-dioxolane)-NQNO) (**2f**)



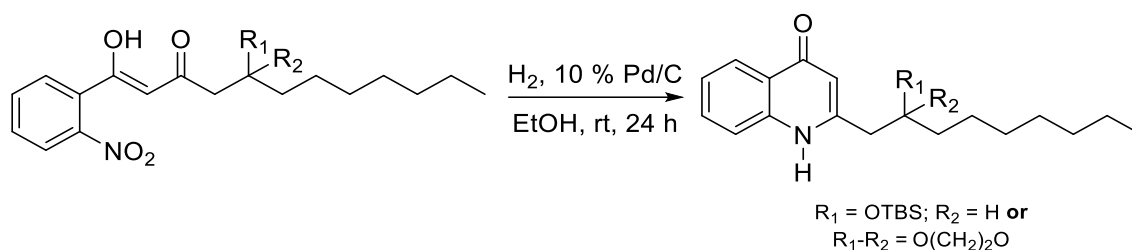
Compound **2f** was synthesized according to the general procedure (described above) using 61.6 mg (163.2 μ mol) of ketone **2d** and 19.0 mg of 10 % Pt/C catalyst. The residue was purified by silica column chromatography with MeOH/EtOAc (1:9) to yield product **2f** as a slightly yellow solid (*m* = 31.4 mg, 90.9 μ mol, 55.7 %). *R_f* = 0.40 (methanol/EtOAc 1:9).

¹H-NMR (MeOH-*d*₄ 399.79 MHz) δ (ppm): 0.86-0.91 (m, 3H, H-9'), 1.24-1.34 (m, 8H, H-5' – H-8'), 1.42-1.48 (m, 2H, H-4'), 1.72-1.76 (m, 2H, H-3'), 3.38 (s, 2H, H-1'), 3.79-3.85 (m, 2H, O-CH₂), 3.88-3.95 (m, 2H, O-CH₂), 6.53 (s, 1H, H-3), 7.51 (ddd, 1H, *J* = 8.2, 7.0, 1.1 Hz, H-6), 7.80 (ddd, 1H, *J* = 8.6, 7.0, 1.5 Hz, H-7), 8.14 (dt, 1H, *J* = 8.7, 0.8 Hz, H-8), 8.26 (ddd, 1H, *J* = 8.2, 1.5, 0.6 Hz, H-5).

¹³C-NMR (MeOH-*d*₄ 100.52 MHz) δ (ppm): 14.40 (C-9'), 23.69, 30.36, 30.80, 32.93 (C-5' – C-8'), 24.61 (C-4'), 38.98 (C-1'), 39.39 (C-3'), 66.42 (O-CH₂-CH₂-O), 110.13 (C-3), 112.15 (C-2'),

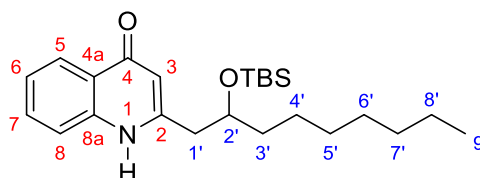
117.26 (C-8), 125.51 (C-4a), 125.82 (C-5), 126.22 (C-6), 133.59 (C-7), 141.80 (C-8a), 151.45 (C-2). C-4 is not visible in ^{13}C -NMR for *N*-oxides due to the electronic effects. ESI-HRMS: $m/z = 346.2003$ $[\text{M}+\text{H}]^+$, calc. for $\text{C}_{20}\text{H}_{27}\text{NO}_4 + \text{H}^+ = 346.2013$.

General procedure 5: Reductive cyclization to form 4-quinolone



The ketone was dissolved in EtOH (42 mL/1 mmol) and 10 % Pd/C catalyst (0.3 mg/1 mg of ketone) was added. The resulting mixture was placed under nitrogen atmosphere and hydrogen gas was supplied from a H_2 -filled balloon. The mixture was allowed to stir for 24 h at room temperature. The reaction mixture was filtered from the catalyst and evaporated to dryness. The residue was purified by silica column chromatography to yield the product.

2-(2'-((*tert*-Butyldimethylsilyl)oxy)nonyl)quinolin-4(1*H*)-one (2'-OTBS-NQ) (**1e**)



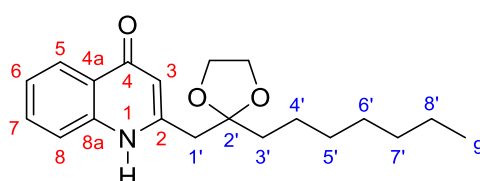
Compound **1e** was synthesized according to the general procedure 5 using 45.8 mg (101.9 μmol) of ketone **1d** and 14.4 mg of 10 % Pd/C catalyst. The residue was purified by silica column chromatography with EtOAc to yield product **1e** as a white solid ($m = 24.1$ mg, 60.0 μmol , 58.9 %). $R_f = 0.56$ (EtOAc).

^1H -NMR (MeOH- d_4 400 MHz) δ (ppm): (-0.35) (s, 3H, Si- CH_3), (-0.02) (s, 3H, Si- CH_3), 0.77 (s, 9H, $\text{C}(\text{CH}_3)_3$), 0.87-0.93 (m, 3H, H-9'), 1.29-1.38 (m, 8H, H-5'-H-8'), 1.42-1.50 (m, 2H, H-4'), 1.55-1.67 (m, 2H, H-3'), 2.71 (dd, 1H, $J = 13.5, 9.4$ Hz, H-1'), 2.93 (dd, 1H, $J = 13.5, 3.5$ Hz, H-1'), 4.13 (dq, 1H, $J = 9.6, 5.3$ Hz, H-2'), 6.26 (s, 1H, H-3), 7.40 (ddd, 1H, $J = 8.1, 6.9, 1.1$ Hz, H-6), 7.57 (d, 1H, $J = 8.3$ Hz, H-8), 7.69 (ddd, 1H, $J = 8.5, 7.0, 1.5$ Hz, H-7), 8.22 (dd, 1H, $J = 8.2, 1.5$ Hz, H-5).

^{13}C -NMR (MeOH- d_4 101 MHz) δ (ppm): (-4.97) (Si- CH_3), (-4.44) (Si- CH_3), 14.44 (C-9'), 18.73 ($\text{C}(\text{CH}_3)_3$), 23.73, 30.38, 30.84, 32.95 (C-5' – C-8'), 25.73 (C-4'), 26.21 ($\text{C}(\text{CH}_3)_3$), 39.29 (C-3'), 42.69 (C-1'), 73.68 (C-2'), 110.27 (C-3), 119.11 (C-8), 125.15 (C-6), 125.64 (C-4a), 126.00 (C-5), 133.38 (C-7), 141.48 (C-8a), 154.94 (C-2), 180.45 (C-4).

ESI-HRMS: $m/z = 402.2813$ $[\text{M}+\text{H}]^+$, calc. for $\text{C}_{24}\text{H}_{39}\text{NO}_2\text{Si} + \text{H}^+ = 402.2823$.

2-(2'-(1,3-Dioxolane))quinolin-4(1*H*)-one (2'-(1,3-dioxolane)-NQ) (**2e**)



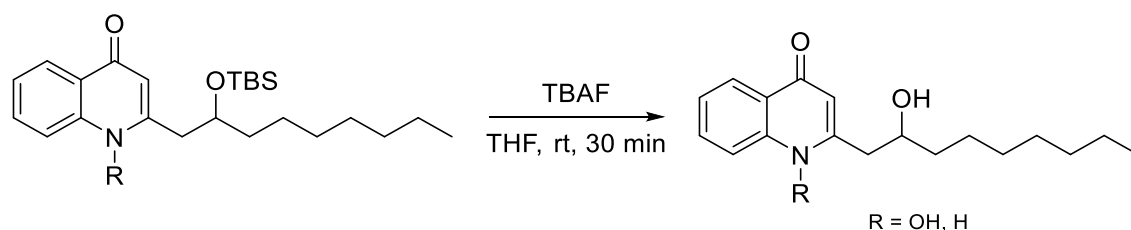
Compound **2e** was synthesized according to the general procedure 5 using 17.5 mg (46.4 μ mol) of ketone **2d** and 6.0 mg of 10 % Pd/C catalyst. The residue was purified by silica column chromatography MeOH/EtOAc 5:95 to yield product **2e** as a white solid (m = 11.8 mg, 35.8 μ mol, 77.3 %). R_f = 0.32 (MeOH/EtOAc 5:95).

$^1\text{H-NMR}$ (MeOH- d_4 400 MHz) δ (ppm): 0.87-0.90 (m, 3H, H-9'), 1.24-1.34 (m, 8H, H-5'-H-8'), 1.41-1.49 (m, 2H, H-4'), 1.67-1.71 (m, 2H, H-3'), 3.03 (s, 2H, H-1'), 3.73-3.81 (m, 2H, O-CH₂), 3.87-3.95 (m, 2H, O-CH₂), 6.33 (s, 1H, H-3), 7.40 (ddd, 1H, J = 8.2, 6.8, 1.2 Hz, H-6), 7.62-7.64 (m, 1H, H-8), 7.70 (ddd, 1H, J = 8.4, 6.8, 1.5 Hz, H-7), 8.22 (dd, 1H, J = 8.3, 1.4 Hz, H-5).

$^{13}\text{C-NMR}$ (MeOH- d_4 101 MHz) δ (ppm): 14.39 (C-9'), 24.68 (C-4'), 23.68, 30.34, 30.80, 32.92 (C-5' – C-8'), 39.48 (C-3'), 43.00 (C-1'), 66.52 (O-CH₂-CH₂-O), 111.67 (C-3), 119.26 (C-8), 125.17 (C-6), 125.55 (C-4a), 125.99 (C-5), 133.40 (C-7), 141.51 (C-8a), 151.85 (C-2), 180.21 (C-4).

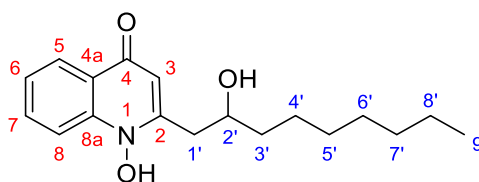
ESI-HRMS: m/z = 330.2053 $[M+H]^+$, calc. for C₂₀H₂₇NO₃ + H⁺ = 330.2064.

General procedure 6: TBS-deprotection



Starting material was dissolved in dry THF (18.5 mL/1 mmol) under nitrogen atmosphere. 1 M TBAF solution in THF (18.5 mL/1 mmol) was added and the mixture was allowed to stir at room temperature for 30 min. Then THF was evaporated under reduced pressure. The residue was dissolved in EtOAc and washed with distilled water 3 times. The organic phase was dried over MgSO₄ and concentrated *in vacuo*. The residue was purified by prep. RP-HPLC (A: H₂O, B: MeOH; 5% B to 95% B) to yield the product.

1-Hydroxy-2-(2'-hydroxynonyl)quinolin-4(1H)-one (2'-OH-NQNO) (**1h**)



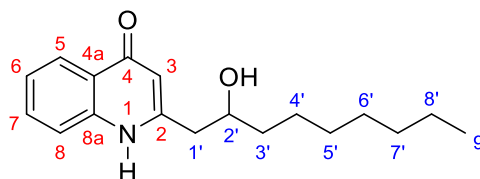
Compound **1h** was synthesized according to the general procedure 6 using 4.5 mg (10.8 μ mol) of starting material **1f** and 0.2 ml of 1 M TBAF solution in THF. The product was obtained as a slightly yellow solid (m = 2.9 mg, 9.6 μ mol, 88.7 %).

$^1\text{H-NMR}$ (MeOH- d_4 400 MHz) δ (ppm): 0.87-0.92 (m, 3H, H-9'), 1.25-1.45 (m, 10H, H-4' – H-8'), 1.55-1.62 (m, 2H, H-3'), 2.97 (dd, 1H, J = 13.8, 8.7 Hz, H-1'), 3.19 (dd, 1H, J = 13.8, 3.9 Hz, H-1'), 4.10 (dddd, 1H, J = 8.9, 7.0, 5.3, 3.9 Hz, H-2'), 6.46 (s, 1H, H-3), 7.53 (ddd, 1H, J = 8.1, 7.0, 1.1 Hz, H-6), 7.83 (ddd, 1H, J = 8.6, 7.0, 1.5 Hz, H-7), 8.16 (d, 1H, J = 8.7 Hz, H-8), 8.27 (dd, 1H, J = 8.2, 1.5 Hz, H-5).

$^{13}\text{C-NMR}$ (MeOH- d_4 101 MHz) δ (ppm): 14.41 (C-9'), 23.71, 30.41, 30.63, 32.98 (C-5' – C-8'), 26.72 (C-4'), 38.77 (C-3'), 40.71 (C-1'), 71.08 (C-2'), 109.07 (C-3), 117.13 (C-8), 125.21 (C-4a), 125.71 (C-5), 126.29 (C-6), 133.63 (C-7), 141.79 (C-8a), 153.40 (C-2), 172.09 (C-4) (visible in HSQC-NMR).

ESI-HRMS: m/z = 304.1896 $[M+H]^+$, calc. for C₁₈H₂₅NO₃ + H⁺ = 304.1908.

2-(2'-Hydroxynonyl)quinolin-4(1*H*)-one (2'-OH-NQ) (**1g**)

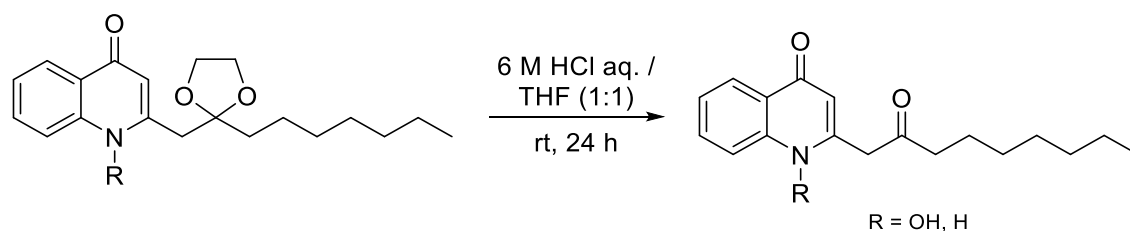


Compound **1g** was synthesized according to the general procedure (described above) using 13.5 mg (33.6 μ mol) of starting material **1e** and 0.3 ml of 1 M TBAF solution in THF. The product was obtained as a white solid ($m = 8.0$ mg, 27.8 μ mol, 82.8 %).

$^1\text{H-NMR}$ (MeOH- d_4 400 MHz) δ (ppm): 0.88-0.91 (m, 3H, H-9'), 1.26-1.44 (m, 10H, H-4' – H-8'), 1.49-1.59 (m, 2H, H-3'), 2.74 (dd, 1H, $J = 14.0, 8.6$ Hz, H-1'), 2.88 (dd, 1H, $J = 14.0, 4.3$ Hz, H-1'), 3.96 (tt, 1H, $J = 8.7, 3.7$ Hz, H-2'), 6.28 (s, 1H, H-3), 7.39 (ddd, 1H, $J = 8.1, 6.9, 1.1$ Hz, H-6), 7.60 (m, 1H, H-8), 7.69 (ddd, 1H, $J = 8.4, 6.9, 1.5$ Hz, H-7), 8.22 (dd, 1H, $J = 8.2, 1.5$ Hz, H-5). $^{13}\text{C-NMR}$ (MeOH- d_4 101 MHz) δ (ppm): 14.40 (C-9'), 23.71, 30.40, 30.64, 32.98 (C-5' – C-8'), 26.73 (C-4'), 38.50 (C-3'), 43.01 (C-1'), 71.64 (C-2'), 110.12 (C-3), 119.20 (C-8), 125.09 (C-6), 125.60 (C-4a), 125.97 (C-5), 133.36 (C-7), 141.51 (C-8a), 154.55 (C-2), 180.52 (C-4).

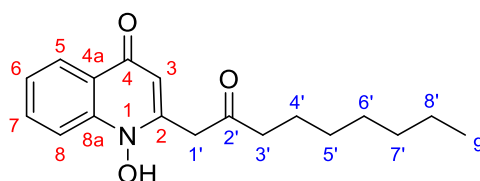
ESI-HRMS: $m/z = 288.1951$ $[\text{M}+\text{H}]^+$, calc. for $\text{C}_{18}\text{H}_{25}\text{NO}_2 + \text{H}^+ = 288.1959$.

General procedure 7: Ketal-deprotection



Starting material was dissolved in THF/6 M HCl aq. solution (1:1) (46.5 mL/1 mmol). The resulting mixture was allowed to stir at room temperature for 24 h. The reaction mixture was diluted with distilled water and extracted 2 times with EtOAc. The combined organic layers were washed with sat. aq. NaHCO_3 and Brine solutions. The organic phase was dried over MgSO_4 and concentrated *in vacuo*. The residue was purified by prep. RP-HPLC or by silica column chromatography to yield the product.

1-Hydroxy-2-(2'-oxononyl)quinolin-4(1*H*)-one (2'-oxo-NQNO) (**2h**)



Compound **2h** was synthesized according to the general procedure 7 using 28.3 mg (85.9 μ mol) of starting material **2f** and 4.0 ml of THF/6 M HCl aq. solution (1:1) mixture. The residue was purified by prep. RP-HPLC (A: H_2O , B: MeOH; 5% B to 95% B) (SPOT PREP Liquid Chromatography by Armen Instrument; Kinetex 5 μ m C18 100 Å LC Column 250 x 21.2 mm, AXIA) to yield product **2h** as a slightly yellow solid ($m = 12.7$ mg, 44.5 μ mol, 52 %).

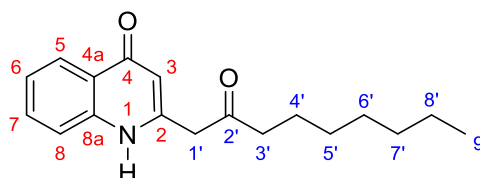
$^1\text{H-NMR}$ (MeOH- d_4 400 MHz) δ (ppm): 0.89-0.92 (m, 3H, H-9'), 1.27-1.42 (m, 8H, H-5' – H-8'), 1.60-1.67 (m, 2H, H-4'), 2.42-2.61 (bs, 2H, H-3'), 6.40 (s, 1H, H-3), 7.52 (t, 1H, J = 7.7 Hz, H-6), 7.82 (ddd, 1H, J = 8.5, 7.0, 1.4 Hz, H-7), 7.95-8.09 (bs, 1H, H-8), 8.29 (dd, 1H, J = 8.3, 1.4 Hz, H-5).

$^{13}\text{C-NMR}$ (MeOH- d_4 101 MHz) δ (ppm): 14.33 (C-9'), 23.63, 30.17, 30.29, 32.85 (C-5' – C-8'), 24.94 (C-4') (visible in HSQC-NMR), 43.59 (C-3') (visible in HSQC-NMR), 125.38, 126.07 (C-5 - C-6), 133.70 (C-7), C-4 is not visible in $^{13}\text{C-NMR}$ for *N*-oxides due to the electronic effects.

Note: Signals H-1', C-2, C-3, C-4, C-4a, C-7, C-8, C-8a, C-1', and C-2' couldn't be detected by NMR using different NMR-solvents (MeOH- d_4 , DMSO- d_6 , MeCN- d_3) at room temperature or 40°C. Low solubility of the compound in common solvents prevented an adequate concentration for measurements.

ESI-HRMS: m/z = 302.1739 $[\text{M}+\text{H}]^+$, calc. for $\text{C}_{18}\text{H}_{23}\text{NO}_3 + \text{H}^+ = 302.1751$.

2-(2'-Oxononyl)quinolin-4(1*H*)-one (2'-oxo-NQ) (**2g**)



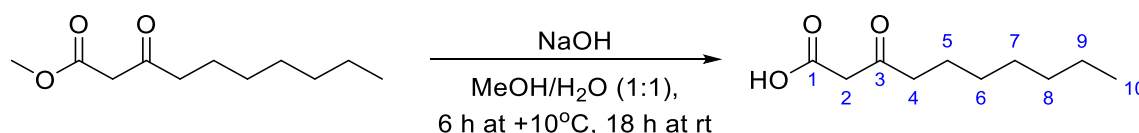
Compound **2g** was synthesized according to the general procedure 7 using 11.6 mg (35.2 μmol) of starting material **2e** and 3.0 mL of THF/6 M HCl aq. solution (1:1) mixture. The residue was purified by silica column chromatography with MeOH/EtOAc (5:95) to yield product **2g** as a white solid (m = 4.8 mg, 16.8 μmol , 47.8 %). R_f = 0.29 (MeOH/EtOAc 5:95).

$^1\text{H-NMR}$ (MeOH- d_4 400 MHz) δ (in keto-form, ppm): 0.89-0.93 (m, 3H, H-9'), 1.26-1.37 (m, 8H, H-5' – H-8'), 1.60-1.66 (m, 2H, H-4'), 2.64 (t, 2H, J = 7.3 Hz, H-3'), 6.23 (s, 1H, H-3), 7.41 (ddd, 1H, J = 8.1, 7.0, 1.1 Hz, H-6), 7.56 (dt, 1H, J = 8.4, 0.8 Hz, H-8), 7.70 (ddd, 1H, J = 8.4, 7.0, 1.5 Hz, H-7), 8.23 (ddd, 1H, J = 8.2, 1.5, 0.6 Hz, H-5). H-1' is in exchange with solvent.

$^{13}\text{C-NMR}$ (MeOH- d_4 101 MHz) δ (in keto-form, ppm): 14.38 (C-9'), 24.58 (C-4'), 23.66, 30.14, 30.20, 32.86 (C-5' - C-8'), 43.51 (C-3'), 47.36 (C-1') (visible in HMBC-NMR), 110.87 (C-3), 119.18 (C-8), 125.21 (C-6), 125.57 (C-4a), 126.04 (C-5), 133.57 (C-7), 141.67 (C-8a), 149.40 (C-2), 180.70 (C-4), 206.74 (C-2').

ESI-HRMS: m/z = 286.1793 $[\text{M}+\text{H}]^+$, calc. for $\text{C}_{18}\text{H}_{23}\text{NO}_2 + \text{H}^+ = 286.1802$.

β -Oxodecanoic acid (**3a**)



Methyl ester **a** (617.0 mg, 3.08 mmol) was dissolved in 4.4 mL of MeOH at +10°C. 4.4 mL of 0.7 M aqueous NaOH solution (3.08 mmol) was added. The resulting mixture was allowed to stir at +10°C for 6 h and at room temperature for 18 h. MeOH was evaporated under reduced pressure ($\leq +30^\circ\text{C}$ for water bath) and the residue was diluted with cooled water (5°C). The resulting mixture was acidified to pH 1-2 with 1 M HCl and product **3a** precipitated. The

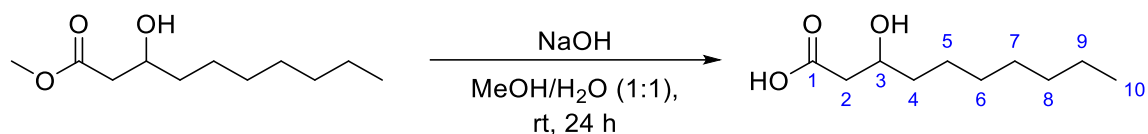
product was separated from water by filtration, dried under reduced pressure, and obtained as a white solid ($m = 461.7$ mg, 2.48 mmol, 80.5 %).

$^1\text{H-NMR}$ (CDCl_3 400 MHz) δ (mixture of enol-/keto-tautomers (1:5), ppm): 0.86-0.90 (m, 3H, H-10), 1.21-1.36 (m, 8H, H-6 - H-9), 1.57-1.66 (m, 2H, H-5), 2.23 (t, 2H, $J = 7.6$ Hz, H-4, enol-form), 2.56 (t, 2H, $J = 7.4$ Hz, H-4, keto-form), 3.52 (s, 2H, H-2, keto-form), 5.04 (s, 1H, H-2, enol-form), 11.86 (s, 1H, COOH).

$^{13}\text{C-NMR}$ (CDCl_3 101 MHz) δ (ppm): 14.02 (C-10), 22.56, 28.90, 28.94, 31.58 (C-9 – C-6), 23.36 (C-5), 43.31 (C-4), 46.72 (C-2, keto-form), 87.84 (C-2, enol-form), 169.06 (C-1), 206.11 (C-3).

ESI-HRMS: $m/z = 231.0967$ [$\text{M-H}+2\text{Na}$] $^+$, calc. for $\text{C}_{10}\text{H}_{17}\text{NaO}_3 + \text{Na}^+ = 231.0968$.

β -Hydroxydecanoic acid (**3b**)



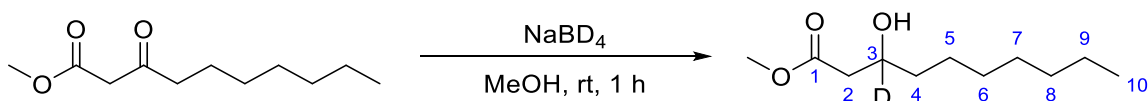
Methyl ester **b** (619.4 mg, 3.06 mmol) was dissolved in 30 mL of MeOH/water (1:1) mixture and NaOH (244.9 mg, 6.12 mmol) was added. The resulting mixture was allowed to stir at room temperature for 24 h. MeOH was evaporated under reduced pressure and the residue was diluted with distilled water. The resulting mixture was acidified to pH 1-2 with 1 M HCl and product **3b** was precipitated at 0°C. The product was separated from water by filtration, dried under reduced pressure, and obtained as a white solid ($m = 382.0$ mg, 2.03 mmol, 66.3 %).

$^1\text{H-NMR}$ (DMSO-d_6 400 MHz) δ (ppm): 0.85-0.87 (m, 3H, H-10), 1.19-1.32 (m, 10H, H-5 – H-9), 1.32-1.39 (m, 2H, H-4), 2.21 (ddd, 1H, $J = 14.8, 7.9, 1.0$ Hz, H-2), 2.29 (ddd, 1H, $J = 14.8, 5.1, 1.0$ Hz, H-2), 3.78 (m, 1H, H-3), 4.57 (bs, 1H, OH), 11.97 (bs, 1H, COOH).

$^{13}\text{C-NMR}$ (DMSO-d_6 101 MHz) δ (ppm): 13.95 (C-10), 22.08, 25.05, 28.69, 29.00, 31.25 (C-9 – C-5), 36.90 (C-4), 42.72 (C-2), 67.04 (C-3), 173.02 (C-1).

ESI-HRMS: $m/z = 233.1123$ [$\text{M-H}+2\text{Na}$] $^+$, calc. for $\text{C}_{10}\text{H}_{19}\text{NaO}_3 + \text{Na}^+ = 233.1125$.

Methyl 3-hydroxydecanoate-d (**3c**)



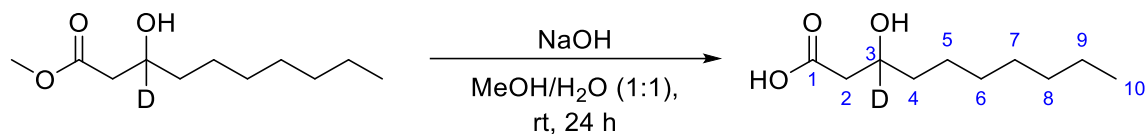
Methyl 3-oxodecanoate (**a**) (1.10 g, 5.49 mmol) was dissolved in dry MeOH (6.8 mL) and added slowly to a suspension of NaBD_4 (298.9 mg, 7.14 mmol) in dry MeOH (19.5 mL). The obtained mixture was stirred for 1 h at room temperature and quenched with 1 M aq. HCl solution. MeOH was evaporated and the remaining water mixture was extracted with diethyl ether 2 times. The combined organic phases were washed with brine solution, dried over MgSO_4 , filtered, and concentrated *in vacuo*. The residue was purified by silica column chromatography (petrol ether/EtOAc 3:1) to yield product **3c** as a colorless oil ($m = 497.5$ mg, 2.42 mmol, 44 %). $R_f = 0.58$ (petrol ether/EtOAc 3:1).

$^1\text{H-NMR}$ (CDCl_3 400 MHz) δ (ppm): 0.85-0.89 (m, 3H, H-10), 1.21-1.54 (m, 12H, H-4 – H-9), 2.37-2.54 (m, 2H, H-2), 3.71 (s, 3H, O-CH $_3$).

^{13}C -NMR (CDCl_3 101 MHz) δ (ppm): 14.20 (C-10), 22.77, 25.59, 29.35, 29.61, 31.93 (C-9 – C-5), 36.58 (C-4), 41.18 (C-2), 51.84 (O-CH₃), 67.77 (C-3), 173.62 (C-1).

ESI-HRMS: m/z = 226.1524 $[\text{M}+\text{Na}]^+$, calc. for $\text{C}_{11}\text{H}_{21}\text{DO}_3 + \text{Na}^+ = 226.1524$.

β -Hydroxydecanoic acid-d (**3d**)



Methyl ester **3c** (98.0 mg, 482 μmol) was dissolved in 5 mL of MeOH/water (1:1) mixture and NaOH (38.6 mg, 964 μmol) was added. The resulting mixture was allowed to stir at room temperature for 24 h. MeOH was evaporated under reduced pressure and the residue was diluted with distilled water. The resulting mixture was acidified to pH 1-2 with 1 M HCl and product **3d** was precipitated at 0°C. The product was separated from water by filtration, dried under reduced pressure, and obtained as a white solid (m = 61.1 mg, 325 μmol , 67.5 %).

^1H -NMR (DMSO-d_6 400 MHz) δ (ppm): 0.84-0.88 (m, 3H, H-10), 1.17-1.39 (m, 12H, H-4 – H-9), 2.24 (q, 2H, J = 14.8 Hz, H-2), 4.55 (s, 1H, OH), 11.97 (s, 1H, COOH).

^{13}C -NMR (DMSO-d_6 101 MHz) δ (ppm): 13.89 (C-10), 22.03, 24.97, 28.64, 28.96, 31.20 (C-9 – C-5), 36.75 (C-4), 42.59 (C-2), 66.53 (C-3) (visible in HMBC-NMR), 172.96 (C-1).

ESI-HRMS: m/z = 212.1369 $[\text{M}+\text{Na}]^+$, calc. for $\text{C}_{10}\text{H}_{19}\text{DO}_3 + \text{Na}^+ = 212.1368$.

4. Supplementary Tables

Table S1. Bacterial strains and their growth conditions.

Strain	Growth conditions
<i>Burkholderia ambifaria</i> AMMD	LB medium; 28°C, 37°C; 180 rpm
<i>Burkholderia thailandensis</i> DSM13276	LB medium; 28°C, 37°C; 180 rpm
<i>Staphylococcus aureus</i> USA300	LB medium; 37°C; 180 rpm
<i>Pseudomonas aeruginosa</i> PAO1	LB medium; 37°C; 180 rpm
<i>Pseudomonas aeruginosa</i> PA14	LB medium; 37°C; 180 rpm

Table S2. Calibration curves for quantification of quinolones. LLOQ = Lower Limit of Quantification. LOD = Limit of Detection (on Dionex Ultimate 3000 UHPLC + Finnigan™ TSQ® Quantum).

Calibration standard	RT	Mass Transitions	Calibration equation; R-value; Concentrations	LLOQ [mg/L]	LOD [mg/L]
2'-OH-NQ	5.71	288/270	Y = 1.68983e+006+7.04084e+006*X; R ² = 0.9883; C = 0.010, 0.025, 0.050, 0.10, 0.25, 0.50, 0.75, 1.00, 2.50, 5.00, 7.50, 10.00 mg/L	0.010	0.001
2'-OH-NQNO	5.96	304/286	Y = 1.56524e+006+4.63466e+006*X; R ² = 0.9867; C = 0.025, 0.10, 0.25, 0.50, 0.75, 1.00, 2.50, 5.00, 7.50, 10.00 mg/L	0.025	0.003
NQ	7.65	272/159	Y = 9.49363e+007+1.96682e+008*X; R ² = 0.9782; C = 0.025, 0.25, 0.75, 1.00, 2.50, 5.00, 7.50, 10.00 mg/L	0.025	0.003
NQNO	7.39	288/159	Y = 3.7495e+007+1.02015e+008*X; R ² = 0.9852; C = 0.025, 0.25, 0.75, 1.00, 2.50, 5.00, 7.50, 10.00 mg/L	0.025	0.003

Table S3a. Relative abundance of detected fragments of each standard after fragmentation by CID at 30 V (on Dionex Ultimate 3000 UHPLC + Finnigan™ TSQ® Quantum).

Standard	RT	Parental mass	Fragments (m/z and relative abundance)
2'-OH-NQ	5.71	288	159 (100%), 270 (55%), 172 (35%), 184 (15%), 198 (10%)
2'-OH-NQNO	5.96	304	159 (100%), 198 (85%), 286 (75%), 184 (50%), 158 (45%), 172 (30%)
2'-oxo-NQ	6.16	286	160 (100%), 159 (25%)
2'-oxo-NQNO	6.23	302	159 (100%), 186 (50%), 158 (55%)
NQ	7.65	272	159 (70%), 172 (35%)

NQNO	7.39	288	159 (100%), 186 (35%), 172 (25%)
Δ^1 -NQ	7.59	270	184 (100%), 159 (30%), 172 (20%), 198 (6%)
Δ^1 -NQNO	6.91	286	198 (100%), 184 (76%), 159 (38%), 158 (28%), 172 (22%)
Δ^2 -NQ	7.27	270	159 (100%), 172 (28%), 184 (16%), 198 (14%)
Δ^2 -NQNO	7.03	286	159 (100%), 158 (88%), 184 (74%), 198 (54%), 172 (50%)

Table S3b. Relative abundance of detected fragments of each standard after fragmentation by CID at 30 V (on Vanquish™ UHPLC + TSQ® Series II Quantum).

Standard	RT	Parental mass	Fragments (m/z and relative abundance)
2'-OH-NQ	4.42	288	159 (100%), 270 (37%), 172 (35%), 184 (20%), 198 (15%)
2'-OH-NQNO	4.77	304	159 (100%), 198 (80%), 286 (65%), 184 (45%), 158 (40%), 172 (32%), 240 (22%), 226 (15%)
2'-oxo-NQ	4.89	286	160 (100%), 159 (15%)
2'-oxo-NQNO	5.04	302	159 (100%), 186 (62%), 158 (38%), 214 (20%), 201 (15%), 176 (12%)
NQ	5.99	272	159 (100%), 172 (42%)
NQNO	6.01	288	159 (100%), 186 (30%), 172 (20%)
Δ^1 -NQ	5.95	270	184 (100%), 159 (25%), 172 (16%), 198 (6%)
Δ^1 -NQNO	5.60	286	198 (100%), 184 (68%), 159 (34%), 158 (24%)
Δ^2 -NQ	5.79	270	159 (100%), 172 (25%), 184 (15%), 198 (12%)
Δ^2 -NQNO	5.75	286	158 (100%), 159 (90%), 184 (70%), 198 (63%), 172 (45%), 212 (18%), 240 (12%)

Table S4. 2'-OH-NQ quantification in *P. aeruginosa* PA14 and PAO1 strains.

Sample	Integrated Area	Sample	Integrated Area
PA14-3h-1	84100	PAO1-3h-1	116317
PA14-3h-2	58009	PAO1-3h-2	304891
PA14-3h-3	81001	PAO1-3h-3	191163
PA14-6h-1	791530	PAO1-6h-1	3504122
PA14-6h-2	831066	PAO1-6h-2	3207211
PA14-6h-3	986640	PAO1-6h-3	2095445
PA14-9h-1	2295687	PAO1-9h-1	5219176
PA14-9h-2	2813816	PAO1-9h-2	3629966
PA14-9h-3	2576780	PAO1-9h-3	4213426
PA14-12h-1	3548713	PAO1-12h-1	5528172

PA14-12h-2	5353730	PAO1-12h-2	4497147
PA14-12h-3	6787559	PAO1-12h-3	6622890
PA14-24h-1	3764432	PAO1-24h-1	3821309
PA14-24h-2	3965460	PAO1-24h-2	3845877
PA14-24h-3	3271938	PAO1-24h-3	5088473

Table S5. 2'-OH-NQNO quantification in *P. aeruginosa* PA14 and PAO1 strains.

Sample	Integrated Area	Sample	Integrated Area
PA14-3h-1	18613	PAO1-3h-1	47554
PA14-3h-2	8439	PAO1-3h-2	118025
PA14-3h-3	16051	PAO1-3h-3	72927
PA14-6h-1	153016	PAO1-6h-1	1309690
PA14-6h-2	169866	PAO1-6h-2	988177
PA14-6h-3	287696	PAO1-6h-3	765605
PA14-9h-1	551553	PAO1-9h-1	1945562
PA14-9h-2	449644	PAO1-9h-2	1398121
PA14-9h-3	423467	PAO1-9h-3	1948738
PA14-12h-1	552234	PAO1-12h-1	2410936
PA14-12h-2	544770	PAO1-12h-2	1345731
PA14-12h-3	853214	PAO1-12h-3	2002962
PA14-24h-1	439808	PAO1-24h-1	1282271
PA14-24h-2	586253	PAO1-24h-2	1320051
PA14-24h-3	396548	PAO1-24h-3	1719640

Table S6. NQ quantification in *P. aeruginosa* PA14 and PAO1 strains.

Sample	Integrated Area	Sample	Integrated Area
PA14-3h-1	501848	PAO1-3h-1	366262
PA14-3h-2	496682	PAO1-3h-2	691942
PA14-3h-3	381478	PAO1-3h-3	564122
PA14-6h-1	27292364	PAO1-6h-1	15042567
PA14-6h-2	30610390	PAO1-6h-2	14316099
PA14-6h-3	39152826	PAO1-6h-3	9160270
PA14-9h-1	207887296	PAO1-9h-1	14851050
PA14-9h-2	228071486	PAO1-9h-2	14349381
PA14-9h-3	218898729	PAO1-9h-3	14760414
PA14-12h-1	145790218	PAO1-12h-1	26663682
PA14-12h-2	184756234	PAO1-12h-2	29582702
PA14-12h-3	202022388	PAO1-12h-3	34178842
PA14-24h-1	115574217	PAO1-24h-1	39157521
PA14-24h-2	174106562	PAO1-24h-2	37789855
PA14-24h-3	176192623	PAO1-24h-3	46101755

Table S7. NQNO quantification in *P. aeruginosa* PA14 and PAO1 strains.

Sample	Integrated Area	Sample	Integrated Area
PA14-3h-1	1971240	PAO1-3h-1	3816139

PA14-3h-2	1020809	PAO1-3h-2	9115471
PA14-3h-3	1559278	PAO1-3h-3	5586308
PA14-6h-1	21267448	PAO1-6h-1	105014203
PA14-6h-2	19080779	PAO1-6h-2	65826758
PA14-6h-3	39783465	PAO1-6h-3	56181928
PA14-9h-1	55971590	PAO1-9h-1	130961624
PA14-9h-2	46297781	PAO1-9h-2	104467959
PA14-9h-3	51258513	PAO1-9h-3	142361611
PA14-12h-1	67280119	PAO1-12h-1	217840225
PA14-12h-2	59189333	PAO1-12h-2	143899586
PA14-12h-3	110715899	PAO1-12h-3	178472465
PA14-24h-1	143371405	PAO1-24h-1	311097746
PA14-24h-2	199897873	PAO1-24h-2	321063006
PA14-24h-3	147173304	PAO1-24h-3	430203776

Table S8. 2'-oxo-NQ quantification in *P. aeruginosa* PA14 and PAO1 strains. ND = Not Detected.

Sample	Integrated Area	Sample	Integrated Area
PA14-3h-1	ND	PAO1-3h-1	ND
PA14-3h-2	ND	PAO1-3h-2	ND
PA14-3h-3	ND	PAO1-3h-3	ND
PA14-6h-1	ND	PAO1-6h-1	ND
PA14-6h-2	ND	PAO1-6h-2	ND
PA14-6h-3	ND	PAO1-6h-3	ND
PA14-9h-1	ND	PAO1-9h-1	ND
PA14-9h-2	ND	PAO1-9h-2	ND
PA14-9h-3	ND	PAO1-9h-3	ND
PA14-12h-1	ND	PAO1-12h-1	ND
PA14-12h-2	ND	PAO1-12h-2	ND
PA14-12h-3	ND	PAO1-12h-3	ND
PA14-24h-1	ND	PAO1-24h-1	ND
PA14-24h-2	ND	PAO1-24h-2	ND
PA14-24h-3	ND	PAO1-24h-3	ND

Table S9. 2'-oxo-NQNO quantification in *P. aeruginosa* PA14 and PAO1 strains. ND = Not Detected.

Sample	Integrated Area	Sample	Integrated Area
PA14-3h-1	ND	PAO1-3h-1	ND
PA14-3h-2	ND	PAO1-3h-2	ND
PA14-3h-3	ND	PAO1-3h-3	ND
PA14-6h-1	ND	PAO1-6h-1	ND
PA14-6h-2	ND	PAO1-6h-2	ND
PA14-6h-3	ND	PAO1-6h-3	ND
PA14-9h-1	ND	PAO1-9h-1	ND

PA14-9h-2	ND	PAO1-9h-2	ND
PA14-9h-3	ND	PAO1-9h-3	ND
PA14-12h-1	ND	PAO1-12h-1	ND
PA14-12h-2	ND	PAO1-12h-2	ND
PA14-12h-3	ND	PAO1-12h-3	ND
PA14-24h-1	ND	PAO1-24h-1	ND
PA14-24h-2	ND	PAO1-24h-2	ND
PA14-24h-3	ND	PAO1-24h-3	ND

Table S10. 2'-OH-NQ quantification in *B. ambifaria* and *B. thailandensis*. ND = Not Detected.

Sample	Integrated Area	Sample	Integrated Area
BA-9h-1	ND	BA-18h-2	ND
BA-9h-2	ND	BA-21h-1	ND
BA-11h-1	ND	BA-21h-2	ND
BA-11h-2	ND	BA-24h-1	ND
BA-12h-1	ND	BA-24h-2	ND
BA-12h-2	ND	BT-7h-1	ND
BA-13h-1	ND	BT-7h-2	ND
BA-13h-2	ND	BT-14h-1	ND
BA-14h-1	ND	BT-14h-2	ND
BA-14h-2	ND	BT-16h-1	ND
BA-18h-1	ND	BT-16h-2	ND

Table S11. 2'-OH-NQNO quantification in *B. ambifaria* and *B. thailandensis*. ND = Not Detected.

Sample	Integrated Area	Sample	Integrated Area
BA-9h-1	ND	BA-18h-2	ND
BA-9h-2	ND	BA-21h-1	ND
BA-11h-1	ND	BA-21h-2	ND
BA-11h-2	ND	BA-24h-1	ND
BA-12h-1	ND	BA-24h-2	ND
BA-12h-2	ND	BT-7h-1	ND
BA-13h-1	ND	BT-7h-2	ND
BA-13h-2	ND	BT-14h-1	ND
BA-14h-1	ND	BT-14h-2	ND
BA-14h-2	ND	BT-16h-1	ND
BA-18h-1	ND	BT-16h-2	ND

Table S12. 2'-oxo-NQ quantification in *B. ambifaria* and *B. thailandensis*. ND = Not Detected.

Sample	Integrated Area	Sample	Integrated Area
BA-9h-1	ND	BA-18h-2	ND
BA-9h-2	ND	BA-21h-1	ND
BA-11h-1	ND	BA-21h-2	ND
BA-11h-2	ND	BA-24h-1	ND
BA-12h-1	ND	BA-24h-2	ND

BA-12h-2	ND	BT-7h-1	ND
BA-13h-1	ND	BT-7h-2	ND
BA-13h-2	ND	BT-14h-1	ND
BA-14h-1	ND	BT-14h-2	ND
BA-14h-2	ND	BT-16h-1	ND
BA-18h-1	ND	BT-16h-2	ND

Table S13. 2'-oxo-NQNO quantification in *B. ambifaria* and *B. thailandensis*. ND = Not Detected.

Sample	Integrated Area	Sample	Integrated Area
BA-9h-1	ND	BA-18h-2	ND
BA-9h-2	ND	BA-21h-1	ND
BA-11h-1	ND	BA-21h-2	ND
BA-11h-2	ND	BA-24h-1	ND
BA-12h-1	ND	BA-24h-2	ND
BA-12h-2	ND	BT-7h-1	ND
BA-13h-1	ND	BT-7h-2	ND
BA-13h-2	ND	BT-14h-1	ND
BA-14h-1	ND	BT-14h-2	ND
BA-14h-2	ND	BT-16h-1	ND
BA-18h-1	ND	BT-16h-2	ND

Table S14. 2'-OH-NQ and 2'-OH-NQ + 1 Da; 2'-OH-NQNO and 2'-OH-NQNO + 1 Da quantification in *P. aeruginosa* PAO1 after β -hydroxydecanoic acid-d (3d) feeding.

Sample	Integrated Area	Sample	Integrated Area
Control-2'-OH-NQ-1	1440413	Control-2'-OH-NQNO-1	1223304
Control-2'-OH-NQ-2	1884944	Control-2'-OH-NQNO-2	1526312
Control-2'-OH-NQ-3	3333398	Control-2'-OH-NQNO-3	1064222
Control-2'-OH-NQ+1Da-1	299708	Control-2'-OH-NQNO+1Da-1	238723
Control-2'-OH-NQ+1Da-2	358985	Control-2'-OH-NQNO+1Da-2	256667
Control-2'-OH-NQ+1Da-3	500500	Control-2'-OH-NQNO+1Da-3	214404

AfterFeeding- 2'-OH-NQ-1	1630731	AfterFeeding- 2'-OH-NQNO-1	1368420
AfterFeeding- 2'-OH-NQ-2	1687804	AfterFeeding- 2'-OH-NQNO-2	1393864
AfterFeeding- 2'-OH-NQ-3	1993007	AfterFeeding- 2'-OH-NQNO-3	1454276
AfterFeeding- 2'-OH-NQ+1Da-1	1604961	AfterFeeding- 2'-OH-NQNO+1Da- 1	1101990
AfterFeeding- 2'-OH-NQ+1Da-2	1500030	AfterFeeding- 2'-OH-NQNO+1Da- 2	1070906
AfterFeeding- 2'-OH-NQ+1Da-3	1784619	AfterFeeding- 2'-OH-NQNO+1Da- 3	1068054

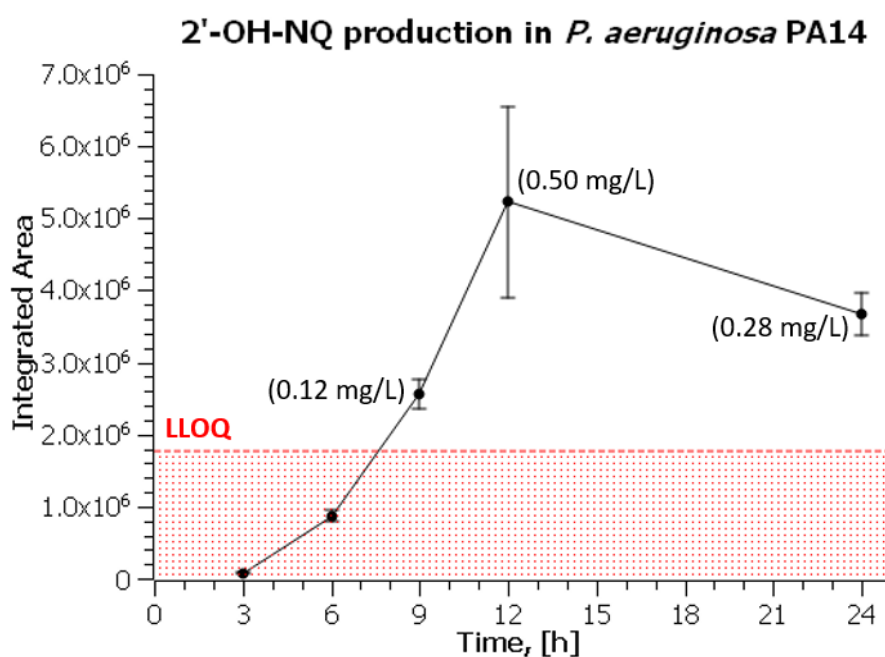
Table S15. 2'-OH-NQ and 2'-OH-NQNO quantification in *P. aeruginosa* PAO1 after β -hydroxydecanoic acid (**3b**) feeding.

Sample	Integrated Area	Sample	Integrated Area
Control-2'-OH-NQ-1	3609549	Control-2'-OH-NQNO-1	2392019
Control-2'-OH-NQ-2	3603880	Control-2'-OH-NQNO-2	2388870
Control-2'-OH-NQ-3	3441893	Control-2'-OH-NQNO-3	2329880
AfterFeeding50 μ M- 2'-OH-NQ-1	12143160	AfterFeeding50 μ M - 2'-OH-NQNO-1	5480479
AfterFeeding50 μ M - 2'-OH-NQ-2	12823687	AfterFeeding50 μ M - 2'-OH-NQNO-2	6123735
AfterFeeding50 μ M - 2'-OH-NQ-3	11926100	AfterFeeding50 μ M - 2'-OH-NQNO-3	6006622
AfterFeeding100 μ M- 2'-OH-NQ-1	9028906	AfterFeeding100 μ M- 2'-OH-NQNO-1	4635033
AfterFeeding100 μ M- 2'-OH-NQ-2	12358925	AfterFeeding100 μ M- 2'-OH-NQNO-2	5885229

AfterFeeding100μM- 2'-OH-NQ-3	12673985	AfterFeeding100μM- 2'-OH-NQNO-3	5658018
AfterFeeding150μM- 2'-OH-NQ-1	13917867	AfterFeeding150μM- 2'-OH-NQNO-1	6378512
AfterFeeding150μM- 2'-OH-NQ-2	10679772	AfterFeeding150μM- 2'-OH-NQNO-2	4974616
AfterFeeding150μM- 2'-OH-NQ-3	11387135	AfterFeeding150μM- 2'-OH-NQNO-3	5437844
AfterFeeding200μM- 2'-OH-NQ-1	13737617	AfterFeeding200μM- 2'-OH-NQNO-1	6219857
AfterFeeding200μM- 2'-OH-NQ-2	11347482	AfterFeeding200μM- 2'-OH-NQNO-2	5337976
AfterFeeding200μM- 2'-OH-NQ-3	9542953	AfterFeeding200μM- 2'-OH-NQNO-3	4430976

5. Supplementary Figures

a)



b)

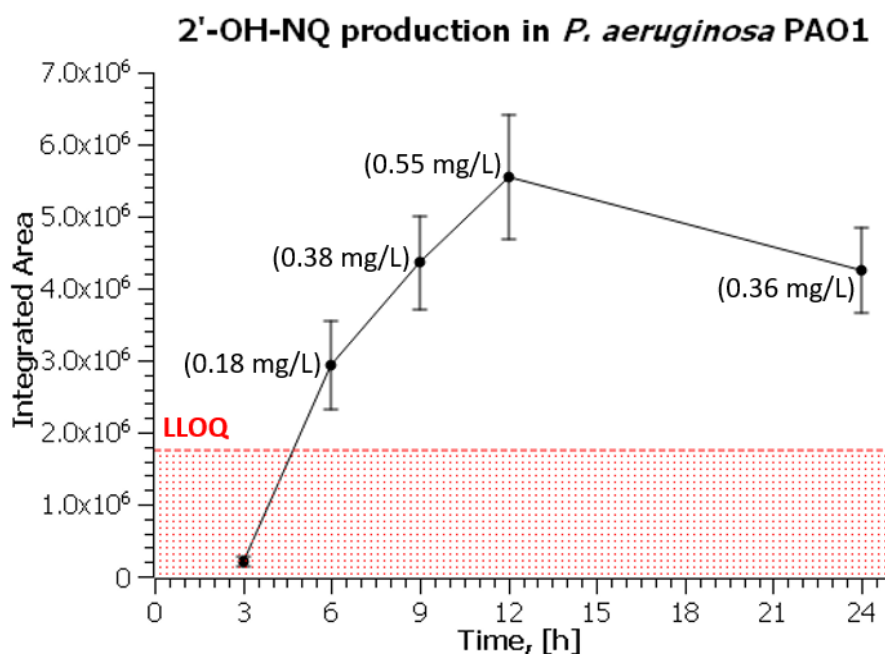


Figure S1. Integrated area of monitored mass transitions of 2'-OH-NQ produced by: **a)** *P. aeruginosa* PA14; **b)** *P. aeruginosa* PAO1 after different incubation times. Quantified concentrations are given in brackets. LLOQ = Lower Limit of Quantification. The error bars represent the population standard deviation.

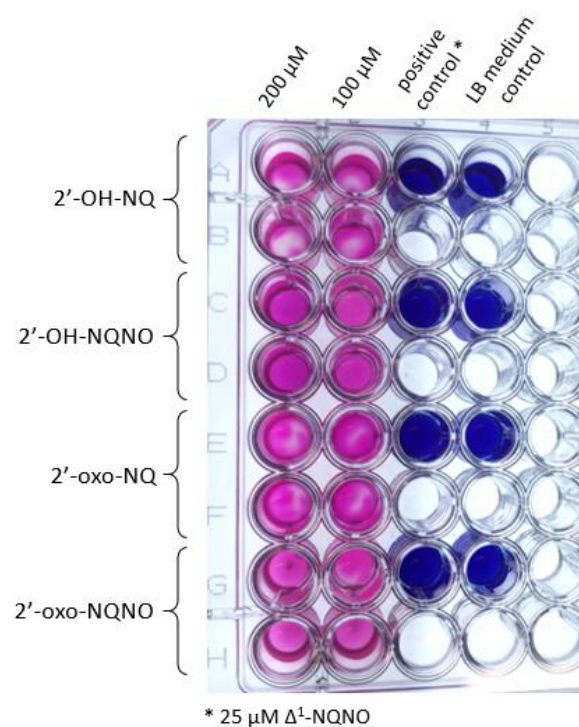


Figure S3. Resazurin assay of *S. aureus* USA300 over an incubation time of 8 h. Δ^1 -NQNO = (E)- Δ^1 -2-(non-1-enyl)-4(1H)-quinolone N-oxide.

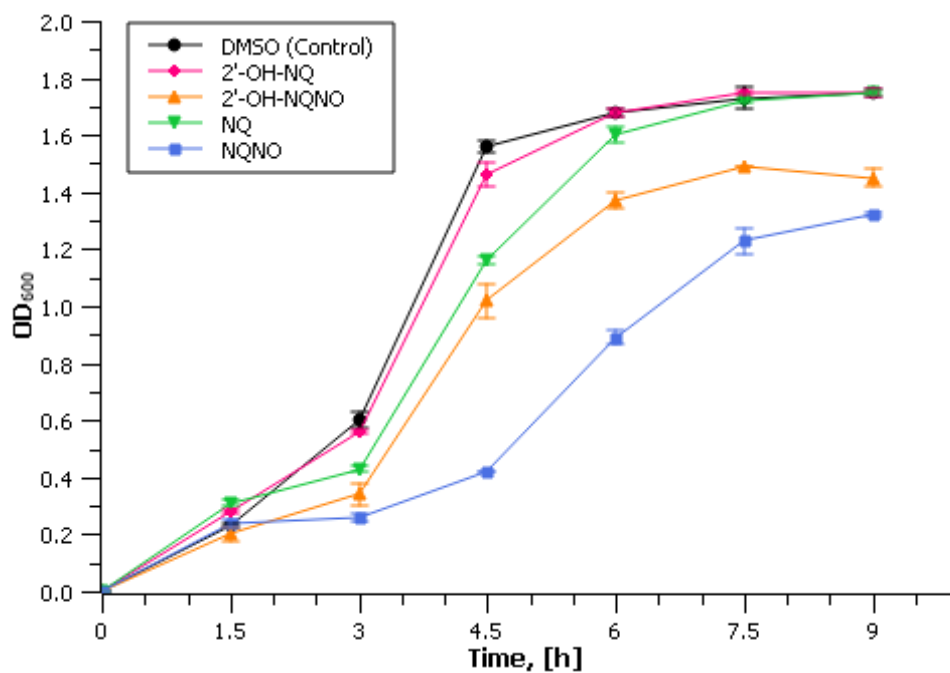
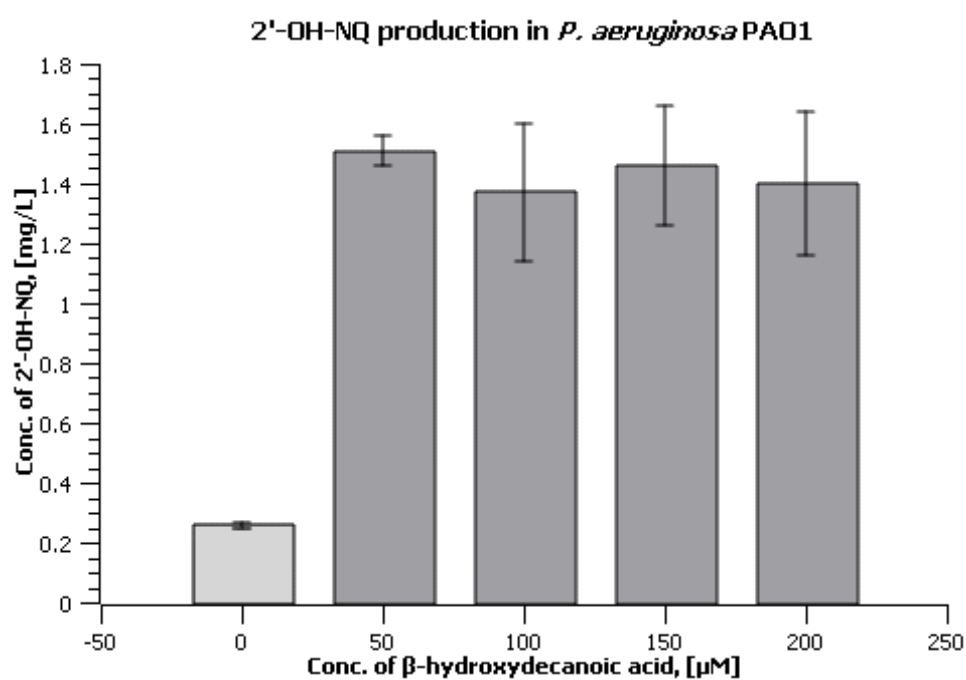


Figure S4. Growth curves of *S. aureus* USA300 in presence of different quinolones (50 μM) and DMSO as a control. The error bars represent the population standard deviation.

a)



b)

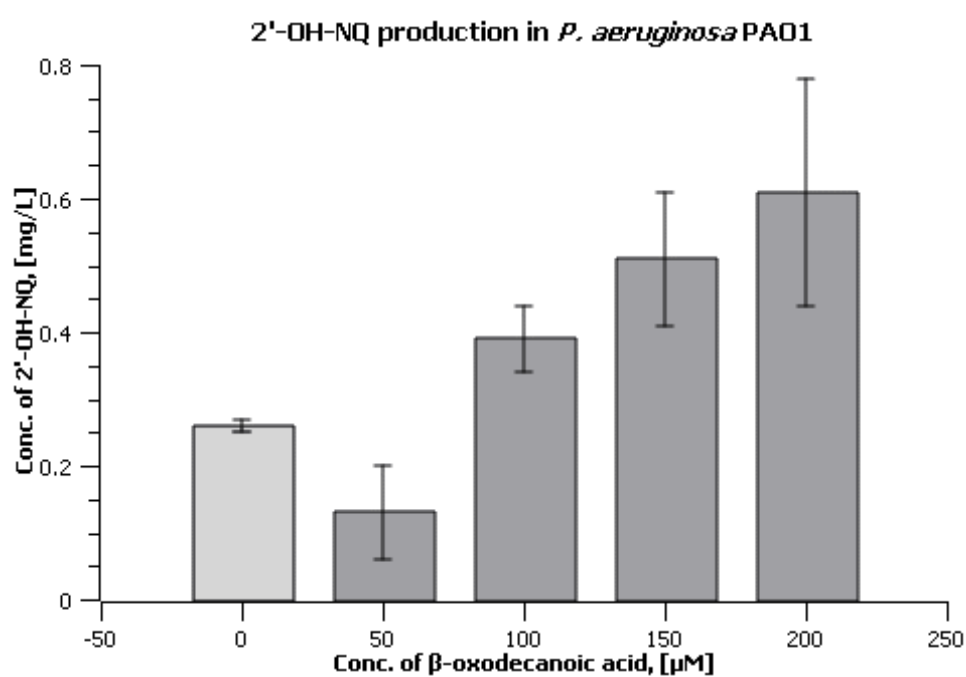
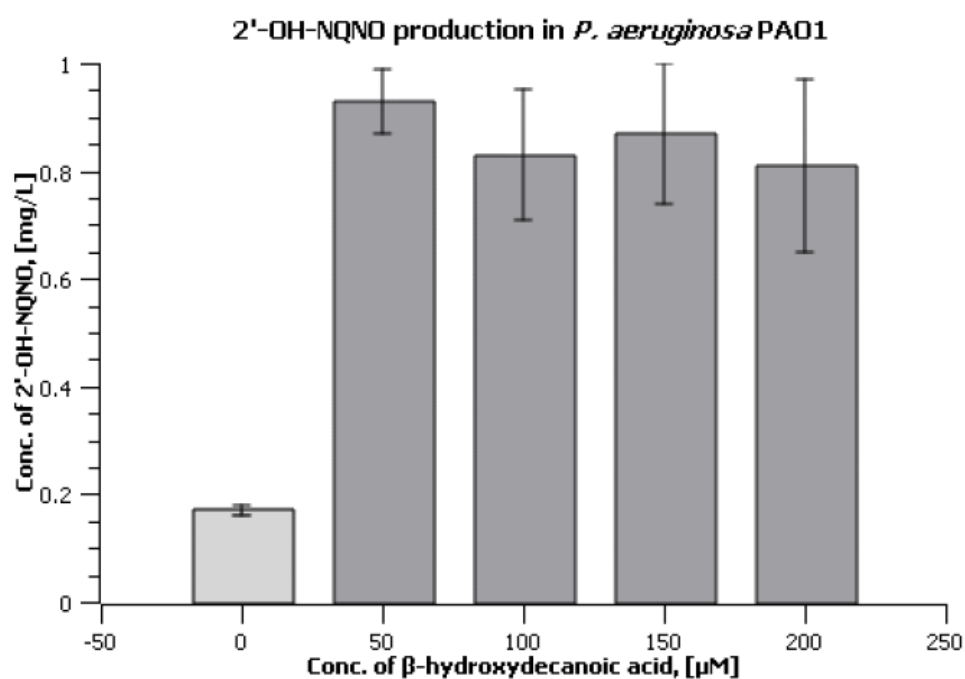


Figure S5. Levels of 2'-OH-NQ production in *P. aeruginosa* PAO1 while feeding:
a) β -hydroxydecanoic acid; **b)** β -oxodecanoic acid to cultures. The error bars represent the population standard deviation.

a)



b)

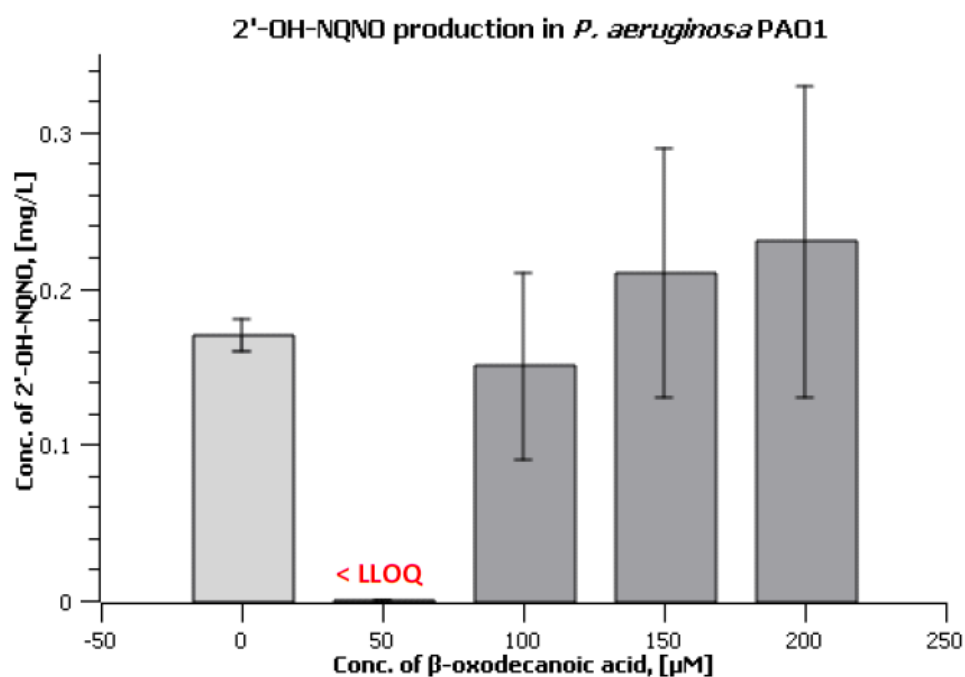
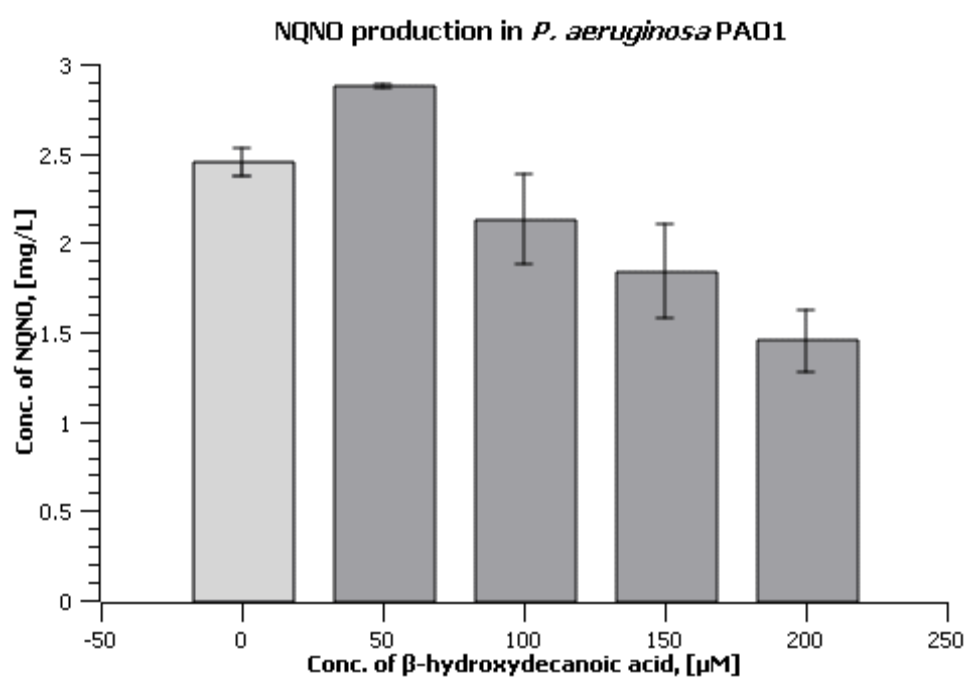


Figure S6. Levels of 2'-OH-NQNO production in *P. aeruginosa* PAO1 while feeding: **a)** β -hydroxydecanoic acid; **b)** β -oxodecanoic acid to cultures. LLOQ = Lower Limit of Quantification. The error bars represent the population standard deviation.

a)



b)

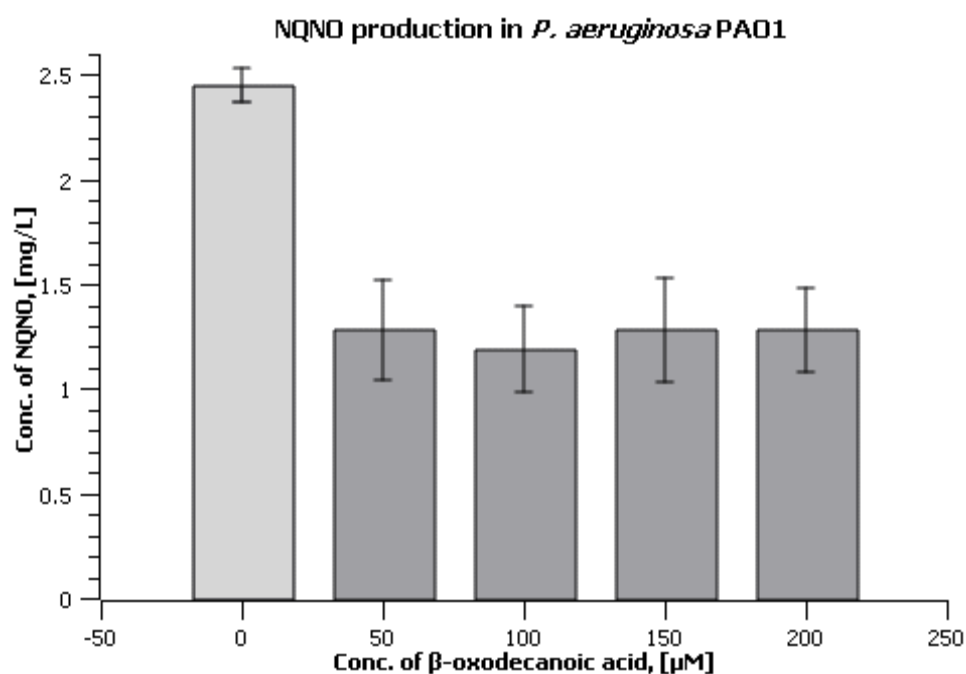


Figure S7. Levels of NQNO production in *P. aeruginosa* PAO1 while feeding:
a) β -hydroxydecanoic acid; **b)** β -oxodecanoic acid to cultures. The error bars represent the population standard deviation.

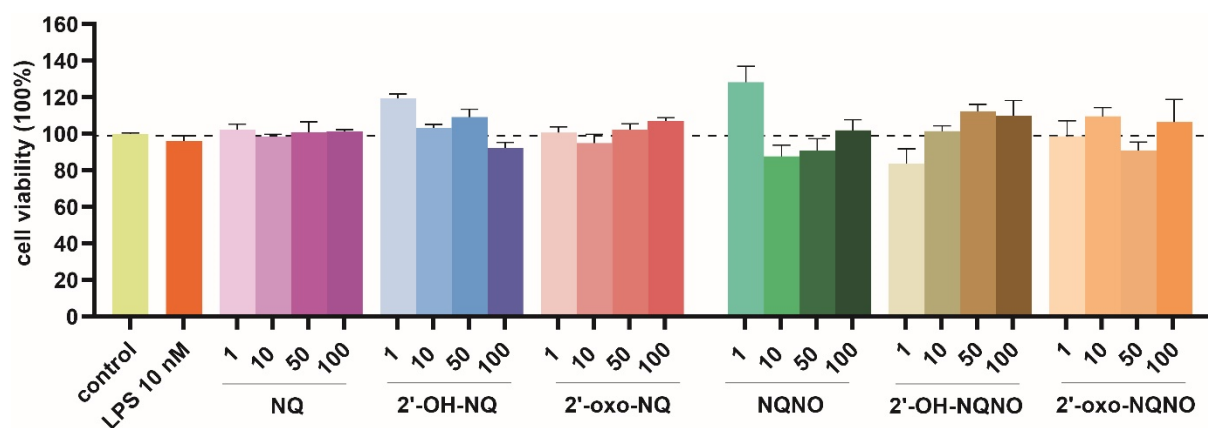


Figure S8. Cell viability of differentiated Caco-2 cells after 24 h of treatment with 10 nM LPS, 1-100 nM NQ, 2'-OH-NQ, 2'-oxo-NQ, NQNO, 2'-OH-NQNO, 2'-oxo-NQNO. Data are presented as Mean $\pm$ SEM of four to five independent biological repetitions in triplicates.

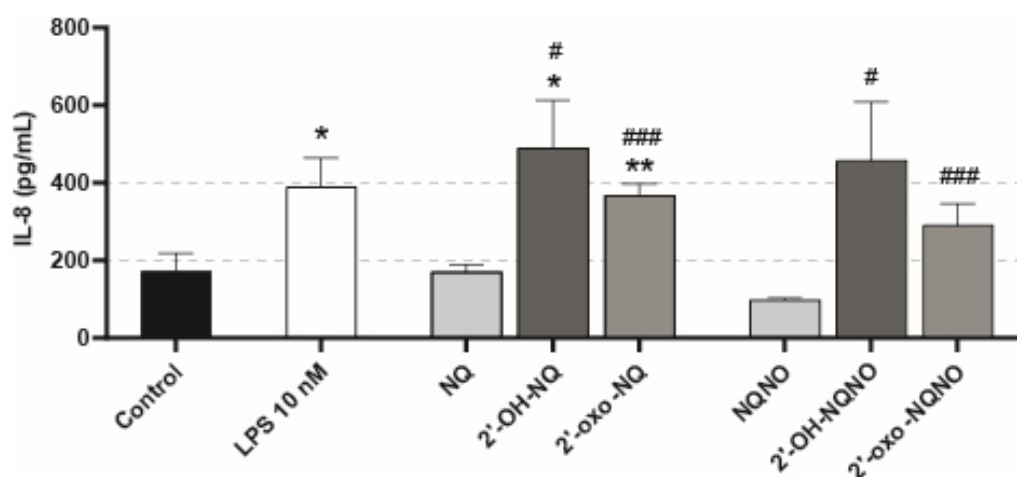


Figure S9. IL-8 release from differentiated Caco-2 cells after 24 h of treatment with medium (Control), 10 nM LPS, 100 nM NQ, 2'-OH-NQ, 2'-oxo-NQ, NQNO, 2'-OH-NQNO, and 2'-oxo-NQNO. Data are presented as Mean $\pm$ SEM of four to five independent biological repetitions in triplicates. * ($p < 0.05$) or ** ($p < 0.01$) indicates statistical significance compared with the control group. # ($p < 0.05$) or ### ($p < 0.001$) indicates statistical significance compared with NQ or NQNO. LPS = Lipopolysaccharides.

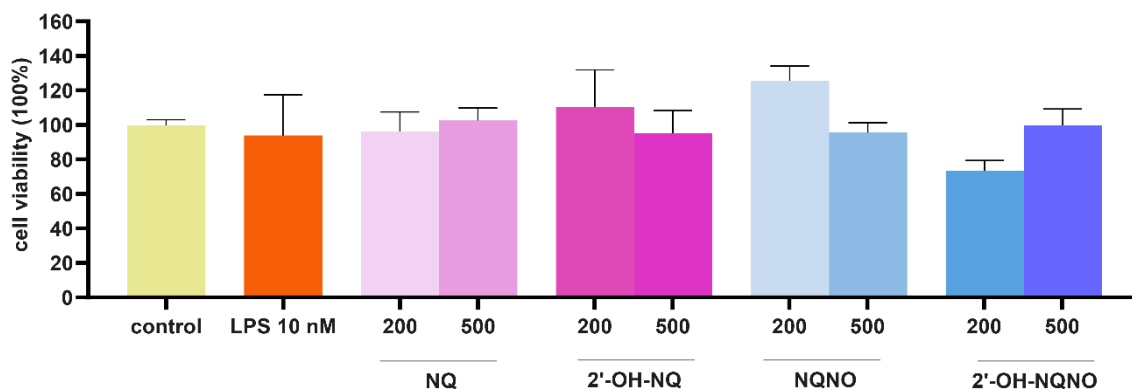


Figure S10. Cell viability of differentiated Caco-2 cells after 24 h of treatment with 10 nM LPS, 200 and 500 nM NQ, 2'-OH-NQ, NQNO, and 2'-OH-NQNO. Data are presented as Mean $\pm$ SEM of four to five independent biological repetitions in triplicates.

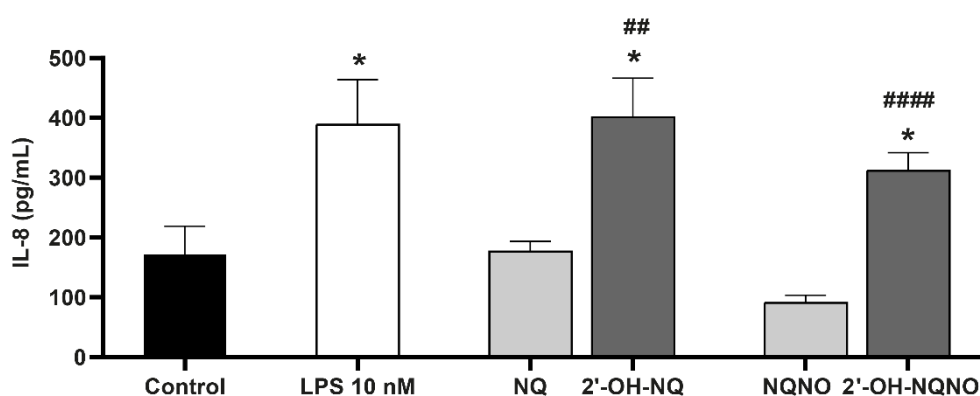
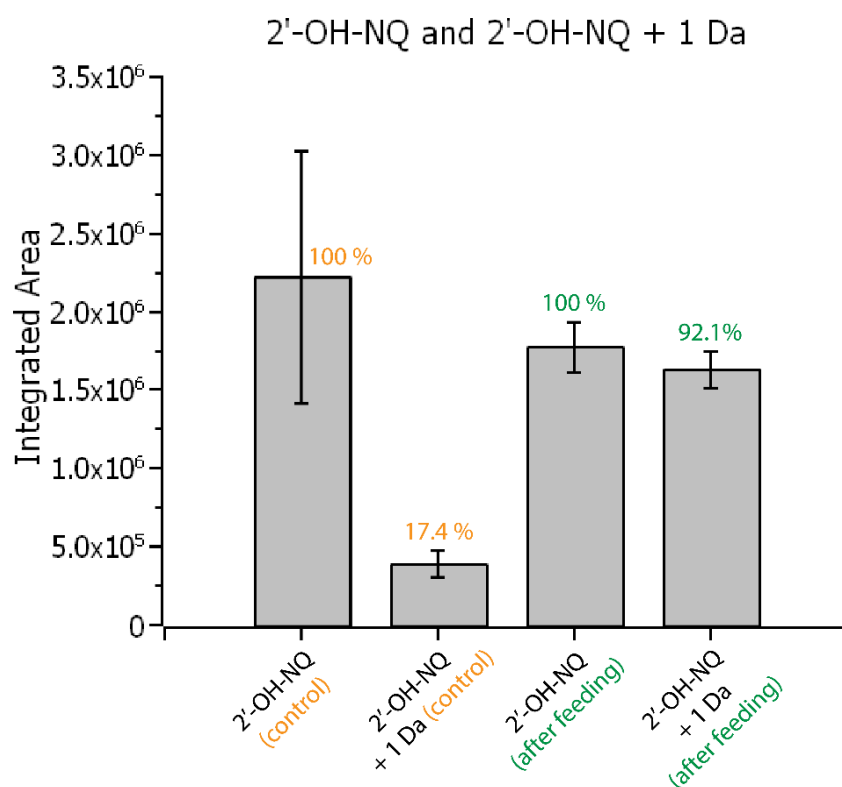


Figure S11. IL-8 release from differentiated Caco-2 cells after 24 h of treatment with medium (Control), 10 nM LPS, 500 nM NQ, 2'-OH-NQ, NQNO, and 2'-OH-NQNO. Data are presented as Mean $\pm$ SEM of four to five independent biological repetitions in triplicates. * ($p < 0.05$) or ** ($p < 0.01$) indicates statistical significance compared with the control group. # ($p < 0.05$) or #### ($p < 0.001$) indicates statistical significance compared with NQ or NQNO. LPS = Lipopolysaccharides.

a)



b)

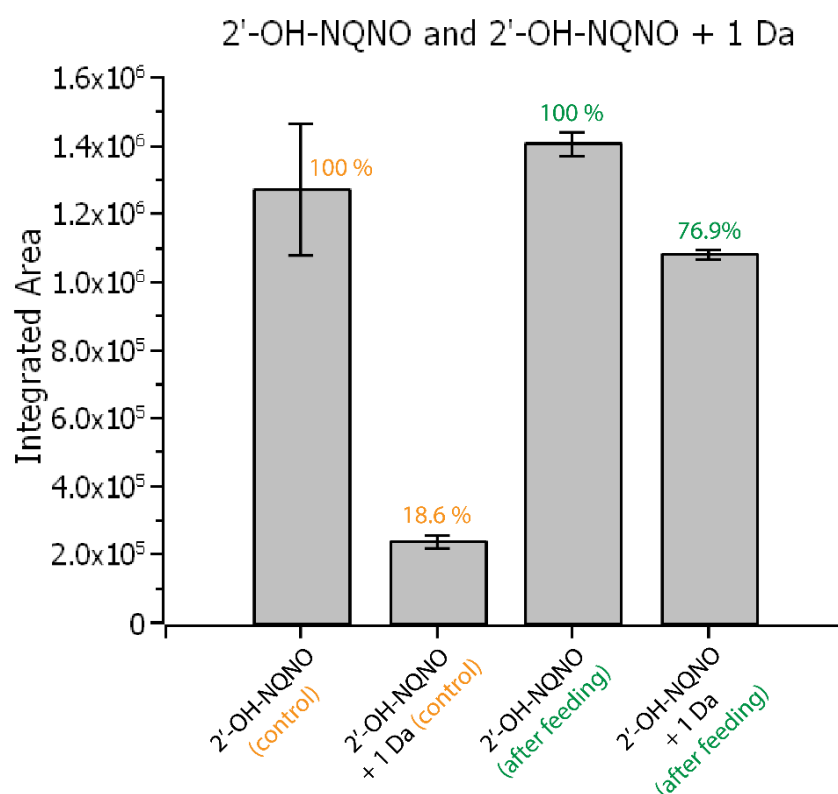


Figure S12. Levels of **a)** 2'-OH-NQ and 2'-OH-NQ + 1 Da; **b)** 2'-OH-NQNO and 2'-OH-NQNO + 1 Da production in *P. aeruginosa* PAO1 in control (orange labels) and after feeding 100 μ M deuterated β -hydroxydecanoic acid (**3d**) to cultures (green labels). The error bars represent the population standard deviation.

6. Supplementary References

- [1] Fulmer *et. al.* Organometallics. 2010, 29, 2176-2179.
- [2] Zheng *et. al.* Bioorg. Med. Chem. 2018, 26, 5934-5943.
- [3] Vleeschouwer *et. al.* Chem. Eur. J. 2014, 20, 7766-7775.
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- [5] Szamosvari *et al.* Chem. Commun., 2020, 56, 6328-6331.

NMR spectra

Biosynthetic flexibility of *Pseudomonas aeruginosa* leads to hydroxylated 2-Alkylquinolones with proinflammatory host response

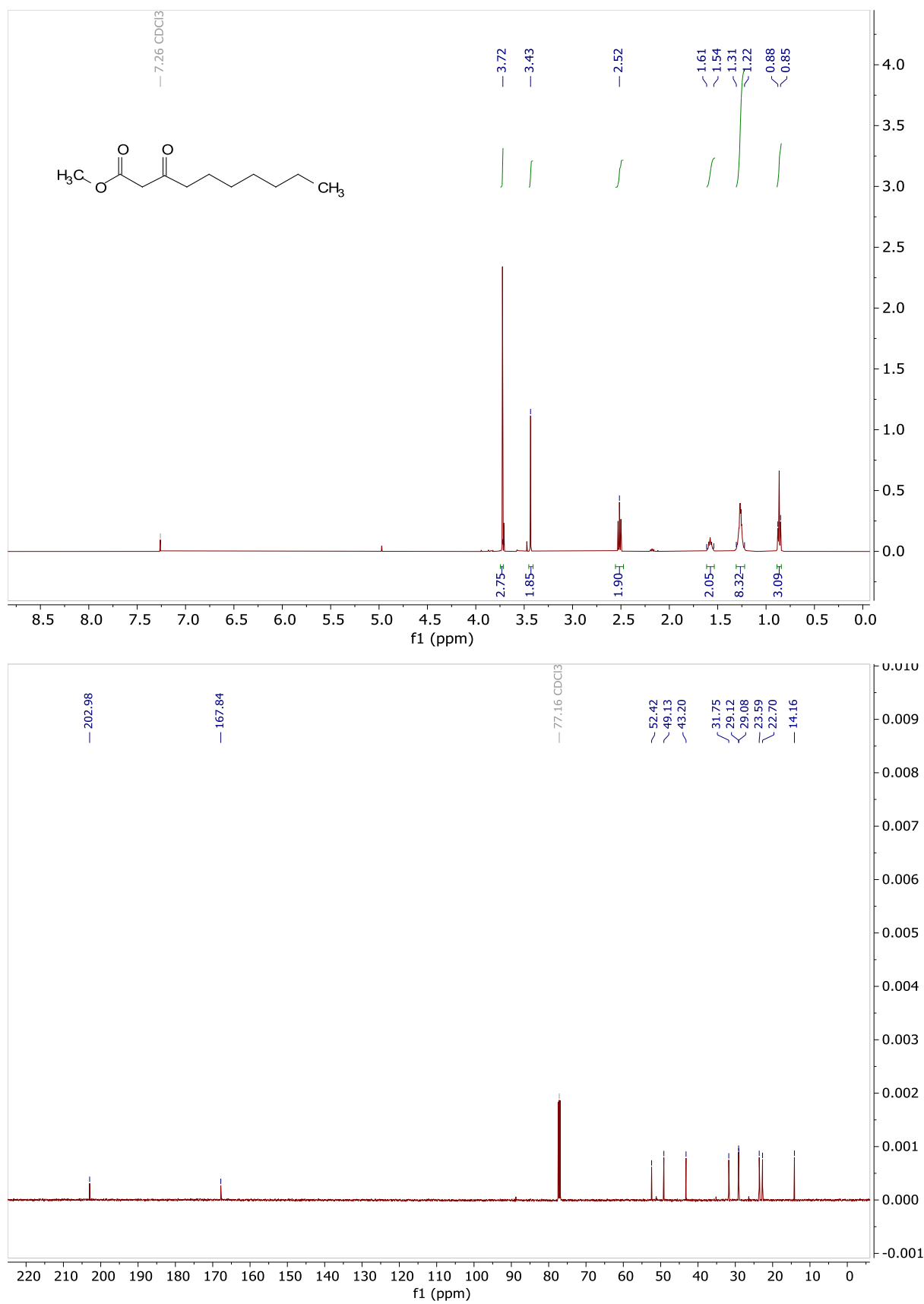
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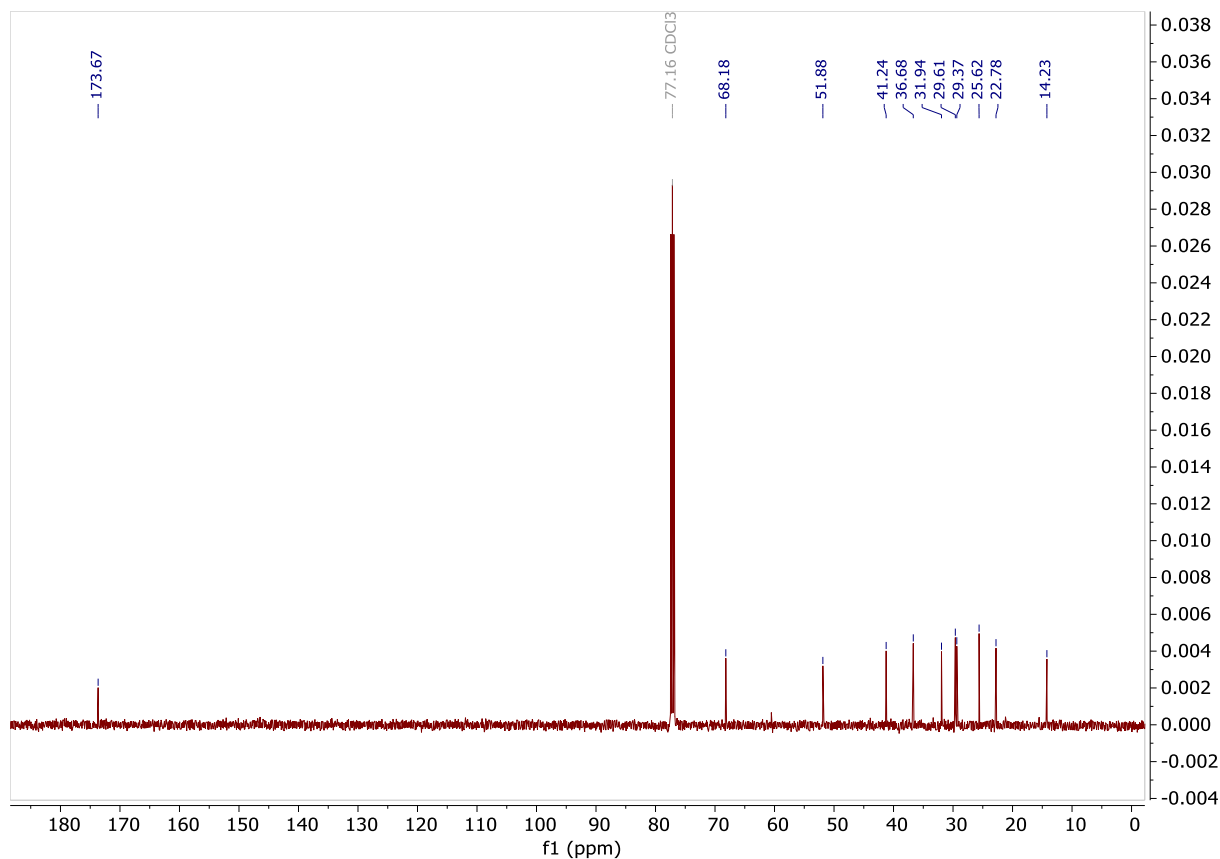
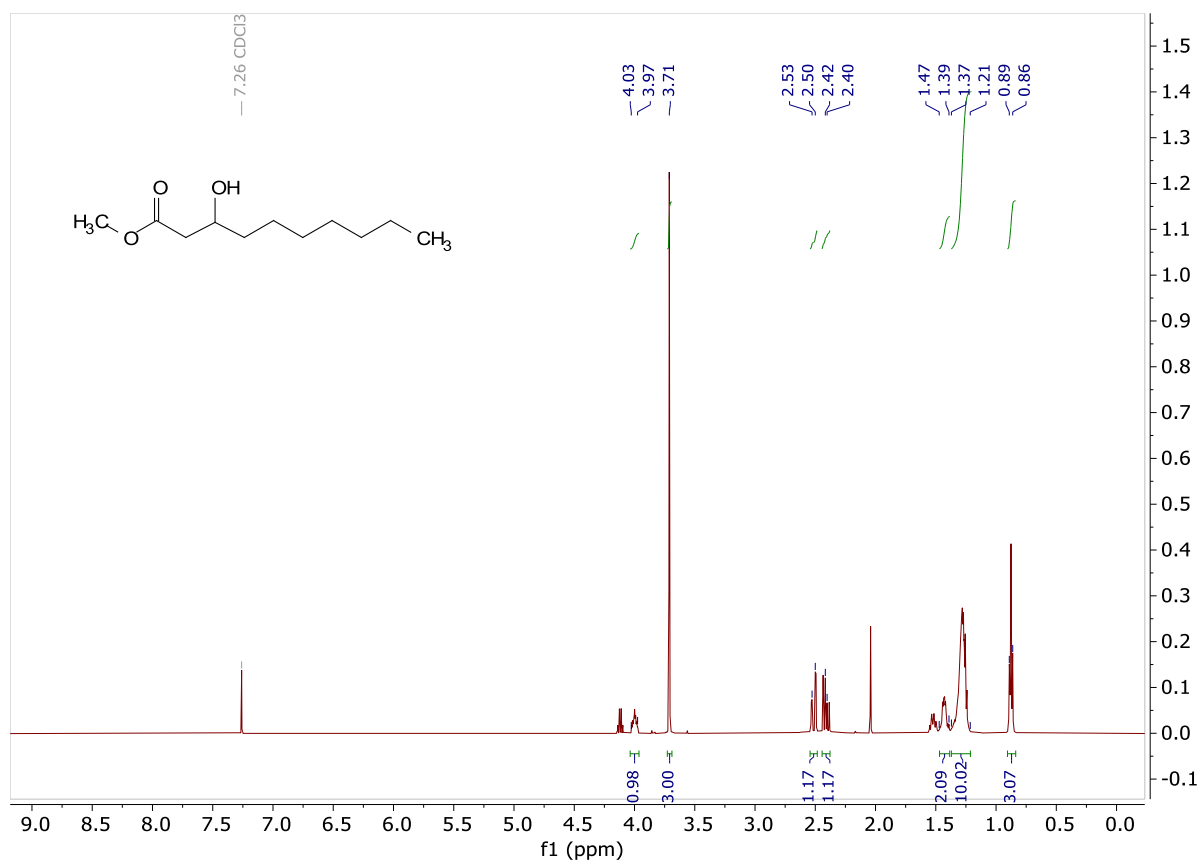
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[c] Faculty of Chemistry, Institute of Physiological Chemistry, University of Vienna, Josef-Holaubek-Platz 2, 1090 Vienna, Austria

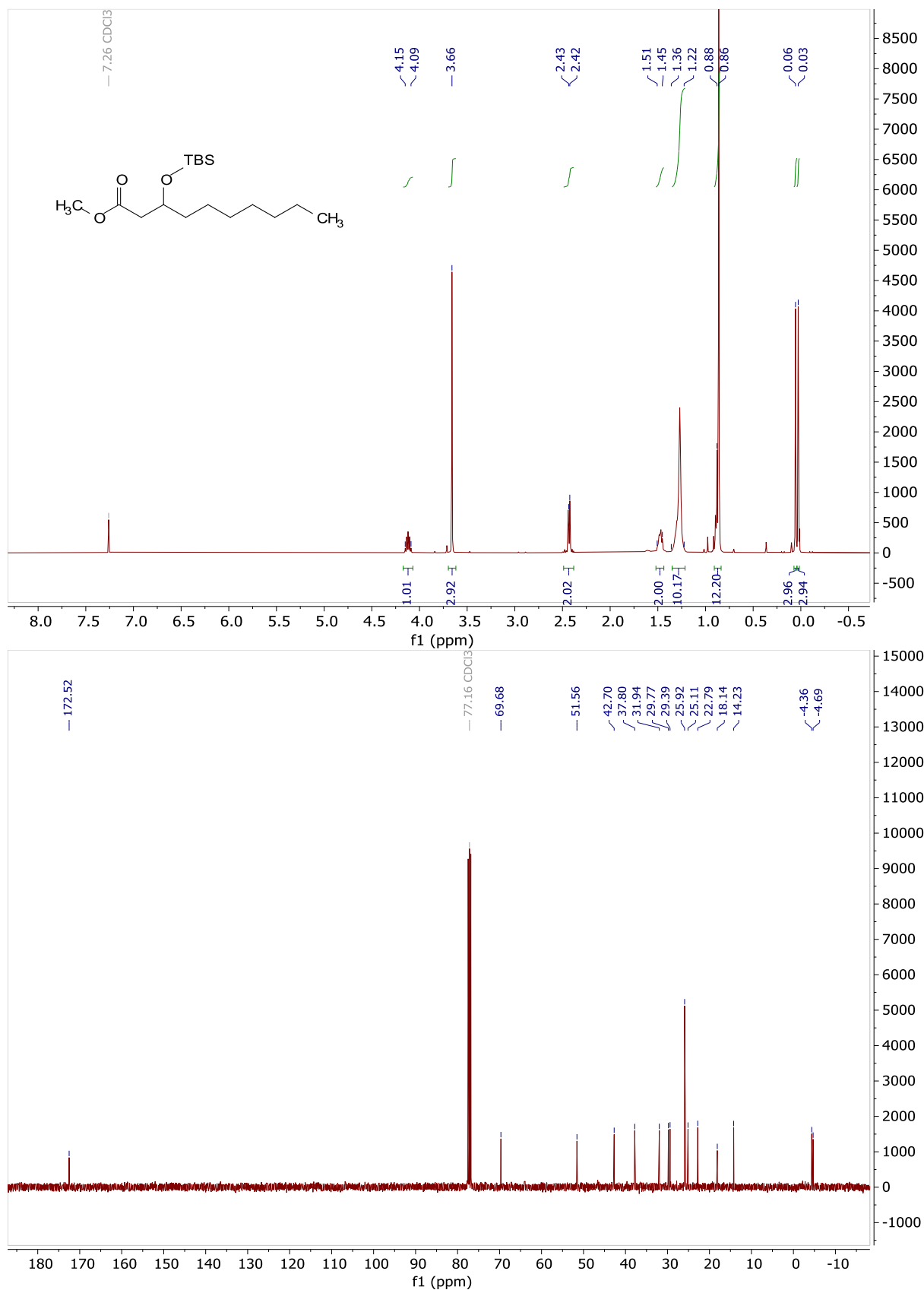
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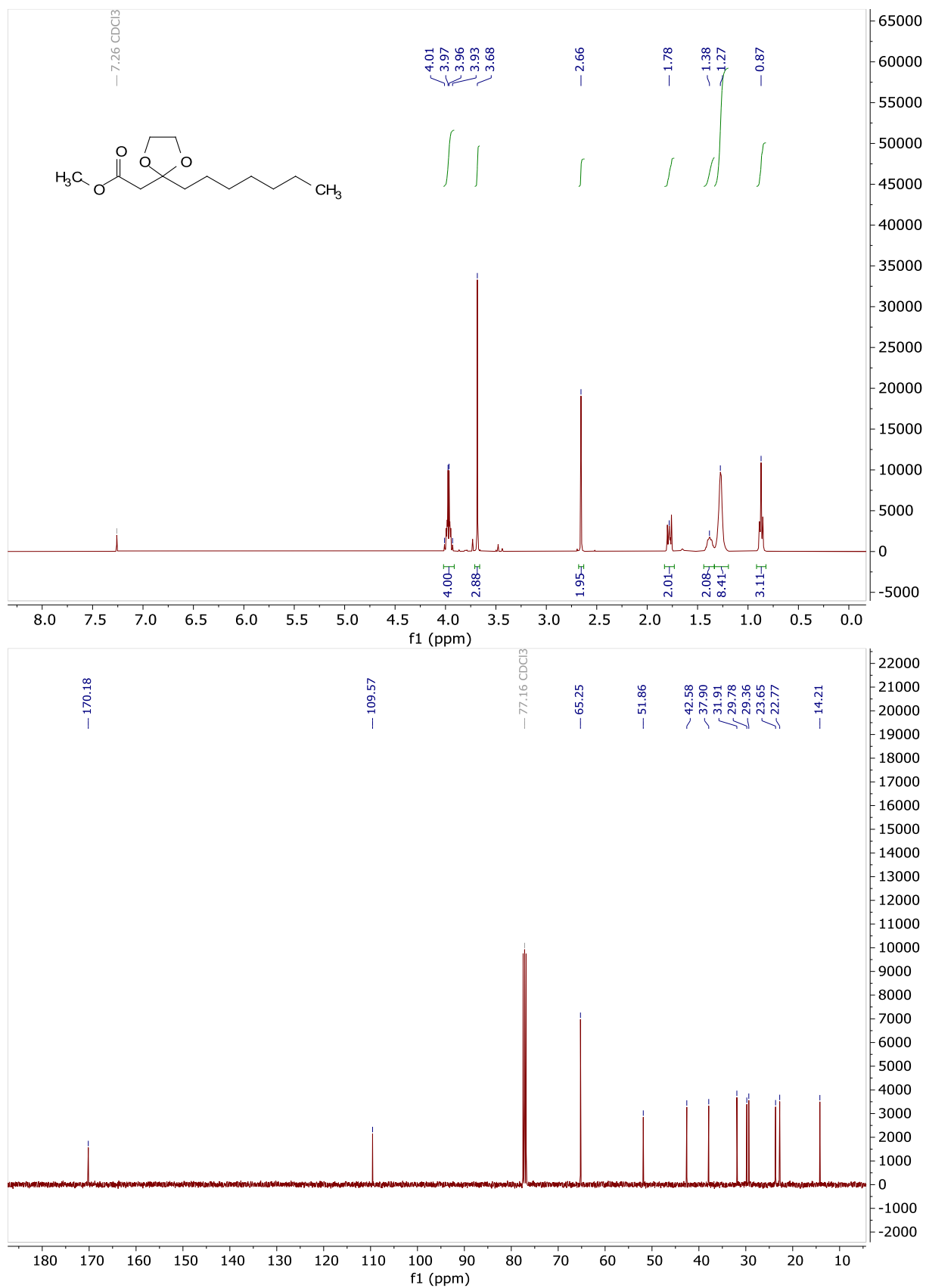
Methyl 3-oxodecanoate (**a**) ¹H-NMR (above) and ¹³C-NMR (below) in CDCl₃



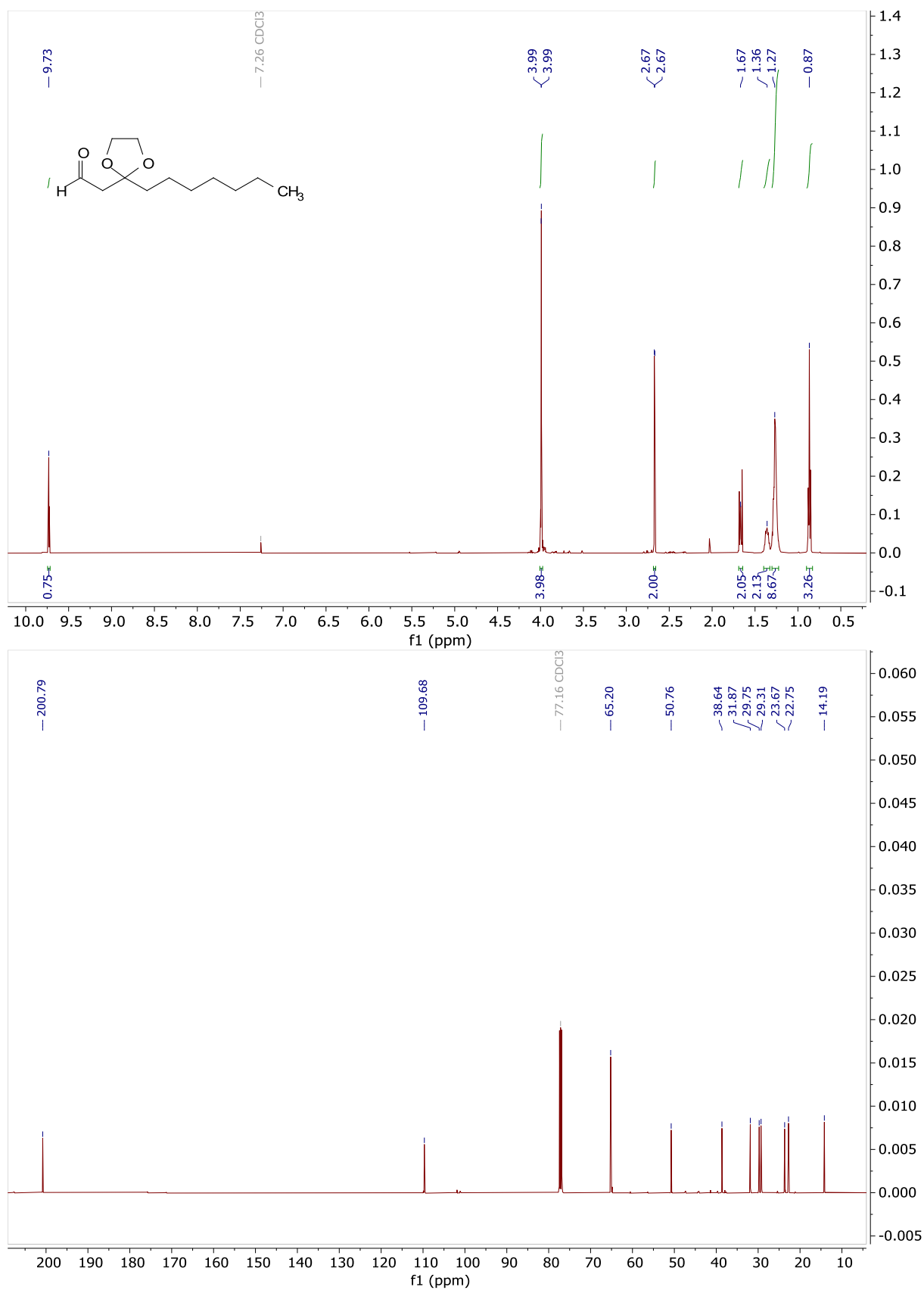
Methyl 3-hydroxydecanoate (**b**) ¹H-NMR (above) and ¹³C-NMR (below) in CDCl₃



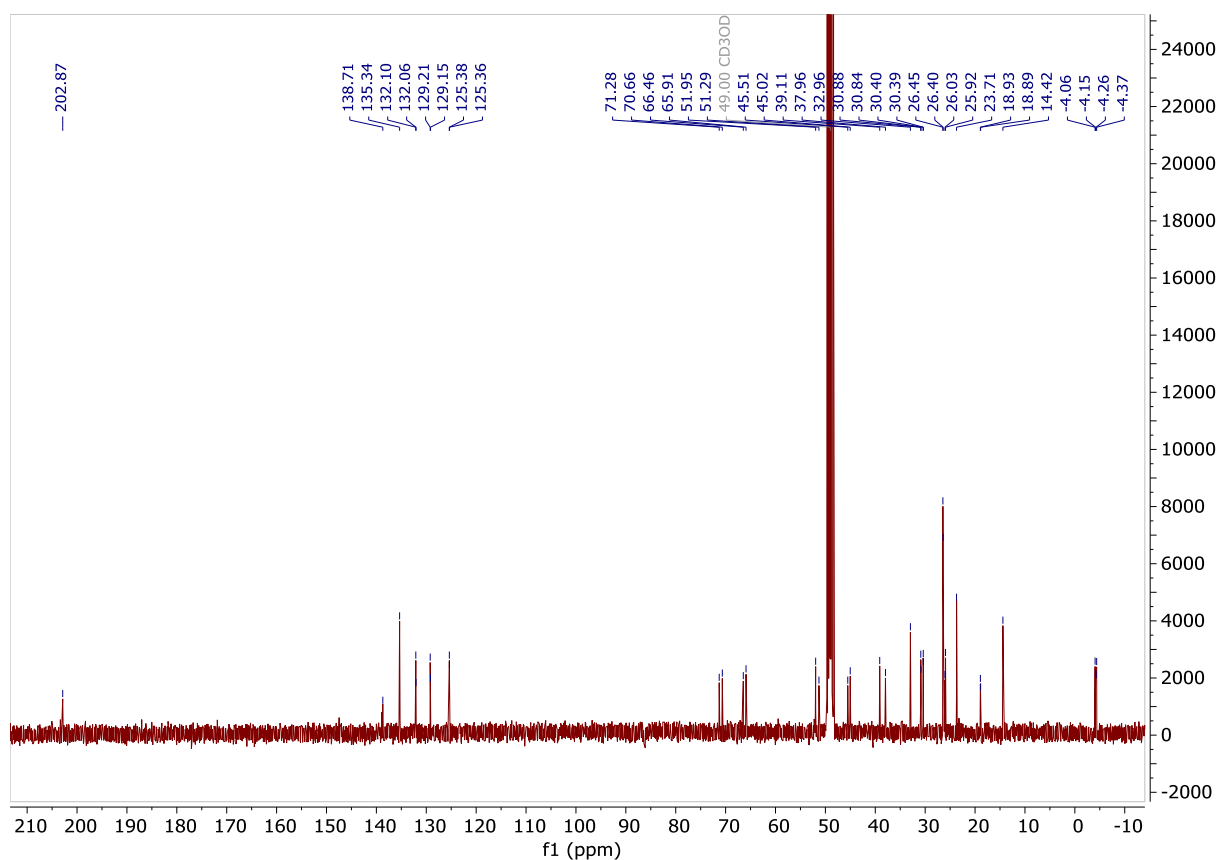
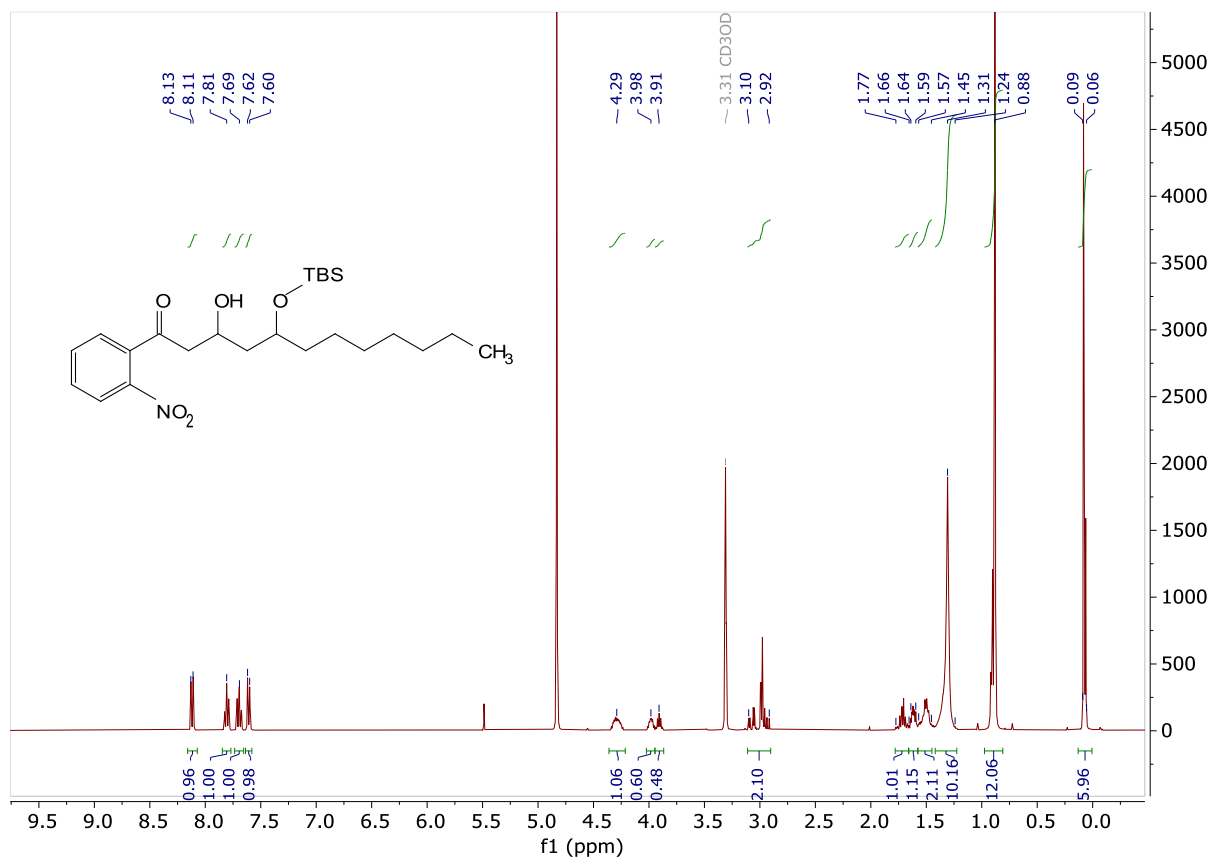
Methyl 3-((*tert*-butyldimethylsilyl)oxy)decanoate (**1a**) ¹H-NMR (above) and ¹³C-NMR (below) in CDCl₃



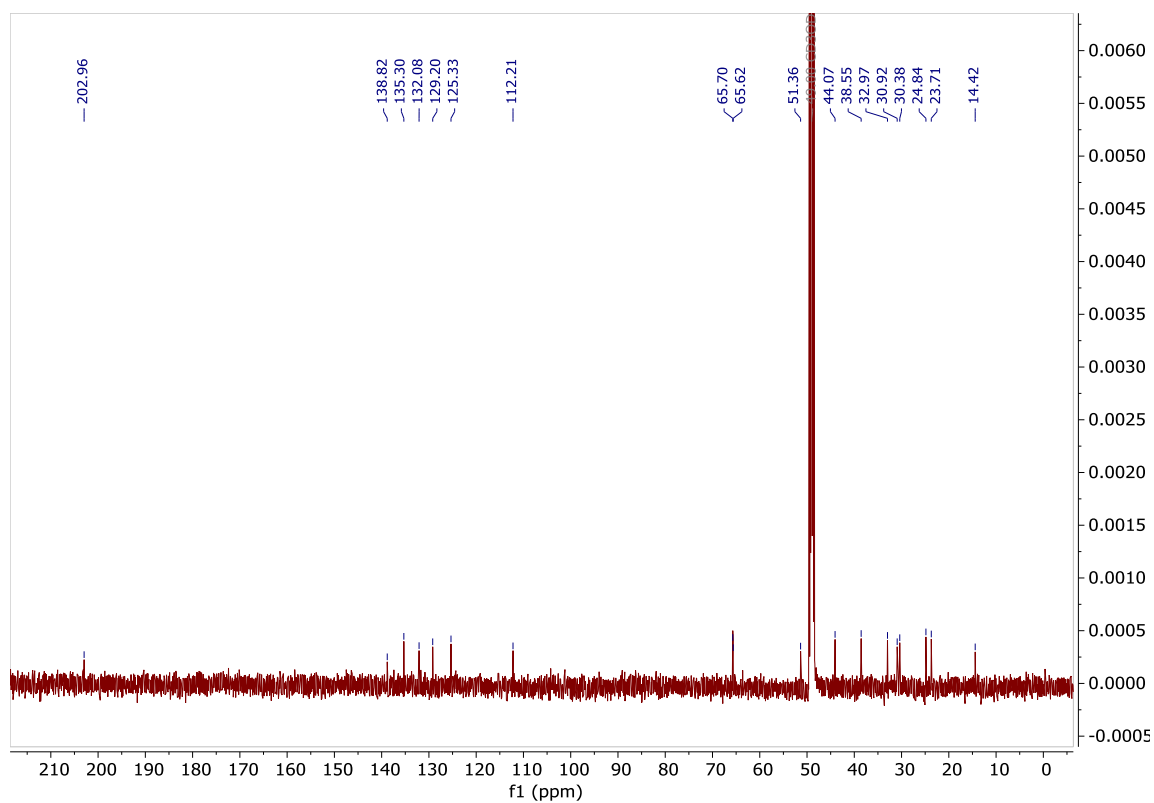
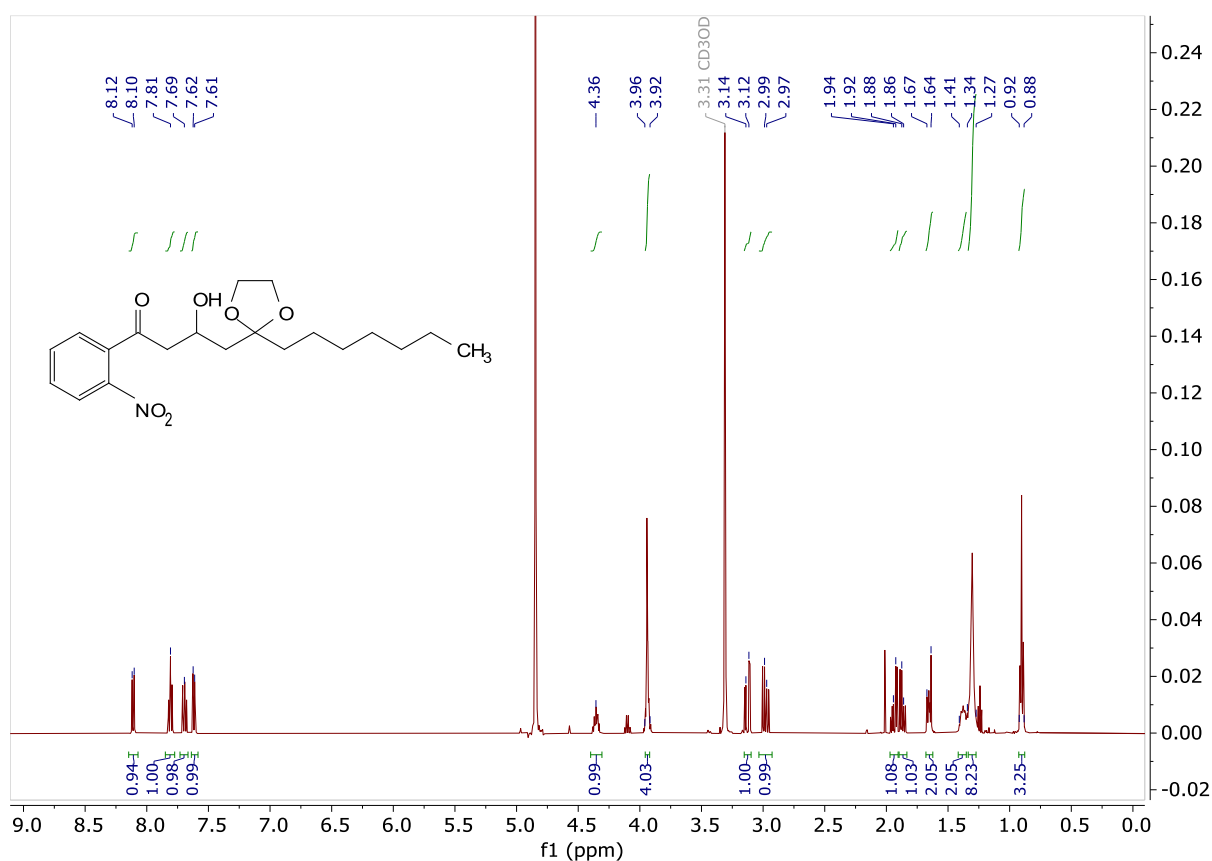
Methyl 3-(1,3-dioxolane)decanoate (**2a**) ¹H-NMR (above) and ¹³C-NMR (below) in CDCl₃



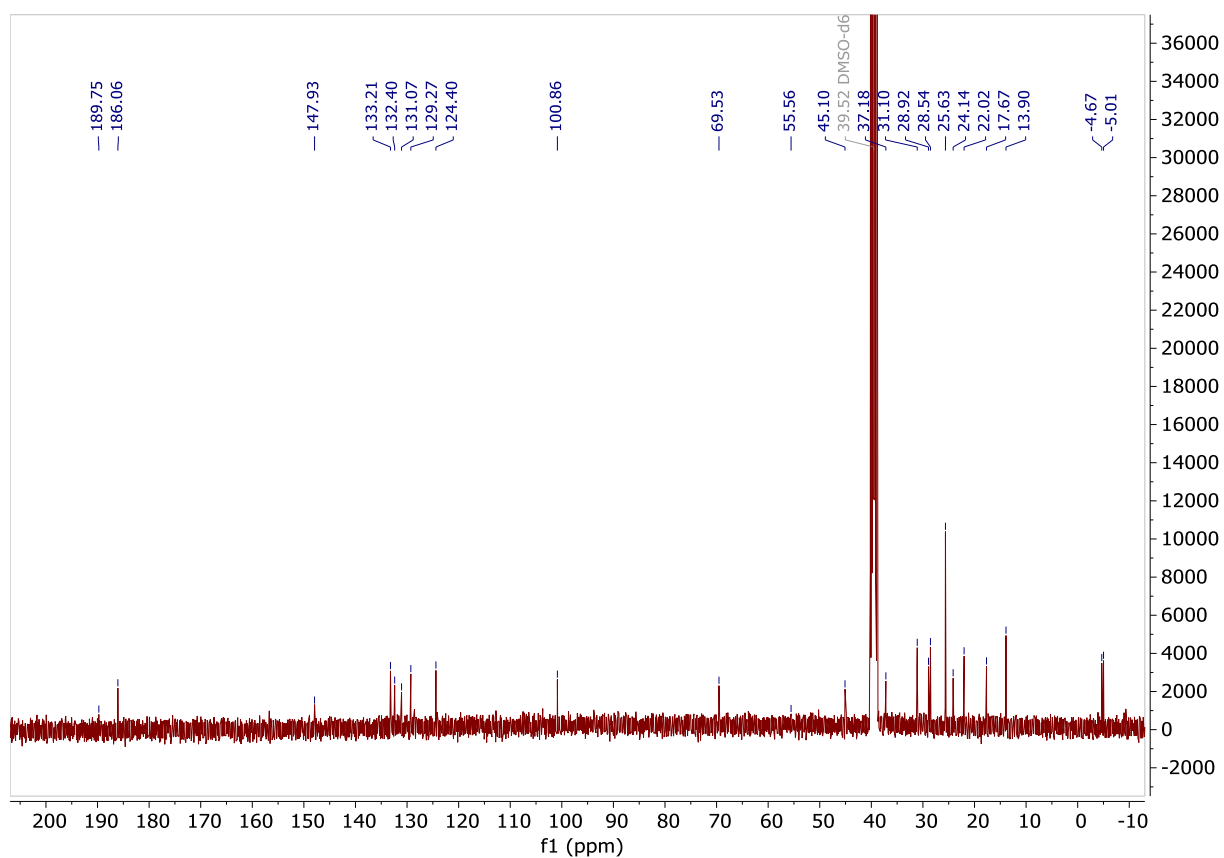
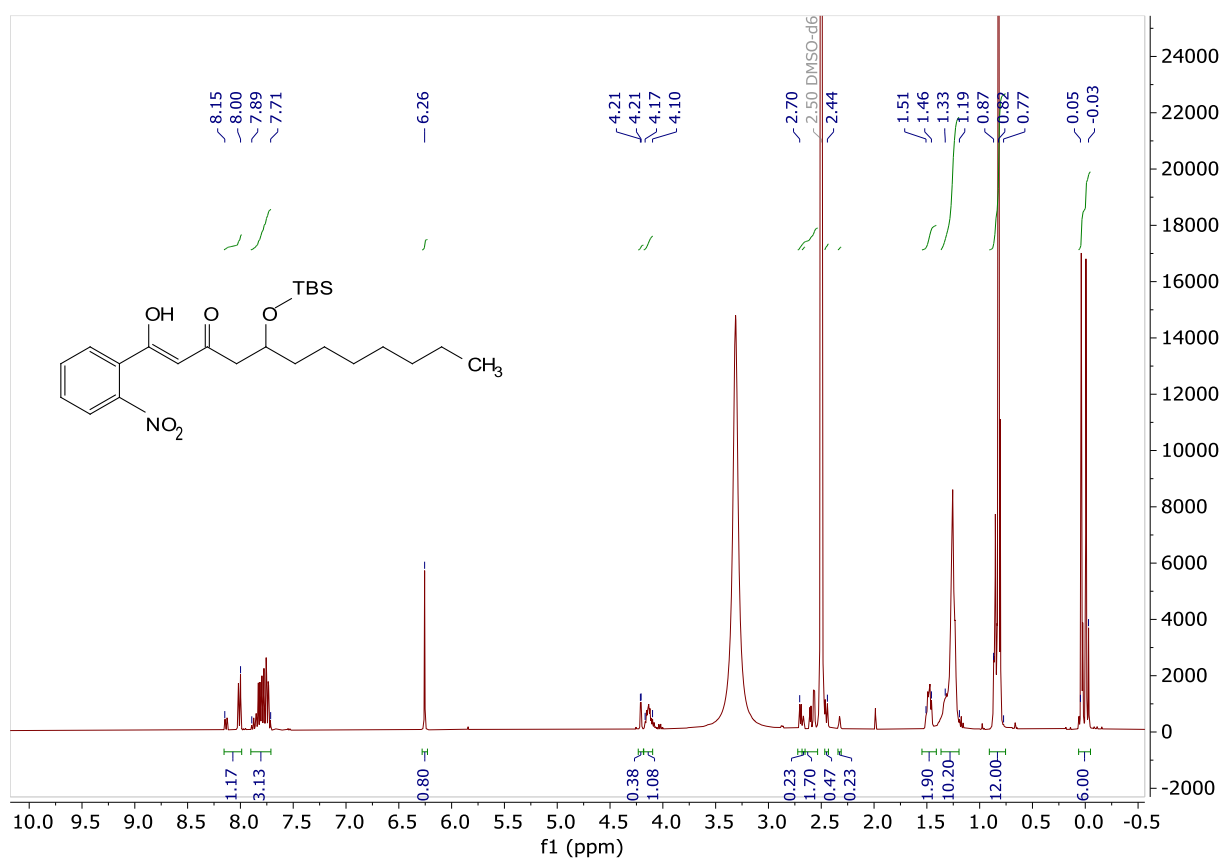
3-(1,3-dioxolane)decanal (**2b**) ¹H-NMR (above) and ¹³C-NMR (below) in CDCl₃



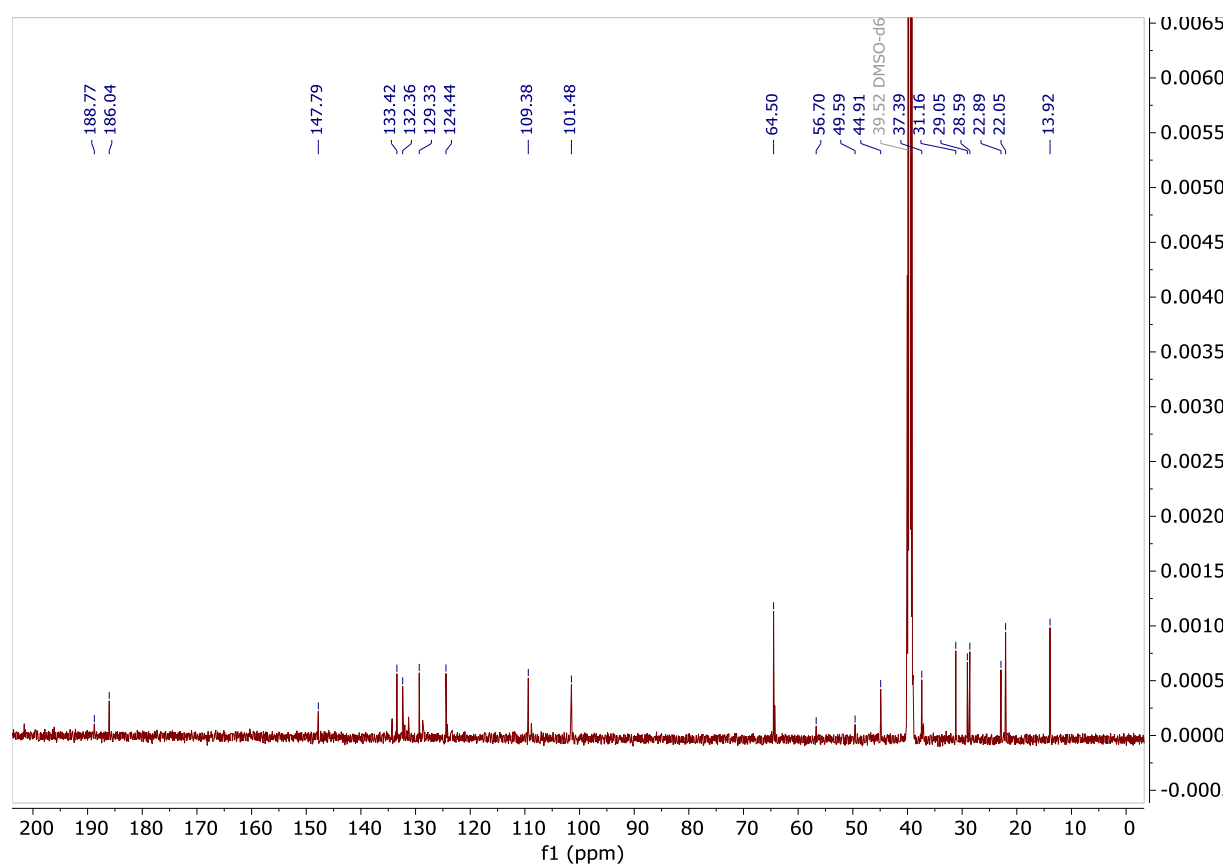
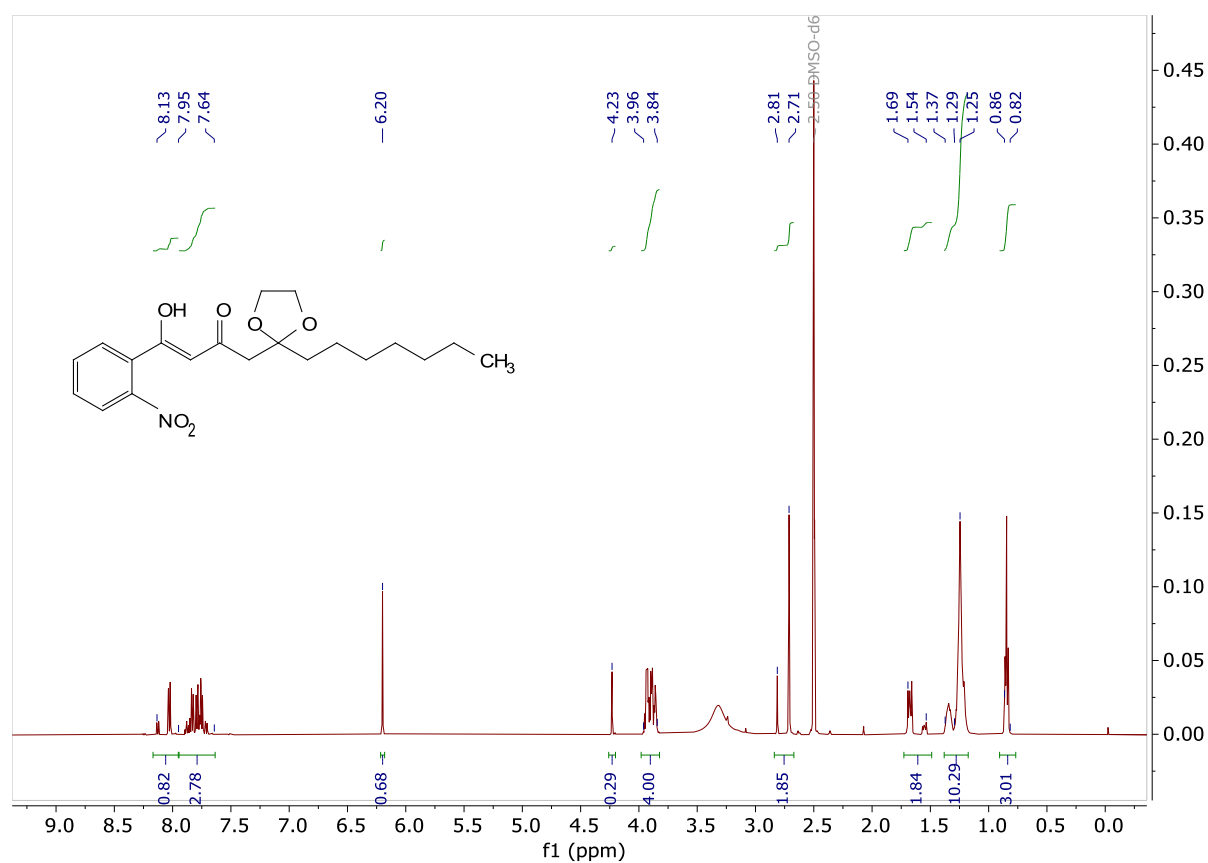
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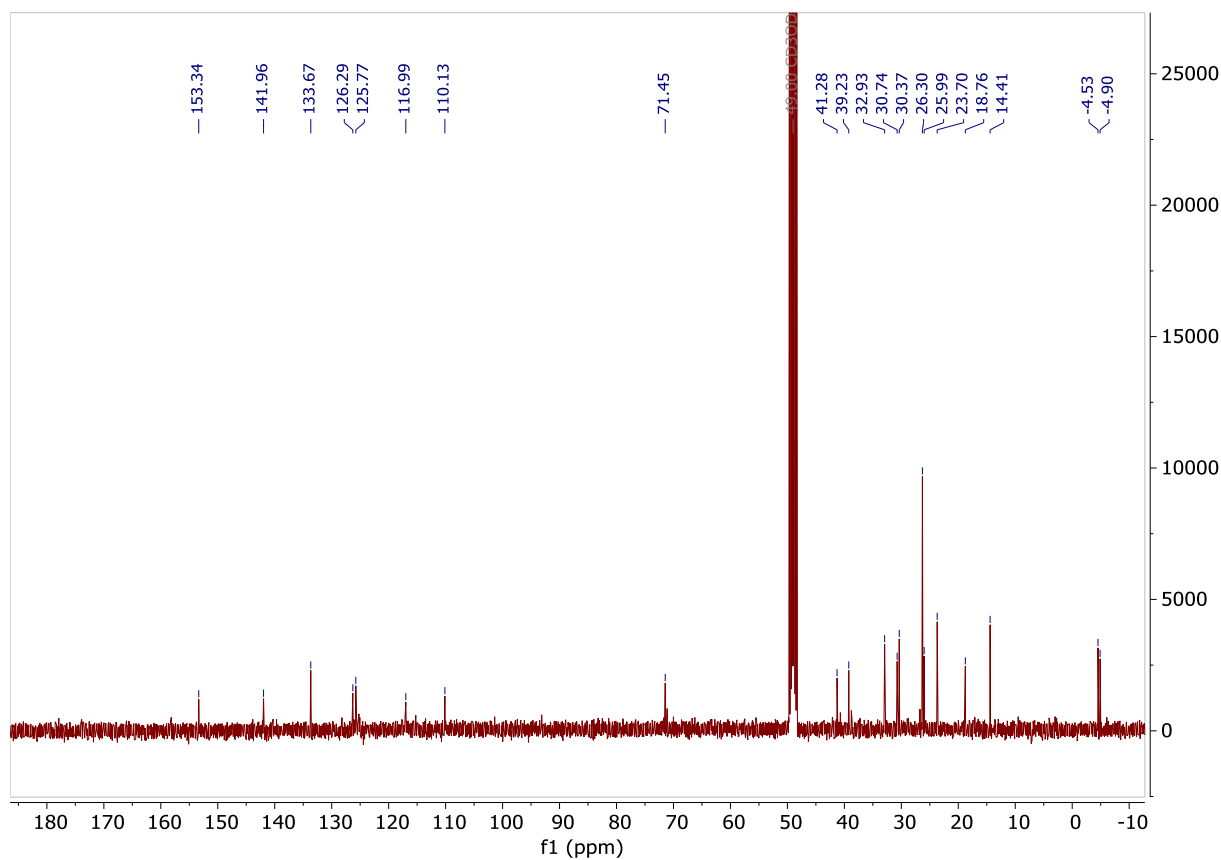
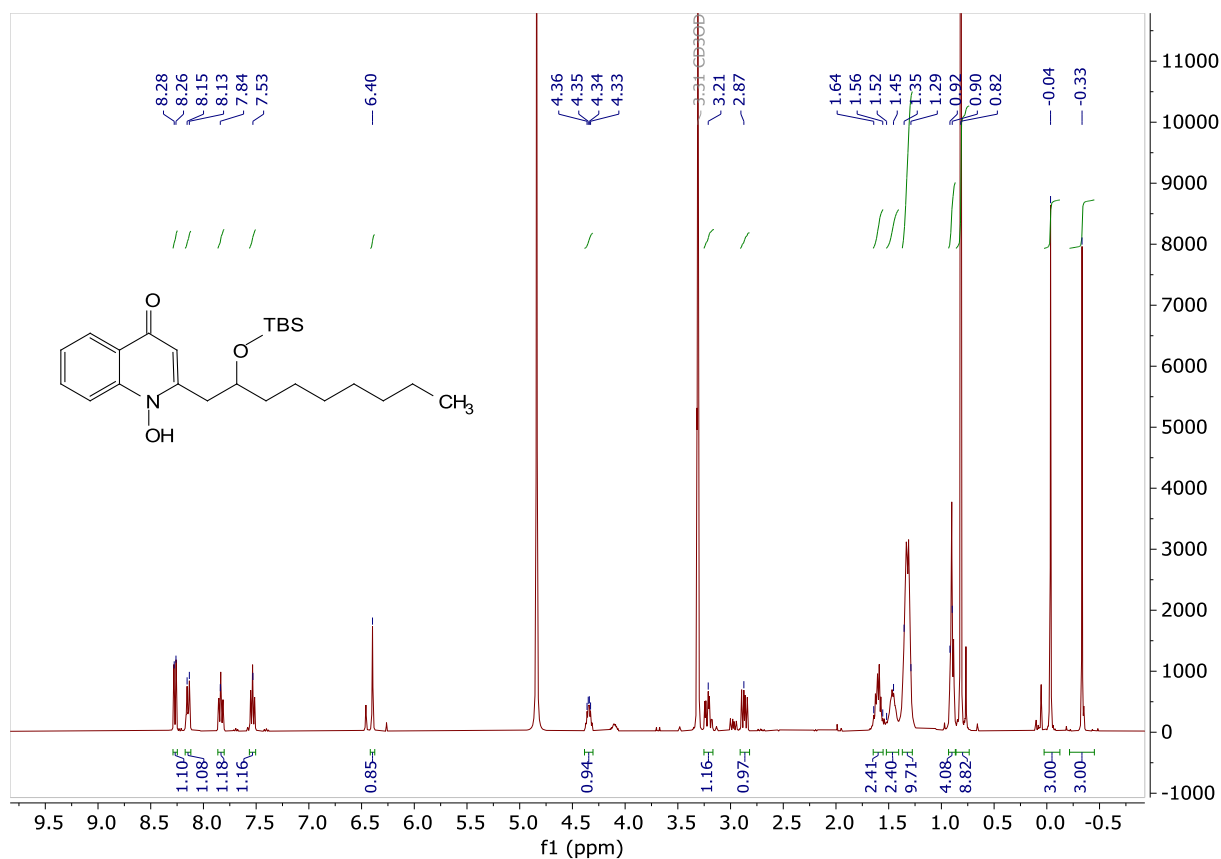
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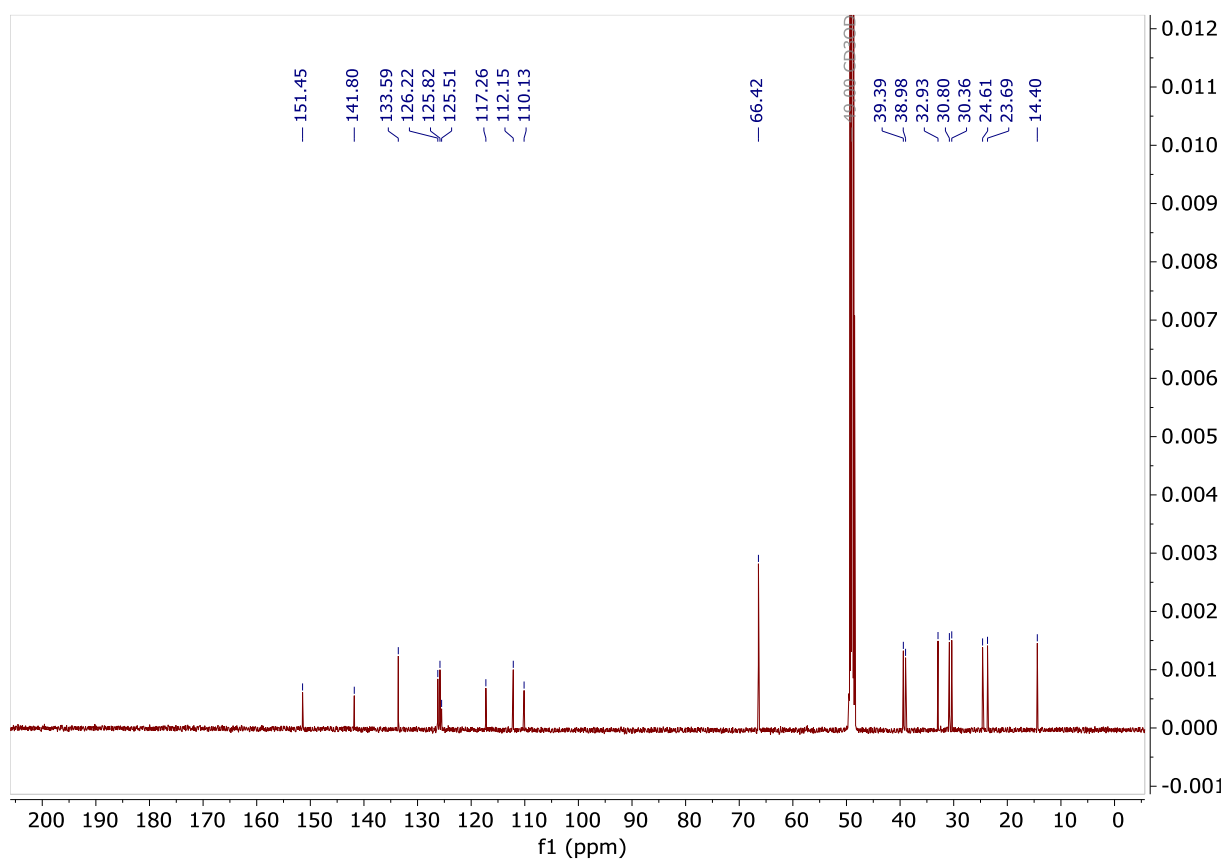
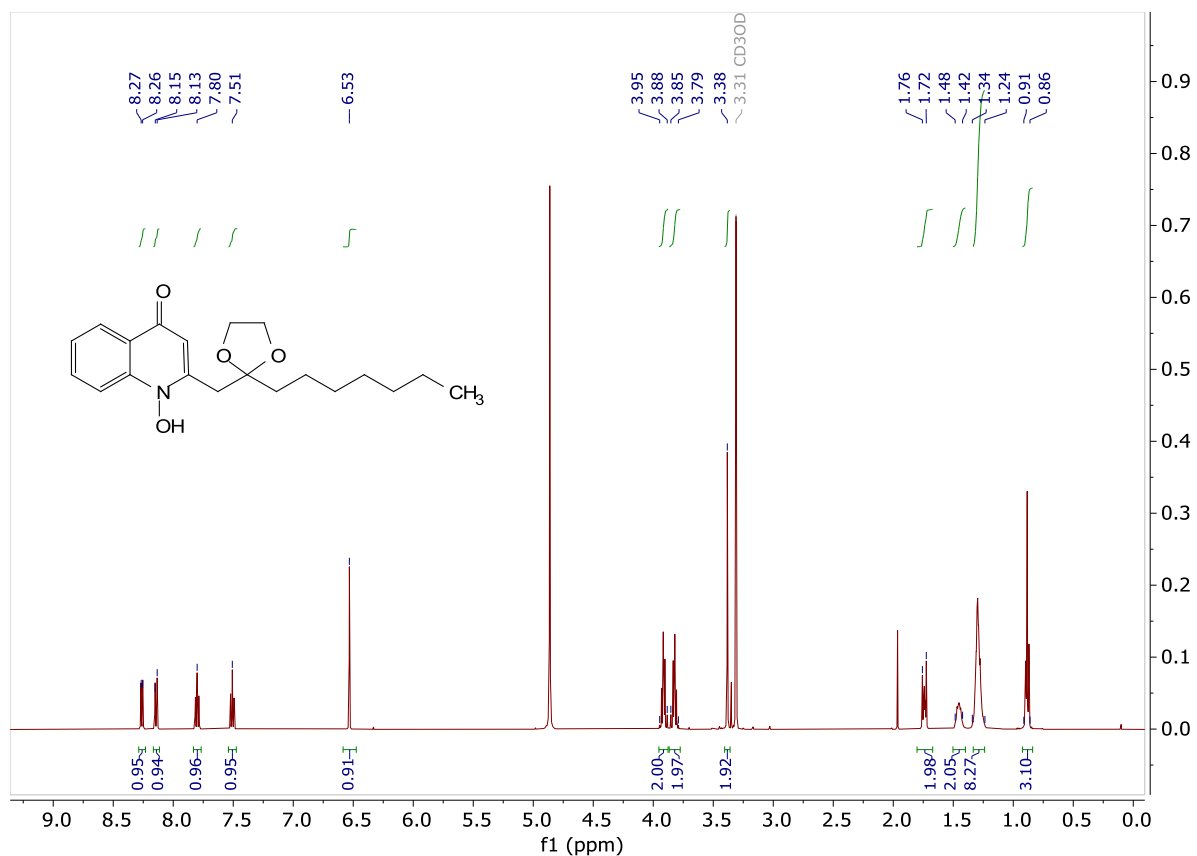
5-((*tert*-butyldimethylsilyl)oxy)-3-oxo-1-(2'-nitrophenyl)dodecan-1-one (**1d**) in ¹H-NMR (above) and ¹³C-NMR (below) in DMSO-d₆



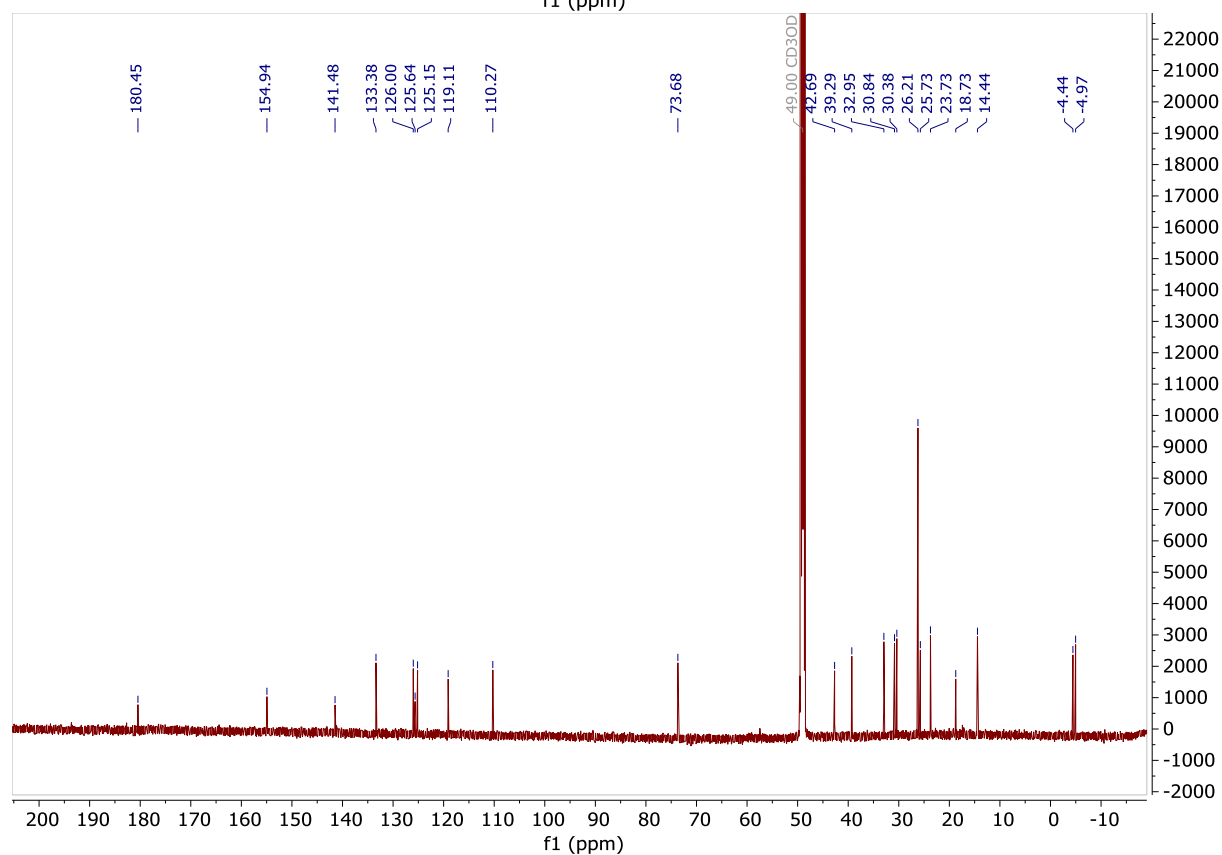
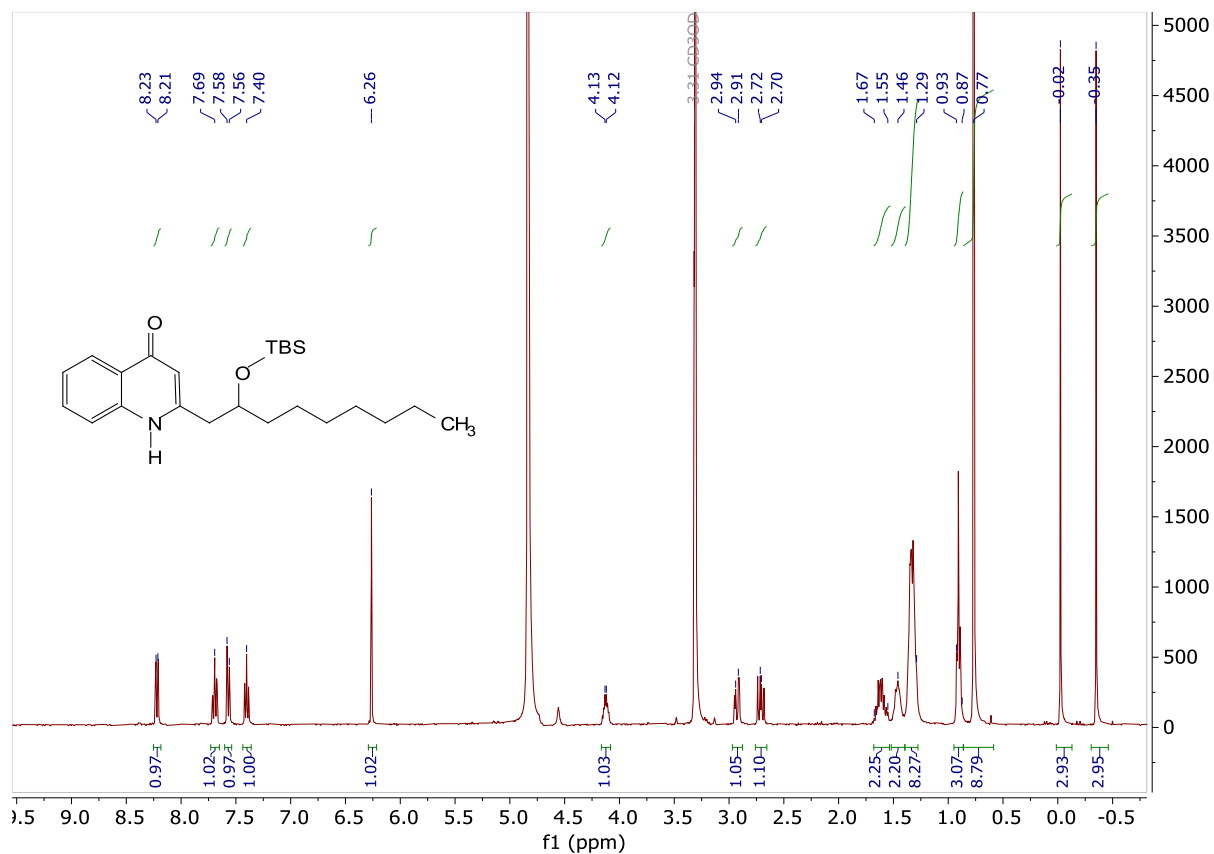
5-(1,3-dioxolane)-3-oxo-1-(2'-nitrophenyl)dodecan-1-one (**2d**) in ¹H-NMR (above) and ¹³C-NMR (below) in DMSO-d₆



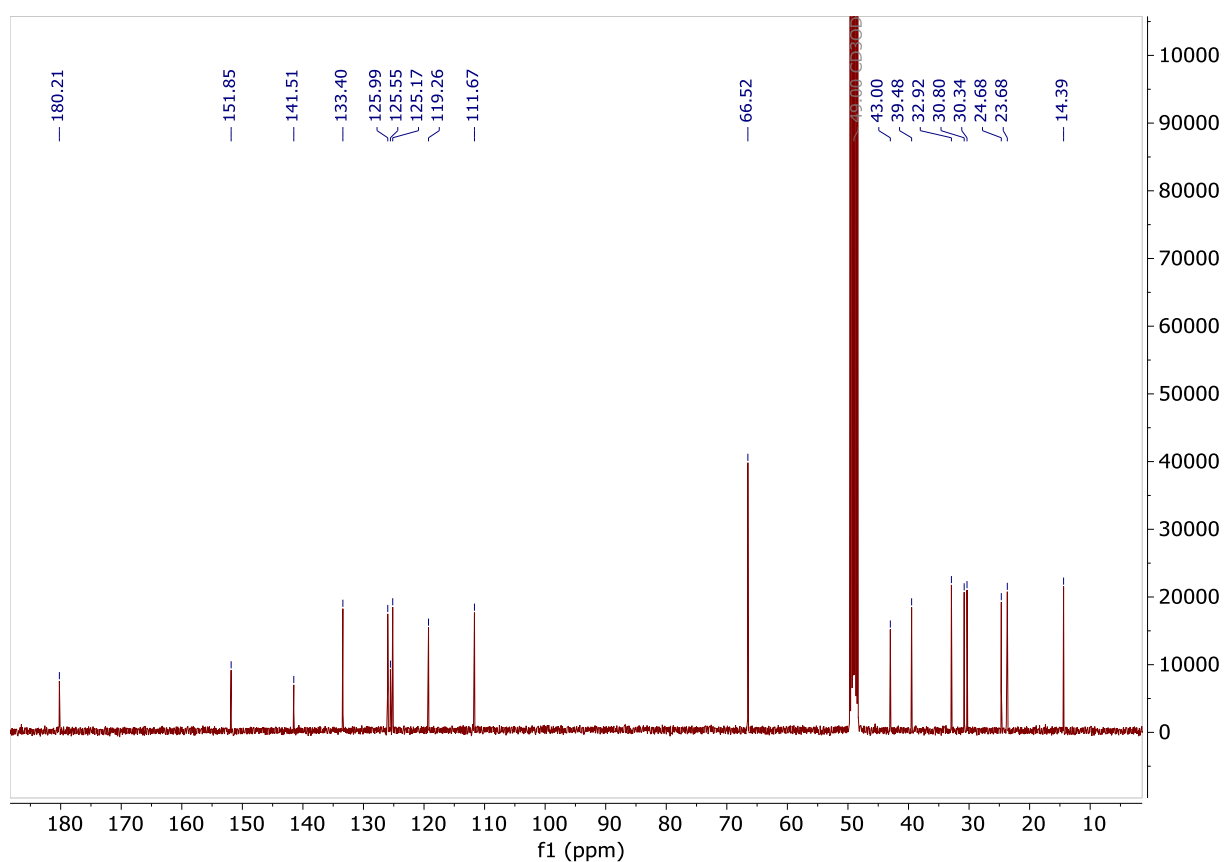
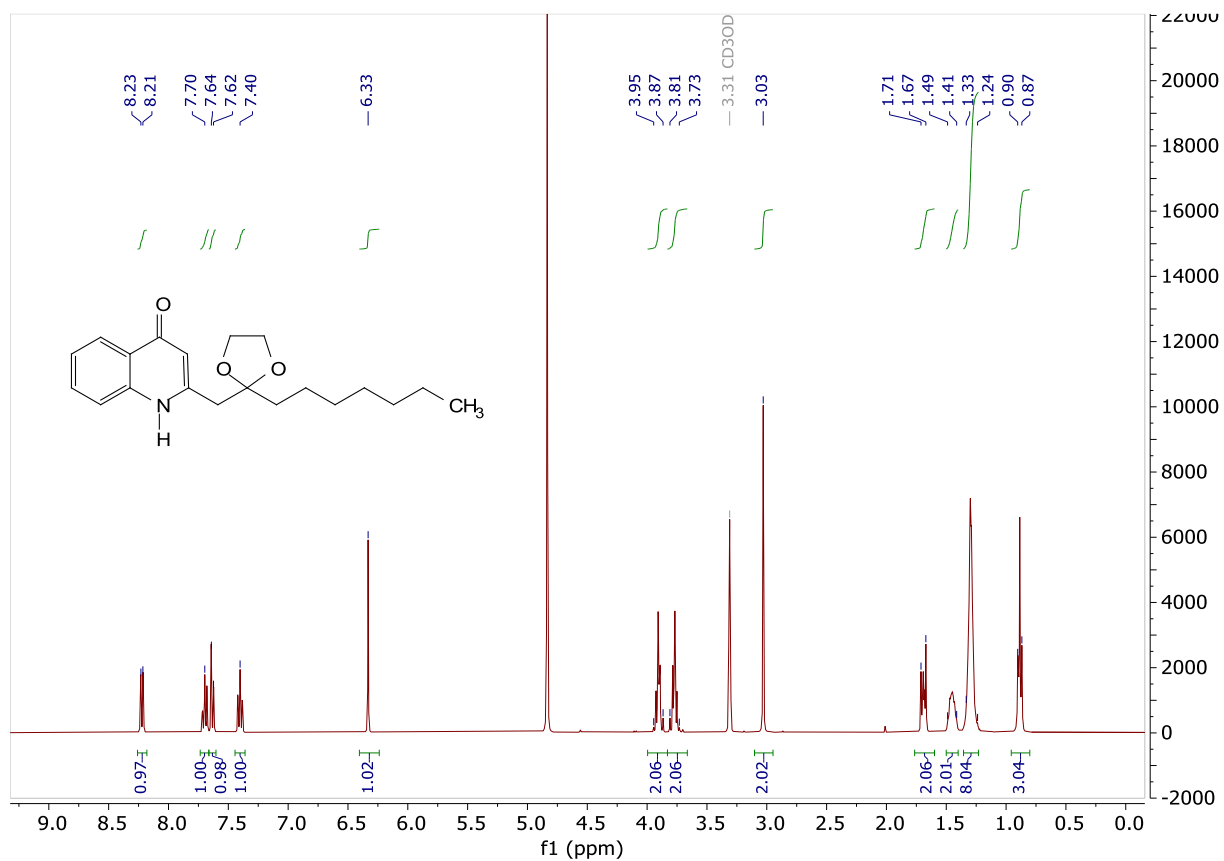
2-(2'-((*tert*-butyldimethylsilyl)oxy)nonyl)-1-hydroxyquinolin-4(1*H*)-one (2'-OTBS-NQNO) (**1f**)
¹H-NMR (above) and ¹³C-NMR (below) in MeOH-d₄



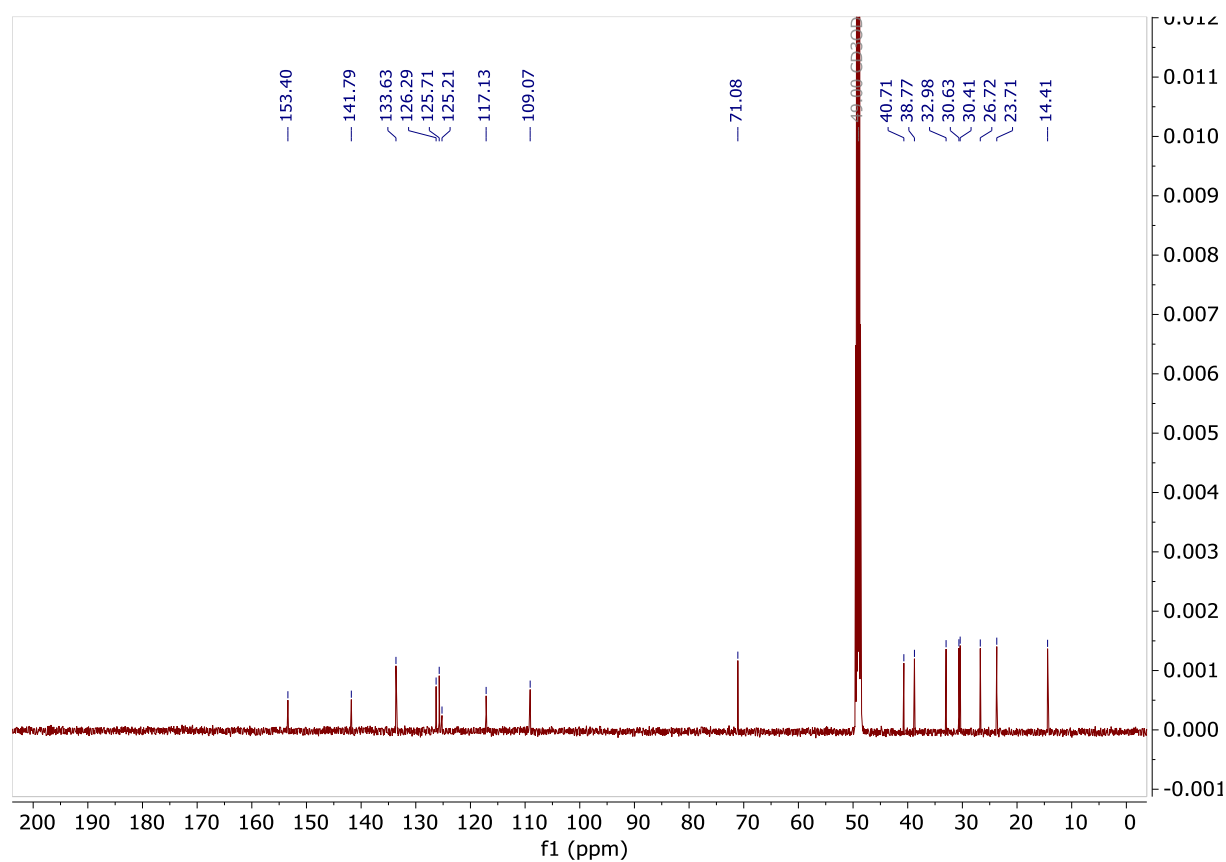
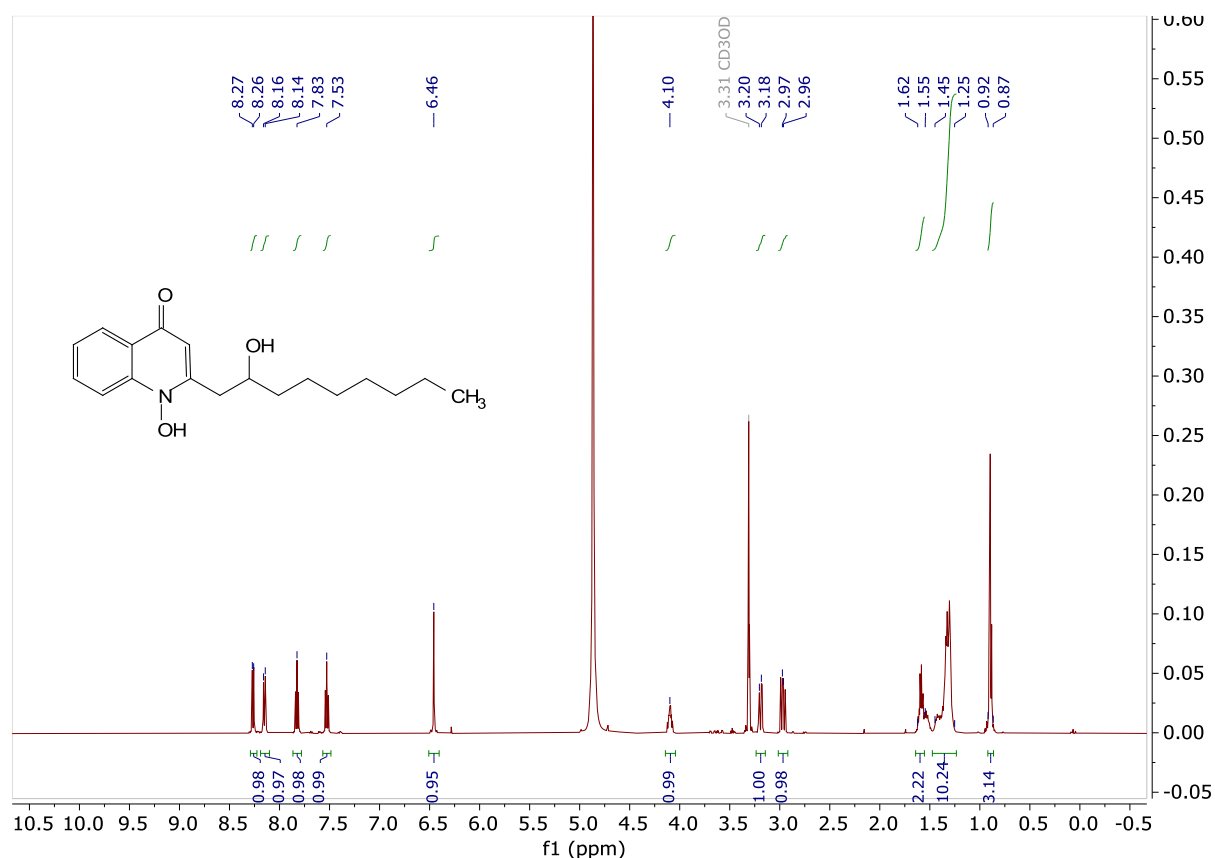
2-(2'-(1,3-dioxolane)-1-hydroxyquinolin-4(1H)-one (2'-(1,3-dioxolane)-NQNO) (**2f**) ¹H-NMR (above) and ¹³C-NMR (below) in MeOH-d₄



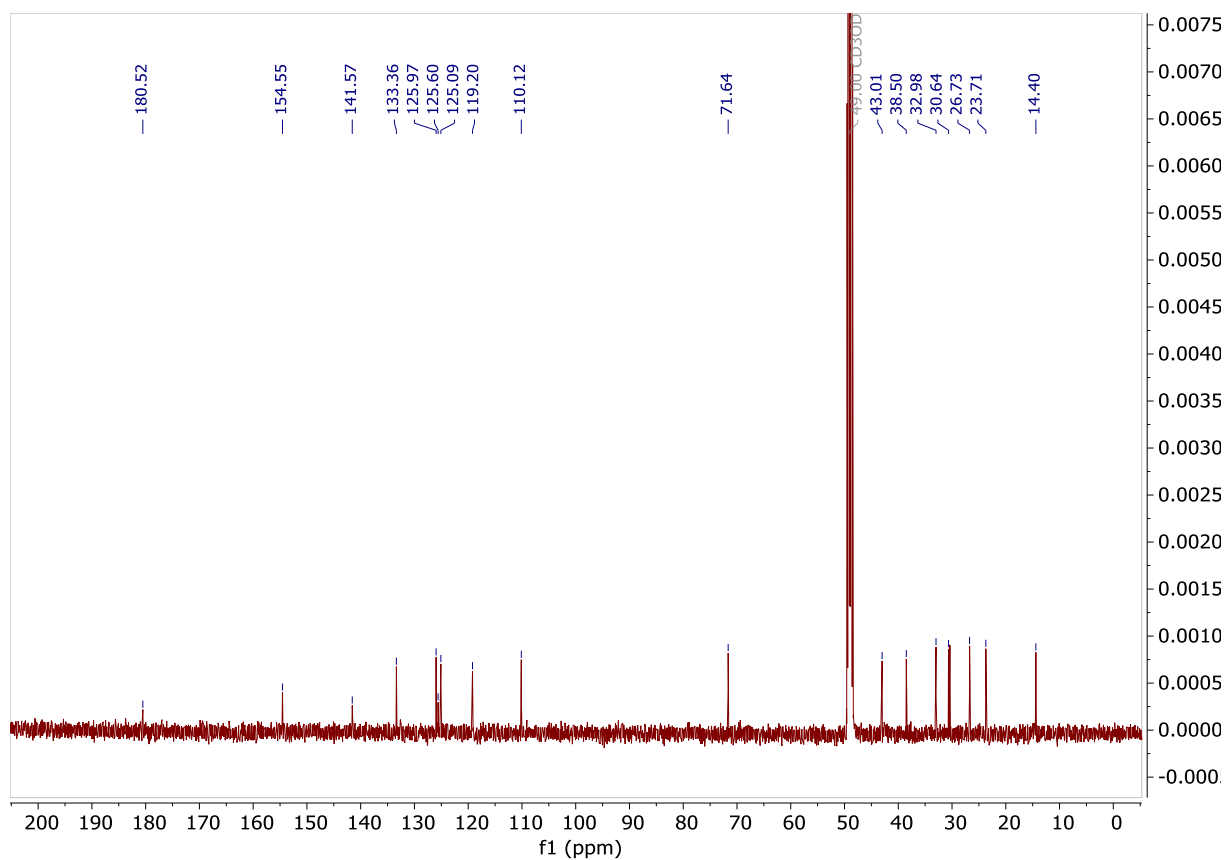
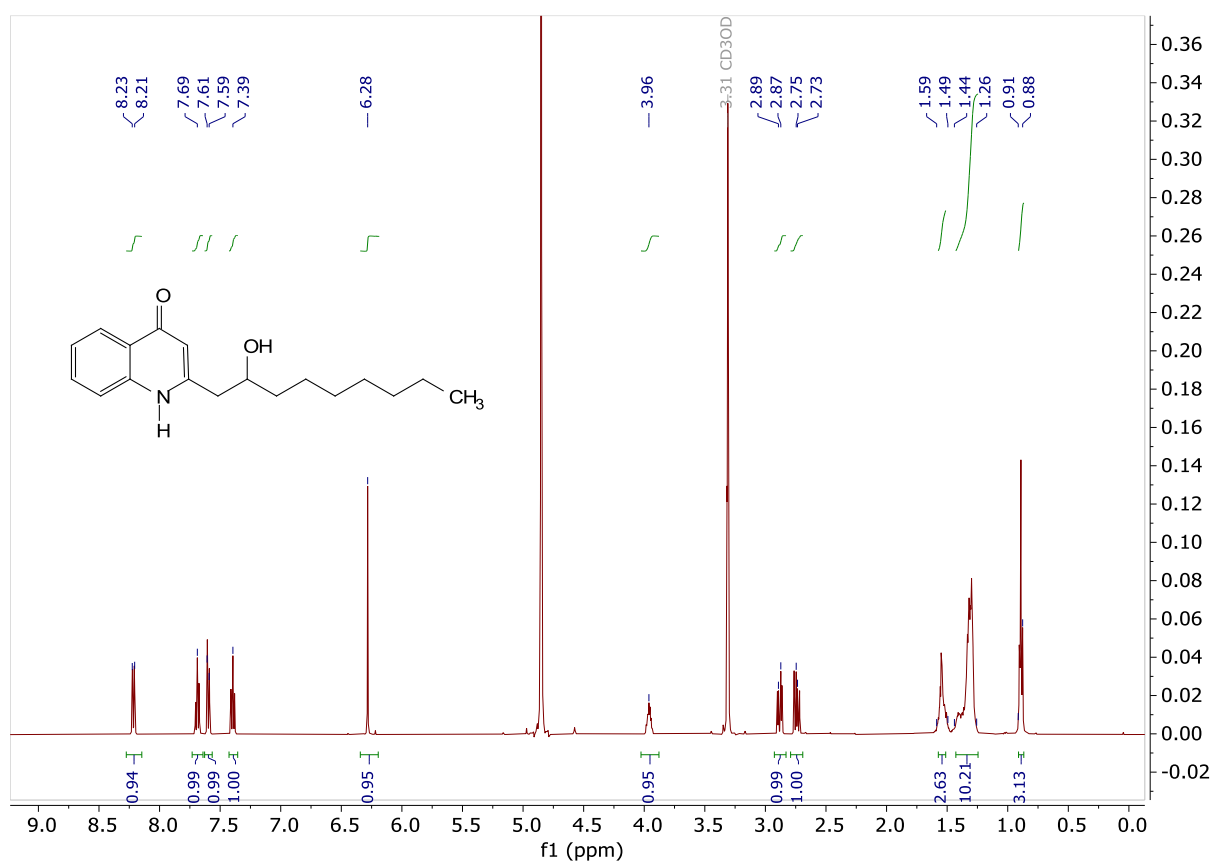
2-(2'-((*tert*-butyldimethylsilyl)oxy)nonyl)quinolin-4(1H)-one (2'-OTBS-NQ) (1e) ¹H-NMR (above) and ¹³C-NMR (below) in MeOH-*d*₄



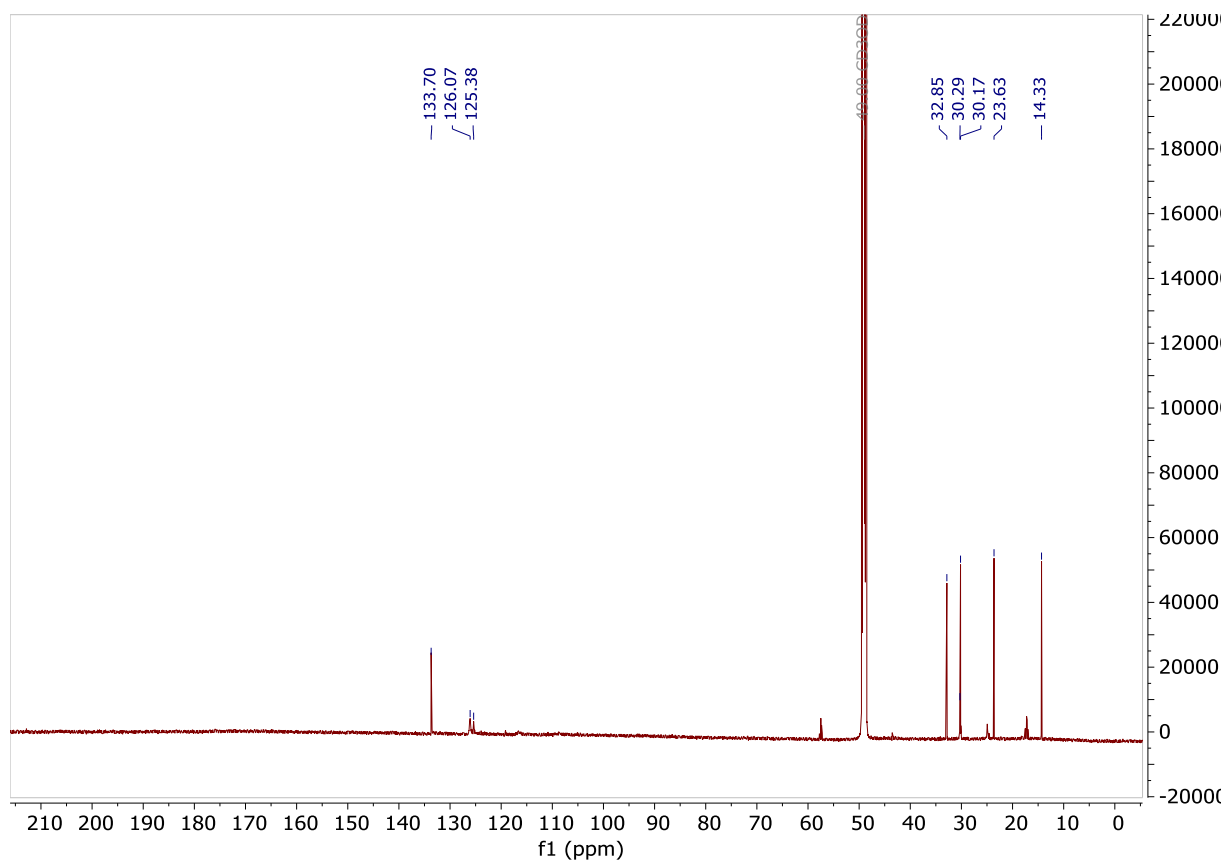
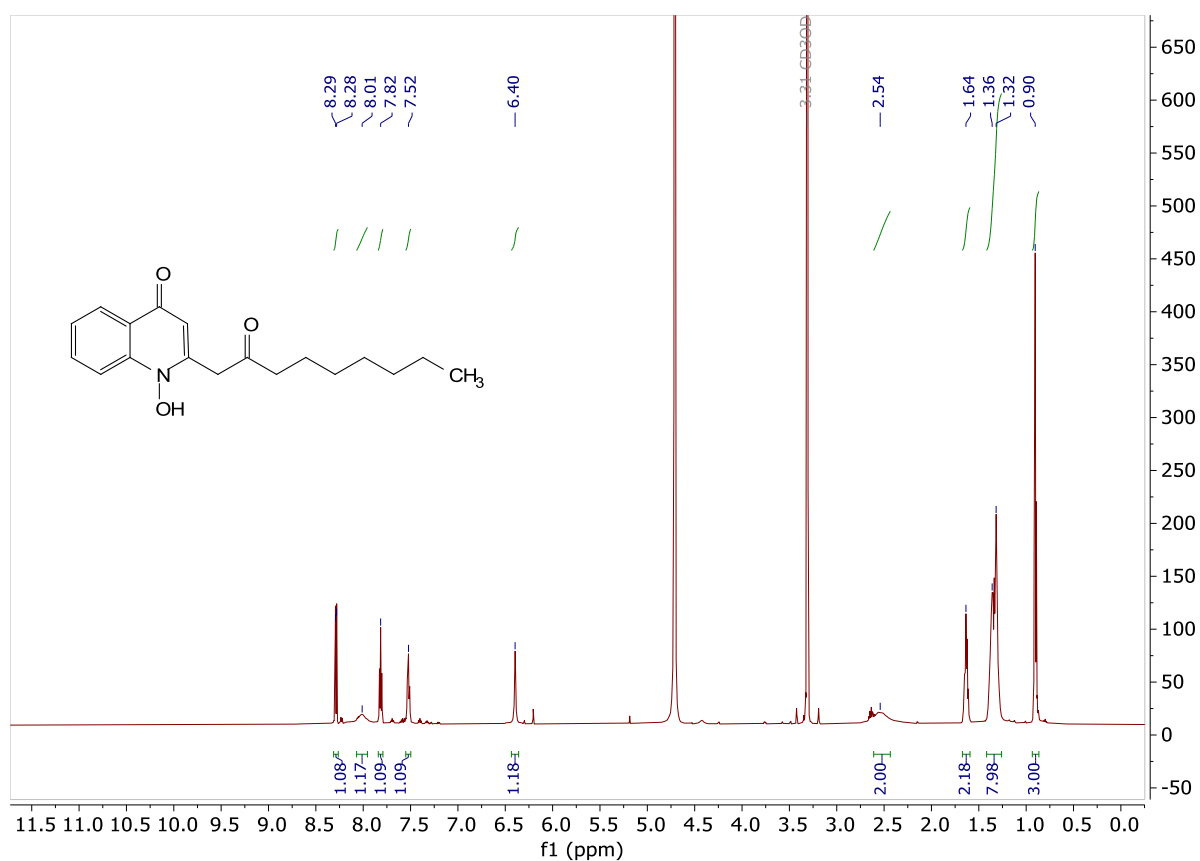
2-(2'-(1,3-dioxolane))quinolin-4(1H)-one (2'-(1,3-dioxolane)-NQ) (**2e**) ¹H-NMR (above) and ¹³C-NMR (below) in MeOH-d₄



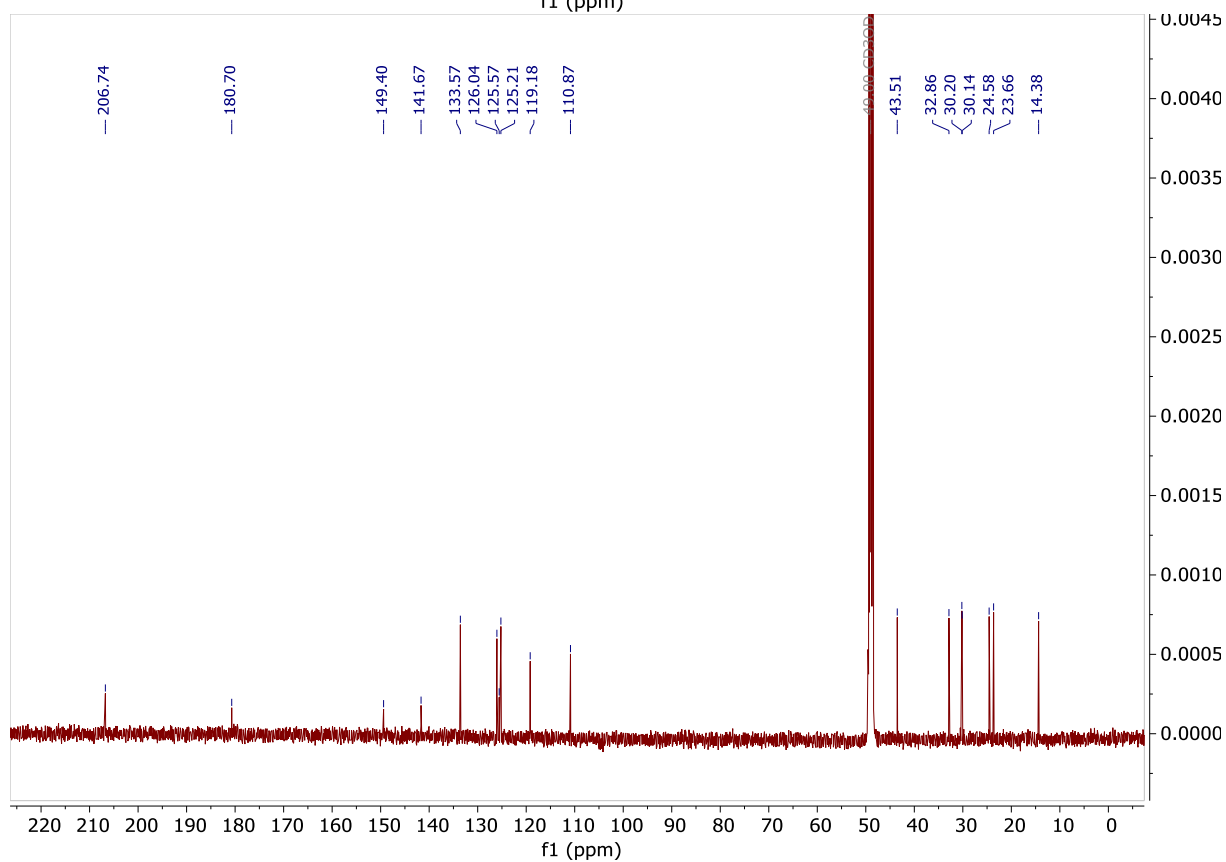
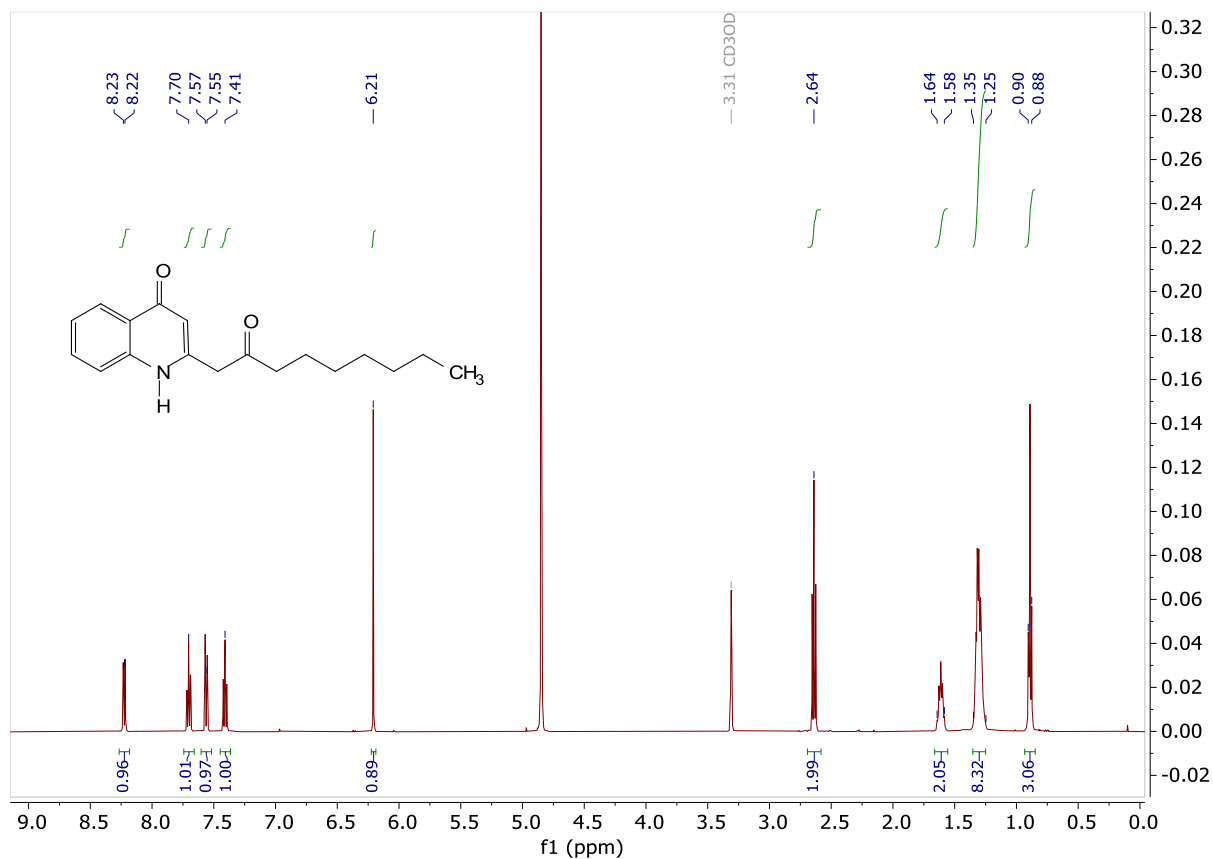
1-hydroxy-2-(2'-hydroxynonyl)quinolin-4(1H)-one (2'-OH-NQNO) (**1h**) ¹H-NMR (above) and ¹³C-NMR (below) in MeOH-d₄



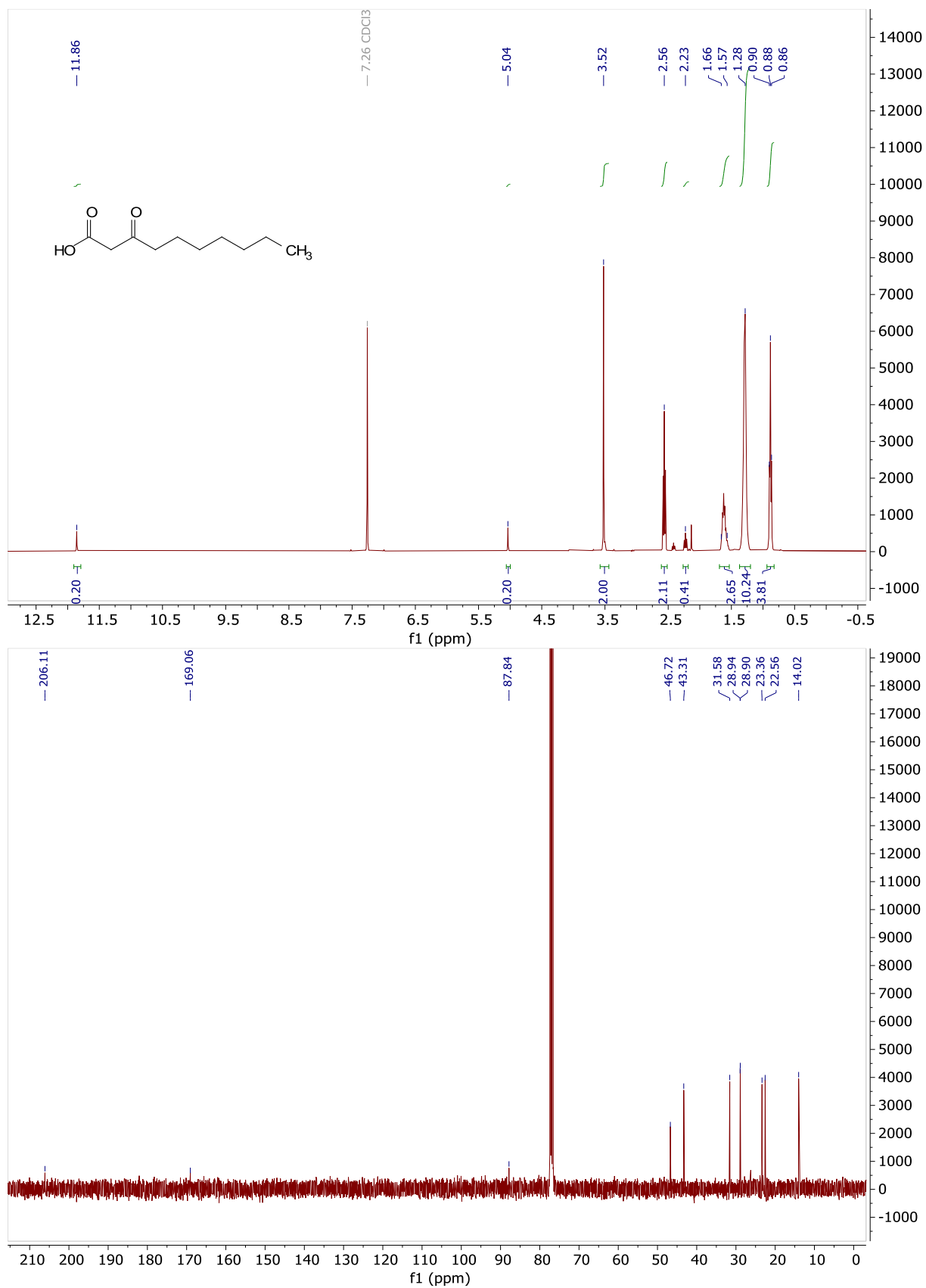
2-(2'-hydroxynonyl)quinolin-4(1H)-one (2'-OH-NQ) (**1g**) ¹H-NMR (above) and ¹³C-NMR (below) in MeOH-d₄



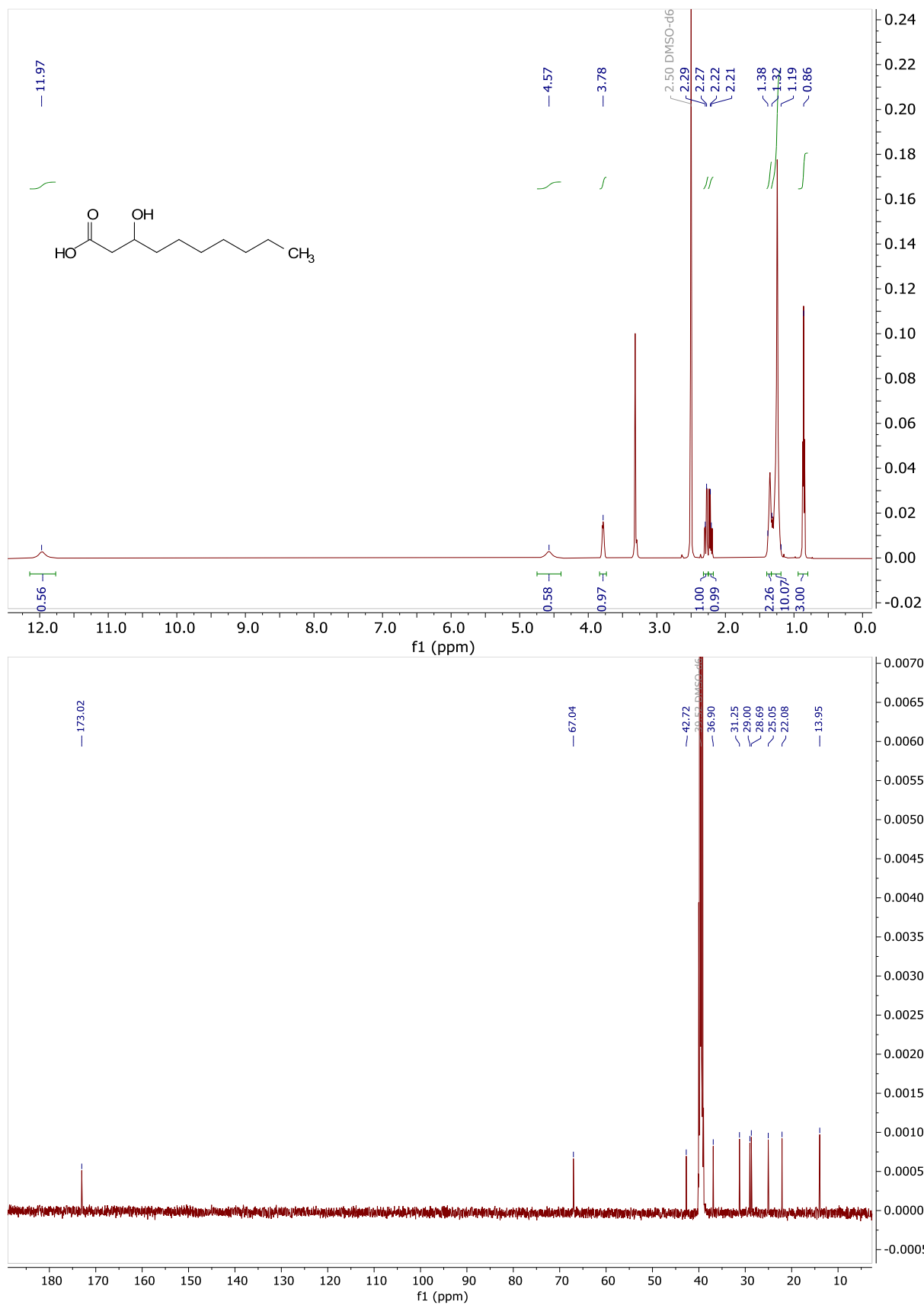
1-hydroxy-2-(2'-oxononyl)quinolin-4(1H)-one (2'-oxo-NQNO) (2h) ¹H-NMR (above) and ¹³C-NMR (below) in MeOH-d₄



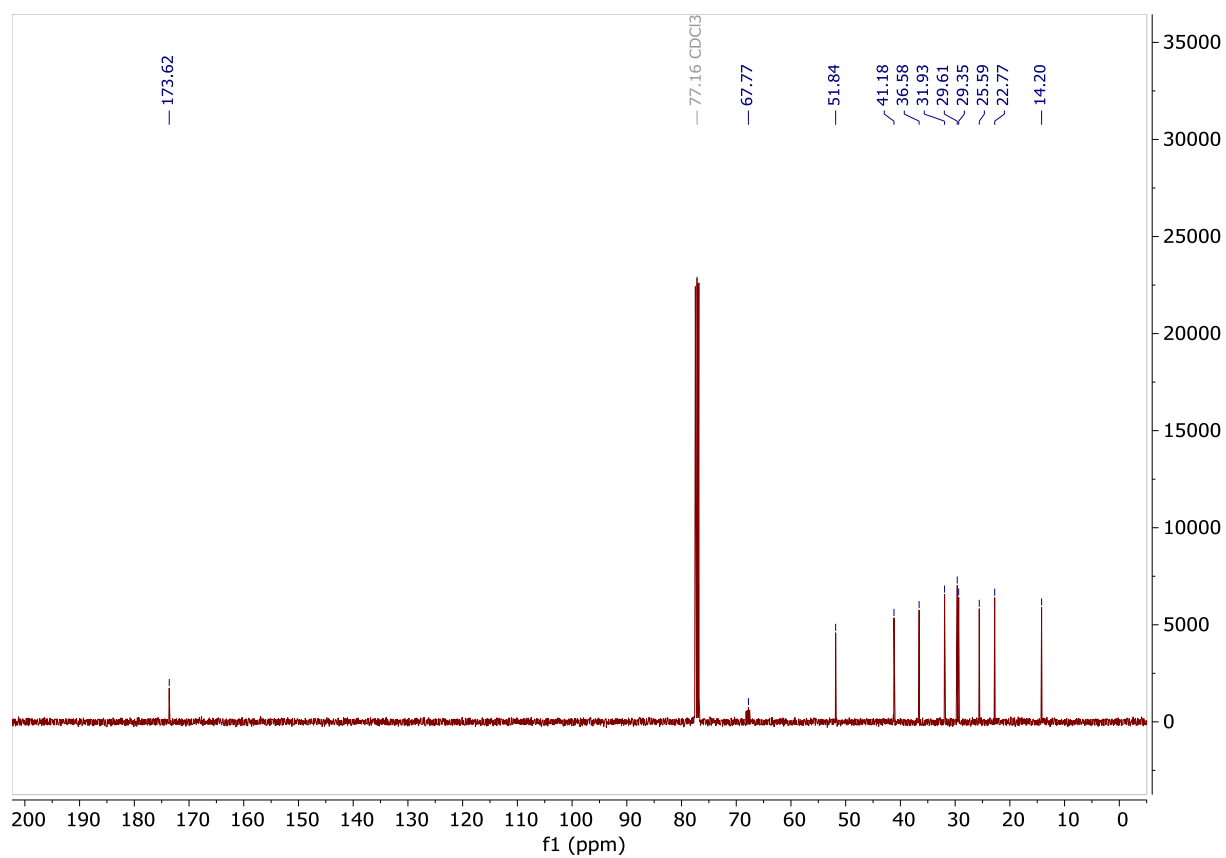
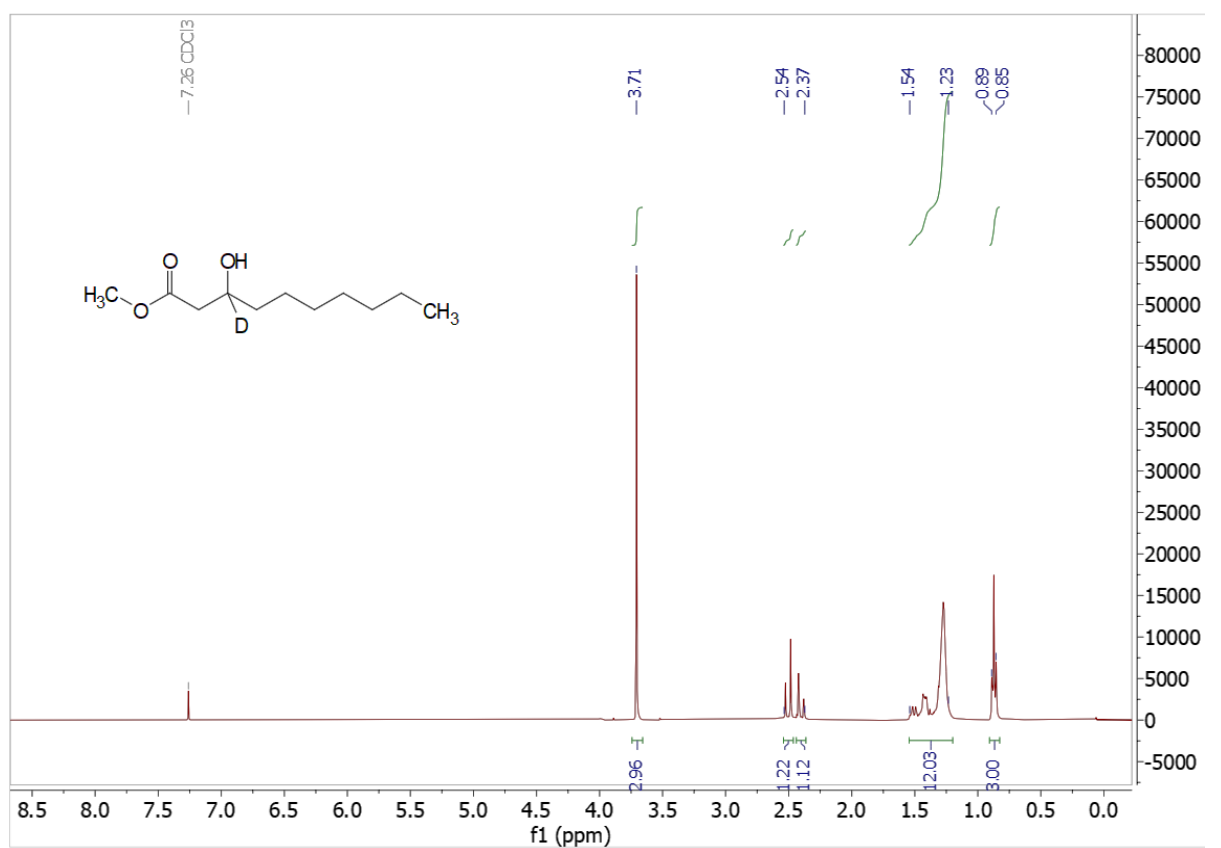
2-(2'-oxononyl)quinolin-4(1H)-one (2'-oxo-NQ) (**2g**) ^1H -NMR (above) and ^{13}C -NMR (below) in MeOH-d_4



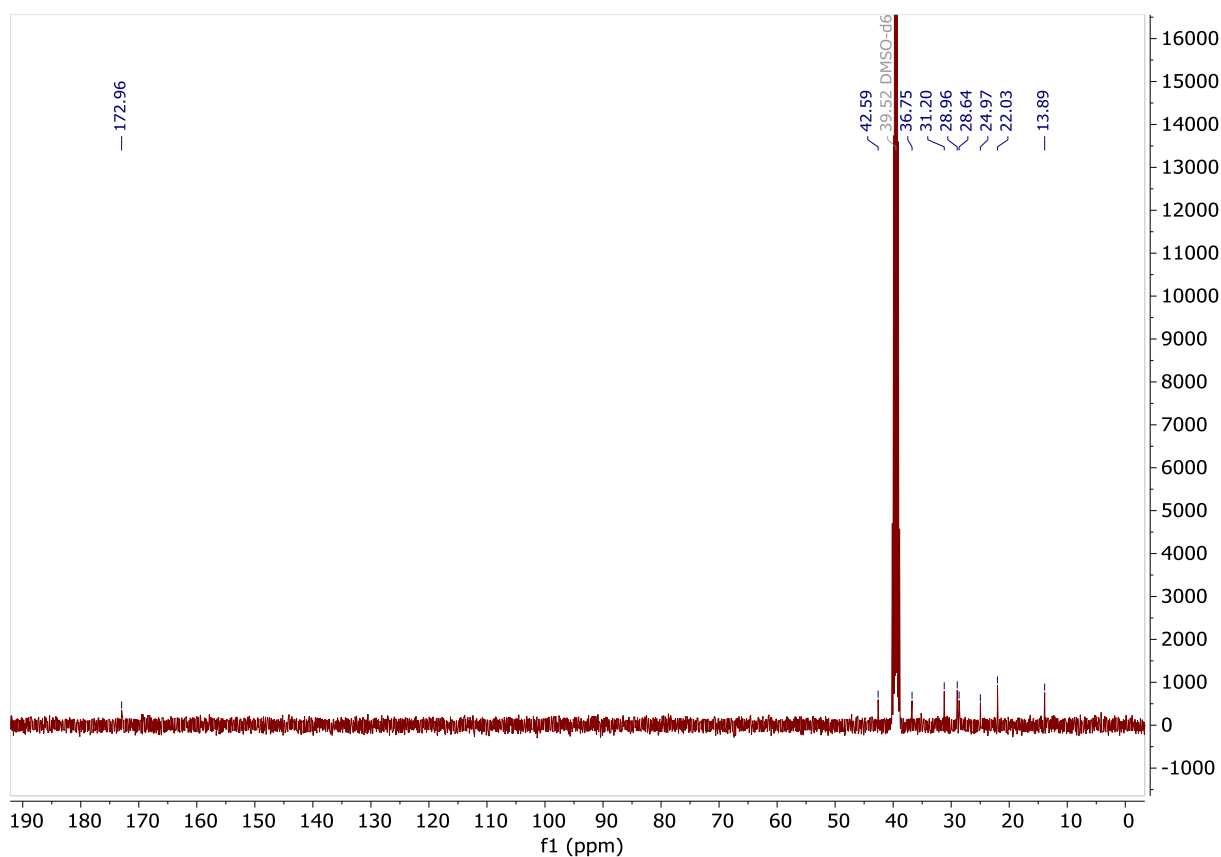
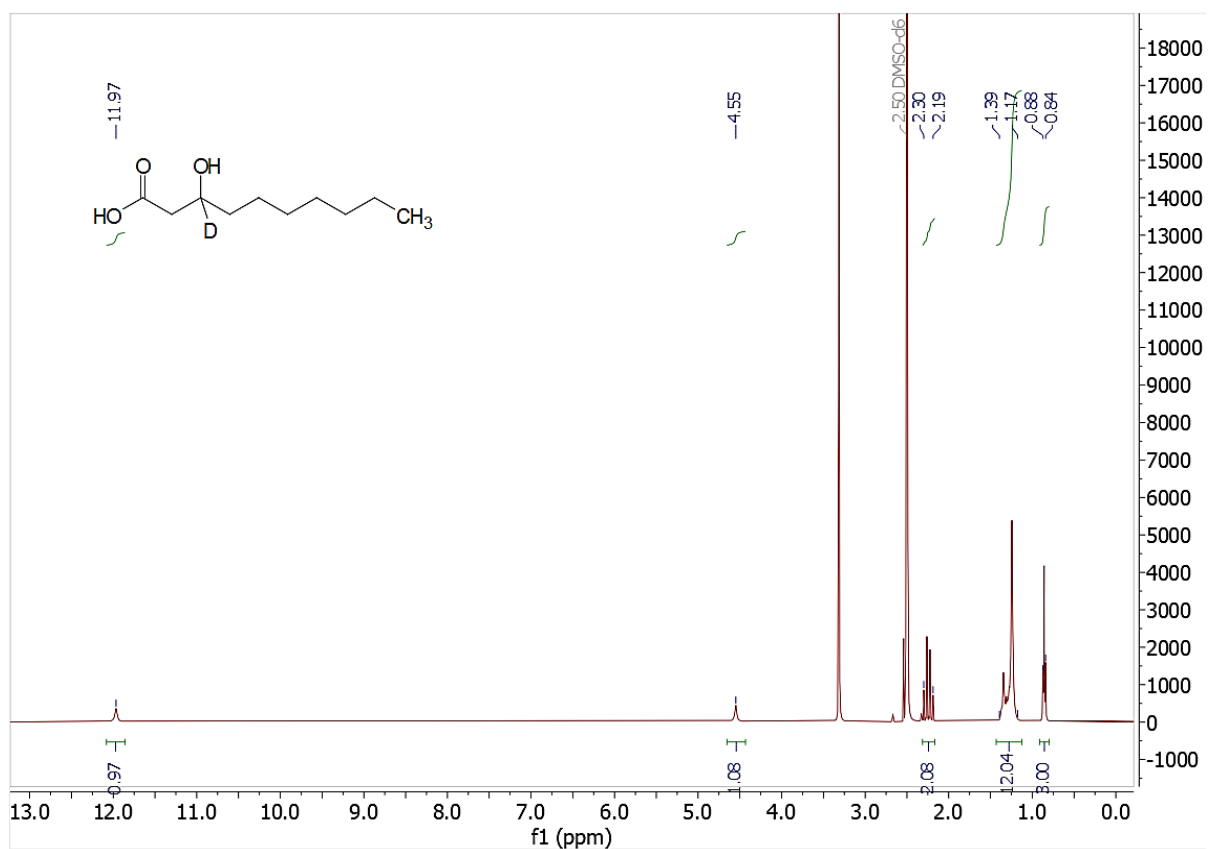
β -oxodecanoic acid (3a) $^1\text{H-NMR}$ (above) and $^{13}\text{C-NMR}$ (below) in CDCl_3



β -hydroxydecanoic acid (**3b**) ^1H -NMR (above) and ^{13}C -NMR (below) in DMSO- d_6



Methyl 3-hydroxydecanoate-d (**3c**) ¹H-NMR (above) and ¹³C-NMR (below) in CDCl₃



β -hydroxydecanoic acid-d (**3d**) $^1\text{H-NMR}$ (above) and $^{13}\text{C-NMR}$ (below) in DMSO- d_6

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Beyond iron: metal-binding activity of the *Pseudomonas* quinolone signal-motif†Dávid Szamosvári,^a Viktoriia Savchenko,^{a,b} Natalie Badouin^c and Thomas Böttcher^{*,a}

The *Pseudomonas* quinolone signal (PQS) is an important quorum sensing signal controlling virulence of the human pathogen *Pseudomonas aeruginosa*. PQS also exhibits multiple additional biological functions for *P. aeruginosa*, including the trapping of ferric iron. The PQS-motif has proven as privileged structure with great potential which is why we here explored the synthesis of two different types of crosslinked dimeric PQS-motifs as potential iron chelators. These compounds indeed chelated ferric iron and produced colorful and fluorescent complexes also with other metal ions. Inspired by these results, we revisited the metal ion binding capabilities of the natural product PQS and were able to detect further metal complexes beyond ferric iron and confirm the complex stoichiometry by mass spectrometry.

Introduction

The opportunistic, nosocomial human pathogen *Pseudomonas aeruginosa* is known for its biosynthesis of a vast variety of small molecules as secondary metabolites and virulence factors that contribute to its survival and pathogenicity in the human host.¹ A prime example of a small molecule with such diverse biological functions is the main quorum sensing molecule of the *Pseudomonas* quinolone signaling system (2-heptyl-3-hydroxy-4(1*H*)-quinolone, PQS). While its biosynthetic precursor 2-heptyl-4(1*H*)-quinolone (HHQ) is primarily acting as a quorum sensing signal, PQS is a multifunctional molecule with many additional functions beyond signaling. For example, PQS is involved in outer membrane vesicle formation

and can trap ferric iron as a bidentate ligand in the vicinity of cells of *P. aeruginosa*. Iron trapping eventually induces the production of siderophores via iron scarcity (Fig. 1).² Chemical modifications of the PQS scaffold already proved PQS to be a promising structural starting point for the generation of compounds with novel bioactive properties.³ Here we report the synthesis of metal chelating compounds with two covalently linked PQS motifs that led to the identification of metal binding capabilities of PQS beyond ferric iron. In addition to the previously known ferric iron complexes of PQS, we could identify so far undescribed PQS-molybdate and PQS-aluminum complexes by LC-HRMS as well as a suspected vanadate complex. The identification of PQS as a versatile metallophore reveals unprecedented insights into the multifunctional roles of PQS for the survival and metal homeostasis of *P. aeruginosa*.

Results and discussion

As one of our research interests revolves around the synthesis of PQS derivatives and analogs to study their bioactivity and influence on bacterial population behavior,^{3,4} we envisioned to synthetically explore structures with a joined dimeric PQS motif. Therefore, we designed scaffolds that represent PQS analogs connected either by a carbon-carbon bond at position 6 (2) or by a shared aromatic system (3). As PQS and derivatives are known to exhibit a generally poor solubility we decided to

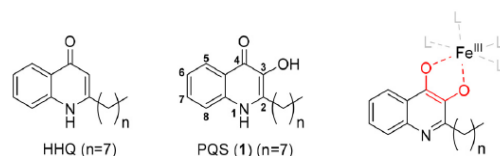


Fig. 1 Structures of the quinolone quorum sensing signals HHQ and PQS of *Pseudomonas aeruginosa* and congeners with different chain lengths. PQS is a bidentate ligand for ferric iron.

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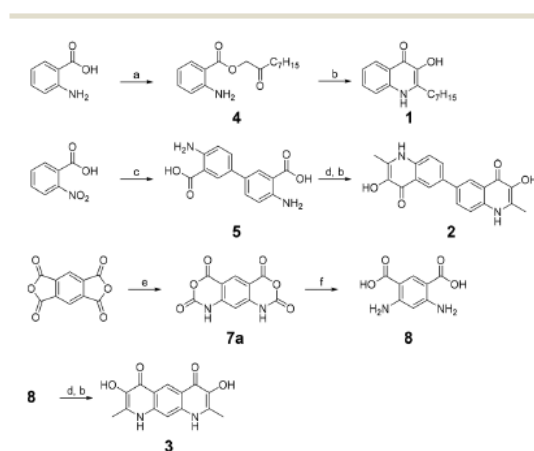
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replace the hydrophobic heptyl side chains of the linked dimeric PQS analogs with methyl groups to improve solubility properties.⁵ The synthesis of these undescribed compounds was adapted from the synthesis of PQS molecules as reported by Hradil *et al.*⁶ where 2-aminobenzoic acid is esterified to 2-oxononyl 2'-aminobenzoate (4) and further cyclized to PQS (1) (Scheme 1). Similarly, we started with the synthesis of compound 2 by the generation of 4,4'-diamino-[1,1'-biphenyl]-3,3'-dicarboxylic acid (5) *via* reduction of 2-nitrobenzoic acid and subsequent benzidine rearrangement.⁷ The 2-aminobenzoic acid motifs thus obtained were esterified with chloroacetone and cyclized to the PQS analog 3,3'-dihydroxy-2,2'-dimethyl-[6,6'-biquinoline]-4,4'-(1*H*,1'*H*)-dione (2). The synthesis of compound 3 started with a double nitrogen insertion in pyromellitic dianhydride by its reaction with trimethylsilyl azide (TMSA) as described for phthalic anhydrides.⁸ The resulting complex mixture of reaction products required extensive purification efforts and only one regioisomer, 1,9-dihydro-2*H*,4*H*-benzo[1,2-*d*:5,4-*d'*]bis[1,3]oxazine-2,4,6,8-tetraone (7a), could be obtained reliably and in practical amounts. Following ring-opening by hydrolysis resulted in 4,6-diaminoisophthalic acid (8) which was esterified and cyclized to 3,7-dihydroxy-2,8-dimethylpyrido[3,2-*g*]quinoline-4,6(1*H*,9*H*)-dione (3) as described for compound 2 (Scheme 1). Since PQS is poorly water soluble and in its native environment secreted in the form of membrane vesicles, we used DMSO as organic solvent system to increase solubility. Qualitative investigations on the metal ion complexing properties of 2 and 3 revealed that both compounds form colorful complexes with Fe³⁺ ions in DMSO.



Scheme 1 Chemical synthesis of 2-heptyl-3-hydroxyquinolin-4(1*H*)-one (PQS, 1), 3,3'-dihydroxy-2,2'-dimethyl-[6,6'-biquinoline]-4,4'-(1*H*,1'*H*)-dione (2) and 3,7-dihydroxy-2,8-dimethylpyrido[3,2-*g*]quinoline-4,6(1*H*,9*H*)-dione (3). Experimental conditions: (a) (1) K₂CO₃, DMF, 90 °C, 1 h, (2) 1-chlorononan-2-one, 30 min at rt, 30 min at 50 °C, 94%; (b) NMP, 2 h, reflux, 84% for 1, 75% for 2, 61% for 3; (c) (1) Zn, NaOH, reflux, 5 h, (2) HCl, reflux, 1 h, 71%; (d) (1) K₂CO₃, DMF, 90 °C, 1 h, (2) 1-chloroacetone, 30 min at rt, 30 min at 50 °C, 51% for 6, 40% for 9; (e) 3 eq. TMSA, 1,4-dioxane, reflux, o/n, 21%; (f) 1 M NaOH, 60 °C, 60 min, 59%.

Similar to PQS, compound 2 formed a blue complex whereas compound 3 formed a greenish-brown color. Upon these initial observations, we investigated the behavior of 2 and 3 with other di- and trivalent metal ions (Al³⁺, Mn²⁺, Cu²⁺, Co²⁺, Zn²⁺, Ni²⁺) and the oxoanions molybdate (MoO₄²⁻) and vanadate (VO₄³⁻). UV absorption spectroscopy showed that compound 2 changes its UV-absorption spectra when incubated with an excess of Fe³⁺, Al³⁺, Cu²⁺, MoO₄²⁻ and VO₄³⁻ although a change in color of the, otherwise colorless, solution of 2 was only observed for Fe³⁺ and MoO₄²⁻ (Fig. 2A and C). Complex formation of 2 with Fe³⁺ and MoO₄²⁻ was also observed by a change in the fluorescence under UV light of 365 nm (Fig. 2C and S8†). In contrast, the otherwise yellow solution of compound 3, with maximum absorption at 445 nm and bright yellow fluorescence under 365 nm, showed a rapid change in color when incubated with the same ions that form a complex with compound 2 (Fig. 2B and D) and a much more pronounced change in the fluorescence was observed when 3 was incubated with Fe³⁺, Al³⁺, MoO₄²⁻ and VO₄³⁻ (Fig. 2D). Complexes with other metal ions were not detected by UV absorption spectroscopy. These results inspired us to revisit the metal chelating capabilities of PQS of *P. aeruginosa* in more detail. Surprisingly no other metal ions or oxoanions of metals, apart from ferric iron, have been described to form complexes with PQS. Therefore, we preselected ions capable to form complexes with 2 and 3 (Fe³⁺, Al³⁺, Cu²⁺, MoO₄²⁻ and VO₄³⁻), and investigated potential complex formation with PQS.

Comparable to 2, a change in color of the otherwise colorless PQS solution was only observed for Fe³⁺ and MoO₄²⁻ while minor changes in fluorescence were observable for all ions tested (Fig. 3F). To identify and confirm possible PQS-complexes, we subsequently used LC-HRMS analysis of the PQS complex formation experiments (Fig. 3). Analysis of the ferric iron spiked PQS sample revealed the *m/z*-values of two distinct PQS-Fe³⁺ complexes (572.2335 Da and 831.3920 Da) that can be assigned to Fe(PQS)₂⁺ and Fe(PQS)₃ + H⁺ (Fig. 3A and B). These complexes have been reported before by LR-MS⁹ and according ferric iron complexes are known for the methyl derivative of PQS (MPQS).⁵ Interestingly, the PQS-Al³⁺ sample showed a similar 2 : 1 and 3 : 1 stoichiometric ratio of PQS and Al³⁺. Here, we detected the *m/z*-values of 543.2797 Da and 802.4307 Da corresponding to Al(PQS)₂⁺ and Al(PQS)₃ + H⁺ (Fig. 3C and D). In the case of MoO₄²⁻, we could detect a mass of 647.2013 Da that represents the MoO₂(PQS)₂ + H⁺ complex (Fig. 3E). Although, the measurement of PQS in a mixture with VO₄³⁻ ions clearly shows the detection of unique mass signals likely corresponding to PQS-vanadate complexes, an unambiguous assignment of the *m/z*-values to a complex structure was not possible (Fig. S7†). Surprisingly, even though measurements of the UV-Vis spectra of 2 and 3 with Cu²⁺ ions indicated complex formation by a significant change in the absorption spectra (Fig. 2), we could not detect the presence of a PQS-Cu²⁺ complex by LC-HRMS. Our results hence demonstrate that PQS is capable of forming complexes with metals beyond ferric iron, including Al³⁺, MoO₄²⁻, and likely VO₄³⁻.

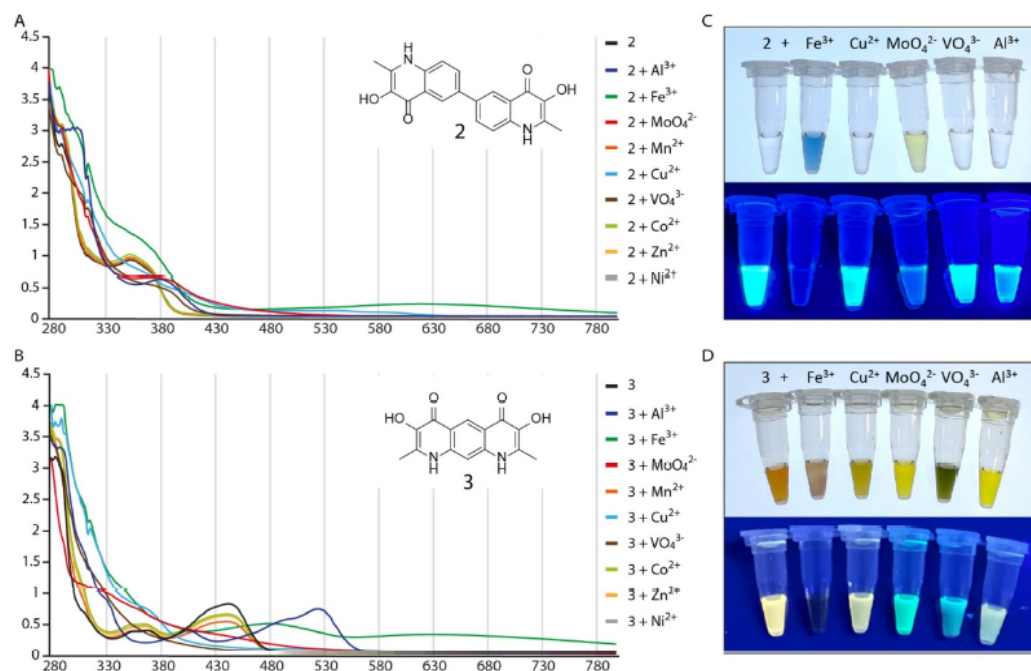


Fig. 2 Overlaid UV-absorption spectra of compound 2 (A) and compound 3 (B) with indicated metal ions in DMSO. Solutions of compound 2 (C) and 3 (D) with metal ions under visible light (top) and under UV light (365 nm, bottom).

Transition metal elements, including Co, Cu, Fe, Mg, Mn, Mo, Ni, and Zn ions, are essential micronutrients for all organisms.¹⁰ As cofactors in enzymes, they facilitate catalytic reactions, contribute to the structural integrity and stability of proteins, and are crucial for electron transport systems.

The low abundance and bioavailability of these trace elements forced bacteria to develop a multitude of acquisition mechanisms like metallophores, metal-recruiting proteins, and metal-complex transporters, to secure a sufficient supply of these nutrients from the environment.¹¹ Especially during a bacterial infection, the host can sequester essential trace metal ions and thereby actively restrict their availability to the bacteria, which is known as nutritional immunity. Overcoming this limitation is decisive for the survival and virulence of the pathogen and the course of the infection.¹² In *P. aeruginosa*, especially the intake mechanisms of ferric and ferrous iron are well characterized.¹³ Thereby, PQS plays an important role by acting as a chelating agent for ferric iron in a proposed 2 : 1 or 3 : 1 complex which we confirmed by our LC-HRMS analysis. Contrary to siderophores, PQS serves as an iron trap since its ferric iron complexes cannot be taken up into the bacteria directly but instead accumulate in the cell envelope and outer membrane vesicles of the bacteria.^{2a,5,14} By trapping ferric iron, PQS can activate an iron starvation response in *P. aeruginosa* and induce the biosynthesis of the siderophores pyochelin and pyoverdine,⁵ and affect the Rhl quorum-sensing

system.⁹ The acquisition mechanisms and roles of other important transition metals in *P. aeruginosa* are known to a lesser extent^{10b} and the chelation by PQS was so far only described for ferric iron. Applying our dimeric PQS analogs 2 and 3 as qualitative chromogenic and fluorogenic PQS-metal ion binding probes allowed us to identify the PQS-motif being capable of complexing MoO₄²⁻, Al³⁺, and likely VO₄³⁻. The existence of PQS-complexes with these ions could finally be confirmed and their stoichiometry elucidated by LC-HRMS as MoO₂(PQS)₂ as well as Al(PQS)₂⁺ and Al(PQS)₃ complexes. In the environment of the bacteria, the trace element molybdenum is mainly present as the oxyanion molybdate (MoO₄²⁻) and essential for the growth of *P. aeruginosa* under anaerobic and microaerophilic conditions. As complex with its ligand molybdopterin, molybdate plays an important role as molybdenum cofactor in the periplasmic nitrate reductase NarGHI that facilitates the reduction of NO₃⁻ to NO₂⁻.¹⁵ A lack of MoO₄²⁻ was shown to significantly reduce the growth of *P. aeruginosa* under anaerobic conditions.¹⁶ To facilitate molybdenum uptake, *P. aeruginosa* uses a high-affinity ATP-binding cassette permease, ModABC to recruit and import molybdate from the environment.^{16,17} Importantly, the molybdate transporter ModABC was found to be essential for anaerobic growth in an *in vivo* animal model of chronic lung infection.¹⁸ ModA was shown to be a molybdate-binding protein and important for bacterial competition and virulence of *P. aeruginosa* in a

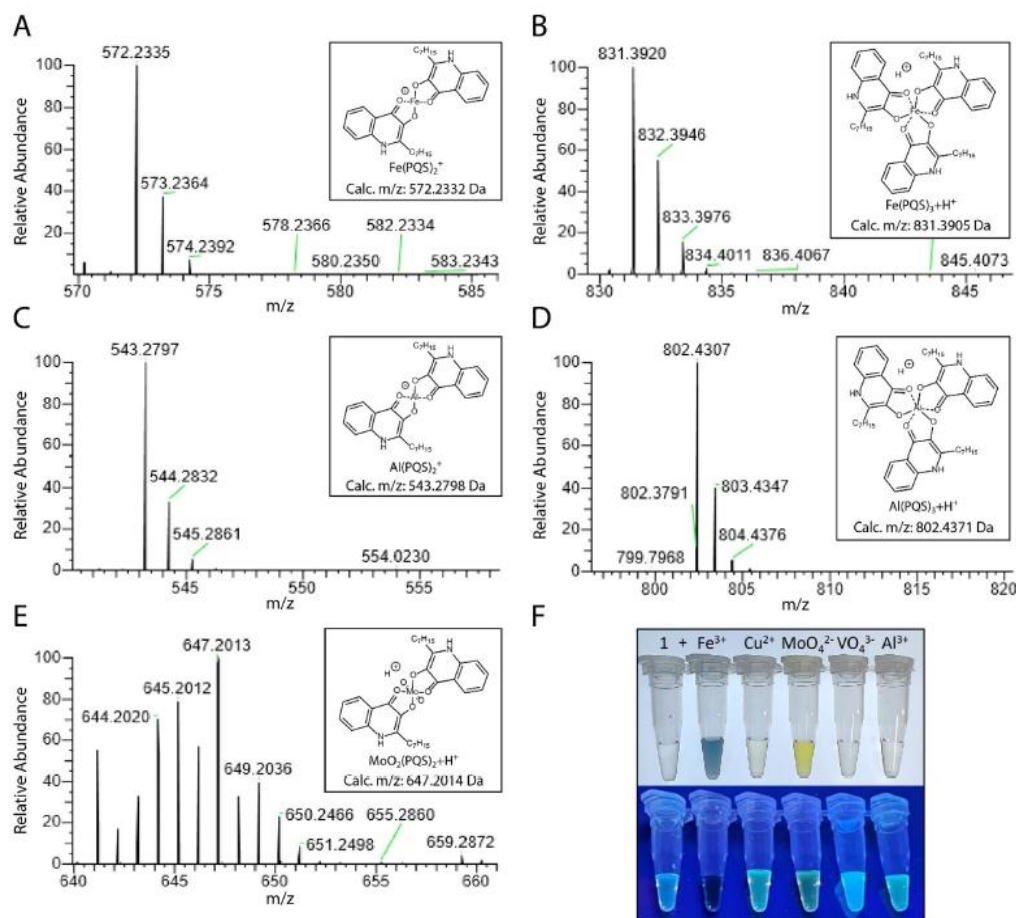


Fig. 3 Identified PQS complexes by HRMS. HRMS spectra of (A) Fe(PQS)₂⁺, (B) Fe(PQS)₃ + H⁺, (C) Al(PQS)₂⁺, (D) Al(PQS)₃ + H⁺, (E) MoO₂(PQS)₂ + H⁺ and (F) solutions of PQS-metal complexes under visible light (top) and under UV light (365 nm, bottom).

mouse model.¹⁹ However, the exact mode of molybdate sequestration from the environment is still unclear and the PQS-MoO₄²⁻ complex could represent a missing link in this transport chain.

Aluminum, in its form as Al³⁺ ion, on the other hand, lacks biological function as a cofactor. Even though the bio-availability of aluminum in an infected host is low, it was shown that aluminum can interfere with the iron acquisition of *P. aeruginosa* by occupying pyoverdine and accumulation in the periplasm of the bacteria.²⁰ Chelation of Al³⁺ by PQS could therefore serve as a detoxification strategy by sequestration of the toxic metal ion to prevent interference with metabolic processes. Similar protection mechanisms by *P. aeruginosa* against antimicrobial and antibiofilm gallium (Ga³⁺) were already observed for its siderophore pyoverdine.²¹ Although a small number of bacteria are known to utilize vanadium as a

cofactor in their enzymes, such as vanadium nitrogenases or vanadate-dependent haloperoxidases,²² vanadate (VO₄³⁻) is generally considered cytotoxic for bacteria due to the structural similarity to the phosphate ion (PO₄³⁻).²³ Vanadate was shown to substitute for phosphate in enzymes such as phosphatases and kinases. Vanadium, in its vanadyl and vanadate forms, was shown to inhibit the growth of *P. aeruginosa*, especially in an iron-limited medium and it was suggested that vanadium can affect different functions in the bacteria depending on the iron content of the cell.²⁴ Interestingly, the siderophores pyochelin and pyoverdine of *P. aeruginosa* have been found to complex vanadium. Even though the underlying mechanisms are still unclear, exposure of *P. aeruginosa* cells to vanadium was shown to cause an increase in reactive oxygen species and, in response, increase superoxide dismutase (SOD) activity in *P. aeruginosa*.²⁴ Although we do not at present have a structure of

the PQS-VO₄³⁻ complexes it is conceivable that PQS serves again as a detoxification agent by complexation of vanadate, similar to aluminum.

Experimental Section

General information: Chemicals and solvents for the synthesis were purchased from Sigma-Aldrich, TCI, Carl Roth or VWR Chemicals and were used without further purification. For silica gel chromatography, distilled technical grade solvents and silica gel 60 A (Carl Roth) were used. Thin layer chromatography (TLC) was performed using aluminum sheets "TLC silica gel 60 F254" from Merck Millipore® and analyzed with UV-light or by permanganate staining. NMR spectra were obtained on Bruker Avance-III 400 and Bruker Avance-III 600 NMR spectrometers at ambient temperature. Multiplicities are given as follows: *s* – singlet, *d* – doublet, *t* – triplet, *q* – quartet, *quint.* – quintet, *m* – multiplet. Chemical shifts (δ) are given in parts per million (ppm) relative to the solvent residual signal with CDCl₃ δ_H = 7.26 ppm and δ_C = 77.16 ppm, DMSO-*d*₆ δ_H = 2.50 ppm and δ_C = 39.52 ppm.²⁵ The data obtained were processed and analyzed with Bruker Topspin 3.5 software. High resolution mass spectrometry data for the synthetic compounds were obtained on an ESI-Orbitrap (Thermo Scientific, LTQ Orbitrap Velos) by direct injection and analyzed with Xcalibur (Thermo Scientific) software. UV-Vis spectroscopy was performed on a TECAN infinite® M200 PRO plate reader using i-control™ software using 96-well plates from Sarstedt (Microtest plates 96-well, flat-bottom, without lid).

Preparation of the metal ion complex solutions: For the qualitative analysis of a complexation event (Fig. 2C, 2D, and 3F), 25 μ L of a 2 mM stock solution of PQS (1), compound 2 or compound 3 in DMSO was added to 25 μ L of a 4 mM stock solution of a metal salt solution in DMSO (the stock solutions for Na₃VO₄ and Na₂MoO₄ were prepared in DMSO and DMSO/H₂O (1 : 1), respectively). For the measurements of the UV-Vis absorption spectra (Fig. 2A and 2B) 50 μ L of a 0.4 mM stock solution of compound 2 or compound 3 in DMSO was added to 50 μ L of a 0.8 mM stock solution of a metal salt solution and incubated for 1 h at room temperature before measurement. For the measurements of the HRMS spectra (Fig. 3A–F and Fig. S1–7†), the metal-PQS complexes were obtained by incubating salt solutions (30 μ M final) with proportional to molar ratio amounts of PQS solution (60–180 μ M final) in 1 mL of Methanol (or Acetone) (Table S1†). Resulting mixtures were kept for at least one hour at +4 °C before subjection to HRMS measurements.

Identification of PQS-metal ion complexes via HRMS: High-resolution mass spectrometry data were obtained on a Hybrid FT Mass Spectrometer LTQ Orbitrap Velos (Thermo Scientific) in positive ESI mode. The structures of PQS-metal complexes were proposed based on their full HRMS spectra and the coordination numbers of metal ions. Detected *m/z* and their distribution for PQS and complexes were com-

pared to the calculated values (ChemDraw Professional 20.0.0.41).

Conclusion

While PQS is mostly considered a quorum sensing molecule of *P. aeruginosa*, its properties as iron chelating molecule indicate further relevance for the pathogen beyond quorum sensing. Our identification of PQS as metallophore of Al³⁺, MoO₄³⁻, and VO₄³⁻ in addition to Fe³⁺ suggests that PQS serves a crucial and more complex role in the metal ion homeostasis of *P. aeruginosa* beyond ferric iron.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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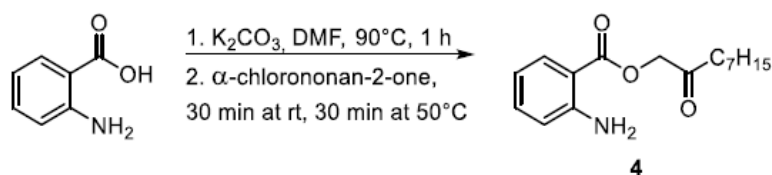
2.2 Publication 2. Supporting Information

Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.
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Supporting Information

Chemical synthesis

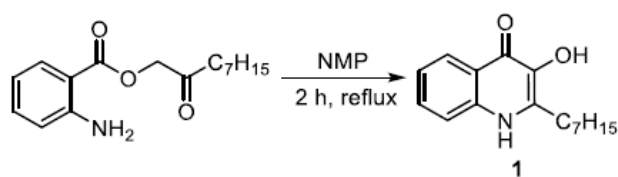
Synthesis of 2-oxononyl 2'-aminobenzoate **4**



Anthranilic acid (3.26 g, 23.8 mmol, 1.2 eq.) was dissolved in DMF (44 mL) and K_2CO_3 (2.93 g, 21.19 mmol, 1.07 eq.) was added. The mixture was heated at 90°C for 1 h. After reaching room temperature, α -chlorononan-2-one (3.5 g, 19.8 mmol, 1.0 eq.) was added. The mixture was stirred for 30 min at room temperature and 30 min at 50°C . After letting cool to room temperature, the mixture was poured in ice-water and the precipitate collected by filtration and dried.

2-oxononyl 2'-aminobenzoate (**4**) was obtained as a white solid (5.15 g, 94 %): $^1\text{H-NMR}$ (CDCl_3 400.13 MHz) δ (ppm): 0.88 (t, $J = 7.0$ Hz, 3H, $-\text{CH}_3$), 1.21 – 1.35 (m, 8H, $-(\text{CH}_2)_4-\text{CH}_3$), 1.63 (m, 2H, $-\text{CO}-\text{CH}_2-\text{CH}_2-$), 2.50 (t, $J = 7.4$ Hz, 2H, $-\text{CO}-\text{CH}_2-\text{CH}_2-$), 4.83 (s, 2H, $\text{COO}-\text{CH}_2-\text{CO}-$), 5.68 (s, br, 2H, $-\text{NH}_2$), 6.66 (m, 2H, H-5 and H-3), 7.29 (m, 1H, H-4), 7.93 (dd, $J = 8.5$ Hz, $J = 1.7$ Hz, 1H, H-6). $^{13}\text{C-NMR}$ (CDCl_3 100.61 MHz) δ (ppm): 14.1 ($-\text{CH}_3$), 22.7, 23.4, 29.1, 29.2, 31.7 ($-(\text{CH}_2)_5-\text{CH}_3$), 39.0 ($-\text{CO}-\text{CH}_2-$), 68.1 ($-\text{COO}-\text{CH}_2-\text{CO}-$), 110.0 (C-1), 116.7 (C-5 and C-3), 131.5 (C-6), 134.7 (C-4), 150.9 (C-2), 167.3 ($-\text{COO}-$), 204.9 ($-\text{CO}-$). ESI-HRMS: $m/z = 278.1752$ [$\text{M}+\text{H}$] $^+$, calc. for $\text{C}_{16}\text{H}_{23}\text{NO}_3 + \text{H}^+ = 278.1751$; $m/z = 300.1574$ [$\text{M}+\text{Na}$] $^+$, calc. for $\text{C}_{16}\text{H}_{23}\text{NO}_3 + \text{Na}^+ = 300.1570$.

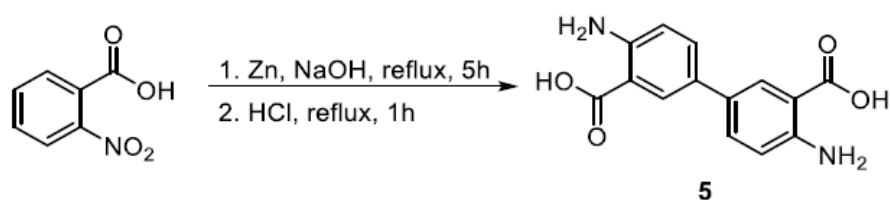
Synthesis of 2-heptyl-3-hydroxy-4-quinolone (**1**)



Compound **4** (1.91 g, 6.9 mmol) was dissolved in NMP (19.2 mL) and the solution was refluxed for 2 h. After reaching room temperature, the solution was poured in ice-water and the precipitated collected by filtration and dried.

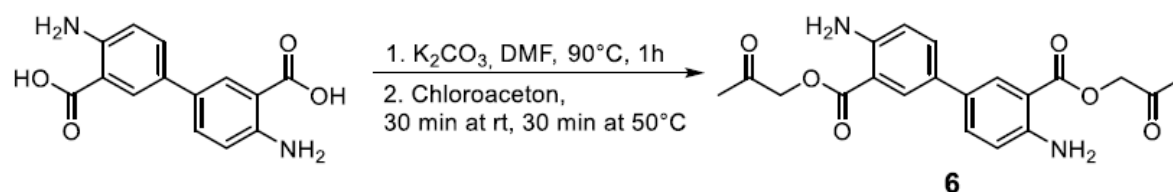
2-heptyl-3-hydroxyquinolin-4(1H)-one (**1**) was obtained as white solid (1.50 g, 84 %): $^1\text{H-NMR}$ ($\text{DMSO}-d_6$ 400.13 MHz) δ (ppm): 0.84 (t, $J = 6.9$ Hz, 3H, $-\text{CH}_3$), 1.20 – 1.37 (m, 8H, $-(\text{CH}_2)_4-\text{CH}_3$), 1.67 (m, 2H, $-\text{CH}_2-(\text{CH}_2)_4-\text{CH}_3$), 2.72 (t, $J = 7.9$ Hz, 2H, $-\text{CH}_2-(\text{CH}_2)_5-\text{CH}_3$), 7.21 (ddd, $J = 2.6$ Hz, 5.4 Hz, 8.1 Hz, 1H, H-6), 7.52 (m, 2H, H-7 and H-8), 8.09 (d, $J = 8.1$ Hz, 1H, H-5), 11.40 (s, 1H, NH). $^{13}\text{C-NMR}$ ($\text{DMSO}-d_6$ 100.61 MHz) δ (ppm): 13.9 ($-\text{CH}_3$), 22.0, 28.4, 28.7, 31.2 ($-(\text{CH}_2)_4-\text{CH}_3$), 27.8 ($-\text{CH}_2-(\text{CH}_2)_4-\text{CH}_3$), 28.1 ($-\text{CH}_2-(\text{CH}_2)_5-\text{CH}_3$), 117.7 (C-8), 121.5 (C-6), 122.1 (C-4a), 124.5 (C-5), 129.9 (C-7), 135.5 (C-2), 137.4 (C-8a), 137.8 (C-3), 168.9 (C-4). ESI-HRMS: $m/z = 260.1651$ [$\text{M}+\text{H}$] $^+$, calc. for $\text{C}_{16}\text{H}_{21}\text{NO}_2 + \text{H}^+ = 260.1645$; $m/z = 282.1470$ [$\text{M}+\text{Na}$] $^+$, calc. for $\text{C}_{16}\text{H}_{21}\text{NO}_2 + \text{Na}^+ = 282.1465$.

Synthesis of 4,4'-diamino-[1,1'-biphenyl]-3,3'-dicarboxylic acid (5)



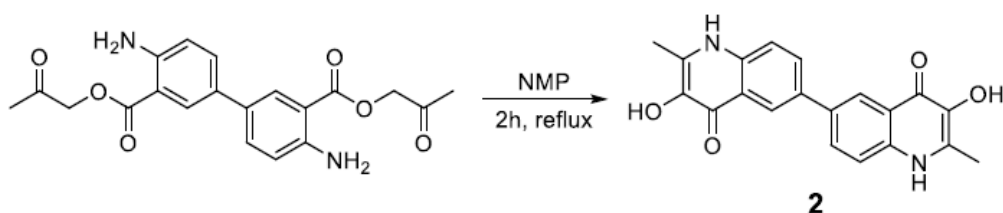
o-Nitrobenzoic acid (6.685 g, 0.04 mol) was dissolved in 200 mL of 12.5 M aq. NaOH to result in a 0.2 M solution. To the solution was added 50 g Zn (0.765 mol) and the mixture heated to 100°C and kept at this temperature for 5 h. After the solution reached room temperature, 100 mL conc. HCl was slowly added and the mixture heated to 100°C for 1 h. After reaching room temperature, the precipitate was isolated by filtration under vacuum and washed with water. The compound was obtained as a green-yellow solid (3.87 g, 71 %). ¹H-NMR (DMSO-*d*₆ 400.1 MHz) δ (ppm): 6.80 (d, 2H, *J* = 8.7 Hz, H-5 and H-5'), 7.57 (dd, 2H, *J* = 8.7 Hz, *J* = 2.3 Hz, H-6 and H-6'), 7.84 (d, 2H, *J* = 2.3 Hz, H-2 and H-2'). ¹³C-NMR (DMSO-*d*₆ 100.6 MHz) δ (ppm): 109.9 (2C, C-3 and C-3'), 117.9 (2C, C-5 and C-5'), 126.6 (2C, C-1 and C-1'), 127.5 (2C, C-2 and C-2'), 131.4 (2C, C-6 and C-6'), 150.1 (2C, C-4 and C-4'), 169.6 (2C, 2xCOOH).

Synthesis of bis(2-oxopropyl) 4,4'-diamino-[1,1'-biphenyl]-3,3'-dicarboxylate (6)



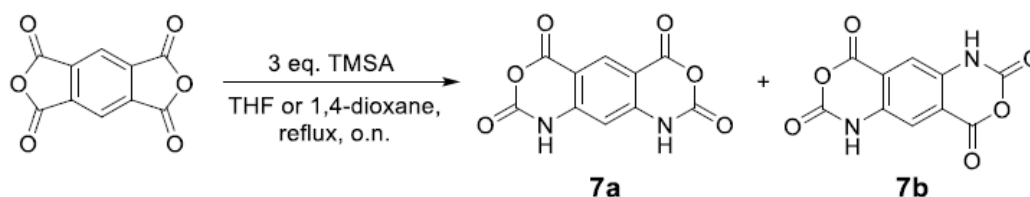
Compound 5 (1.718 g, 6.31 mmol) was dissolved in 25 mL DMF and K₂CO₃ (9.465 mmol, 1.5 eq.) was added. The mixture was heated at 90°C for 1 h. After reaching room temperature, chloroacetone (1.75 mL, 3 eq.) was added. The mixture was stirred for 30 min at room temperature and 30 min at 50°C. At room temperature, the mixture was poured in ice-water. The mixture was extracted with ethyl acetate, the combined organic phases washed with brine, filtered and the solvent evaporated leaving a brown solid. The solid was suspended in a small volume of ethyl acetate and collected by filtration. The solid was washed with small volumes of ethyl acetate and dried leaving the clean product as a brown solid (1.25 g, 51.5%). ¹H-NMR (DMSO-*d*₆ 400.1 MHz) δ (ppm): 2.16 (s, 6H, 2x -CH₃), 4.98 (s, 4H, 2x -CH₂-), 6.67 (s, br, 4H, 2x -NH₂), 6.87 (d, 2H, *J* = 8.6 Hz, H-5 and H-5'), 7.52 (dd, 2H, *J* = 8.6 Hz, *J* = 2.3 Hz, H-6 and H-6'), 7.90 (d, 2H, *J* = 2.3 Hz, H-2 and H-2'). ¹³C-NMR (DMSO-*d*₆ 100.6 MHz) δ (ppm): 25.9 (2C, 2x -CH₃), 68.3 (2C, 2x -CH₂-), 108.3 (2C, C-3 and C-3'), 117.4 (2C, C-5 and C-5'), 126.5 (2C, C-1 and C-1'), 126.9 (2C, C-2 and C-2'), 132.1 (2C, C-6 and C-6'), 150.2 (2C, C-4 and C-4'), 166.5 (2C, 2x COOH), 202.2 (2C, 2x -CO-). ESI-HRMS: *m/z* = 385.1396 [M+H]⁺, calc. for C₂₀H₂₀N₂O₆ + H⁺ = 385.1394; *m/z* = 407.1217 [M+Na]⁺, calc. for C₂₀H₂₀N₂O₆ + Na⁺ = 407.1214.

Synthesis of 3,3'-dihydroxy-2,2'-dimethyl-[6,6'-biquinoline]-4,4'-(1*H*,1'*H*)-dione (**2**)



Compound **6** (1.0 g, 2.6 mmol) was dissolved in 10 mL NMP and the solution was refluxed for 2 h. After reaching room temperature, the solution was poured in ice-water and the precipitated collected by filtration. The slightly impure product (780 mg) was purified by washing with THF (5x 15 mL). The pure product was obtained as a light brown solid (680 mg, 75%). ¹H-NMR (DMSO-*d*₆ 600.2 MHz) δ (ppm): 2.40 (s, 6H, 2x -CH₃), 7.63 (d, 2H, *J* = 8.7 Hz, H-8 and H-8'), 7.96 (dd, 2H, *J* = 8.7 Hz, *J* = 2.1 Hz, H-7 and H-7'), 8.16 (s, br, 2H, 2x -OH), 8.40 (d, 2H, *J* = 2.1 Hz, H-5 and H-5'), 11.62 (s, br, 2x -NH). ¹³C-NMR (DMSO-*d*₆ 100.6 MHz) δ (ppm): 14.1 (2C, 2x -CH₃), 118.6 (2C, C-8 and C-8'), 121.5 (2C, C-5 and C-5'), 122.6 (2C, C-4a and C-4a'), 128.6 (2C, C-7 and C-7'), 131.7 (2C, C-2 and C-2'), 132.8 (2C, C-6 and C-6'), 136.5 (2C, C-8a and C-8a'), 138.3 (C-3 and C-3'), 168.7 (C-4 and C-4'). ESI-HRMS: *m/z* = 349.1184 [M+H]⁺, calc. for C₂₀H₁₆N₂O₄ + H⁺ = 349.1183; *m/z* = 371.1007 [M+Na]⁺, calc. for C₂₀H₁₆N₂O₄ + Na⁺ = 371.1002; *m/z* = 719.2117 [2M+Na]⁺, calc. for C₄₀H₃₂N₄O₈ + Na⁺ = 719.2112.

Synthesis of 1,9-dihydro-2*H*,4*H*-benzo[1,2-*d*:5,4-*d'*]bis([1,3]oxazine)-2,4,6,8-tetraone (**7a**) and 1,6-dihydrobenzo[1,2-*d*:4,5-*d'*]bis([1,3]oxazine)-2,4,7,9-tetraone (**7b**)



Pyromellitic dianhydride (1 eq.) was suspended in THF or 1,2-dioxane (0.8 M) and TMSA (3eq.) was added dropwise. The colourless mixture was refluxed overnight and turned yellow/orange. The mixture was allowed to cool to room temperature and the precipitated collected by centrifugation. The precipitate was washed with THF or 1,4-dioxane and dried under vacuum.

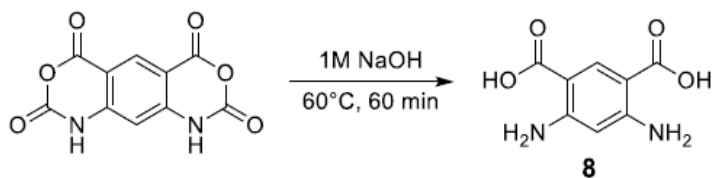
The reaction in THF resulted in a yellow solution with light-yellow precipitate containing a mixture of isomers **7a**:**7b** in a ratio of 1:0.85 (23 %) that could not be separated.

The reaction in 1,4-dioxane gave an orange reaction solution with white precipitate. After extensive washing of the precipitate with 1,4-dioxane, pure **7a** was obtained as white solid (21%). The supernatants were combined, the solvent evaporated and the precipitate washed again extensively with 1,4-dioxane. After several repetitions of this method a small amount of pure **7b** could be isolated as yellow solid for characterization.

1,9-dihydro-2*H*,4*H*-benzo[1,2-*d*:5,4-*d'*]bis([1,3]oxazine)-2,4,6,8-tetraone (**7a**): ¹H-NMR (DMSO-*d*₆ 400.13 MHz) δ (ppm): 6.79 (s, 1H, H-10), 8.31 (s, 1H, H-5), 12.09 (s, br, 2H, 2x -NH). ¹³C-NMR (DMSO-*d*₆ 100.6 MHz) δ (ppm): 99.4 (C-10), 106.7 (2C, C-4a and C-5a), 131.9 (C-5), 146.7 (2C, C-9a and C-10a), 146.8 (2C, C-2 and C-8), 158.4 (2C, C-4 and C-6). ESI-HRMS: *m/z* = 249.0149 [M+H]⁺, calc. for C₁₀H₄N₂O₆ + H⁺ = 249.0142.

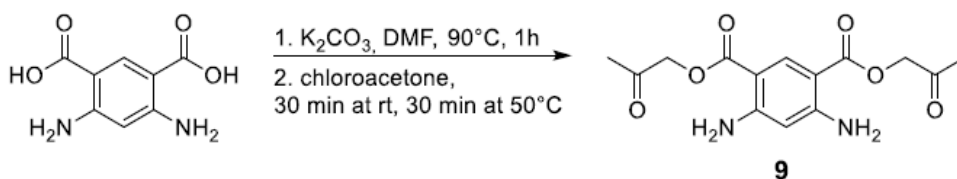
1,6-dihydrobenzo[1,2-d:4,5-d']bis([1,3]oxazine)-2,4,7,9-tetraone (**7b**): $^1\text{H-NMR}$ (DMSO- d_6 400.13 MHz) δ (ppm): 7.60 (s, 2H, H-5 and H-10), 11.83 (s, 2H, 2x -NH). $^{13}\text{C-NMR}$ (DMSO- d_6 100.6 MHz) δ (ppm): 114.7 (2C, C-5 and C-10), 117.3 (2C, C-5a and C-10a), 136.0 (2C, C-4a and C-9a), 146.4 (2C, C-2 and C-7), 159.1 (2C, C-4 and C-9).

Synthesis of 4,6-diaminoisophthalic acid **8**



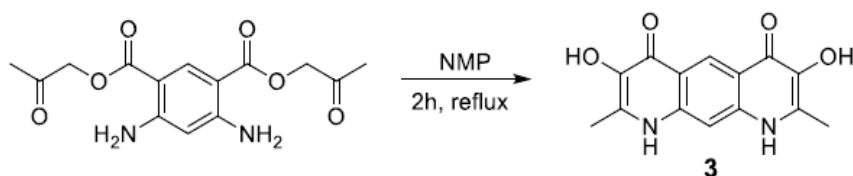
The compound **7a** was dissolved in 1 M NaOH (250 mM) while the reaction was warmed up to 60°C. The reaction was stirred at 60°C for 1 h and afterwards cooled with an ice-bath and brought to pH 4 with acetic acid. The precipitated was filtered, washed with water, and dried under vacuum. The product was obtained as a white solid (59 %). $^1\text{H-NMR}$ (DMSO- d_6 400.13 MHz) δ (ppm): 5.84 (s, 1H, H-5), 6.89 (s, 4H, 2x -NH $_2$), 8.29 (s, 1H, H-2). $^{13}\text{C-NMR}$ (DMSO- d_6 100.6 MHz) δ (ppm): 97.2 (C-5), 100.7 (2C, C-1 and C-3), 137.9 (C-2), 154.9 (2C, C-4 and C-6), 168.9 (2C, 2x -COOH). ESI-HRMS: m/z = 197.0558 [M+H] $^+$, calc. for $\text{C}_8\text{H}_8\text{N}_2\text{O}_4 + \text{H}^+$ = 197.0557; m/z = 219.0377 [M+Na] $^+$, calc. for $\text{C}_8\text{H}_8\text{N}_2\text{O}_4 + \text{Na}^+$ = 219.0376; m/z = 415.0860 [2M+Na] $^+$, calc. for $\text{C}_{16}\text{H}_{16}\text{N}_4\text{O}_8 + \text{Na}^+$ 415.0860.

Synthesis of bis(2-oxopropyl) 4,6-diaminoisophthalate (**9**)



4,6-Diaminoisophthalic acid (**8**) (0.358 g, 1.825 mmol) was dissolved in 8 mL DMF and 378 mg K_2CO_3 (2.73 mmol, 1.5 eq.) was added. The mixture was heated at 90°C for 1 h. After reaching room temperature, 440 μL chloroacetone (5.475 mmol, 3.0 eq.) was added. The mixture was stirred for 30 min at room temperature and 30 min at 50°C. At room temperature, the mixture was poured in water and extracted with ethyl acetate. The combined organic phases were washed with water, dried over Na_2SO_4 , filtered and the solvent evaporated. The residue was dissolved in small amounts of DCM and added on a silica gel column equilibrated with petrol ether/ethyl acetate 1:1. The residue was purified by column chromatography with petrol ether/ethyl acetate 1:1 and 1:2. The product was obtained as slightly yellow solid (226 mg, 40%). R_f = 0.23 (petrol ether/ethyl acetate 1:1). $^1\text{H-NMR}$ (DMSO- d_6 400.13 MHz) δ (ppm): 2.12 (s, 6H, 2x -CH $_3$), 4.89 (s, 4H, 2x -CH $_2$ -), 5.94 (s, 1H, H-5), 6.92 (s, 4H, 2x -NH $_2$), 8.47 (s, 1H, H-2). $^{13}\text{C-NMR}$ (DMSO- d_6 100.6 MHz) δ (ppm): 25.8 (2C, 2x -CH $_3$), 67.9 (2C, 2x -CH $_2$ -), 97.1 (C-5), 99.9 (2C, C-1 and C-3), 137.5 (C-2), 154.8 (2C, C-4 and C-6), 165.7 (2C, 2x -COO-), 202.4 (2C, 2x -CO-). ESI-HRMS: m/z = 331.0904 [M+Na] $^+$, calc. for $\text{C}_{14}\text{H}_{16}\text{N}_2\text{O}_6 + \text{Na}^+$ = 331.0901; m/z = 639.1905 [2M+Na] $^+$, calc. for $\text{C}_{28}\text{H}_{32}\text{N}_4\text{O}_{12} + \text{Na}^+$ 639.1909.

Synthesis of 3,7-dihydroxy-2,8-dimethylpyrido[3,2-g]quinoline-4,6(1H,9H)-dione (**3**)



Bis(2-oxopropyl) 4,6-diaminophthalate **9** (1.46 g, 4.736 mmol) was dissolved in 20 mL NMP and refluxed for 2 h. The reaction was cooled to room temperature and the precipitate collected by filtration, washed with water, and dried. The product was obtained as a yellowish-brown solid ($m = 789$ mg, 61.2%). $^1\text{H-NMR}$ (DMSO- d_6 400.13 MHz) δ (ppm): 2.37 (s, 6H, 2x -CH $_3$), 7.47 (s, 1H, H-10), 8.06 (s, br, 2H, 2x -OH), 8.95 (s, 1H, H-5), 11.33 (s, 2H, 2x -NH). $^{13}\text{C-NMR}$ (DMSO- d_6 100.6 MHz) δ (ppm): 14.3 (2C, 2x -CH $_3$), 101.3 (C-10), 118.9 (2C, C-4a and C-5a), 123.6 (C-5), 133.4 (2C, C-2 and C-8), 136.2 (2C, C-3 and C-7), 138.2 (2C, C-9a and C-10a), 170.0 (2C, C-4 and C-6). ESI-HRMS: $m/z = 295.0690$ $[\text{M}+\text{Na}]^+$, calc. for $\text{C}_{14}\text{H}_{12}\text{N}_2\text{O}_4 + \text{Na}^+ = 295.0689$; $m/z = 567.1482$ $[2\text{M}+\text{Na}]^+$, calc. for $\text{C}_{28}\text{H}_{24}\text{N}_4\text{O}_8 + \text{Na}^+ = 567.1486$.

Table S1. Conditions used for formation experiments and resulting PQS-metal ion complexes.

Salt	Final molarity of salt*, μM	Final molarity of PQS*, μM	Detection of metal-PQS complexes in MeOH	Detection of metal-PQS complexes in Acetone
CuCl ₂	30	60	-	-
ZnCl ₂	30	60	-	-
NiCl ₂	30	60	-	-
CoCl ₂	30	60	-	-
MnCl ₂	30	60	-	-
AlCl ₃	30	90	+	+
FeCl ₃	30	90	+	+
Na ₃ VO ₄	30	150	+	+
Na ₂ MoO ₄	30	180	+	+

* Stock solutions were prepared in DMSO (DMSO/H₂O (1:1) for Na₂MoO₄).

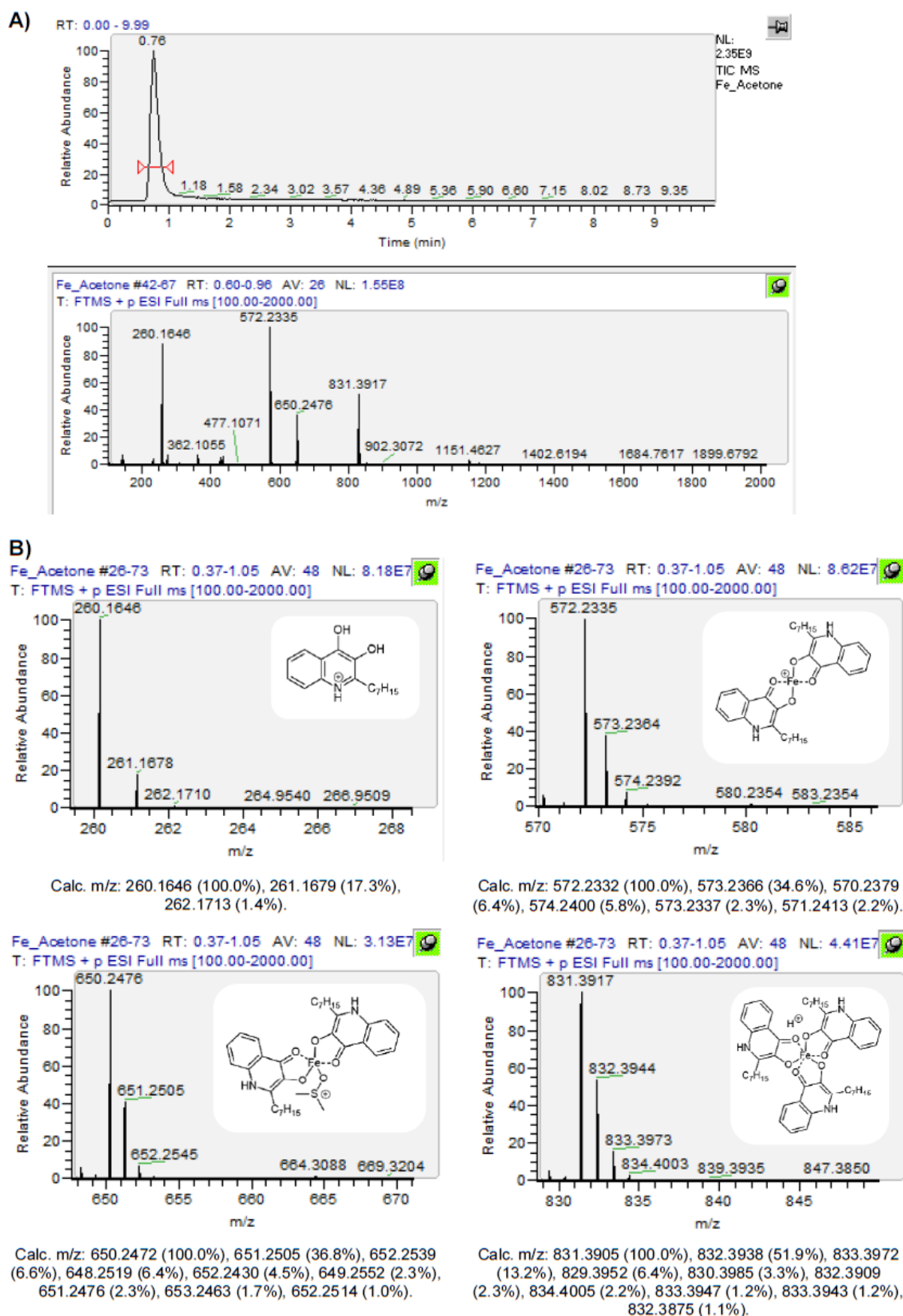


Figure S1. A) Full HRMS-spectrum of Fe(III)-PQS complexes in acetone. B) Detected and calculated m/z for PQS and complexes. Insets: proposed complexes structures.

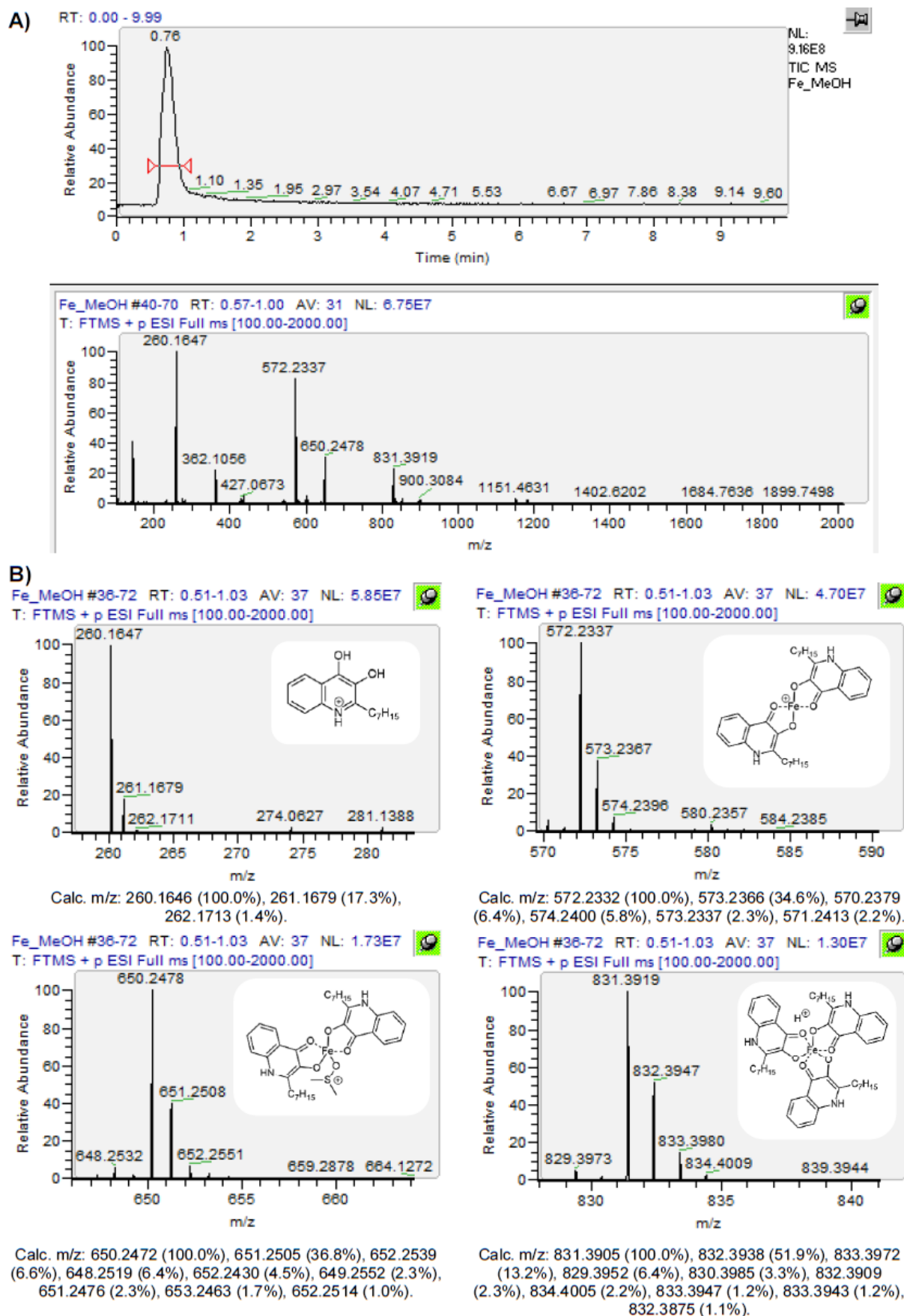
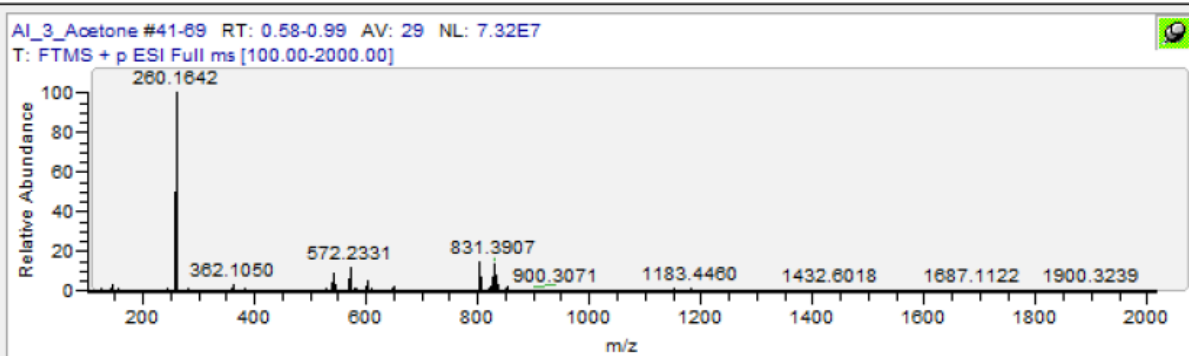
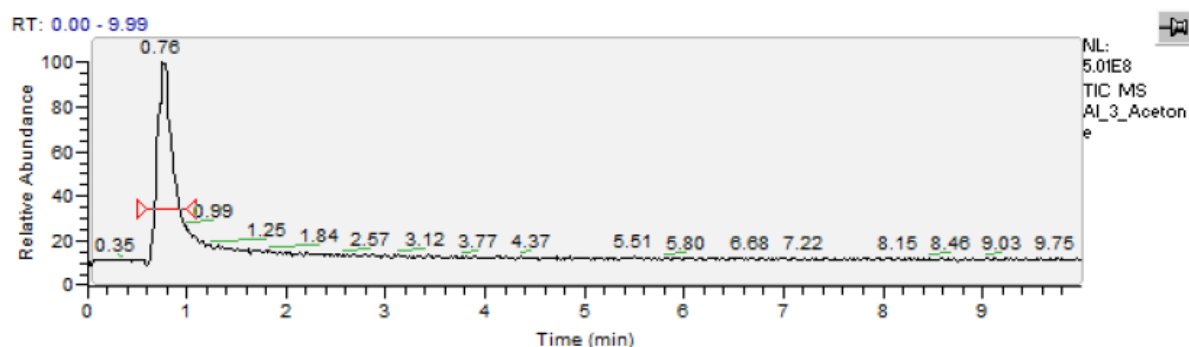


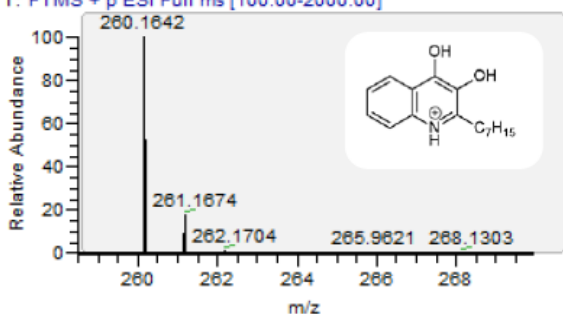
Figure S2. A) Full HRMS-spectrum of Fe(III)-PQS complexes in methanol. B) Detected and calculated m/z for PQS and complexes. Insets: proposed complexes structures.

A)



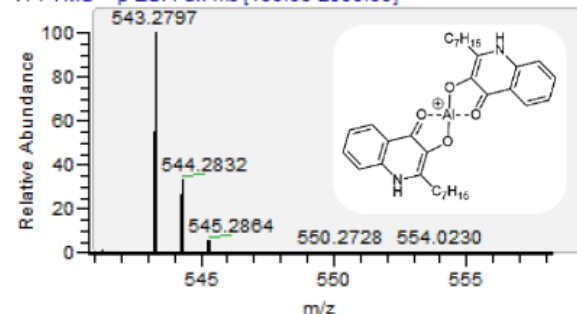
B)

Al_3_Aceton #38-72 RT: 0.54-1.03 AV: 35 NL: 6.42E7
T: FTMS + p ESI Full ms [100.00-2000.00]



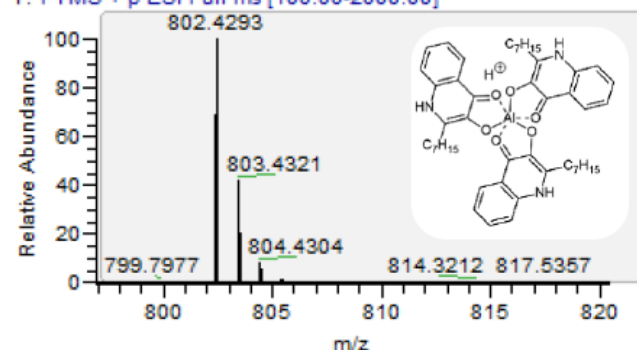
Calc. m/z: 260.1646 (100.0%), 261.1679 (17.3%),
262.1713 (1.4%).

Al_3_Aceton #38-72 RT: 0.54-1.03 AV: 35 NL: 5.54E6
T: FTMS + p ESI Full ms [100.00-2000.00]



Calc. m/z: 543.2798 (100.0%), 544.2832 (34.6%),
545.2866 (5.8%).

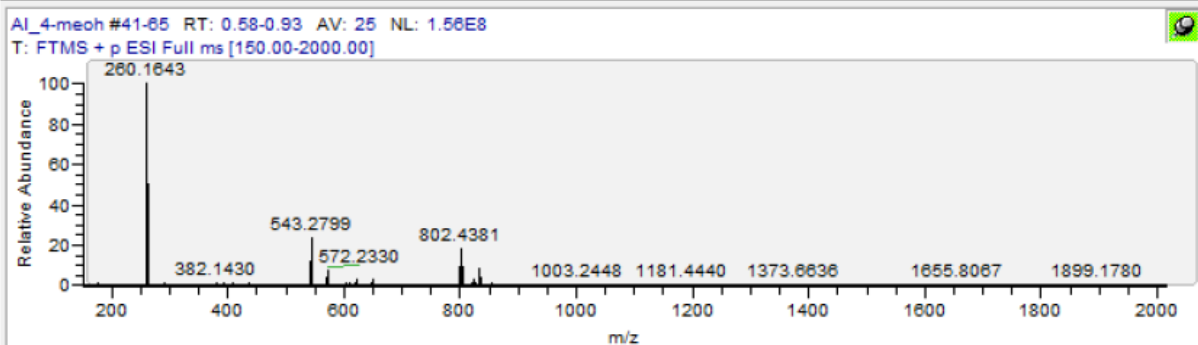
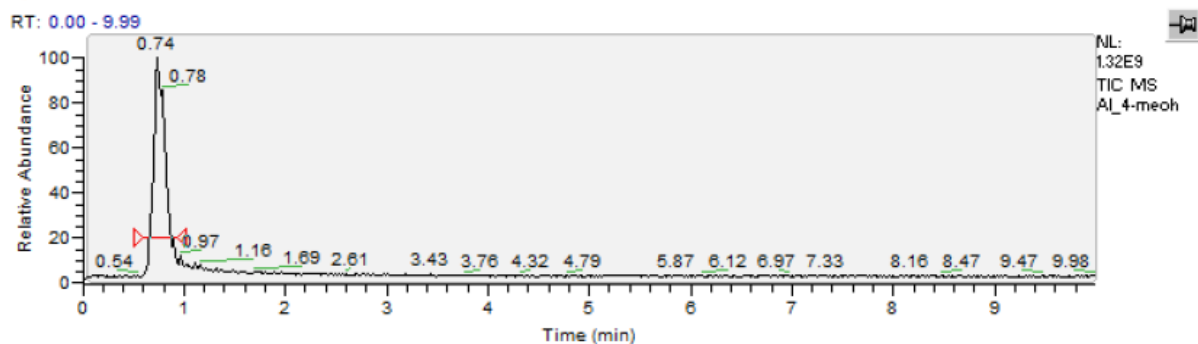
Al_3_Aceton #31-79 RT: 0.44-1.13 AV: 49 NL: 6.31E6
T: FTMS + p ESI Full ms [100.00-2000.00]



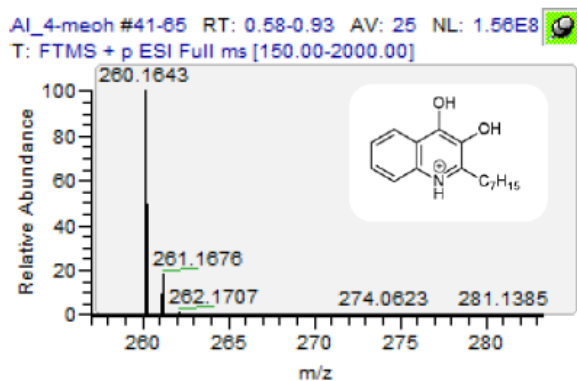
Calc. m/z: 802.4371 (100.0%), 803.4404 (51.9%), 804.4438 (13.2%),
805.4471 (2.2%), 804.4413 (1.2%), 803.4341 (1.1%).

Figure S3. A) Full HRMS-spectrum of Al-PQS complexes in acetone. B) Detected and calculated m/z for PQS and complexes. Insets: proposed complexes structures.

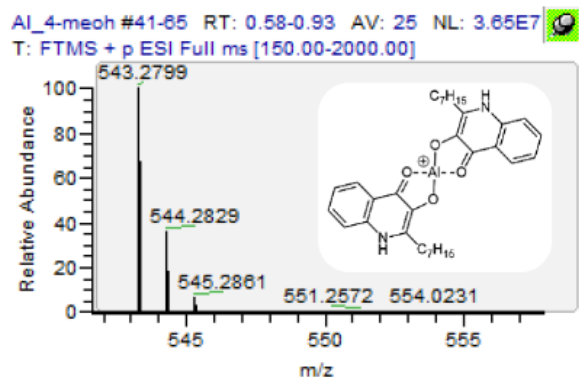
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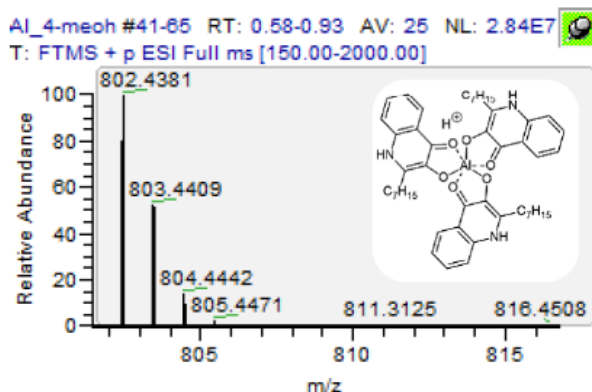
B)



Calc. m/z: 260.1646 (100.0%), 261.1679 (17.3%),
262.1713 (1.4%).



Calc. m/z: 543.2798 (100.0%), 544.2832 (34.6%),
545.2866 (5.8%).



Calc. m/z: 802.4371 (100.0%), 803.4404 (51.9%), 804.4438 (13.2%),
805.4471 (2.2%), 804.4413 (1.2%), 803.4341 (1.1%).

Figure S4. A) Full HRMS-spectrum of Al-PQS complexes in methanol. B) Detected and calculated m/z for PQS and complexes. Insets: proposed complexes structures.

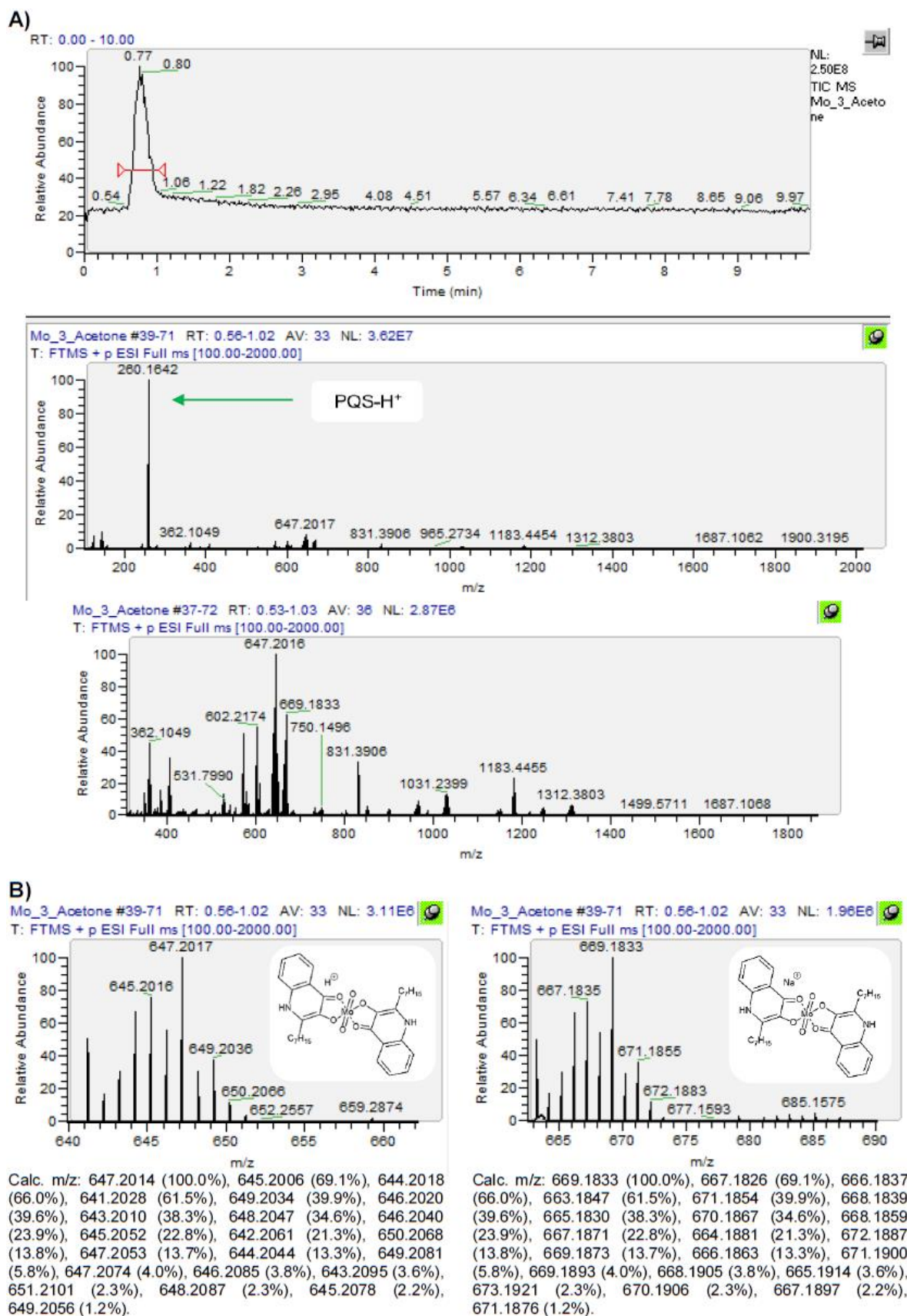


Figure S5. A) Full HRMS-spectrum of Mo(VI)-PQS complexes in acetone. **B)** Detected and calculated m/z for complexes and their proposed structures.

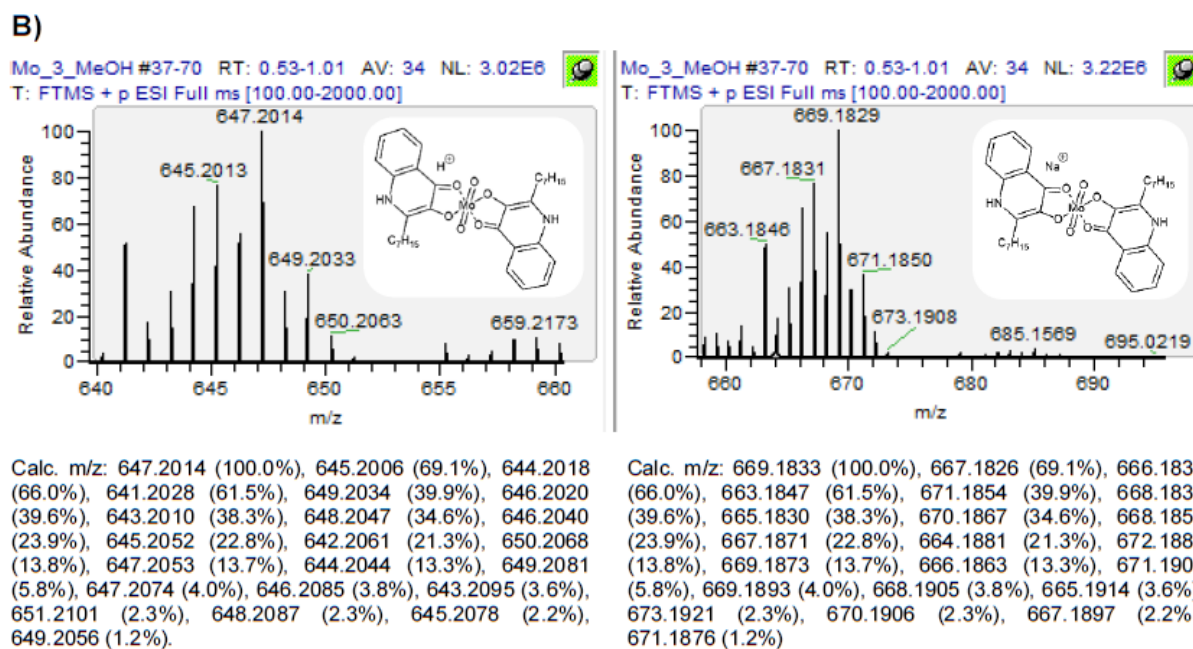
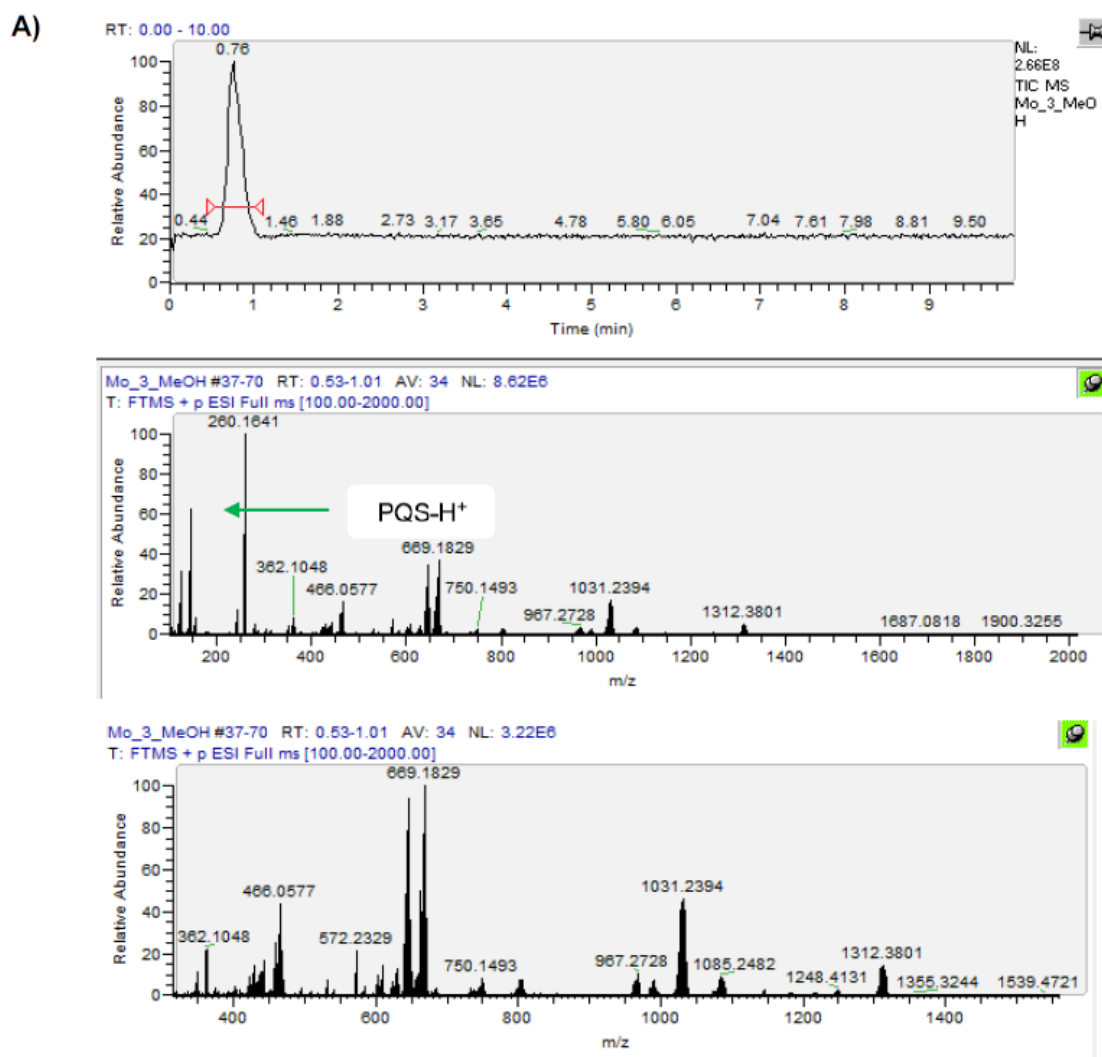
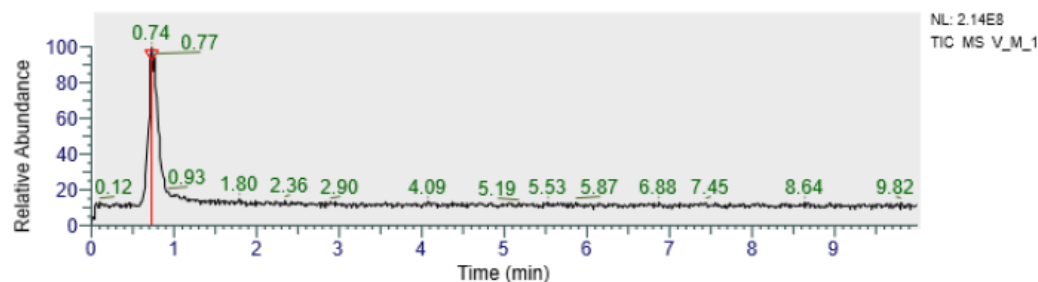


Figure S6. A) Full HRMS-spectrum of Mo (VI)-PQS complexes in methanol. **B)** Detected and calculated m/z for complexes and their proposed structures.

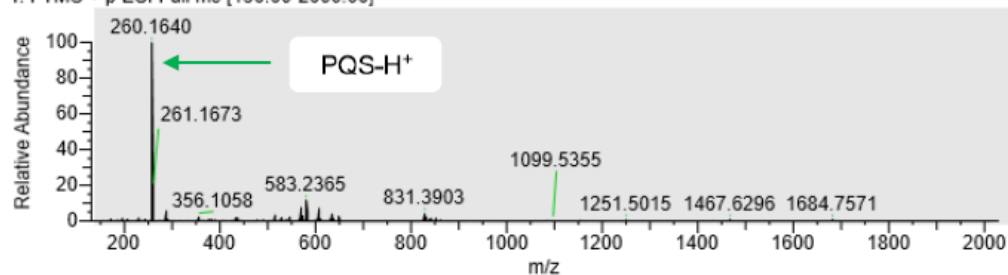
A)

RT: 0.00-9.99

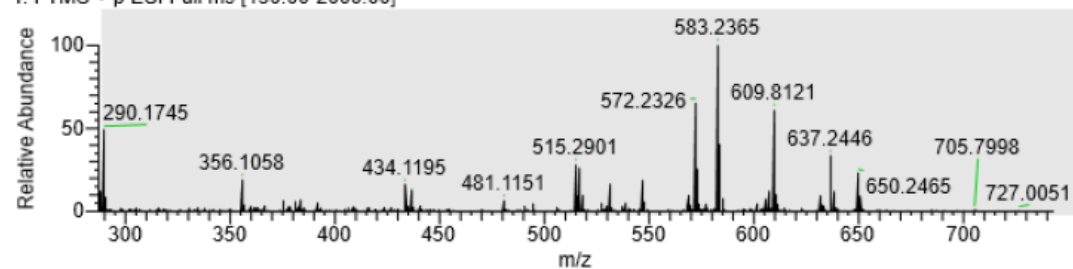


~Spectrum 1 51 - V_M_1 - C1T1

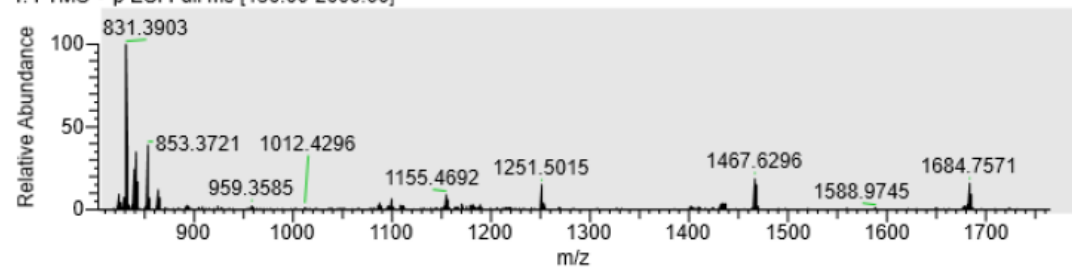
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T: FTMS + p ESI Full ms [150.00-2000.00]



V_M_1 #51 RT: 0.74 AV: 1 NL: 8.01E6
T: FTMS + p ESI Full ms [150.00-2000.00]

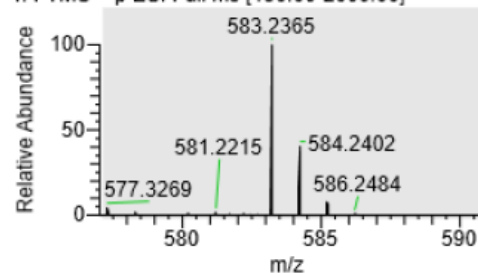


V_M_1 #51 RT: 0.74 AV: 1 NL: 2.85E6
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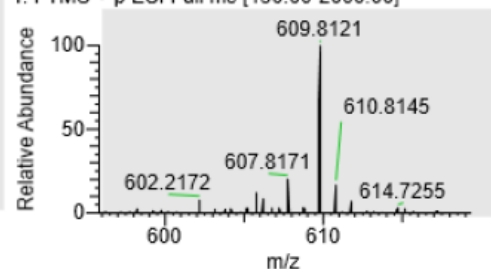


B)

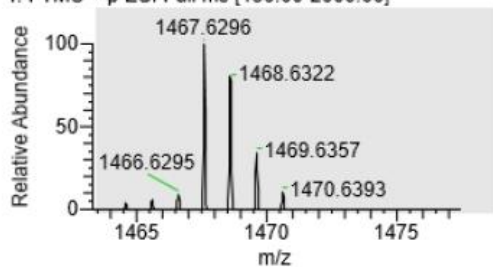
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V_M_1 #51 RT: 0.74 AV: 1 NL: 4.88E6
T: FTMS + p ESI Full ms [150.00-2000.00]



V_M_1 #51 RT: 0.74 AV: 1 NL: 5.31E5
T: FTMS + p ESI Full ms [150.00-2000.00]



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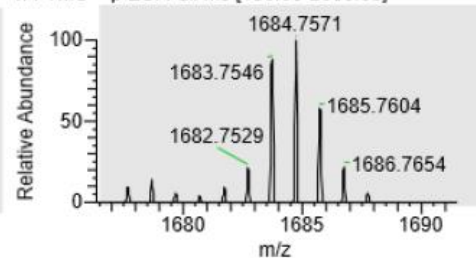


Figure S7. A) Full HRMS-spectrum of V (V)-PQS complexes in methanol. B) Detailed excerpts of unidentified V (V)-PQS complexes.

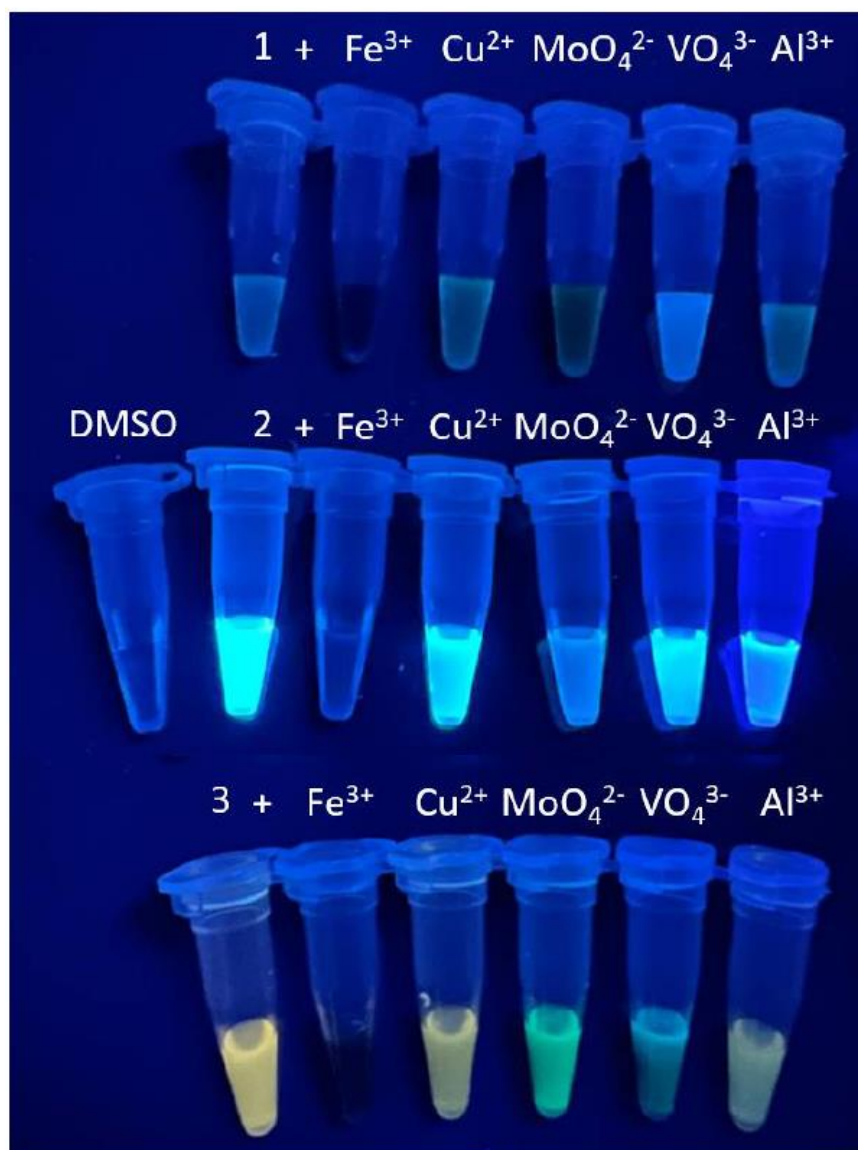
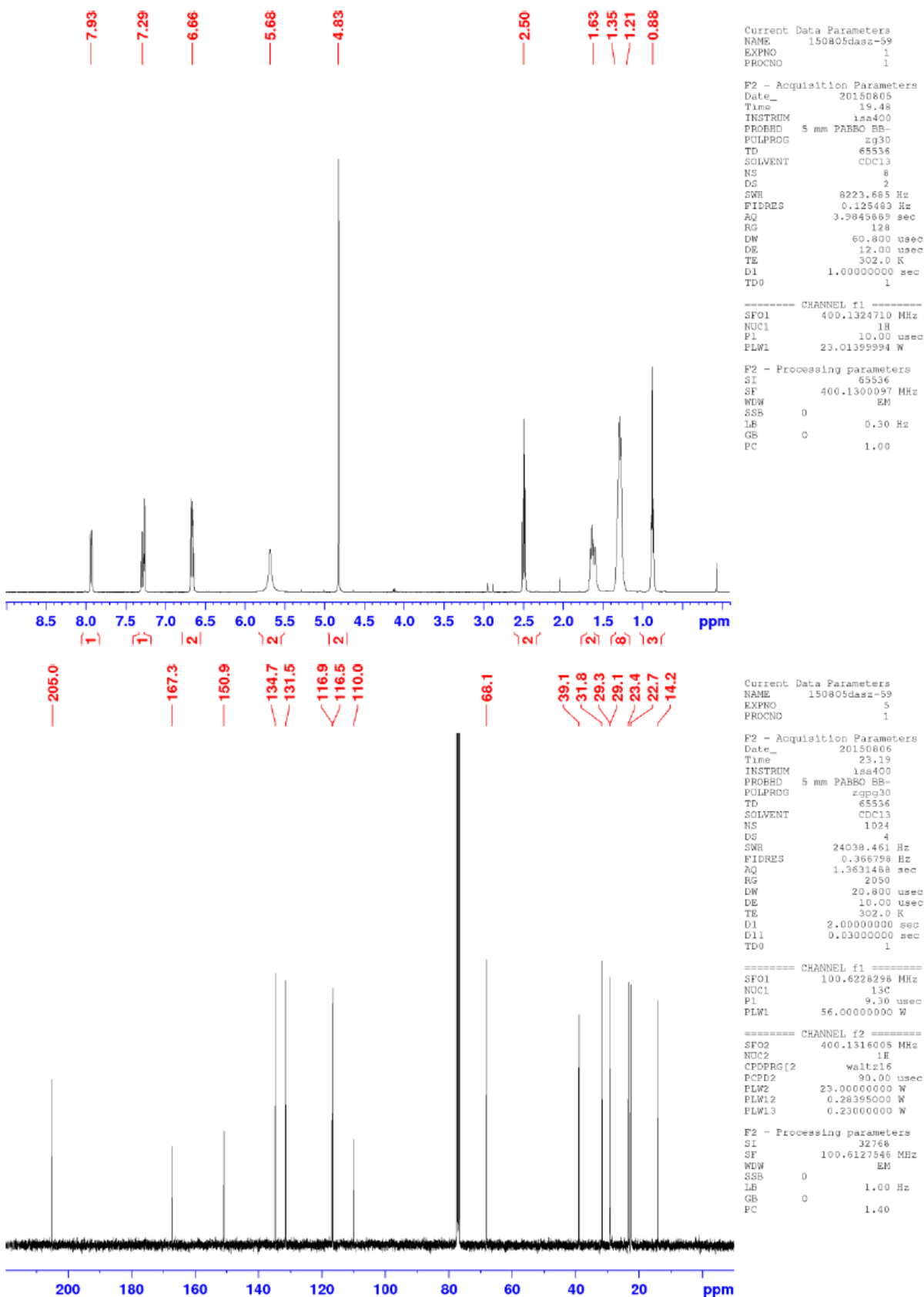


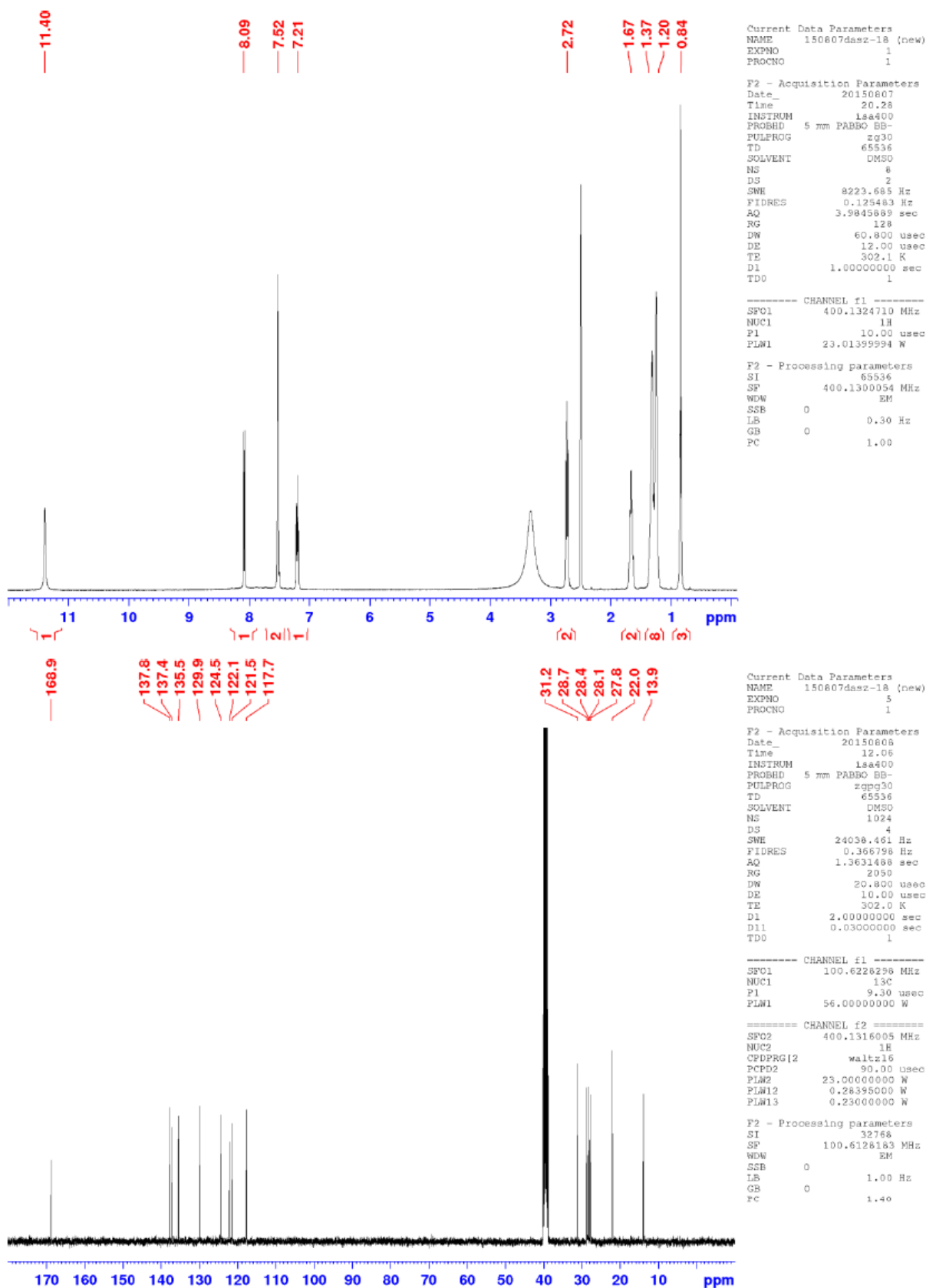
Figure S8. DMSO solutions of compound 1 (top), 2 (middle) and 3 (bottom) with metal ions under UV light (365 nm).

NMR spectra

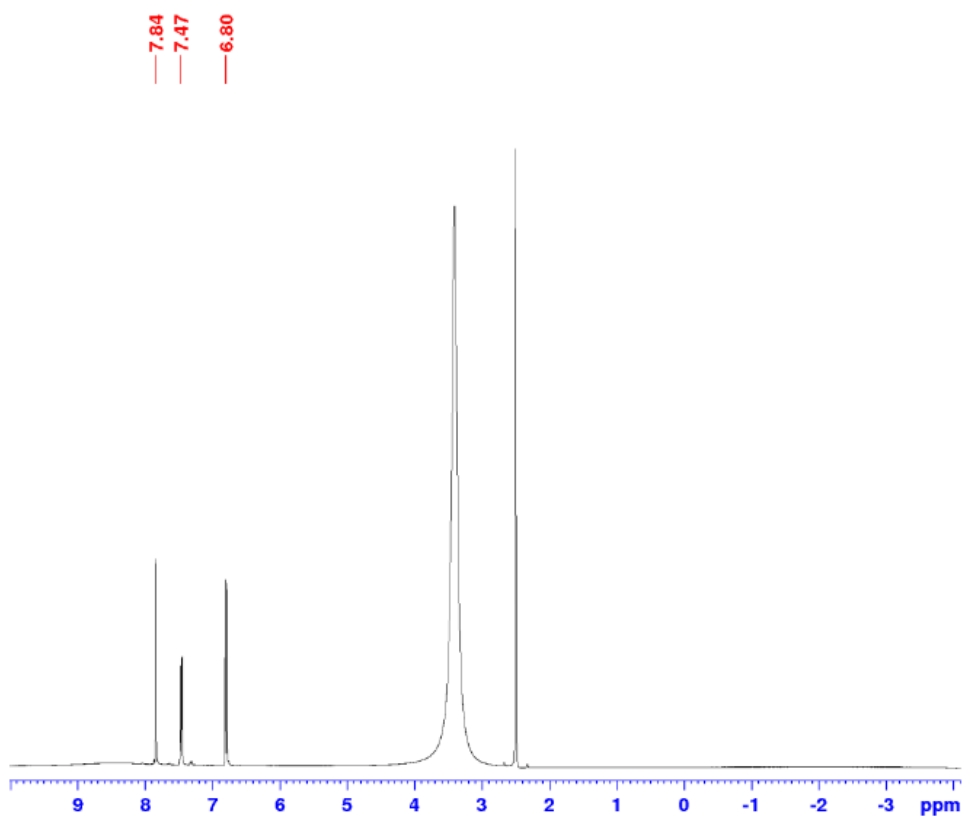
¹H- and ¹³C-NMR spectra of 4 in CDCl₃



^1H - and ^{13}C -NMR spectra of **1** in $\text{DMSO}-d_6$



^1H - and ^{13}C -NMR spectra of 5 in DMSO-d_6



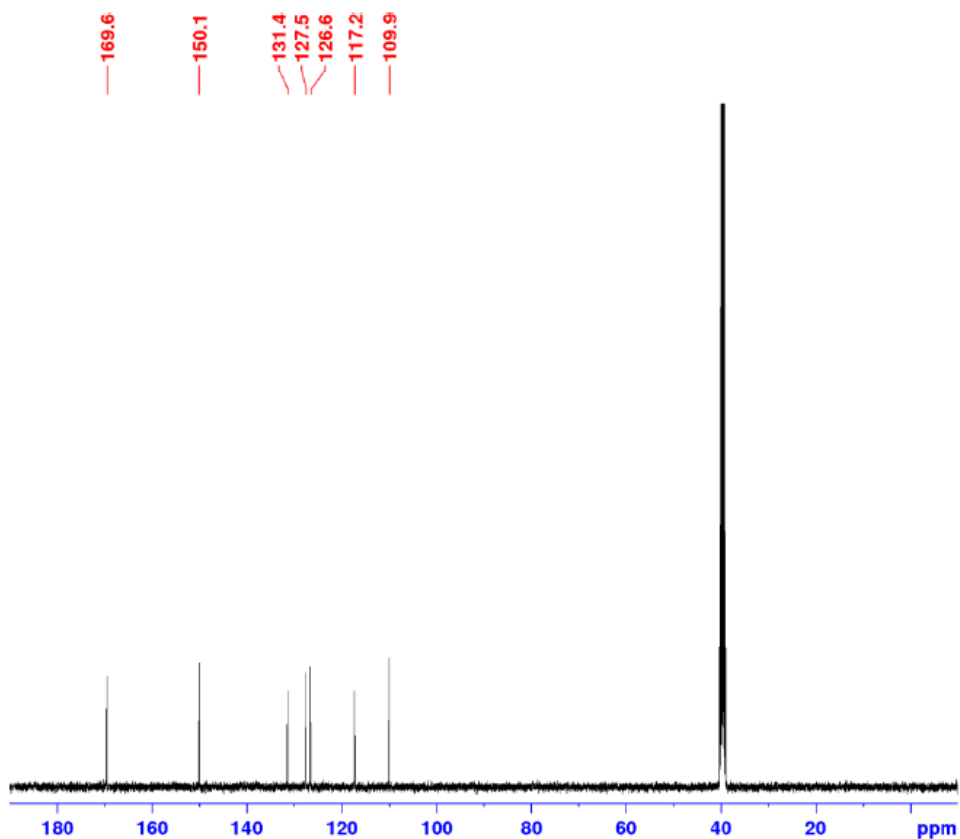
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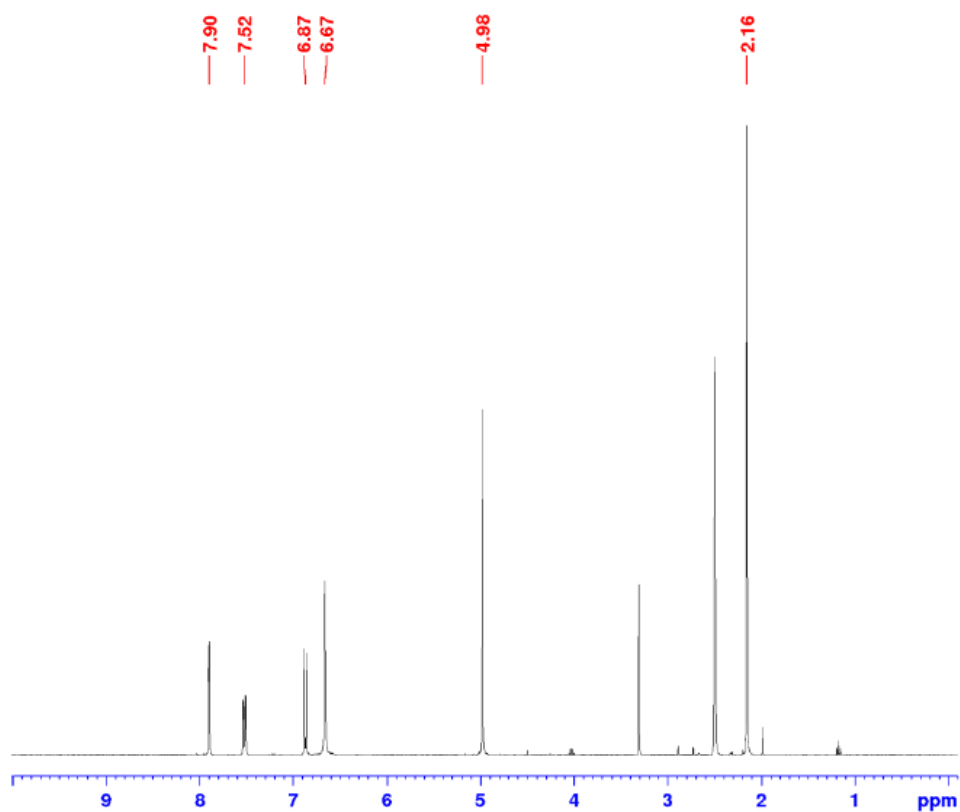
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¹H- and ¹³C-NMR spectra of 6 in DMSO-d₆

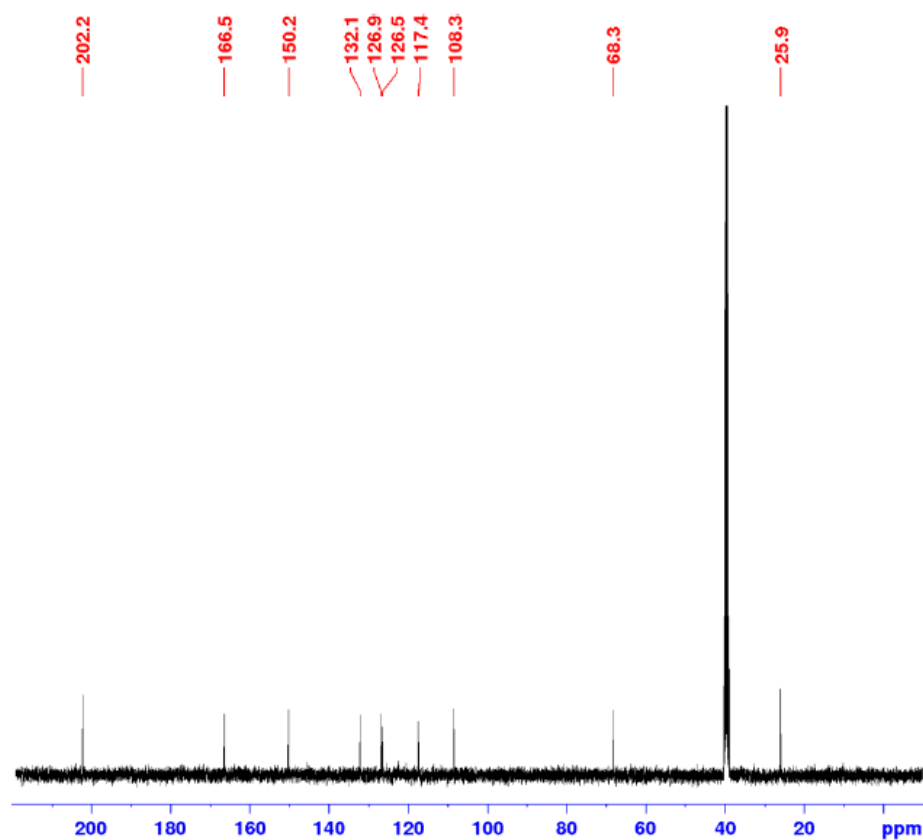


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TD0 1

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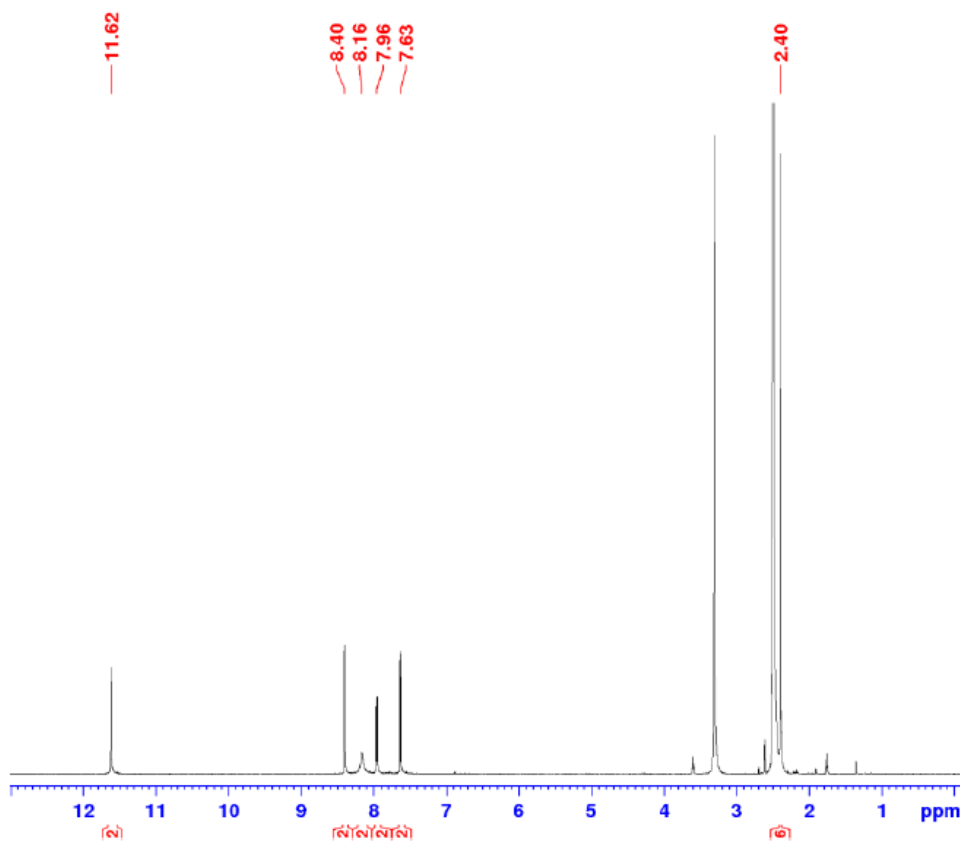
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TD0 1

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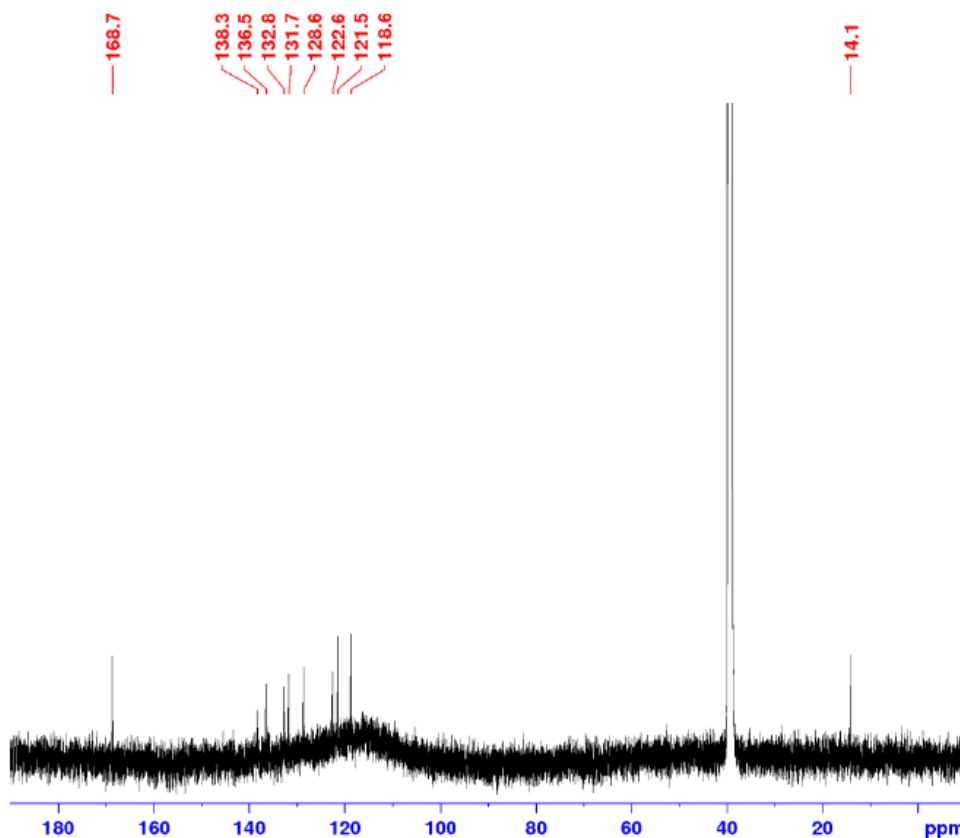
^1H - and ^{13}C -NMR spectra of **2** in DMSO-d_6



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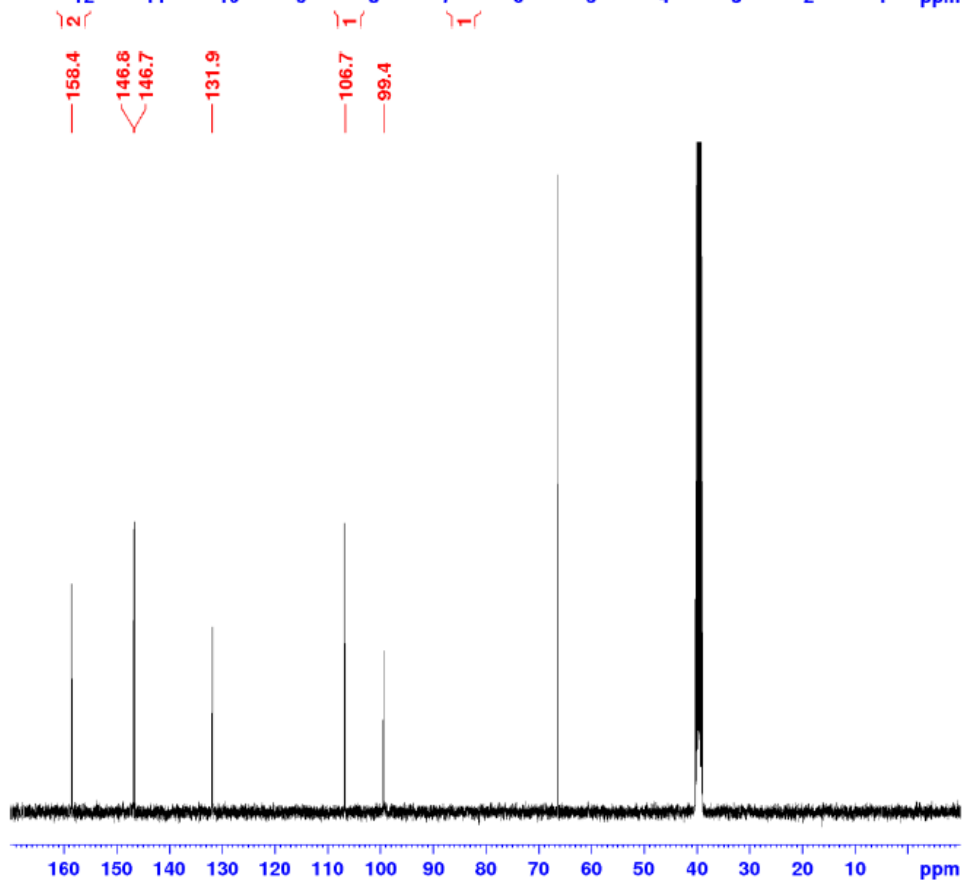
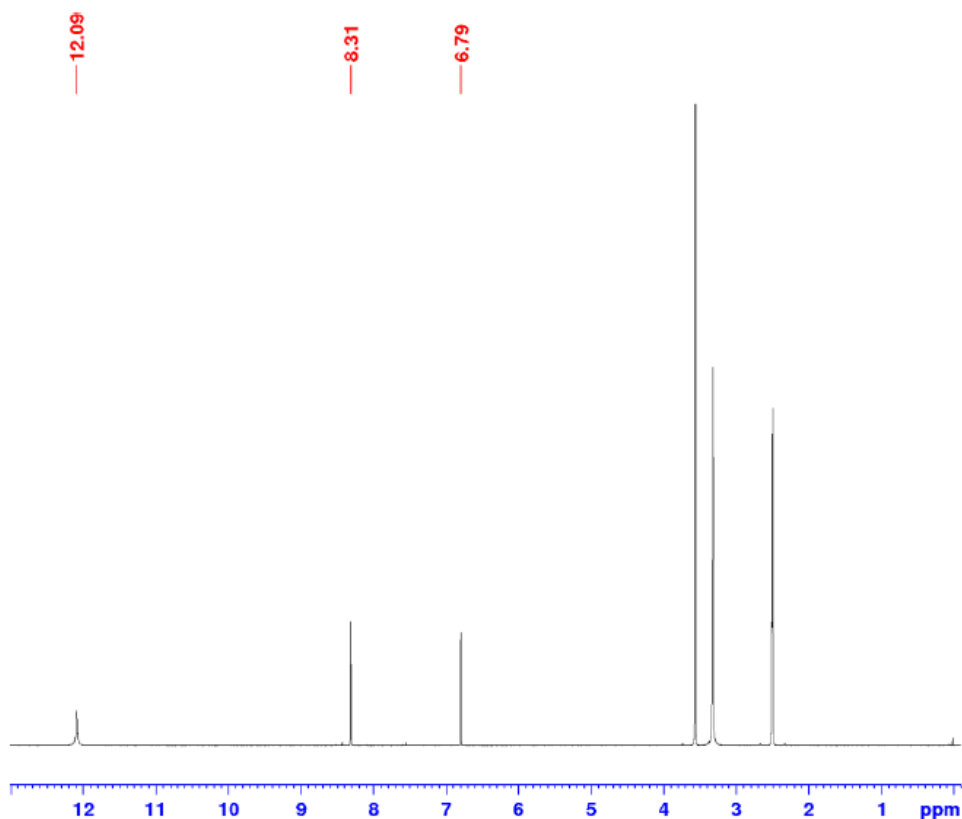


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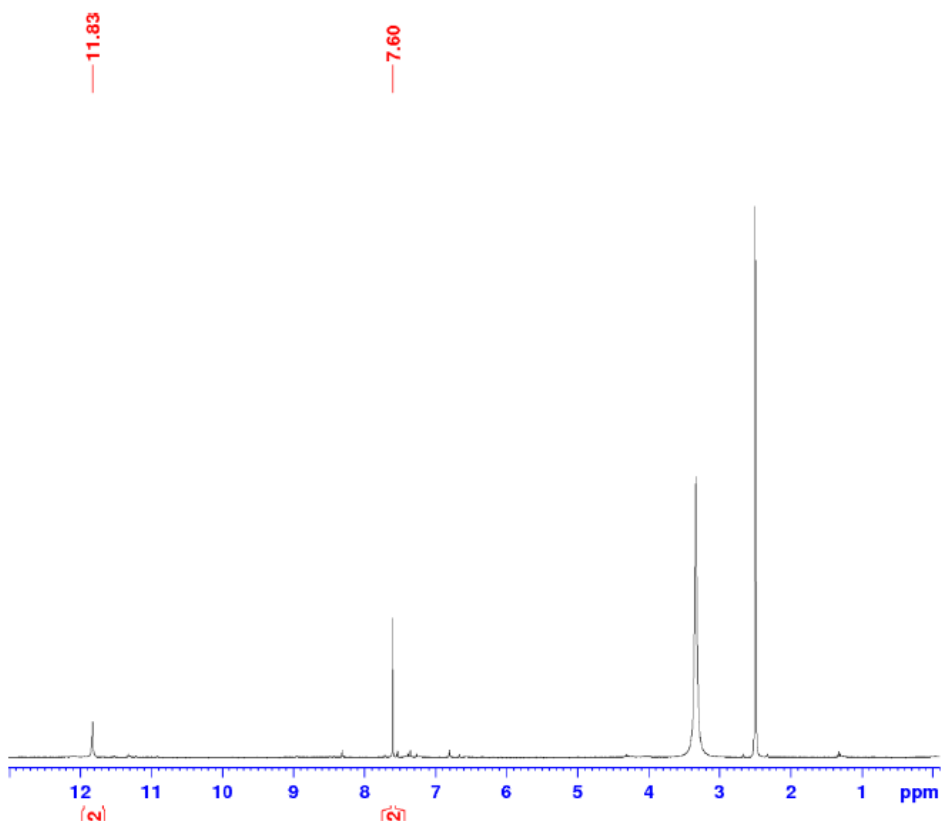
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¹H- and ¹³C-NMR spectra of 7a in DMSO-d₆



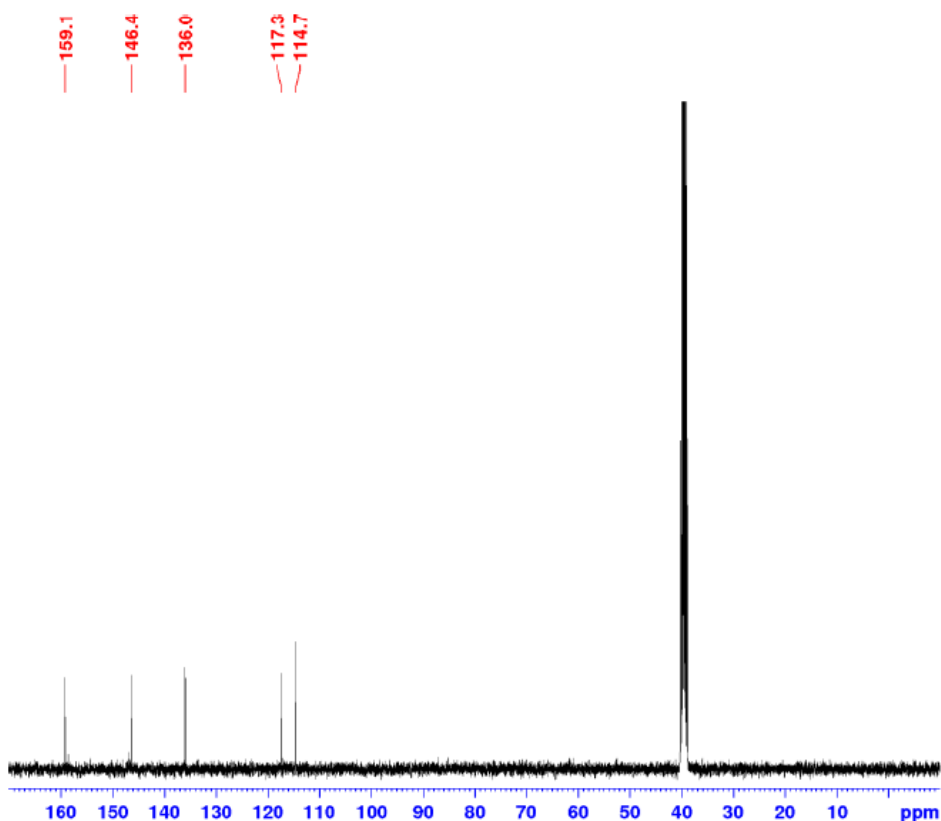
^1H - and ^{13}C -NMR spectra of 7b in DMSO- d_6



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PROCNO 1

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NUC1 1H
P1 12.90 usec
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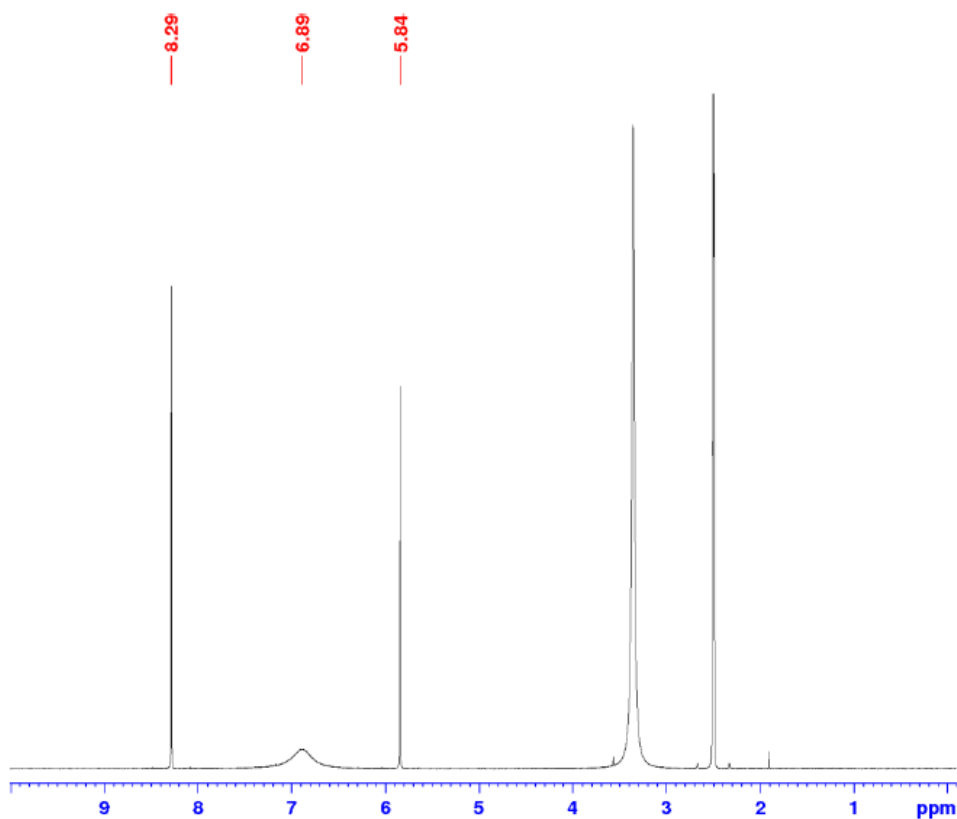


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DE 12.38 usec
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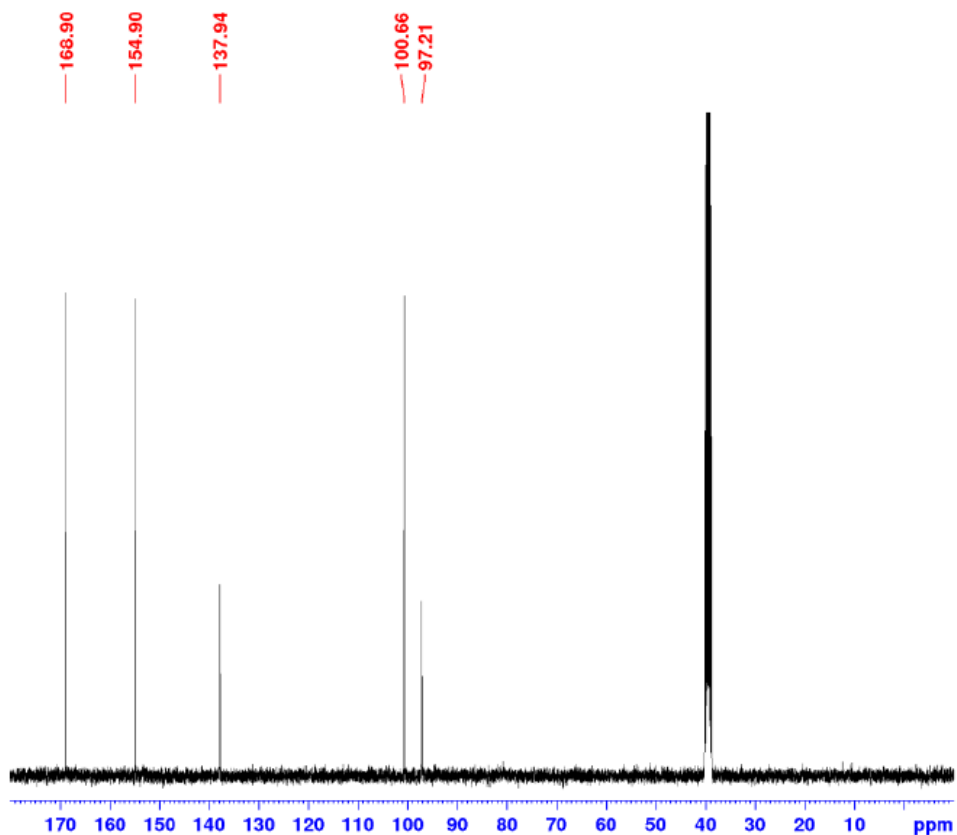
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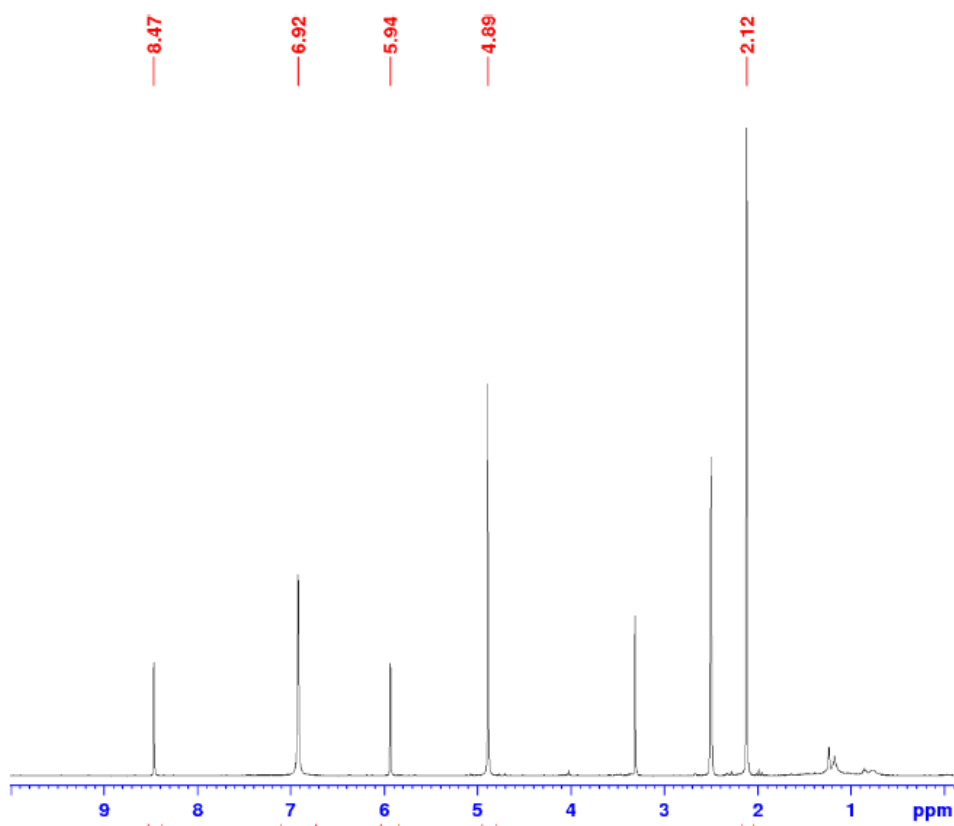


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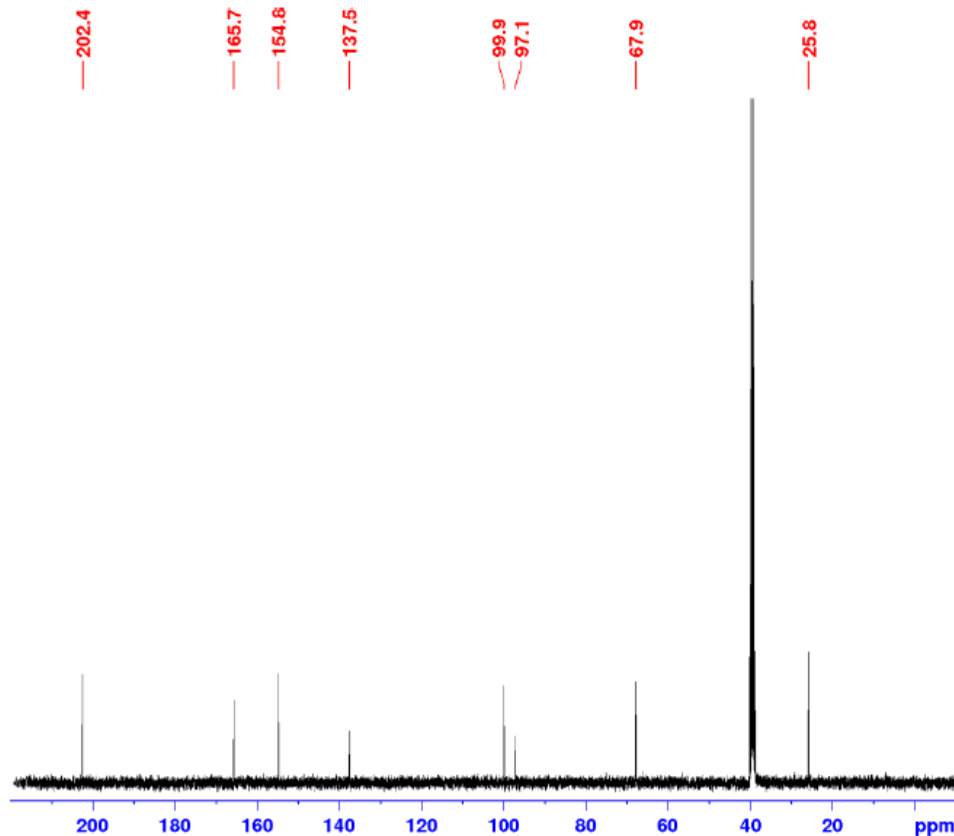


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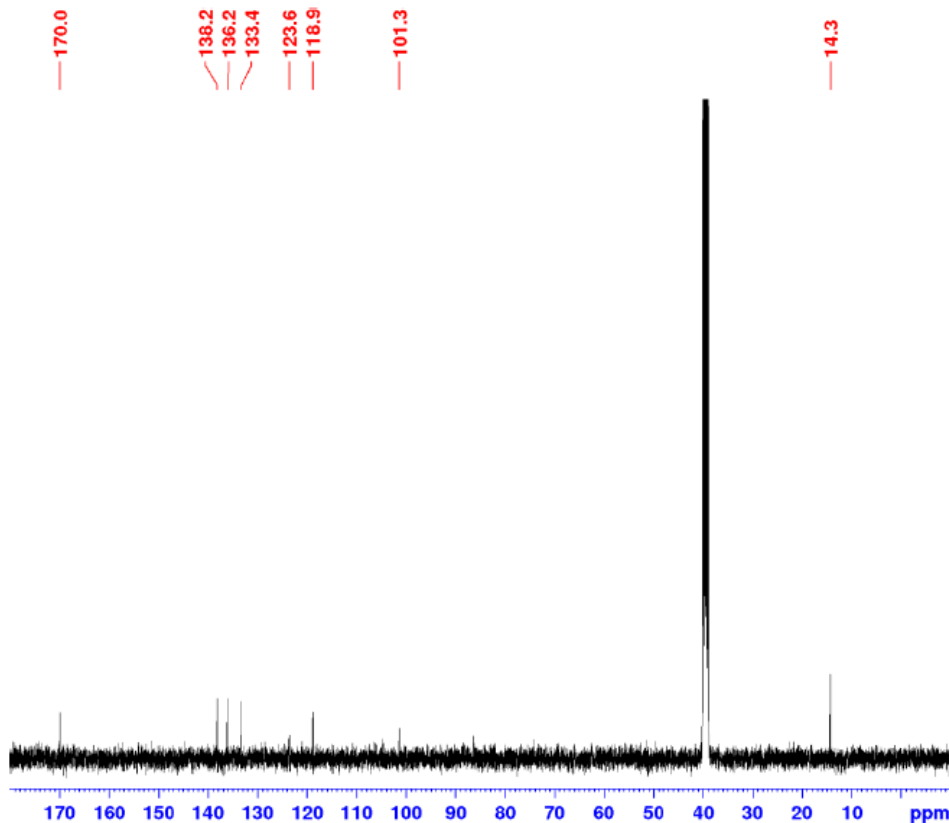
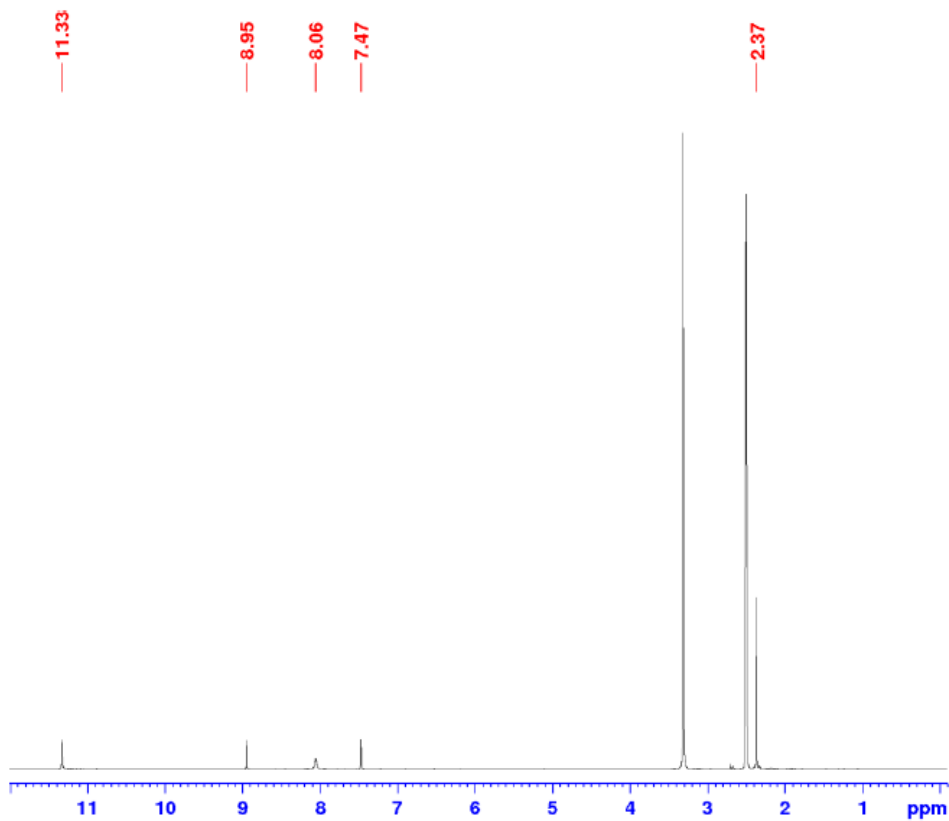
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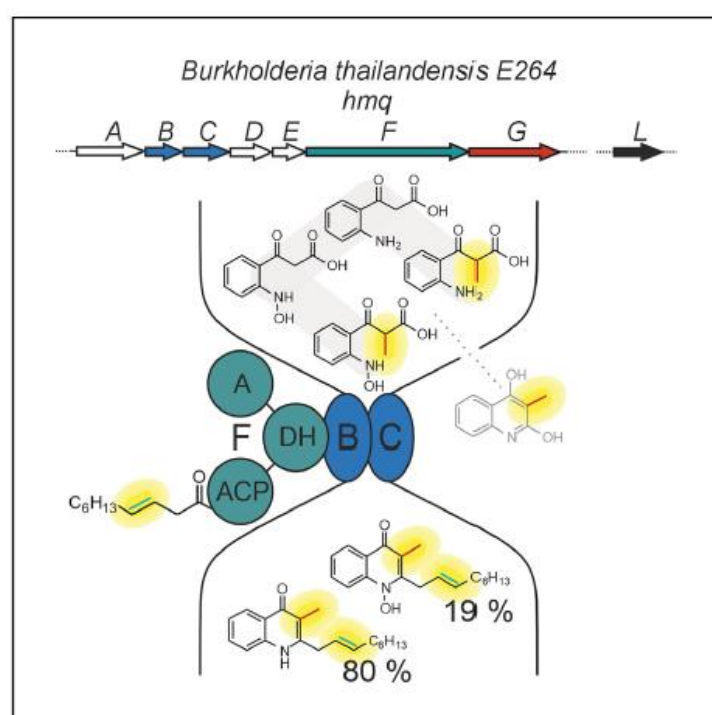
^1H - and ^{13}C -NMR spectra of 3 in DMSO- d_6



2.3 Publication 3. Manuscript

Article

Unraveling key steps in the biosynthesis of antimicrobial methylated unsaturated 2-alkyl-4-quinolones of *Burkholderia thailandensis*



Bacterial 2-alkyl-4-quinolones act as signals and antibiotics in various ecological niches. In contrast to other quinolone producers, *Burkholderia thailandensis* predominantly produces 3-methylated 2-nonenylquinolones. Savchenko and Jaegers et al. uncovered the exact biosynthetic sequence along with enzymatic specificities and metabolic fluxes that determine the product spectrum.

Viktoriia Savchenko, Miriam Jaegers, René Rasche, ..., Thomas Böttcher, Susanne Fetzner, Simon Ernst

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Highlights

Biochemical rationale for production of 3-methylated quinolones

Identification and synthesis of elusive pathway intermediates

Specific and direct interaction between biosynthetic enzymes

Enzyme specificities and bottlenecks tightly control metabolic flux

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Article

Unraveling key steps in the biosynthesis of antimicrobial methylated unsaturated 2-alkyl-4-quinolones of *Burkholderia thailandensis*

Viktoriia Savchenko,^{1,2,7} Miriam Jaegers,^{3,7} René Rasche,⁴ Eric Herrmann,⁴ Simone König,⁵ Daniel Kümmel,⁴ Thomas Böttcher,¹ Susanne Fetzner,³ and Simon Ernst^{3,6,8,*}

SUMMARY

The 2-alkyl-4(1H)-quinolone family of natural products comprises a diverse set of compounds acting as signals and antibiotics. The 2-alkyl-4(1H)-quinolone biosynthetic pathway of *Burkholderia thailandensis* exhibits a strong preference for the production of 3-methylated quinolones with *trans*- Δ^2 -unsaturated alkyl chains. Here, we complete the description of the pathway and decipher the biochemical rationale for this preference. Our data suggest that highly efficient methylation of the intermediate 2-aminobenzoylacetate to 2-(2'-aminobenzoyl)propionate (2-ABP), combined with substrate preference of the final condensing enzyme HmqBC for 2-ABP and a 3-alkenoyl donor, is the major factor determining the product pattern. Surprisingly, 2-ABP appears to largely decompose to 4-hydroxy-3-methyl-2(1H)-quinolone, indicating an enzymatic bottleneck created by HmqBC. While the diversity of quinolone products acting as a multitarget antibiotic cocktail may be advantageous, key enzymes of the pathway nevertheless have evolved toward promoting the production of congeners that are active especially toward gram-positive bacteria and fungi and, moreover, resist C3-targeted detoxification.

INTRODUCTION

Among the plethora of chemically diverse microbial natural products, compounds with a 2-alkyl-4(1H)-quinolone (AQ) scaffold play important roles in cell-cell signaling, interspecies competition, and bacteria-host interactions.^{1–3} They are primarily known as extracellular metabolites of the opportunistic pathogen *Pseudomonas aeruginosa*, acting as quorum-sensing signal molecules and/or antimicrobials. *P. aeruginosa* produces a series of AQs, 3-hydroxylated AQs, and AQ N-oxides (AQNOs) but does not form 3-methylated congeners (MAQs).⁴ Members of the genus *Burkholderia*, including environmental and pathogenic species, also synthesize a broad but distinct range of AQ-type compounds.^{4–6} In contrast to *P. aeruginosa*, *Burkholderia thailandensis* predominantly produces MAQs, mainly with a *trans*- Δ^2 unsaturation in the C₉ alkyl side chain, and their N-oxides.^{5,6}

AQ-type secondary metabolites can exhibit antibacterial, antifungal, and cytotoxic activities.^{5,7–15} Differences in the substitution pattern and in alkyl chain length and saturation strongly affect their antimicrobial activity. 2-Heptyl-4(1H)-quinolone (HHQ), for example, known as a *P. aeruginosa* quorum-sensing signal molecule, also exhibits species-specific bacteriostatic activity against members of the Vibrionaceae and anti-biofilm activity against *Bacillus subtilis* and *Candida albicans*.^{12,13}

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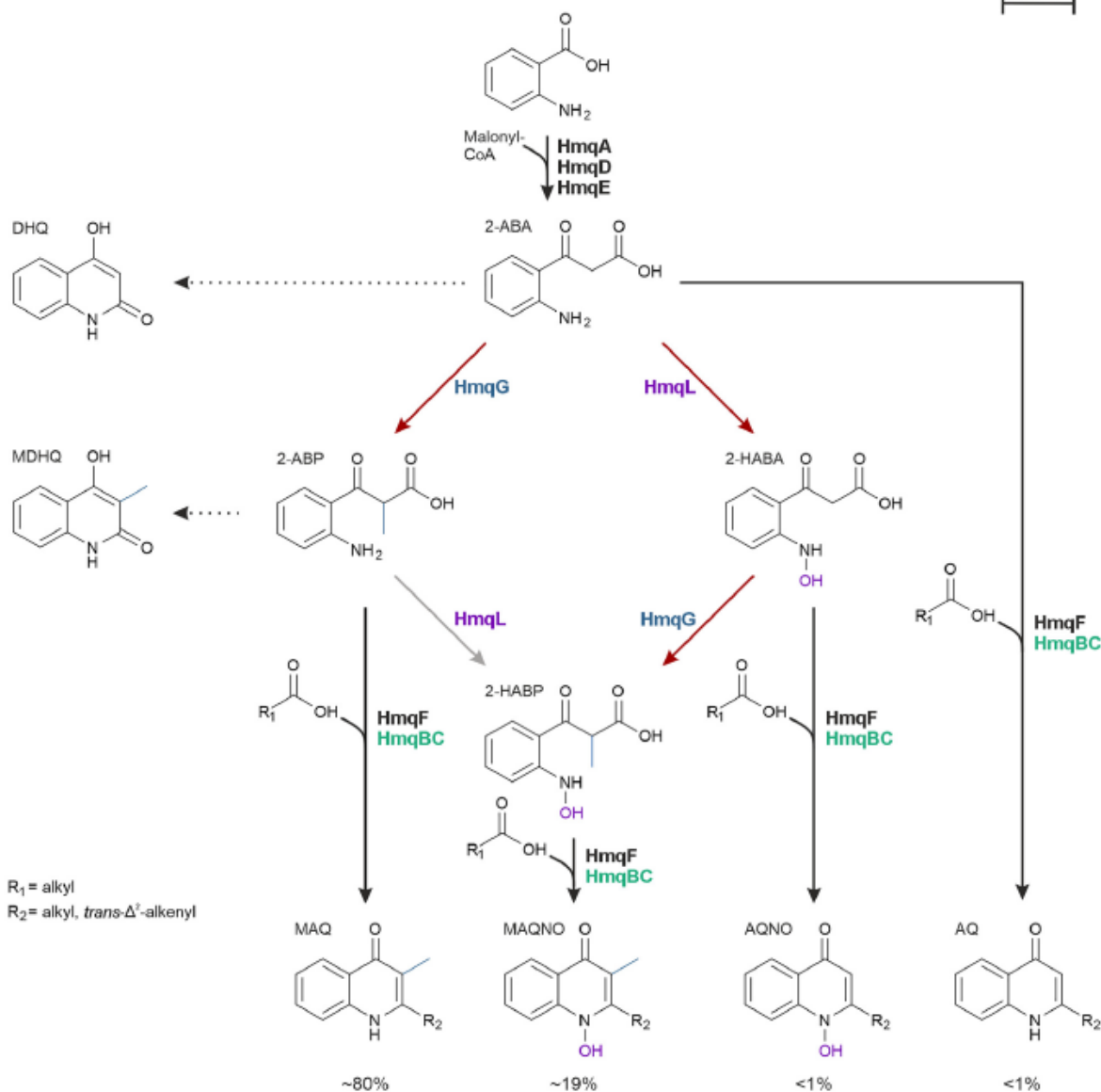
HHQ N-oxide (HQNO), one of the most abundant AQ-type metabolites of *P. aeruginosa*, mimics respiratory quinones/quinols and thus targets respiratory electron transfer.^{16–19} Moreover, HQNO and also *trans*- Δ^2 -3-methyl-2-nonenyl-4(1*H*)-quinolone (Δ^2 -MNQ) act as inhibitors of pyrimidine biosynthesis, and the latter additionally dissipates the proton-motive force via a monensin-like mechanism.²⁰ By interfering with energy metabolism via two distinct mechanisms and simultaneously inhibiting pyrimidine biosynthesis, HQNO and Δ^2 -MNQ act synergistically to inhibit bacterial growth.²⁰ Interestingly, methylation at C3 and Δ^2 -unsaturation of AQs and their N-oxides confers significant inhibitory potency against gram-positive bacteria, e.g., *Staphylococcus aureus* and *B. subtilis*.^{5,21} These structural features also correlate with antifungal and anti-oomycete activity, as plant pathogens such as *Verticillium dahlia*, *Pyricularia oryzae*, *Cochliobolus miyabeanus*, *Rhizoctonia solani*, and *Phytophthora capsici* were shown to be inhibited mainly by MAQs with 2-heptenyl and 2-nonenyl side chains.^{22–24} Moreover, 3-methylation of AQs may confer resistance to bacterial detoxification and degradation pathways, which—to the present knowledge—are initiated by 3-hydroxylation of the quinolone ring.^{25–27} Introduction of a hydroxy group at C3 of HQNO by *S. aureus* already decreases its toxicity.²⁸

Notably, the AQ biosynthetic pathway of *B. thailandensis* is poised to strongly favor the production of MAQs and MAQNOs over the production of non-methylated congeners, thereby pushing the AQ product spectrum toward more potent antimicrobials against gram-positive bacteria and fungi and toward congeners that may be recalcitrant to detoxification and degradation. However, the question of the rationale for the preference toward MAQ(NO) formation is so far unresolved.

According to alignment analyses, about 97% of the *Burkholderia pseudomallei* strains and 79% of the *B. thailandensis* strains included in the *Burkholderia* genome database contain the *hmqABCDEF*G gene cluster encoding the MAQ biosynthetic enzymes.²⁹ Competition assays suggested that *B. pseudomallei* relies on the products of the *hmqA–G* and *hmqL* genes for competitive fitness against environmental gram-positive bacteria.¹¹ In members of the *Burkholderia cepacia* complex, the *hmq* gene cluster was identified in about one-third of the sequenced members.²⁹ Interestingly, the *hmqABCDEF*G cluster is more prevalent in environmental strains associated with the rhizosphere than in clinical strains and plays a beneficial role in plant growth.³⁰

The biosynthetic enzymes encoded by the *hmqABCDE* genes of *B. thailandensis* are similar to those encoded by the AQ biosynthetic genes *pqsABCDE* of *P. aeruginosa*.⁶ Analogous to PqsA, HmqA was proposed to act as an anthranilate coenzyme A (CoA) ligase in the initial step of the biosynthetic pathway (Figure 1). The putative 3-oxoacyl-[acyl-carrier-protein] synthase HmqD, an ortholog of PqsD, is thought to subsequently catalyze the condensation of anthraniloyl-CoA and malonyl-CoA to form 2-aminobenzoylacetyl-CoA (2-ABA-CoA). HmqE, which is able to restore the AQ phenotype of a *P. aeruginosa* *pqsE* mutant, likely acts as 2-ABA-CoA thioesterase to form 2-aminobenzoylacetate (2-ABA) and thus channels the short-lived intermediate 2-ABA-CoA, which tends to undergo intramolecular cyclization to form the side product 4-hydroxy-2(1*H*)-quinolone (2,4-dihydroxyquinoline, DHQ) toward AQ synthesis.³¹ In *P. aeruginosa*, 2-ABA is the central branching point of the pathway: as the key step toward AQNO biosynthesis, PqsL catalyzes the hydroxylation of 2-ABA to 2-hydroxylaminobenzoylacetate (2-HABA).³² 2-ABA and 2-HABA are also substrates of the heterodimeric FabH-like enzyme PqsBC, which mediates a decarboxylative Claisen condensation with an acyl-CoA to form AQs and AQNOs, respectively.^{32–34} Based on their similarity to the respective subunits

*Burkholderia
thailandensis*
E264



(A) The biosynthetic genes *hmqA-G* form a predicted operon, whereas *hmqL* is a monocistronic gene located elsewhere in the genome. (B) The branched pathway mediates formation of four classes of quinolones, namely, 2-alk(enyl)-4(1H)-quinolones (AQs), 2-alk(enyl)-3-methyl-4(1H)-quinolones (MAQs), and the corresponding *N*-oxide derivatives (AQNOs and MAQNOs, respectively). The given percentages represent the approximate proportions of total alk(enyl) quinolones in tryptic soy broth (Szamosvári et al.,⁵ Vial et al.,⁶ and our own data). For detailed descriptions, see the text. Genes/enzymes investigated in this study are colored. Dashed arrows indicate spontaneous reactions, gray arrows indicate hypothetical reactions, and red arrows highlight findings of this study.

of PqsBC, HmqB and HmqC are presumed to form the heterodimeric enzyme HmqBC²⁹; however, biochemical data have not been reported yet.

HmqF and HmqG are unique to the AQ biosynthetic pathway of members of the genus *Burkholderia*. HmqF consists of an adenylation (A_{HmqF}) domain, a dehydrogenase (DH_{HmqF}) domain, and an acyl carrier protein (ACP_{HmqF}) domain. It catalyzes the activation of C_6 – C_{14} (preferably C_{10}) fatty acids in an ATP-dependent manner and loads the activated fatty acid to its phosphopantetheinylated ACP domain, where the DH domain introduces the *trans*- Δ^2 unsaturation into the acyl chain.³⁵ HmqF presumably delivers the unsaturated alkyl moiety to HmqBC for the condensation reaction. HmqG is an *S*-adenosylmethionine (SAM)-dependent methyltransferase required for the synthesis of all MAQs.⁶ Based on the identification of the metabolite 4-hydroxy-3-methyl-2(1*H*)-quinolone (MDHQ) in cultures of *Burkholderia* sp. strain 3Y-MMP,³⁶ which should result from intramolecular cyclization of methylated 2-ABA, the methylation reaction can be assumed to precede the condensation reaction mediated by HmqBC. However, the observation that a *Burkholderia ambifaria* *hmqG* mutant can produce AQs⁶ indicates that, at least in this species, methylation of the pathway intermediate is not a prerequisite for the HmqBC reaction. MAQNO biosynthesis in *B. thailandensis*, in addition to the HmqA–F proteins, requires HmqL,²¹ a flavoprotein monooxygenase, which, like PqsL, is capable of catalyzing the *N*-hydroxylation of 2-ABA.³⁷

Analogous to AQ and AQNO biosynthesis of *P. aeruginosa*, 2-ABA appears to be a central branching point also in the biosynthetic pathway of *Burkholderia* spp. In principle, three enzymes likely compete for 2-ABA: HmqL can convert 2-ABA to 2-HABA,³⁷ the methyltransferase HmqG is presumed to act on 2-ABA to form 2-(2'-aminobenzoyl) propionate (2-ABP), and HmqBC, analogous to PqsBC of *P. aeruginosa*, may condense 2-ABA with an activated fatty acid to form AQs. However, 2-ABP and 2-HABA may also be substrates of HmqL and HmqG, respectively. To produce all AQ compound classes, four substrates would need to compete for HmqBC, namely 2-ABA, 2-HABA, 2-ABP, and 2-(2'-hydroxylaminobenzoyl) propionate (2-HABP), to form AQs, AQNOs, MAQs, and MAQNOs, respectively (Figure 1).

In the MAQ(NO) biosynthetic pathway, enzymes at branching points compete for the same substrate, and substrate promiscuities (of HmqG and HmqL) might additionally contribute to the complexity of the pathway. In the absence of spatial or temporal compartmentalization, the substrate preferences and kinetic properties of enzymes at branching points are major determinants for the proportion between pathway products. Our study aims to define the preferred branches of the AQ biosynthetic pathway of *B. thailandensis* and to characterize the rationale for the bias toward MAQ and MAQNO synthesis.

RESULTS AND DISCUSSION

HmqG is active toward 2-ABA

Mutagenesis studies have revealed that the SAM-dependent methyltransferase HmqG is required for the synthesis of MAQs and MAQNOs.⁶ However, biochemical data on HmqG are lacking. Interestingly, MDHQ was previously identified as an extracellular metabolite of *Burkholderia* sp. 3Y-MMP,³⁶ suggesting methylation of the pathway intermediate 2-ABA. To characterize the methyltransferase, we produced HmqG in *E. coli* and purified the 79.5 kDa protein in high yields of approximately 80 mg/L of bacterial culture (Figure S1). First, we addressed the substrate scope of HmqG and, to this end, incubated the enzyme with SAM and different

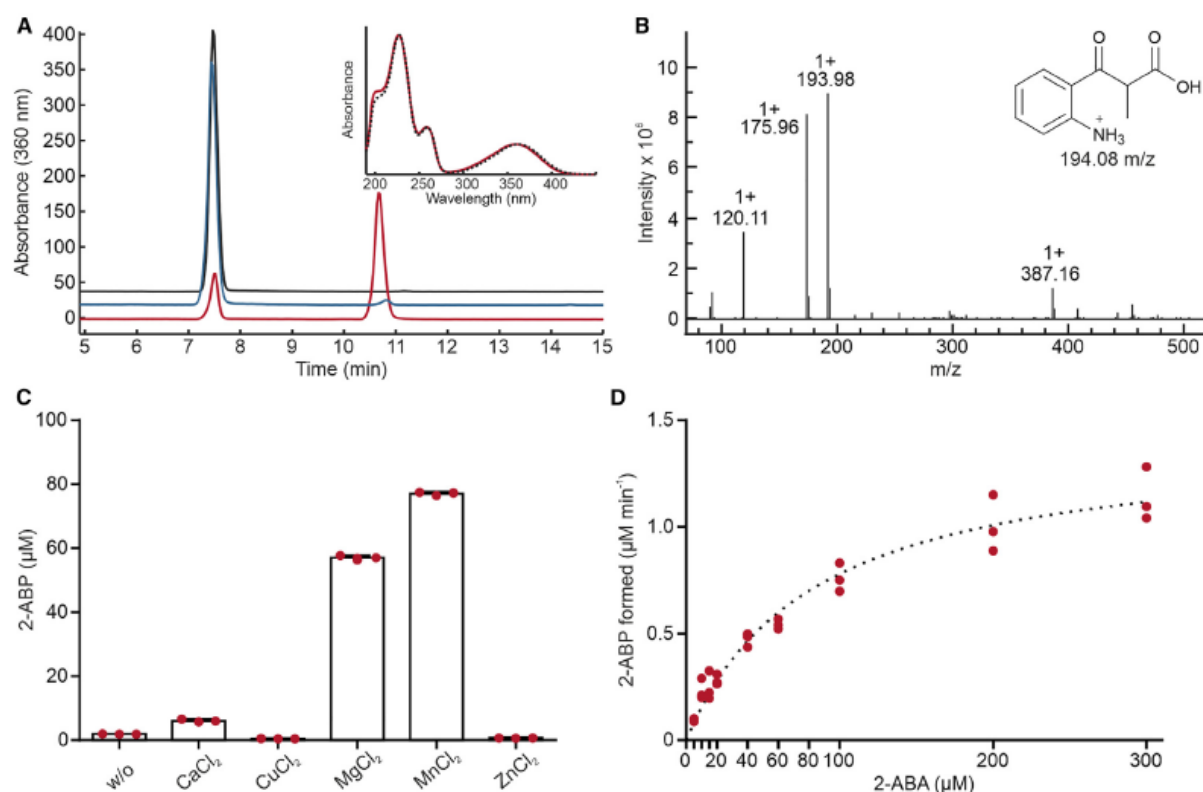


Figure 2. Enzymatic activity of HmqG

(A) HPLC chromatograms of 2-ABA reaction mixtures without HmqG (black), with HmqG (blue), and with HmqG and 3 mM MgCl₂ (red). The inset shows normalized UV-vis absorbance spectra of 2-ABA (black dashed line) and enzymatically produced 2-ABP (red solid line) in 10 mM ammonium bicarbonate (pH 8.8).

(B) Mass spectrum of enzymatically produced 2-ABP from LC-MS analysis.

(C) Cation dependence of HmqG. The assay contained the indicated salt at 3 mM and 100 μM 2-ABA. Samples were subjected to HPLC after 10 min of incubation. Error bars represent the standard deviation of three independent experiments.

(D) Steady-state kinetics of HmqG. The reaction mixture contained 3 mM MnCl₂ and varying concentrations of 2-ABA. Samples were analyzed after 1 min of incubation. All activity assays of HmqG were performed with 10 μM HmqG in 20 mM Tris-HCl (pH 8.0) and 1 mM SAM in 100 μL total volume and at 25°C. Red dots indicate individual data points of three independent experiments. The dashed line represents the non-linear fit including all data points. Data were analyzed using GraphPad Prism 6.

available AQ pathway metabolites (HHQ and its C5/C9 congeners, HQNO, DHQ, anthranilic acid, and 2-ABA) and analyzed the mixtures by high-performance liquid chromatography (HPLC) (Figure 2A). We observed the formation of a product when HmqG was incubated with 2-ABA, whereas no additional peaks were detected in the presence of any of the other substrates tested. The ultraviolet-visible (UV-vis) absorption spectrum of the product was highly similar to that of 2-ABA (Figure 2A, inset), and mass spectrometry (MS) analysis (Figure 2B) was in good agreement with the addition of a methyl group to 2-ABA, suggesting the formation of 2-ABP. Tandem MS (MS/MS) fragmentation analysis (Figure S2) verified the incorporation of a methyl group into 2-ABA by HmqG. Moreover, liquid chromatography (LC) retention times, UV-vis absorbance spectra, fragmentation patterns, and high-resolution MS data (Data S1) of synthetic 2-ABP were identical to those of the enzymatic product. Since many methyltransferases require divalent cations for activity,³⁸ we tested the reaction in the presence of Mg²⁺, which drastically increased the product yield (Figure 2A). Addition of Mn²⁺ was even more beneficial for the reaction

(Figure 2C). The spectral similarities of substrates (SAM and 2-ABA) and products (S-adenosyl homocysteine and 2-ABP) precluded the use of continuous spectroscopic assays to monitor reaction kinetics. Nevertheless, to determine steady-state parameters of HmqG, enzyme assays containing varying concentrations of 2-ABA were stopped during the linear phase of the reaction by the addition of acetonitrile and directly subjected to HPLC analysis. The amount of 2-ABP formed was used to calculate the apparent reaction velocity and steady-state kinetics (Figure 2D). HmqG appears to be rather slow, with a k_{cat} of $0.14 \pm 0.01 \text{ min}^{-1}$, a K_m of $82.7 \pm 10.4 \mu\text{M}$, and a specificity constant of $28.2 \text{ s}^{-1} \text{ M}^{-1}$.

HmqG and HmqL compete for 2-ABA

The flavoprotein *N*-hydroxylase HmqL was reported to be essential for the biosynthesis of MAQNOs,²¹ and we demonstrated in a previous study that HmqL is indeed isofunctional to the 2-ABA *N*-hydroxylase PqsL of *P. aeruginosa*.³⁷ In *B. thailandensis*, the sequential order of reactions leading to the formation of MAQNOs is unclear. We hypothesized that either HmqG is promiscuous and able to methylate 2-ABA, as well as its *N*-hydroxylated congener 2-HABA, or HmqL accepts both 2-ABA and 2-ABP as substrates. To determine the substrate scope of HmqG and HmqL, we performed a series of experiments in which we added the respective enzymes individually or sequentially to 2-ABA and analyzed the reaction by high-performance liquid chromatography-mass spectrometry (HPLC-MS) (Figure 3). As described above, the presence of HmqG alone and incubation for 30 min resulted in the complete conversion of 2-ABA to 2-ABP (Figures 3A and 3B). Likewise, when HmqL was individually added, only 2-HABA was produced. Due to the instability of 2-HABA ($t_{1/2} = 16.2 \text{ min}$)³² and the efficient reaction of HmqL,³⁷ we analyzed the product formation of reactions that involved HmqL after 4 min incubation. Interestingly, when HmqL was added to the mixture after completion of the HmqG reaction, no additional compound other than 2-ABP was observed. Addition of HmqL to synthetic 2-ABP led to equivalent results. We conclude that, in contrast to previously published pathway schemes,²¹ HmqL is specific for 2-ABA and does not convert its methylated congener 2-ABP. However, when 2-ABA was incubated with HmqG and HmqL simultaneously, we observed the formation of 2-HABA, 2-ABP, and a third substance, presumably 2-HABP. Analogous to 2-ABA and 2-ABP, the UV-vis absorption spectrum of 2-HABP is almost identical to that of 2-HABA (Figure 3C). LC-MS analysis of the respective peak revealed a major ion with $[M + H]^+$ of 191.98 Da, which is consistent with the m/z of 2-HABP upon loss of water (Figure 3D). The pronounced in-source fragmentation or cyclization is likely a result of the instability of 2-HABP and especially the hydroxylamine group. Similar MS spectra were observed for 2-HABA in the same experiment (Data S1). To further verify the identity of 2-HABP, we derivatized enzymatically produced 2-HABP using acetic anhydride according to previously described procedures.³² LC coupled to high-resolution MS (LC-HRMS) revealed a signal with $[M + Na]^+$ of 274.0686 Da, which is in agreement with the expected product (Figure S3). The presented data indicate that HmqG and HmqL compete for 2-ABA within the MAQ(NO) biosynthetic pathway. HmqL is specific for 2-ABA, whereas HmqG is capable of methylating both 2-ABA and 2-HABA. However, the determination of steady-state kinetics of HmqG with 2-HABA as substrate was not feasible, since it was impossible to handle 2-HABA/2-HABP without major decomposition.

Interaction between HmqBC and HmqF enables selective delivery of unsaturated acyl moieties

During AQ biosynthesis, the previously described reaction products are condensed with an acyl moiety by the heterodimeric enzyme HmqBC to generate the final

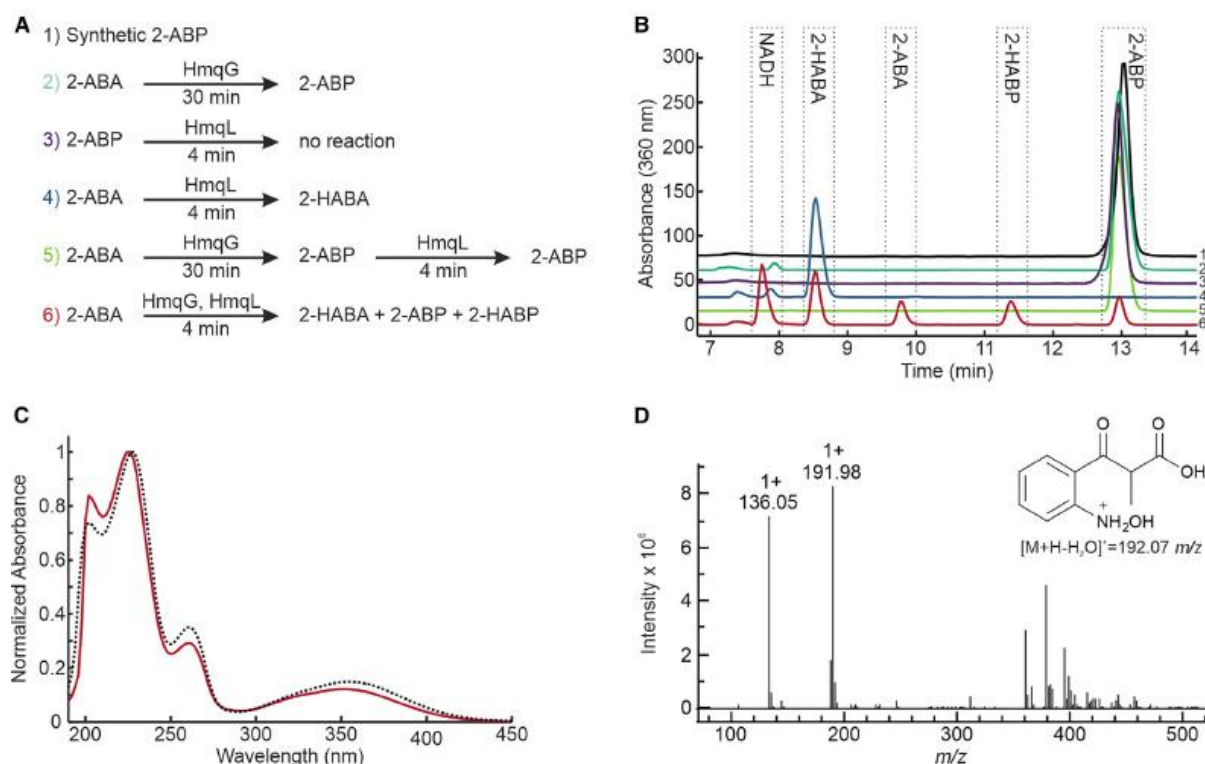


Figure 3. The reaction sequence of HmqG and HmqL during MAQ biosynthesis

(A) Simplified scheme of the experiment. Enzyme concentrations of HmqG and HmqL were 20 and 10 μM , respectively. Moreover, reactions employing HmqG contained 1 mM SAM. When the activity of HmqL was assayed, 5 μM FAD, 400 μM NADH, and 0.2 μM flavin reductase HpaC were added to provide the reduced FAD cofactor, and 94 U mL^{-1} catalase was supplemented to remove H_2O_2 . All reactions were performed in 20 mM Tris-HCl, 3 mM MnCl_2 , 100 μM 2-ABA (pH 8.0); with shaking at 1,000 rpm; and at 25°C.

(B) Representative HPLC chromatograms at 360 nm. Samples were directly subjected to HPLC after the indicated time points.

(C) UV-vis spectra of 2-HABA (black dashed line) and 2-HABP (red solid line) in 10 mM ammonium bicarbonate (pH 8.8).

(D) Mass spectrum of enzymatically produced 2-HABP from LC-MS analysis.

quinolone product. For this, it was proposed that HmqF specifically provides the required *trans*- Δ^2 unsaturated acyl moiety to HmqBC, possibly by protein-protein interaction.³⁵ To test this hypothesis, we initially generated a multimeric structural model of HmqBC and HmqF using AlphaFold2 in multimer mode. HmqF consists of three individual domains, an A_{HmqF} domain, a DH_{HmqF} domain, and an ACP_{HmqF} domain, connected by two putatively disordered linker regions ("loops"). This likely results in a high degree of interdomain flexibility (Figures S4A and S4B). Two possible interfaces between HmqF and HmqBC are proposed (Figure S4C): the formation of one high-confidence interface involving a helix of A_{HmqF} (residues 440–468) and three helices of HmqC (residues 34–40, 236–247, and 279–287) can be identified based on low predicted alignment errors (Figures S4D and S4F). A second proposed binding site has a rather low confidence score and includes residues 1,060–1,068 of the DH_{HmqF} domain and residues 37–42 of HmqB (Figures S4E and S4F). We addressed the predicted protein-protein interactions by biolayer interferometry (BLI) and pull-down assays (Figure 4). We prepared HmqBC according to previously described procedures for PqsBC,³³ resulting in yields of 6 mg of the heterodimer per liter of bacterial culture (Figure S1). We also individually purified the A_{HmqF} domain, the DH_{HmqF} domain, and the ACP_{HmqF} domain, since full-length HmqF was reported to be not soluble.³⁵ Biotinylated HmqBC was used as bait, whereas A_{HmqF} , DH_{HmqF} , and ACP_{HmqF} were used as prey proteins in both BLI and

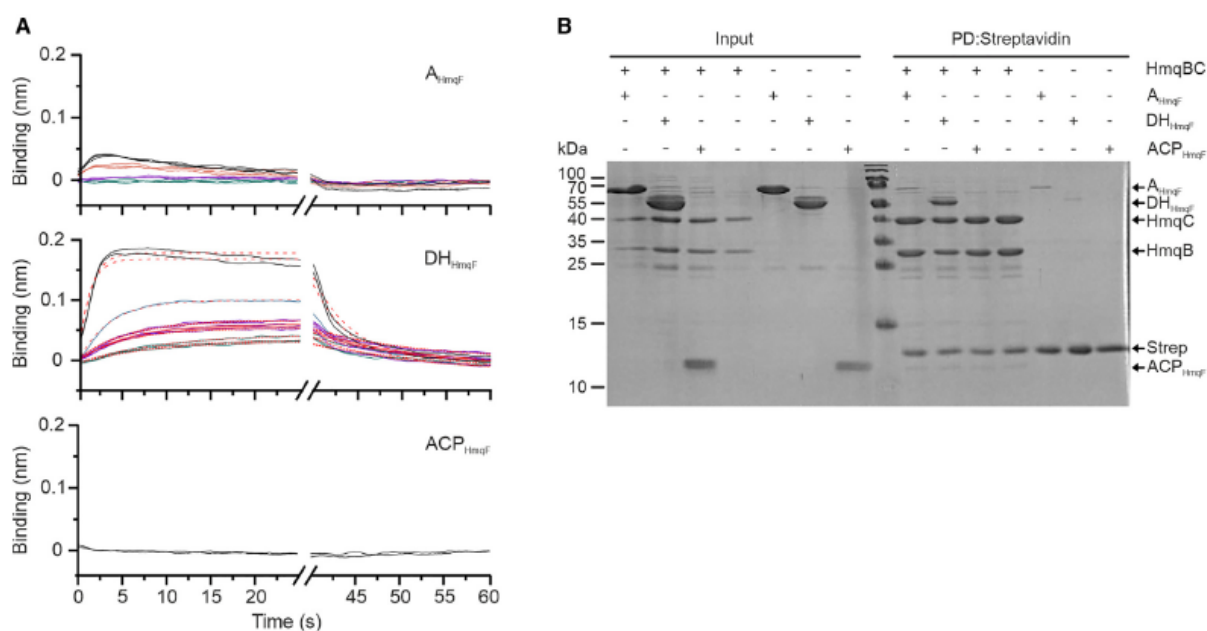


Figure 4. Interaction of HmqBC with HmqF

(A) Biolayer interferometry of biotinylated HmqBC with A_{HmqF} (top), DH_{HmqF} (middle), and ACP_{HmqF} (bottom). Interaction was monitored at 0.05 (green lines), 0.1 (purple lines), 0.25 (blue lines), 0.5 (orange lines), and 1 μ M (black lines) concentrations of the respective domain of HmqF. The raw data were filtered using a Savitzky-Golay filter for plotting. Non-linear fits are shown as red dashed lines. Experiments were repeated at least twice. Data were analyzed using Octet Analysis Studio (version 13.0.1.35).

(B) Pull-down (PD) assay of biotinylated HmqBC with individual HmqF domains using streptavidin Sepharose. "Input" represents the proteins that were applied to the streptavidin Sepharose.

pull-down experiments. In BLI (Figure 4A), we observed specific binding of DH_{HmqF} to HmqBC at all tested concentrations and calculated a K_D of $0.17 \pm 0.11 \mu$ M ($k_{on} = 0.77 \pm 0.02 \mu$ M⁻¹ s⁻¹, $k_{off} = 0.12 \pm 0.06$ s⁻¹). The addition of A_{HmqF} resulted in a small and short-lived increase in layer thickness at high concentrations (0.5 and 1 μ M), which we attributed to non-specific binding. ACP_{HmqF} did not interact with HmqBC. The findings from BLI were corroborated by a pull-down assay (Figure 4B), which moreover revealed that A_{HmqF} non-specifically binds to streptavidin and thereby explains the increase in layer thickness observed in BLI. In the pull-down assay, DH_{HmqF} co-eluted with HmqBC from the streptavidin beads, verifying a physical interaction between the two proteins. These results show that it is plausible that HmqF interacts with HmqBC *in vivo*, possibly via the interface between DH_{HmqF} and HmqB. The linker regions between the HmqF domains may provide the necessary flexibility to deliver the acyl moiety from the ACP_{HmqF} domain to the HmqBC active site. Direct interaction between the DH domain of HmqF and HmqBC might promote the installation of a specific unsaturated side chain into the final product and could explain the strong bias for the production of AQs with *trans*- Δ^2 -nonenyl side chains by *B. thailandensis*.

Substrate specificities of HmqBC promote the formation of unsaturated MAQs

As a possible rationale for the predominant production of MAQs by *B. thailandensis*, we hypothesized that either a high activity of HmqG *in situ* or the substrate specificity of HmqBC might be the determining factor for the observed quinolone composition. We therefore aimed to analyze the reactivity and substrate preference of HmqBC *in vitro*. Initial activity assays containing 2-ABA/2-ABP and decanoyl-CoA as

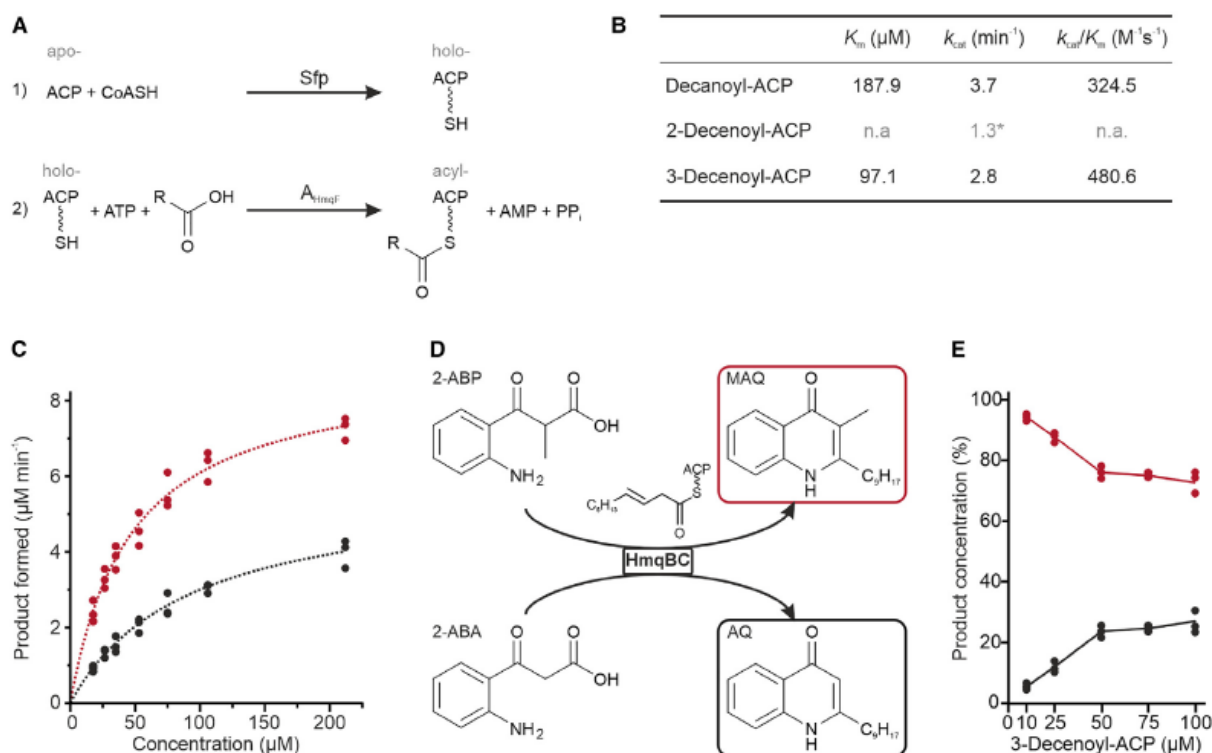


Figure 5. Substrate preference of HmqBC

(A) Enzymatic one-pot acylation of ACP_{HmqF}. R, C₉H₁₉, trans-C₉:1 Δ², or trans-C₉:1 Δ³. Wavy line represents phosphopantetheine arm.

(B) Steady-state kinetics of HmqBC with different acyl donors. Reactions contained 5 μM HmqBC, 100 μM 2-ABP, and varying concentrations of the indicated acyl-ACPs. The reaction with trans-2-decenoyl-ACP_{HmqF} does not follow Michaelis-Menten kinetics. The asterisk indicates the apparent reaction velocity at 273 μM substrate. n.a., not applicable.

(C) HmqBC steady-state kinetics with 2-ABA (black) and 2-ABP (red). Dashed lines indicate non-linear fits.

(D) Reaction scheme of the 2-ABA/2-ABP competition assay. Two substrates, 2-ABA and 2-ABP, compete for HmqBC. Depending on the substrate preference, HmqBC preferably converts either 2-ABA or 2-ABP to AQ (black box) or MAQ (red box), respectively, until 3-decenoyl-ACP_{HmqF} is depleted.

(E) The dependence on 3-decenoyl-ACP_{HmqF} concentrations reveals that 2-ABP is preferred over 2-ABA. Depicted are the final concentrations of AQ (black) and MAQ (red) in the assay shown in (D). The assays contained 10 μM HmqBC, 300 μM 2-ABA, 300 μM 2-ABP, and varying concentrations of 3-decenoyl-ACP (10–100 μM). All experiments were performed in triplicates and data were analyzed using MATLAB R2018b and GraphPad Prism 6.

substrates revealed the formation of only minute amounts of AQs or MAQs, respectively, over the course of 2 h, indicating a general functionality as condensing enzyme. However, the activities were unphysiologically low.

Next, we aimed to provide fatty-acid-loaded ACP_{HmqF} as a substrate for HmqBC to characterize the reaction of the latter (Figure S5). A_{HmqF} and the phosphopantetheinyl transferase Sfp were employed to enzymatically load ACP_{HmqF} with decanoic acid, trans-2-decenoic acid, or trans-3-decenoic acid (Figure 5A) in a multienzyme reaction. Here, the phosphopantetheine moiety of CoA is transferred to ACP_{HmqF} by Sfp to generate holo-ACP_{HmqF}. The respective fatty acid is activated in an ATP-dependent manner and attached to holo-ACP_{HmqF} by A_{HmqF}, resulting in the final fatty acyl-ACP_{HmqF}. The intact apo-ACP_{HmqF} and fatty acyl-ACPs_{HmqF} were measured with MS for the determination of their molecular masses and the degree of acylation. Protein spectra are shown in Figure S6. The mass of the unmodified apoprotein was determined to be 11,911 Da, which is 48 Da more than the expected 11,863 Da for the theoretical mass of apo-ACP_{HmqF}. This mass shift is likely due to

the modification of the three methionine residues in the protein sequence, which is known to often occur during sample handling. The main protein species was accompanied by two minor protein forms detected at 11,776 and 11,734 Da. The first likely resulted from the loss of the N-terminal methionine residue, which is also a common process. The reason for the third stable protein species is not entirely clear, but it could be due to losses of small groups such as HCOOH during MS measurement. The signals of all three proteins shifted due to acylation, indicating that all apoprotein forms were successfully modified to acylated holoproteins. This shift of the charge-state distribution is indicated in Figure S6. The deconvoluted mass of decanoyl-S-ACP_{HmqF} was determined to be 12,407 Da, verifying the addition of only one unit of the modifier group per apo-ACP_{HmqF}. The acylation appeared to be nearly complete, but small protein signals detected in the acylated sample at the location of the signals for the unmodified protein indicated some residual apoprotein. Analogous results were obtained for *trans*-2-decenoyl-S-ACP_{HmqF} and *trans*-3-decenoyl-S-ACP_{HmqF}, respectively. The derivatization yield was estimated from these data to be at least 90% and likely better, allowing the use of the fatty acyl-ACPs_{HmqF} in HmqBC activity assays.

In steady-state kinetics (Figure 5B), HmqBC showed an overall preference for *trans*-3-decenoyl-ACP_{HmqF} generating *trans*- Δ^2 -MNQ, which is in agreement with the predominant production of *trans*- Δ^2 -MAQ(NO)s by cultures of *B. thailandensis*. Decanoyl-ACP_{HmqF} was converted with a higher k_{cat} , but the enzyme also exhibited a higher K_m for the saturated substrate. Surprisingly, *trans*-2-decenoyl-ACP_{HmqF} was converted to *trans*- Δ^1 -MNQ hardly at all, indicating a specific substrate recognition of 3-unsaturated fatty-acid-ACPs by HmqBC and supporting the notion that HmqBC, in conjunction with HmqF, is responsible for determining the nature of the alk(en)yl side chain of the final quinolones.

The availability of *trans*-3-decenoyl-ACP_{HmqF} as an acyl donor further allowed us to characterize the kinetic parameters of HmqBC for the aromatic substrates 2-ABA and 2-ABP (Figure 5C). The data obtained show a considerable preference for 2-ABP ($K_m = 47 \pm 4 \mu\text{M}$, $k_{cat} = 1.75 \pm 0.06 \text{ min}^{-1}$, $k_{cat}/K_m = 621 \text{ M}^{-1} \text{ s}^{-1}$) over 2-ABA ($K_m = 92 \pm 11 \mu\text{M}$, $k_{cat} = 1.12 \pm 0.06 \text{ min}^{-1}$, $k_{cat}/K_m = 204 \text{ M}^{-1} \text{ s}^{-1}$). To further substantiate the substrate preference, we performed a competition assay in which 2-ABA and 2-ABP were offered to HmqBC in excess, whereas 3-decenoyl-ACP_{HmqF} was limited (Figure 5D). Varying the concentration of acyl donor can then provide further information on which substrate is preferred. Similar experimental approaches and calculations were previously successfully used to understand enzyme kinetics with multiple alternative substrates.^{39–42} The results verify the preference for 2-ABP, which is remarkably pronounced at low concentrations of *trans*-3-decenoyl-ACP_{HmqF} (94.5% MAQ to 5.5% AQ) and levels off at a 75%:25% (MAQ/AQ) ratio at higher concentrations (Figure 4E), which is in good agreement with the 3-fold higher specificity constant of the enzyme for 2-ABP over 2-ABA.

Our data suggest that HmqBC not only prefers 2-ABP over 2-ABA, but also exerts control over the installed acyl moiety. In conjunction with the preference of HmqF for decanoic acid over other fatty acids³⁵ and the prominent role of its DH domain in the interaction with HmqBC, the substrate specificity of HmqBC provides a biochemical rationale for the abundant production of Δ^2 -MNQ(NO) by *B. thailandensis*.

High expression levels of *hmqG* drive efficient methylation *in situ*

During routine analyses of *B. thailandensis* cultures, we noticed considerable amounts of the side products DHQ (1.44 μM) and MDHQ (9.81 μM) (Figure 6A),

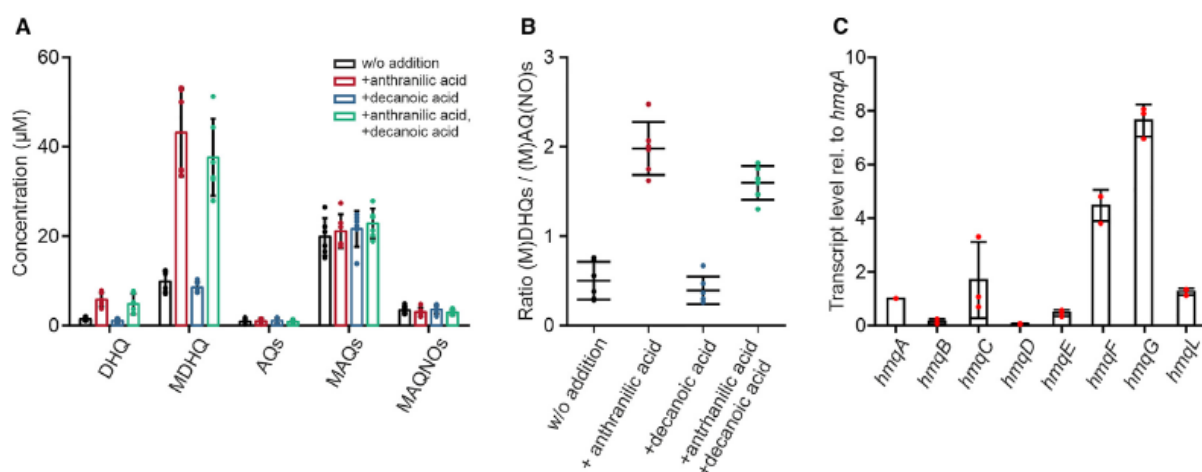


Figure 6. Quinolone production and expression profile of *hmq* genes in *B. thailandensis*

(A) Major quinolones in bacterial cultures after 10 h of incubation. AQs include C₇-AQ, C₉-AQ, Δ²-C₇-AQ, and Δ²-C₉-AQ; MAQs comprise C₇-MAQ, C₉-MAQ, Δ²-C₇-MAQ, Δ²-C₉-MAQ, Δ²-C₁₀-MAQ, and Δ²-C₁₁-MAQ; MAQNOs encompass Δ²-C₉-MAQNO and Δ²-C₁₁-MAQNO. Data were gathered from two independent experiments with three biological replicates each. Bars represent averages, error bars indicate standard deviation.

(B) Mean of (M)DHQ to (M)AQ(NO)s ratios from (A). Error bars indicate standard deviation.

(C) Expression of *hmq* genes normalized to *hmqA*. Bars represent averages of three independent experiments, error bars indicate standard deviation.

which originate from a non-catalyzed intramolecular cyclization of 2-ABA/HABA and its methylated congeners 2-ABP/2-HABP (Figure 1). This prompted us to probe the metabolic fluxes and identify potential bottlenecks of the pathway. To this end, we supplemented *B. thailandensis* cultures with anthranilic acid and decanoic acid and quantified the major products. Interestingly, supplementation of anthranilic acid caused a remarkable increase in DHQ and even more so MDHQ levels, whereas (M)AQ concentrations were almost unaltered, shifting the (M)DHQ to (M)AQ(NO) ratio from 0.5 to 2 (Figure 6B). These results are indicative of an accumulation of pathway intermediates due to a bottleneck created by low abundances and/or low activities of HmqBC or HmqF, combined with efficient methylation of 2-ABA/2-HABA before cyclization occurs. Addition of decanoic acid resulted in only minor changes in the quinolone profile, supporting the notion that substrate availability for HmqF is not a limiting factor in AQ biosynthesis.

In addition to the biochemical level, the AQ composition is presumably also influenced by transcriptional regulation of the *hmq* genes. We therefore performed RT-qPCR to relatively quantify transcript levels of each individual *hmq* gene (Figure 6C). Remarkably, *hmqF* and even more so *hmqG* show high transcript levels compared to all other *hmq* genes. An increased expression of *hmqF* is likely beneficial for the pathway to prevent acyl donor availability to become a limiting factor for MAQ(NO) biosynthesis. Further, we assume that elevated expression levels of *hmqG* constitute one of the main reasons for the high abundance of MDHQ, MAQ, and MAQNO and lay the foundation for a biosynthetic pathway optimized to produce predominantly methylated metabolites. Differential gene expression within operons is a widespread mechanism in bacteria and allows fine-tuned regulation of the biosynthetic activity on the transcriptional level. For example, Wang and colleagues identified multiple internal promoters within the biosynthetic operon of epothilone in *Myxococcus xanthus*.⁴³ Related to our study, a similar phenomenon was also described for the *pqs* operon of *P. aeruginosa*. Here, a promoter upstream of *pqsD* was proposed to be responsible for an internal transcript

containing *pqsD*, *pqsE*, and *phnAB*, ensuring the production of anthranilic acid and *Pseudomonas* quinolone signal (PQS)-related virulence under nutrient-limiting conditions.⁴⁴

Limitations of the study

There are some limitations of the study concerning the complex biology of *B. thailandensis*. First, *in vitro* activities of enzymes do not necessarily reflect their activities *in situ*, which may define the metabolic pathway within the bacterial cell. Further, affinity tags might affect enzymatic activities *in vitro*. Second, assays employing HmqF cannot be conducted with full-length HmqF due to its insolubility, and the kinetics of HmqBC were instead determined using acyl-ACP_{HmqF}. The impact of the interaction between DH_{HmqF} and HmqBC on the reaction catalyzed by HmqBC could therefore not be tested in activity assays. Third, even though internal promoters in biosynthetic gene clusters are not uncommon, the reasons for fine-tuned gene expression of the *hmqG* locus remain enigmatic and further work is needed to experimentally verify internal promoters or other regulatory elements within the *hmqABCDEFG* operon. Last, the question of whether DHQ and especially MDHQ are of physiological relevance for *Burkholderia* remains unresolved.

EXPERIMENTAL PROCEDURES

Resource availability

Lead contact

Requests for further information and resources and reagents should be directed to and will be fulfilled by the lead contact, Simon Ernst (Simon.Ernst@Beiersdorf.com), upon reasonable request.

Materials availability

Plasmids generated in this study are available upon reasonable request. Synthesized chemicals can be produced upon reasonable request.

Data and code availability

- This paper does not report original code.
- Additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

Method details

Bacterial strains

B. thailandensis E264 DSM 13276 was obtained from the German Collection of Microorganisms and Cell Cultures and routinely grown in tryptic soy broth at 37°C with shaking at 180 rpm. *E. coli* BL21(DE) was obtained from Merck Novagen and *E. coli* LMG194 (ATCC 4709) from the American Type Culture Collection. *E. coli* was routinely cultivated in lysogeny broth (Miller) at 37°C with shaking at 180 rpm.

Structure prediction

Structural models for *B. thailandensis* HmqB, HmqC, and HmqF were generated via AlphaFold2^{45,46} v.2.3.0 in multimer mode on a local cluster. Afterward, the structures were visualized with ChimeraX.⁴⁷

Plasmids and cloning procedures

The pBAD-based shuttle vector pHERD30T⁴⁸ was used for inducible overexpression of *hmqBC* (BTH_II1934, BTH_II1933), *hmqG* (BTH_II1929), and *hmqL* (BTH_II1576). Plasmids used in this study were constructed using either restriction-free cloning procedures⁴⁹ or the NEBuilder HiFi DNA Assembly Tool and Master Mix (New

England Biolabs). During this process, the sequence encoding an N-terminal octahistidine tag and a tobacco etch virus (TEV) protease recognition site was attached to *hmqC*, resulting in pHERD30T::*hmqB-NHis₈-hmqC*. Likewise, *hmqG* was equipped with a sequence encoding an N-terminal octahistidine tag and a TEV protease recognition site, leading to the plasmid pHERD30T::*NHis₈-hmqG*. *hmqL* was fused to sequences encoding an N-terminal hexahistidine tag and a Ser-Ala-Gly linker (pHERD30T::*NHis₆-hmqL*).³⁷ The A, DH, and ACP domain sequences of *hmqF* were cloned separately into pET28b as described previously,³⁵ leading to plasmids pET28b::*NHis₆-hmqF-A*, pET28b::*NHis₆-hmqF-DH*, and pET28b::*NHis₆-hmqF-ACP*. Coding sequences of *hmqB*, *hmqC*, *hmqF*, *hmqG*, and *hmqL* were amplified from genomic DNA of *B. thailandensis* E264 using Q5 High-Fidelity DNA polymerase (New England Biolabs) according to the manufacturer's protocols.

Quinolone production of *B. thailandensis* E264

For quinolone production in *B. thailandensis*, liquid cultures were inoculated to an initial OD₆₀₀ of 0.05 from liquid overnight pre-cultures and cultivated for 10 h. Metabolites were extracted by liquid-liquid extraction as described below.

Synthesis of 2-ABP

The synthesis of 2-ABP started from 2-nitrobenzoic acid. Carbonyl activation as acyl imidazole and subsequent condensation with benzyl potassium malonate yielded benzyl 2-nitrobenzoyl acetate. Methylation with iodomethane gave benzyl 2-(2'-nitrobenzoyl) propionate, which was in one step reduced and deprotected to 2-ABP as detailed in the [supplemental information](#).

RT-qPCR

For analysis of relative *hmq* transcript levels in *B. thailandensis* E264, bacteria were grown to an OD₆₀₀ of 3.3 in tryptic soy broth at 37°C, 180 rpm. Approximately 5 × 10⁸ cells were harvested by centrifugation (5 min, 20,000 × g, 4°C), frozen in liquid nitrogen, and stored at −80°C until further use. Total RNA was extracted using the Monarch Total RNA Miniprep Kit (New England Biolabs) according to the manufacturer's protocol. RT-qPCR was performed with 200 ng of total RNA and the Luna Universal Probe One-Step RT-qPCR Kit (New England Biolabs) according to the manufacturer's recommendations. Relative expression levels of *hmq* genes were determined employing the 2-ΔΔc(T) method⁵⁰ using *gyrA* (BTH_11632) as reference gene.

Protein production and purification

E. coli LMG194 was used throughout the study for heterologous production of HmqBC, HmqG, and HmqL, whereas *E. coli* BL21(DE3) was used for production of individual domains of HmqF. Bacteria carrying the respective plasmid were cultivated in Terrific broth medium supplemented with 30 μg mL^{−1} gentamicin (pHERD30T) or 100 μg mL^{−1} kanamycin (pET28b) at 37°C, 160 rpm, until the culture reached an OD₆₀₀ of approximately 0.4 and then cooled to 20°C for 1 h before gene expression was started by addition of 0.02% (w/v) L-arabinose (pHERD30T) or 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (pET28b). Cells were harvested by centrifugation, frozen in liquid nitrogen, and stored at −80°C until further use.

Preparation of HmqBC, A_{HmqF}, DH_{HmqF}, ACP_{HmqF}, HmqG, and HmqL followed the same procedure, except for the pH of buffers used. For HmqBC and for HmqF domains, the pH was adjusted to 8.0, for HmqG to pH 7.8, and for HmqL to pH 8.3. For protein preparation, cells were resuspended in 30 mM Tris-HCl, 150 mM NaCl, 10 mM imidazole, 5% (v/v) glycerol, and 0.1% (v/v) Nonidet P-40 and

Table 1. BLI measurement cycle and parameters

Step	Time	
(1) Baseline	60 s	assay buffer + 0.05% Tween 20
(2) Loading	700 s	protein ($2 \mu\text{g mL}^{-1}$) in assay buffer + 1 mM MgCl_2
(3) Baseline	60 s	assay buffer
(4) Association	40–60 s	assay buffer + analyte
(5) Dissociation	60 s	assay buffer + 0.05% Tween 20

disrupted by sonication. Cell debris and insoluble components were removed by centrifugation ($40,000 \times g$, 4°C) and subsequent filtration ($0.45 \mu\text{m}$ polyvinylidene fluoride [PVDF] membrane). Cell extracts containing soluble protein were applied to a 5 mL Protino Ni-NTA column (Macherey-Nagel) with a flow rate of 1 mL min^{-1} . The column was washed with 15 column volumes of buffer A (30 mM Tris-HCl, 300 mM NaCl, 5% [v/v] glycerol, 20 mM imidazole) before the protein was eluted with a linear gradient to 100% buffer B (30 mM Tris-HCl, 300 mM NaCl, 5% [v/v] glycerol, 300 mM imidazole) over 10 column volumes. Fractions containing the protein of interest were pooled and dialyzed against storage buffer (30 mM Tris-HCl, 50 mM NaCl, 10% [v/v] glycerol, 1 mM DTT) overnight at 8°C . Subsequently proteins were concentrated to $20\text{--}30 \text{ mg mL}^{-1}$ using centrifugal concentrators and flash frozen in liquid nitrogen. The flavin reductase HpaC and the *B. subtilis* phosphopantetheinyl transferase Sfp were purified as described.^{51,52} Protein purity was verified by SDS-PAGE.

BLI and pull-down assay

Free amines from HmqBC were biotinylated and used for BLI kinetics analysis. To this end, 2 mg HmqBC was mixed with $100 \mu\text{g}$ (+)-biotin-*N*-hydroxysuccinimide ester (Sigma-Aldrich) in 30 mM HEPES (pH 8.2) and incubated for 1 h at room temperature. Free (+)-biotin-*N*-hydroxysuccinimide ester was removed using a 5 mL Hi-Trap desalting column (Cytiva) according to the manufacturer's recommendations. Labeling efficiency was monitored by MS analysis. Octet SA Biosensors were loaded stepwise with $2 \mu\text{g mL}^{-1}$ of biotinylated protein. An unloaded sensor was used as reference. Data were collected using an Octet R2 Protein Analysis System (Sartorius) at 30°C and 1,000 rpm shaking using the pattern given in Table 1. Steps 3–5 were repeated to generate replicates. For the association step, the sensors were incubated with the tested HmqF domains as analyte at different concentrations. For the lower concentrations of DH_{HmqF} (0.05 and $0.1 \mu\text{M}$), the results from four replicates were used. At higher concentrations (0.25 and $1 \mu\text{M}$), only one and two measurements could be fitted confidently. For A_{HmqF} , at least three replicates per concentration were measured. For ACP_{HmqF} , no binding was observed at the highest concentration. All measurements were performed in assay buffer (30 mM HEPES [pH 8.0], 50 mM NaCl) supplemented with 0.05% Tween 20.

BLI data were analyzed with the Octet Analysis Studio (version 13.0.1.35). The buffer control signal was subtracted from the traces, the y axis was aligned to the average of the baseline step (55–60 s), and the interstep correction was performed to the association step. The raw data were denoised using the implemented Savitzky-Golay filter. The association (0–25 s) and dissociation (0–60 s) steps were fitted separately (partial fitting) for each replicate. The association rate (k_{on}) and dissociation rate (k_{off}) were derived from these fits, and the dissociation constant K_D was calculated as the quotient of k_{off} and k_{on} . Mean value and standard deviation were calculated over all concentrations.

For the pull-down assay, proteins were diluted in assay buffer (30 mM HEPES [pH 8.0], 50 mM NaCl) supplemented with 0.1% Tween 20 and incubated with equilibrated streptavidin Sepharose beads (Cytiva). Final concentrations of 5 μ M biotinylated HmqBC and 25 μ M A_{HmqF}, DH_{HmqF}, and ACP_{HmqF} were used. The mixture was incubated 20 min at 4°C, 5 rpm. Supernatant was removed (500 $\times$ g, 1 min), 60 μ L was mixed with 20 μ L 4 $\times$ SDS loading dye as input fraction, and the beads were washed three times with assay buffer (+0.1% Tween 20). Beads were resuspended in 50 μ L 1 $\times$ SDS loading dye as the pull-down fractions. Both fractions were analyzed using SDS-PAGE and Coomassie staining.

Generation, purification, and MS analysis of fatty-acid-loaded ACP

ACP_{HmqF} was loaded with fatty acids as described previously³⁵ with minor modifications. In brief, a 250 μ L reaction mixture contained 4 mM CoA, 4 mM ATP, 1 mM DTT, 4 mM decanoic acid or 4 mM *trans*-2- or 3-decenoic acid, 0.8 mM ACP_{HmqF}, 10 μ M Sfp, and 25 μ M A_{HmqF} in buffer (50 mM Tris-HCl, 50 mM NaCl, 5 mM MgCl₂, 5% [v/v] glycerol [pH 8.0]). The mixture was incubated at 25°C for 3 h. Subsequently, precipitants were removed by centrifugation (20,000 $\times$ g, 4°C, 10 min), and the supernatant was subjected to size-exclusion chromatography using a Superdex 75 Increase 10/300 GL size-exclusion column. ACP_{HmqF} was eluted using 20 mM Tris-HCl and 150 mM NaCl (pH 8.0) at a constant flow rate of 0.6 mL min⁻¹ at 12°C. Fractions containing ACP_{HmqF} were pooled and concentrated to approximately 5 mg mL⁻¹ using centrifugal concentrators with a 5 kDa cutoff. After concentration, ACP_{HmqF} was frozen in liquid nitrogen and stored at -80°C until further use.

For MS analysis of native proteins, samples were desalted using Omix C18 tips (Agilent Technologies) and directly injected into the source of a Q-TOF Premier (Waters) at 100 pmol μ L⁻¹. Data were accumulated for 3 min. Spectra were deconvoluted using the maximum entropy function MaxEnt1 in the MassLynx 4.1 instrument driver software.

Activity assays of HmqL

Activity assays of HmqL (10 μ M) were performed in 20 mM Tris-HCl, 5 μ M FAD, and 400 μ M NADH (pH 8.0) at 25°C, 1,000 rpm, in open reaction tubes. The 2-ABA/2-ABP concentrations were 100 μ M. To continuously generate the reduced FAD cofactor, 0.2 μ M flavin reductase HpaC was added. Hydrogen peroxide produced by unproductive oxidation of reduced flavin was removed by supplementation of 94 U mL⁻¹ catalase. After 4 min, the reaction mixtures were directly subjected to HPLC.

Activity assays of HmqG

The cation dependence of HmqG was tested using 20 μ M HmqG in 20 mM Tris-HCl, 1 mM SAM, and 100 μ M 2-ABA (pH 8.0) at 25°C. Salts (CaCl₂, CuCl₂, MgCl₂, MnCl₂, and ZnCl₂) were added at a concentration of 3 mM. Reaction products were analyzed by HPLC after 10 min incubation.

For determination of steady-state parameters, 3 mM MnCl₂ was added to all assays, and the 2-ABA concentration was varied between 5 and 300 μ M. After 1 min, the reactions were stopped by addition of 50% acetonitrile and directly analyzed by HPLC. Reaction velocities were calculated based on the amount of 2-ABP formed. Data were analyzed and mean values with standard deviations were calculated using GraphPad Prism 6.

Activity assays of HmqBC

Activity assays of HmqBC were conducted using 5 μ M HmqBC, 50 μ M 3-decenoyl-HmqF-ACP, and varying concentrations of 2-ABA/2-ABP (5–300 μ M) or 100 μ M 2-ABP and varying concentrations of fatty acyl-ACPs (19–273 μ M) in 20 mM

Tris-HCl and 50 mM NaCl (pH 8.0) at room temperature. Substrate consumption was monitored at a wavelength of 360 nm using a V-750 UV-vis spectrophotometer (Jasco). A molar extinction coefficient of $\epsilon_{360} = 5,050 \text{ M}^{-1} \text{ cm}^{-1}$ for both 2-ABA and 2-ABP was used to calculate enzyme activities. To determine the molar extinction coefficient of 2-ABP, we enzymatically converted a defined amount of 2-ABA to 2-ABP using HmqG and measured the UV-vis absorbance spectra. Full conversion of 2-ABA was verified by HPLC analysis. The obtained spectra were compared to the previously published spectra of 2-ABA.³³ Data were analyzed, and mean values with standard deviations were calculated using GraphPad Prism 6.

For 2-ABP/2-ABA competition experiments, the reaction mixtures (100 μL) contained 5 μM HmqBC, 300 μM both 2-ABP and 2-ABA, and varying concentrations of 3-decenoyl-HmqF-ACP (10–100 μM) in 20 mM Tris-HCl and 50 mM NaCl (pH 8.0). Mixtures were incubated for 3 h at 25°C followed by extraction of MAQ and AQ products and HPLC analysis. Quantification of MAQs and AQs was achieved using standard curves generated with authentic reference compounds.

Coupled enzyme assays

Enzyme assays combining HmqG and HmqL were performed using 20 μM HmqG and 10 μM HmqL in 20 mM Tris-HCl, 3 mM MnCl_2 , 1 mM SAM, 5 μM FAD, 400 μM NADH, 0.2 μM HpaC, and 94 U mL^{-1} catalase (pH 8.0) at 25°C, 1,000 rpm, in open reaction tubes. The concentration of 2-ABA was 100 μM . Reaction products 2-ABP, 2-HABA, and 2-HABP were immediately analyzed by HPLC or HPLC-MS after the indicated time points (4 and 30 min).

Derivatization of 2-HABP

Unstable 2-HABP produced in a coupled enzyme assay was derivatized as described by Drees et al.³² The assay was performed in 20 mM HEPES (pH 8.6) at 25°C. After 5 min of incubation, acetic anhydride was added to a final concentration of 1% (v/v), and the resulting mixture was kept on ice for 30 min. Precipitated protein was removed by centrifugation for 4 min at 4,500 rpm and 4°C. The supernatant was then filtered through a 0.2 μm syringe filter and analyzed with LC-HRMS. LC-HRMS: $m/z = 274.0686$ $[\text{M} + \text{Na}]^+$, 234.0761 $[\text{M}-\text{H}_2\text{O} + \text{H}]^+$, 256.0582 $[\text{M}-\text{H}_2\text{O} + \text{Na}]^+$; calc. for $\text{C}_{12}\text{H}_{13}\text{O}_5\text{N} + \text{Na}^+ = 274.0686$ (Figure S3).

Extraction of quinolone metabolites

Quinolones were extracted from bacterial cultures and enzyme assays by vigorous mixing with 1 vol of acidic ethyl acetate (0.1% acetic acid) for 15 s, followed by centrifugation (20,000 $\times g$, 1 min, room temperature) and removal of the organic layer. Extraction was repeated a total of three times. Organic layers were pooled and evaporated to dryness *in vacuo*. Dried samples were stored at -20°C until further use.

HPLC and LC-MS analysis of small molecules

HPLC experiments were performed with an Agilent 1100 series instrument with autosampler, binary pump, and diode array detector. Analysis of 2-ABA, 2-HABA, 2-ABP, and 2-HABP was performed using Knauer Eurospher II 100-3 3 $\times$ 150 mm C18H columns. Eluents were aqueous 10 mM NH_4HCO_3 (pH 8.8) (A) and acetonitrile (B). Separation was achieved by 1 min 99% A, followed by a linear gradient from 99% to 85% A over 15 min at a flow rate of 0.5 mL min^{-1} and 35°C.

Knauer Eurospher II 100-3 3 $\times$ 150 mm C18 columns were used for HPLC of AQ reaction products. AQs were separated by 15 min of isocratic flow of 80% methanol and 20% water with 0.1% (w/v) citric acid at a flow rate of 0.5 mL min^{-1} and 35°C.

LC-MS analysis of 2-ABA and derivatives thereof was performed using a Thermo Scientific Ultimate 3000/Bruker Amazon speed instrument. LC parameters were the same as described above. Electrospray ionization parameters were varied according to the manufacturer's recommendations to increase the sensitivity for detecting labile compounds.

Quinolones were identified and quantified using a Waters Acquity Premier UPLC equipped with quaternary pump, temperature-controlled sample manager, and PDA and QDa detectors. Metabolites were separated on a Waters Acquity Premier BEH C18 column, 1.7 μm particle size, 2.1 $\times$ 50 mm, at 40°C using 50% A for 0.65 min, followed by a linear gradient from 22% to 10% A over 2.2 min. Subsequently, the column was reequilibrated for 1.16 min with 50% A. Solution A was 0.1% acetic acid in water, solution B was methanol. The flow rate was kept constant at 0.43 mL min⁻¹. MS analysis was performed in positive mode according to the manufacturer's recommendations, a cone voltage of 15 V, and a capillary voltage of 0.8 kV.

MS/MS analysis of small molecules

MS/MS analysis was performed by a TSQ Series II Quantis (Thermo Fisher Scientific) mass spectrometer using direct injection. The flow rate was 10 $\mu\text{L min}^{-1}$, and electrospray ionization (ESI) was used as an ion source. The ion spray voltage was 3,500 V (positive mode), vaporizer temperature 20°C, ion transfer tube temperature 275°C, sheath gas pressure 5 psi, ion sweep gas pressure 0 psi, and aux gas 2 psi. The fragmentation pattern of analyzed compounds was acquired in a product ion scan mode using a collision energy of 10, 15, 20, or 30 V to fragment the corresponding precursor ion before recording the fragments in a mass range of m/z 50–250. The scan rate was 1,000 Da s⁻¹ and CID gas was fixed at 1.5 mTorr.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xcrp.2024.102100>.

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AUTHOR CONTRIBUTIONS

S.E. conceived the project. S.E. designed and performed most of the experiments and analyzed the data. V.S. designed the synthetic methodology and performed chemical synthesis, NMR, small-molecule HR-MS, and MS/MS analysis and analyzed the data. M.J. conducted RT-qPCR experiments and analyzed the data. M.J. and R.R. performed protein-protein interaction experiments and analyzed the data. E.H. and D.K. performed structural modeling and analysis. S.K. performed protein HR-MS with Synapt G2 Si and analyzed the data. S.E., S.F., T.B., and V.S. wrote the manuscript. All the authors read, edited, and contributed to the manuscript.

DECLARATION OF INTERESTS

The authors declare no conflicts of interest.

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2.3 Publication 3. Supporting Information

Cell Reports Physical Science, Volume 5

Supplemental information

**Unraveling key steps in the biosynthesis
of antimicrobial methylated unsaturated 2-alkyl-
4-quinolones of *Burkholderia thailandensis***

Viktoriia Savchenko, Miriam Jaegers, René Rasche, Eric Herrmann, Simone König, Daniel Kümmel, Thomas Böttcher, Susanne Fetzner, and Simon Ernst

Supplemental Information

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Supplemental Figures
SDS-PAGE

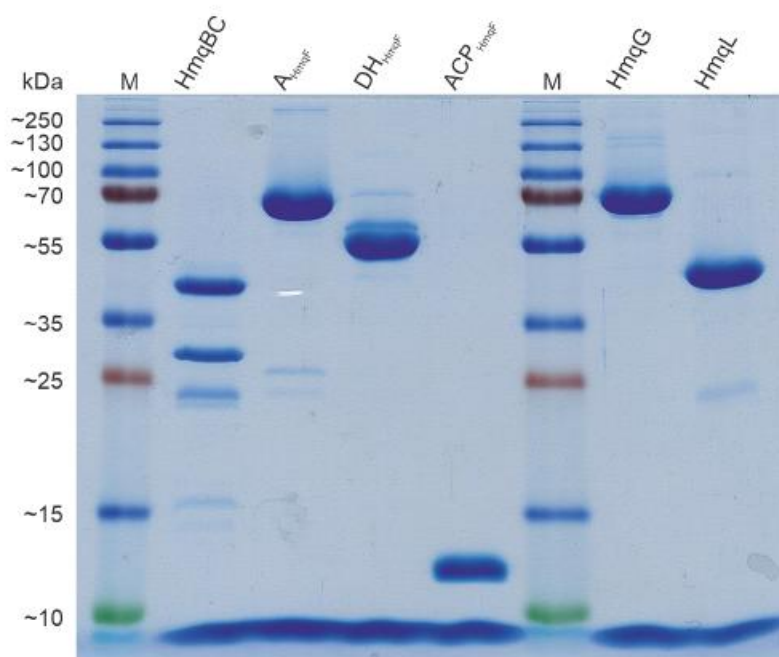


Figure S1: SDS PAGE of Hmq proteins. Loaded are 8 μ g total protein per lane on a discontinuous 12.5% gel. Theoretical masses are HmqB – 30.3 kDa, HmqC – 42.8 kDa, Adenylation domain of HmqF (A_{HmqF}) – 72 kDa, dehydrogenase domain of HmqF (DH_{HmqF}) – 60.6 kDa, acyl carrier protein domain of HmqF (ACP_{HmqF}) – 11.9 kDa, HmqG – 79.5 kDa, HmqL – 44 kDa.

Fragmentation of 2-ABA and 2-ABP

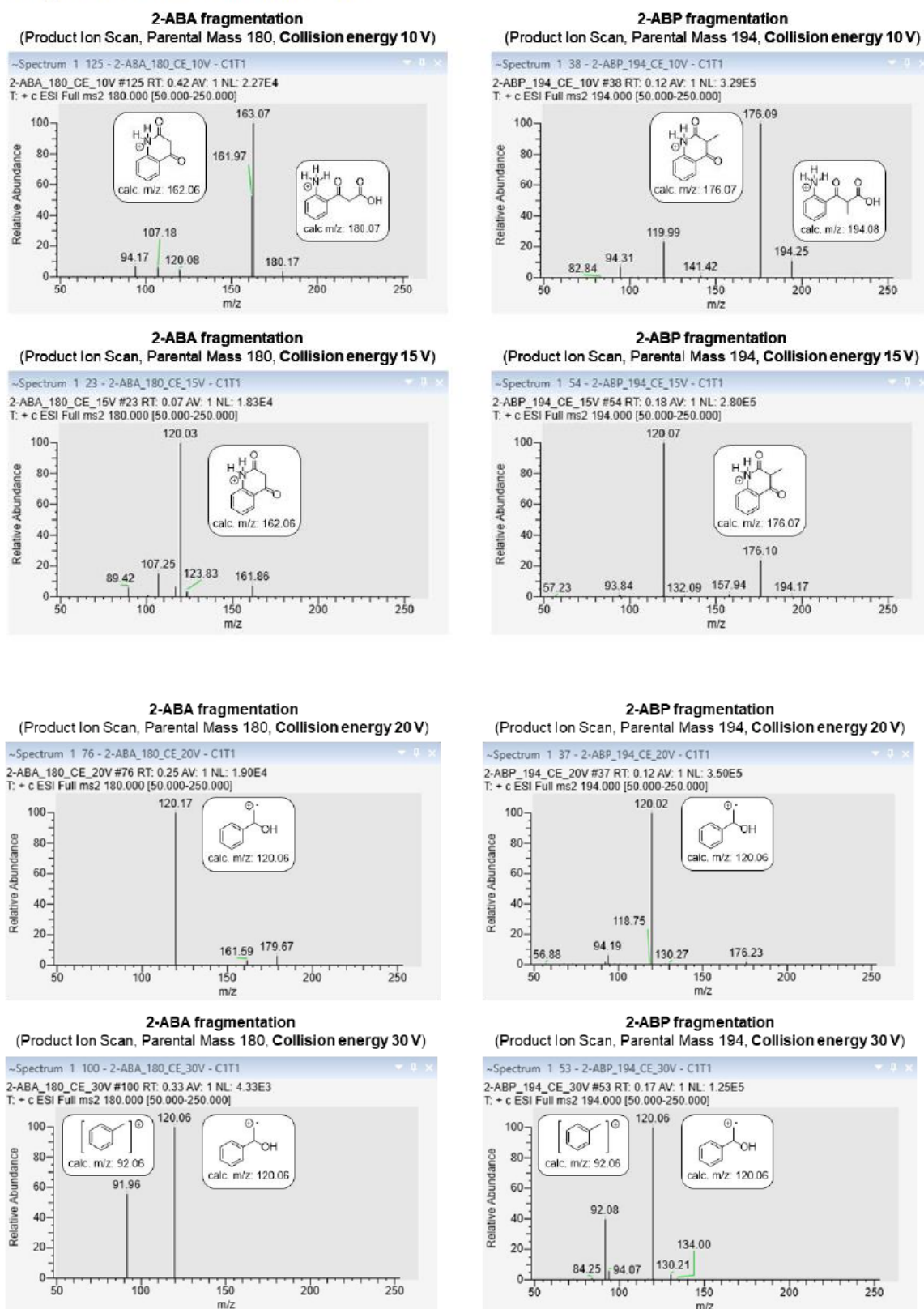


Figure S2. Fragmentation of 2-ABA and 2-ABP. MS/MS fragmentation pattern and proposed structures of fragment ions of synthetic 2-ABA (left column) and enzymatic 2-ABP (right column) at different collision energies (10 V, 15 V, 20 V, or 30 V).

LC-HRMS of derivatized 2-HABP

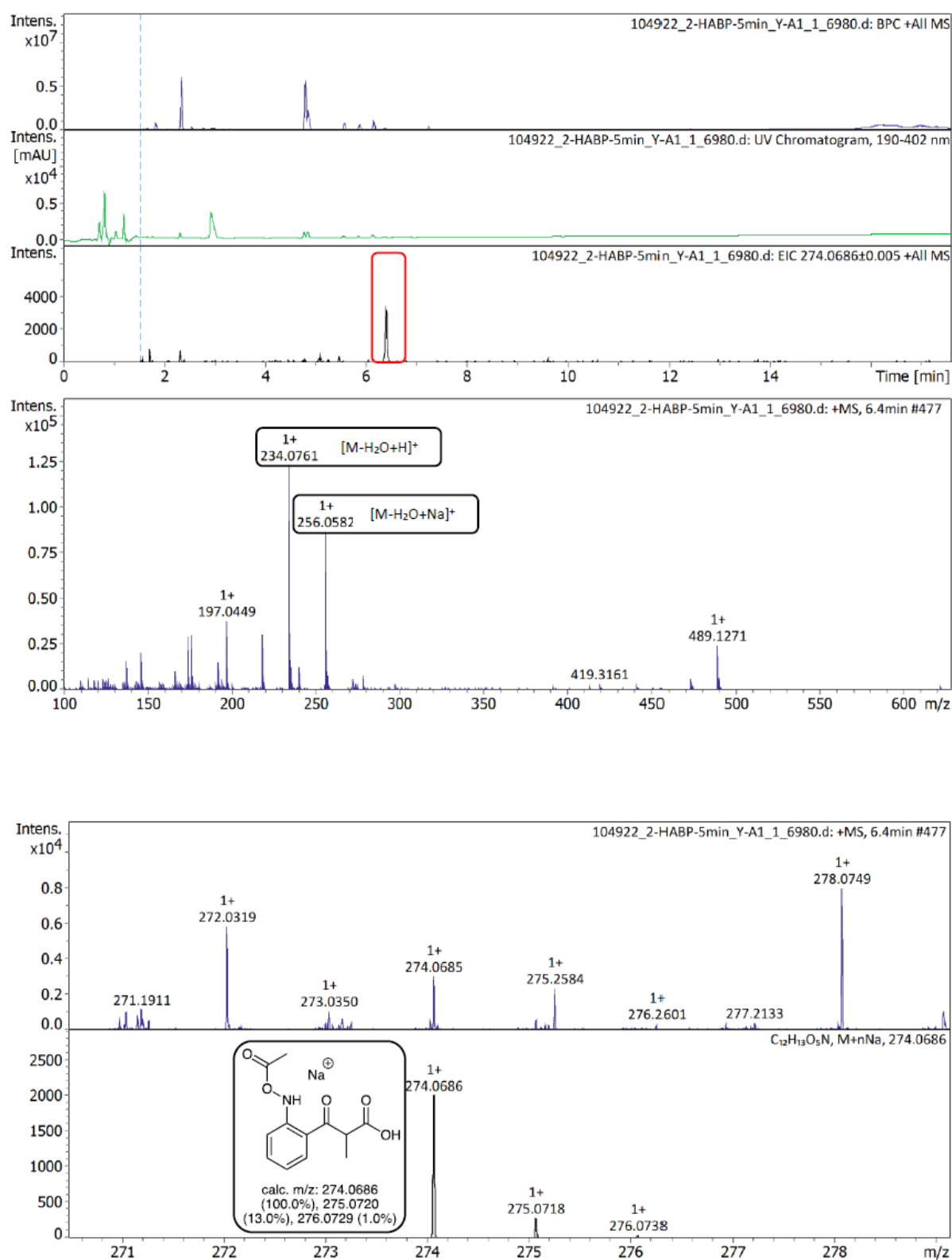


Figure S3: LC-HRMS of derivatized, enzymatically produced 2-HABP.

AlphaFold models and predictions of HmqBC / HmqBC – HmqF

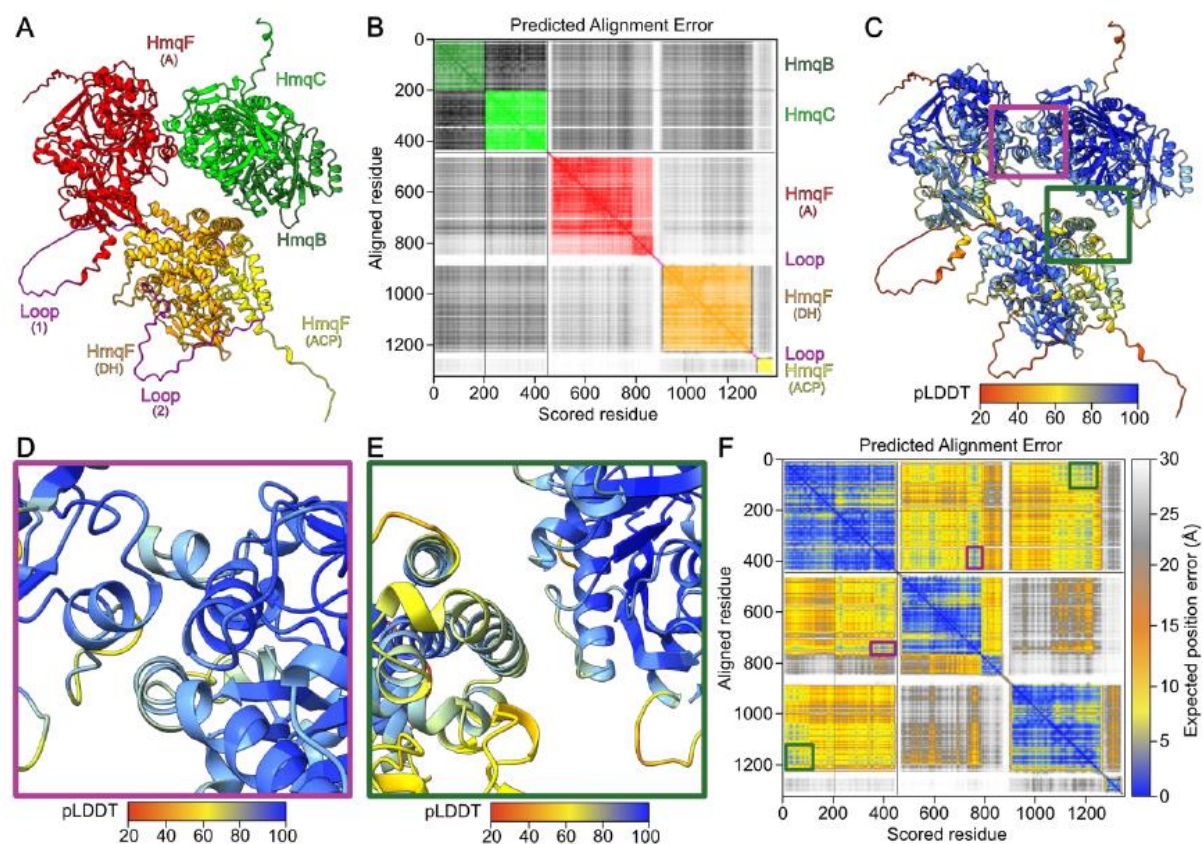


Figure S4: AlphaFold2 structure prediction of the HmqF-HmqBC complex. (A) Overview of the AlphaFold2 predictions. HmqB and HmqC are colored dark green and light green, respectively. A_{HmqF} , DH_{HmqF} , and ACP_{HmqF} domains are colored in red, orange, and yellow, respectively. Two linker regions are highlighted in magenta. (B) PAE (predicted align error) plot for each individual protein domain. (C) Model of the HmqBCF complex colored by pLDDT (predicted local distance difference test) score. Possible interaction interfaces between HmqF and HmqBC are marked by boxes. (D) Close-up of the interface between A_{HmqF} and HmqC. (E) Close-up of the interface between DH_{HmqF} and HmqB. (F) PAE plot with highlighted interaction regions of HmqF and HmqBC.

Proposed mechanism of action for HmqBC

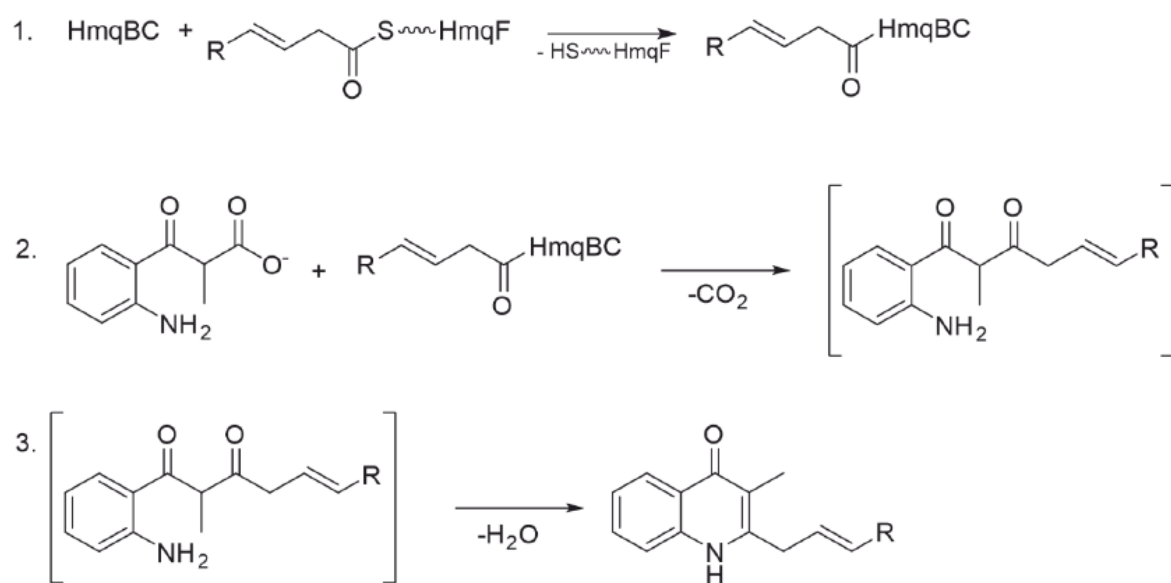


Figure S5: Reaction mechanism of HmqBC. Proposed mechanism of the reaction between fatty acyl-loaded HmqF and HmqBC toward the formation of MAQ. The winding line indicates the phosphopantetheine moiety attached to ACP_{HmqF}.

Protein mass spectrometry

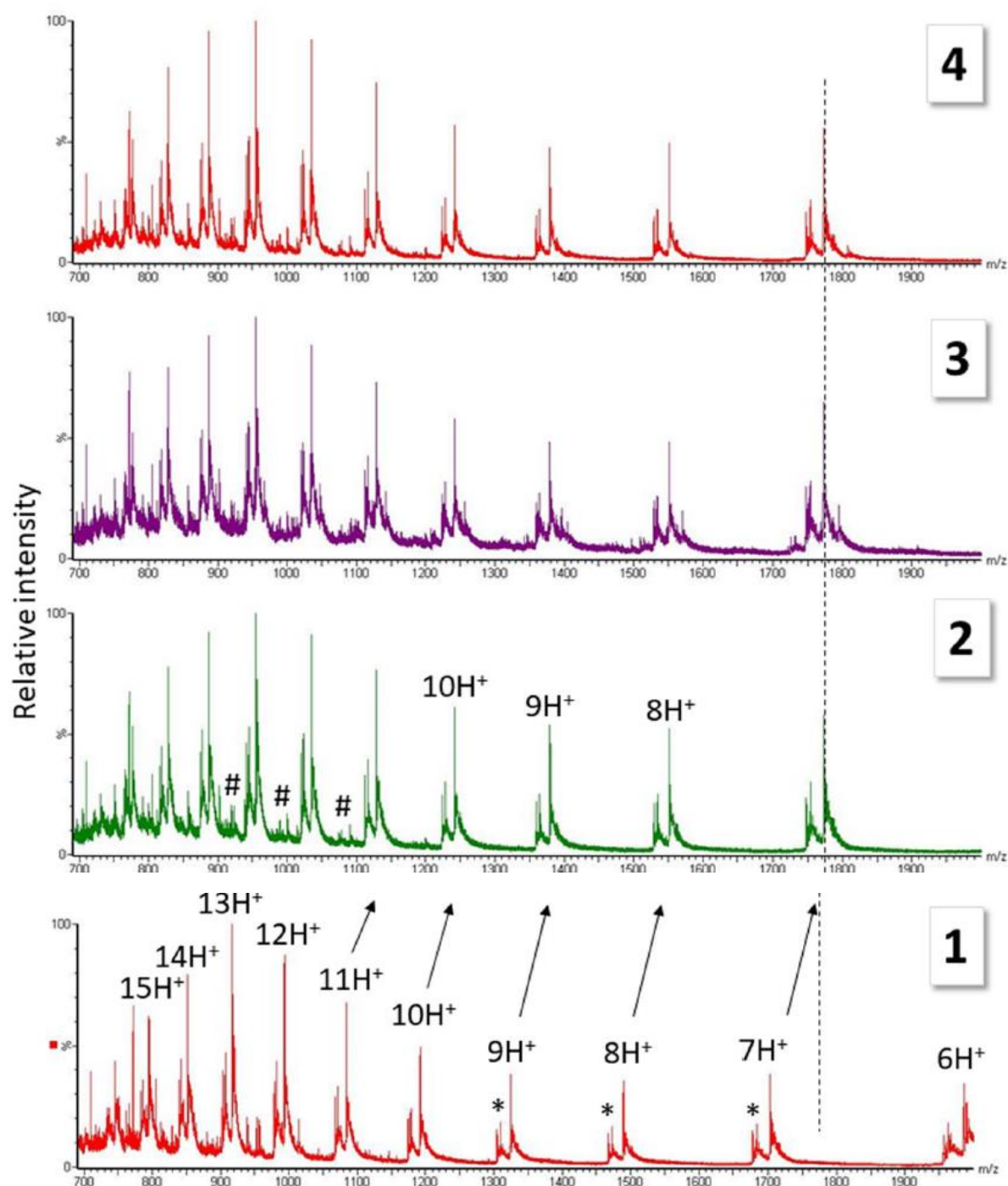


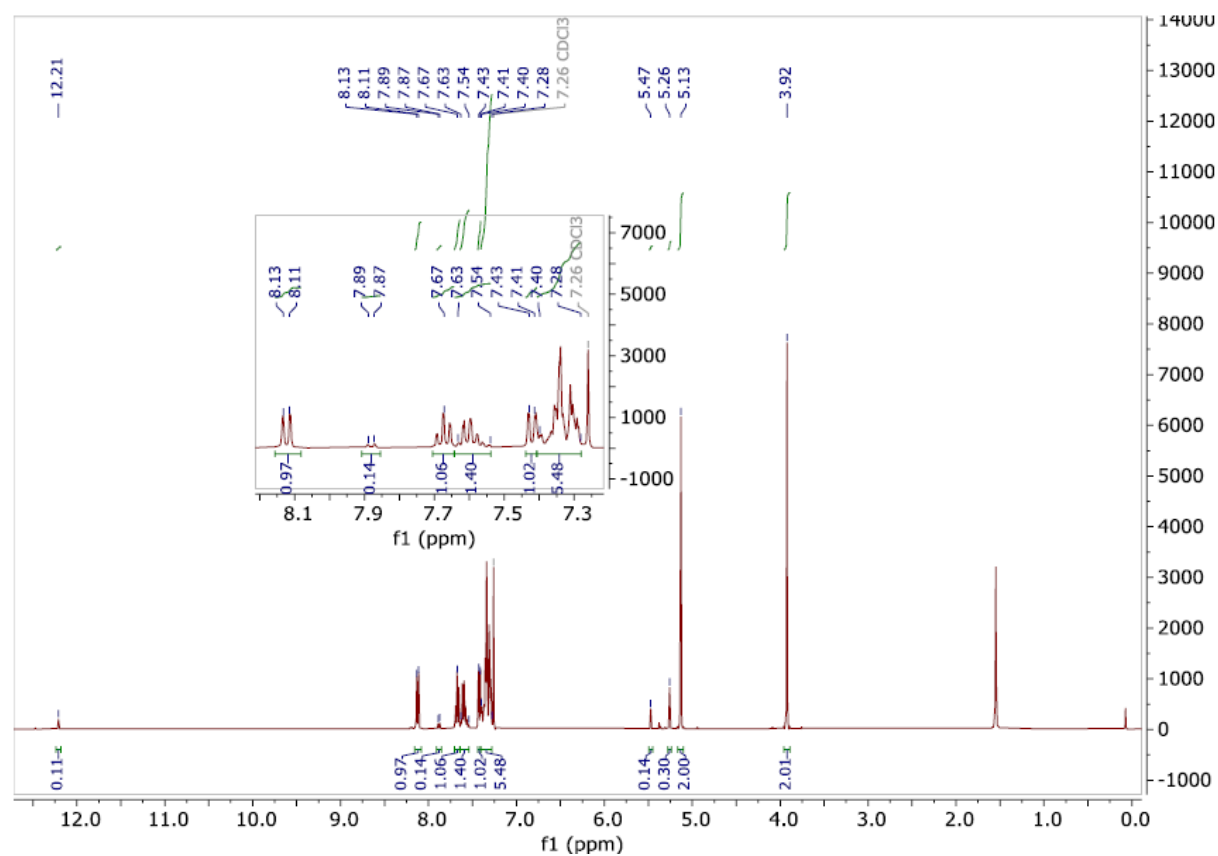
Figure S6: Protein mass spectrometric analysis of ACP_{HmqF} . Mass spectrometric analysis of ACP_{HmqF} (1) and ACP_{HmqF} loaded with decanoic acid (2), *trans*-2-decanoic acid (3) or *trans*-3-decanoic acid (4). The labels (e.g. $10H^+$) indicate the charge state distribution, which is shifted for the modified proteins (2-4) as indicated by the arrows and further visualized by a vertical dotted line. Stars point to satellite peaks generated by protein forms of slightly lower mass than the main species. Crosses highlight peaks, which likely originate from residues of unmodified protein.

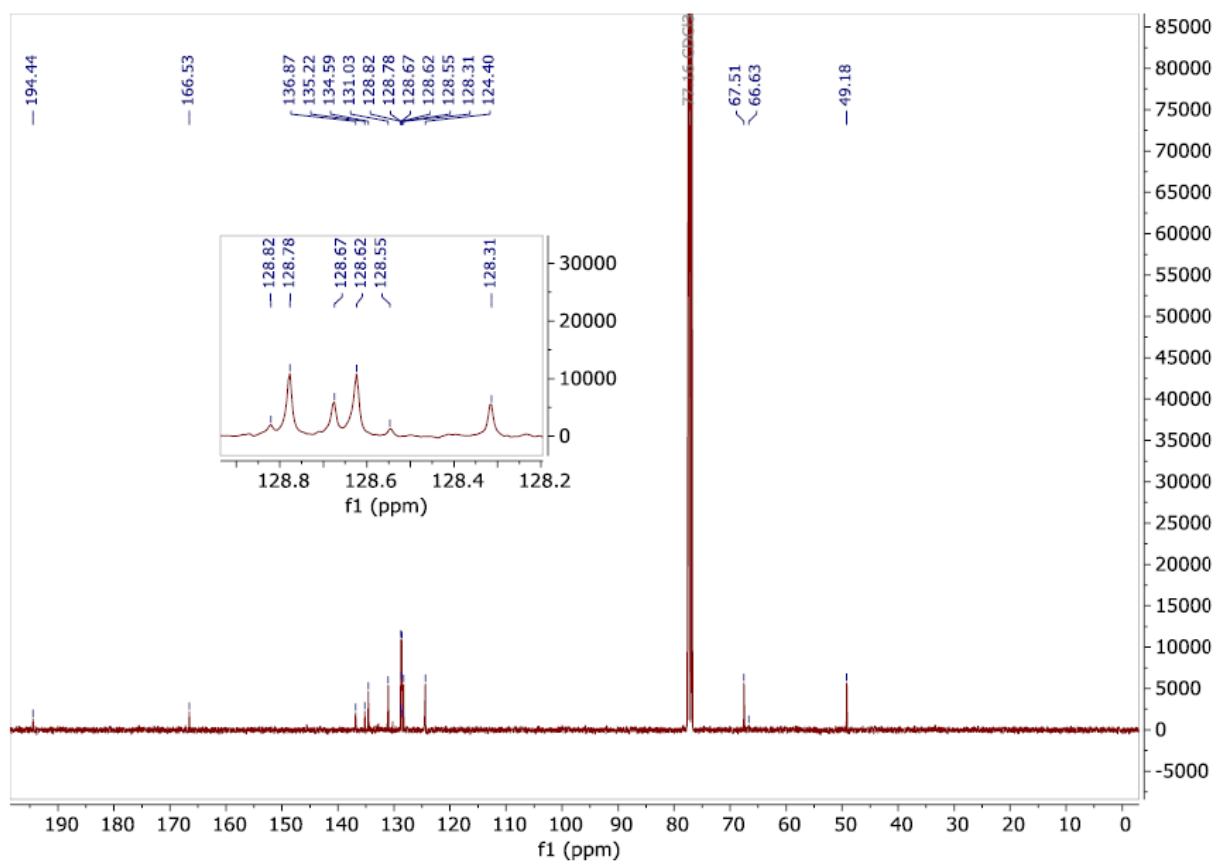
Supplemental Data

Data S1: Nuclear magnetic resonance and (high-resolution) mass spectrometry spectra

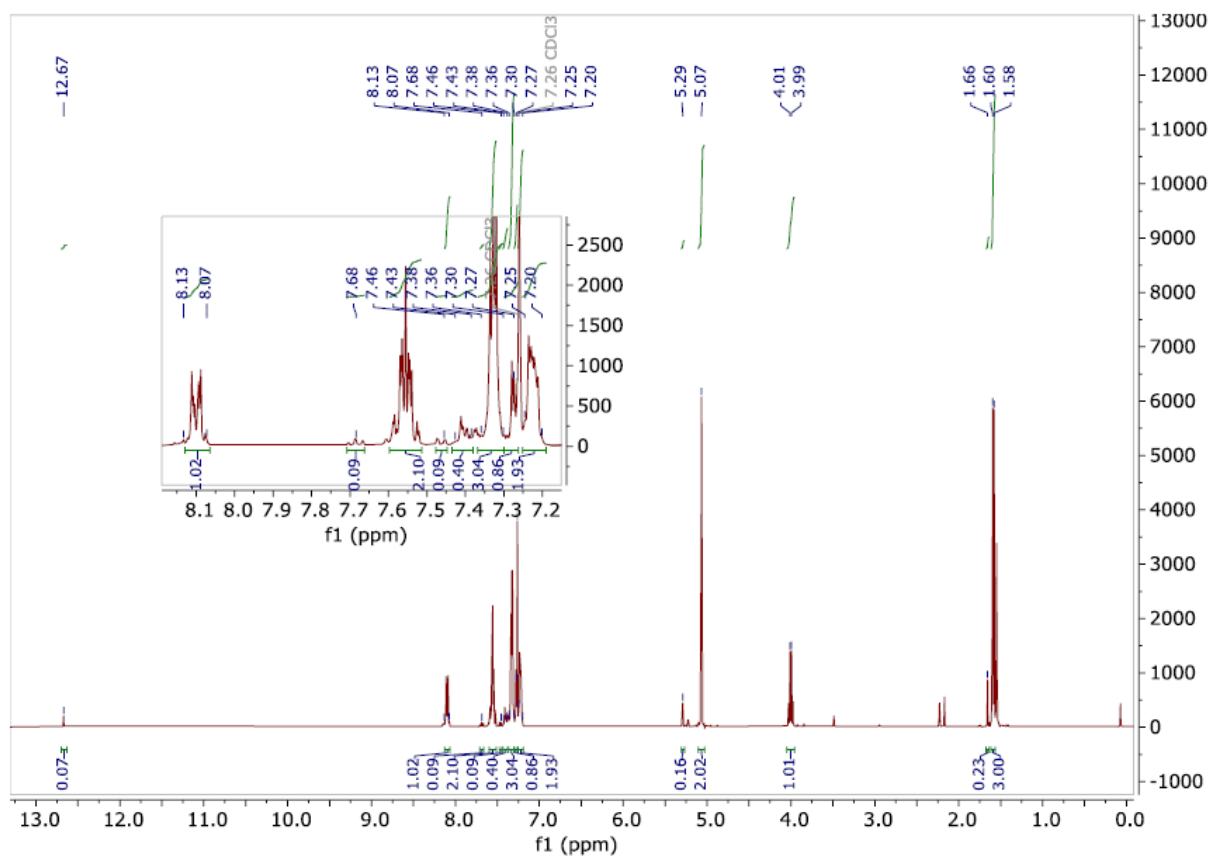
NMR spectra

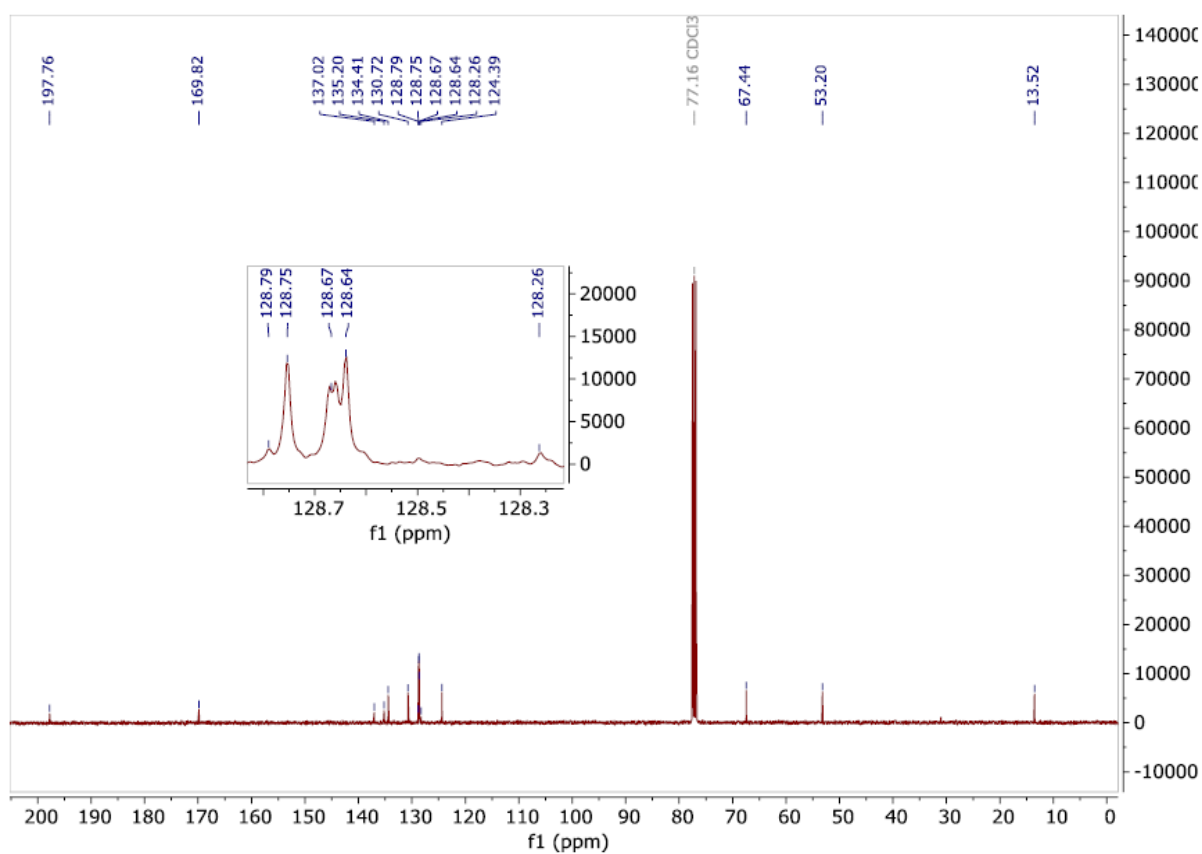
Benzyl 2-nitrobenzoyl acetate (**1a**) ^1H -NMR (above) and ^{13}C -NMR (below) in CDCl_3



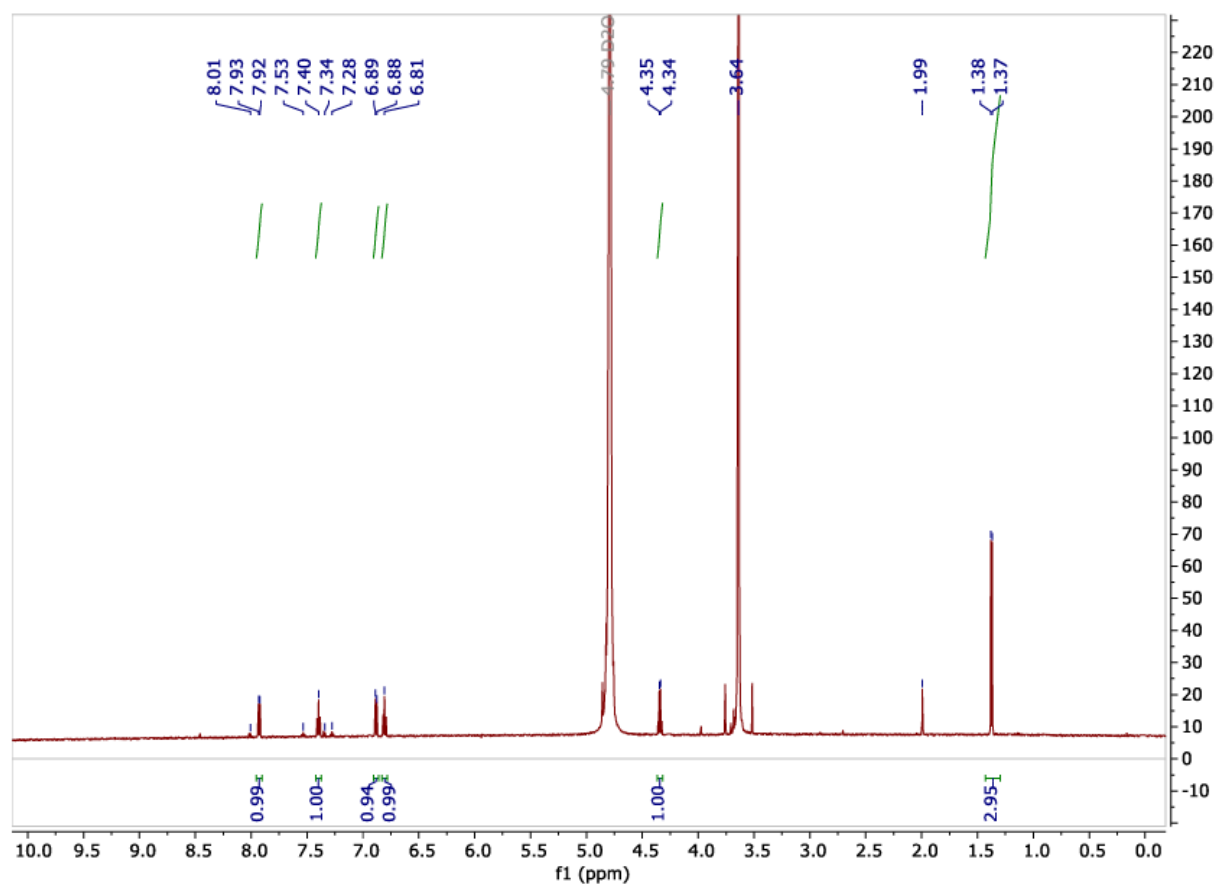


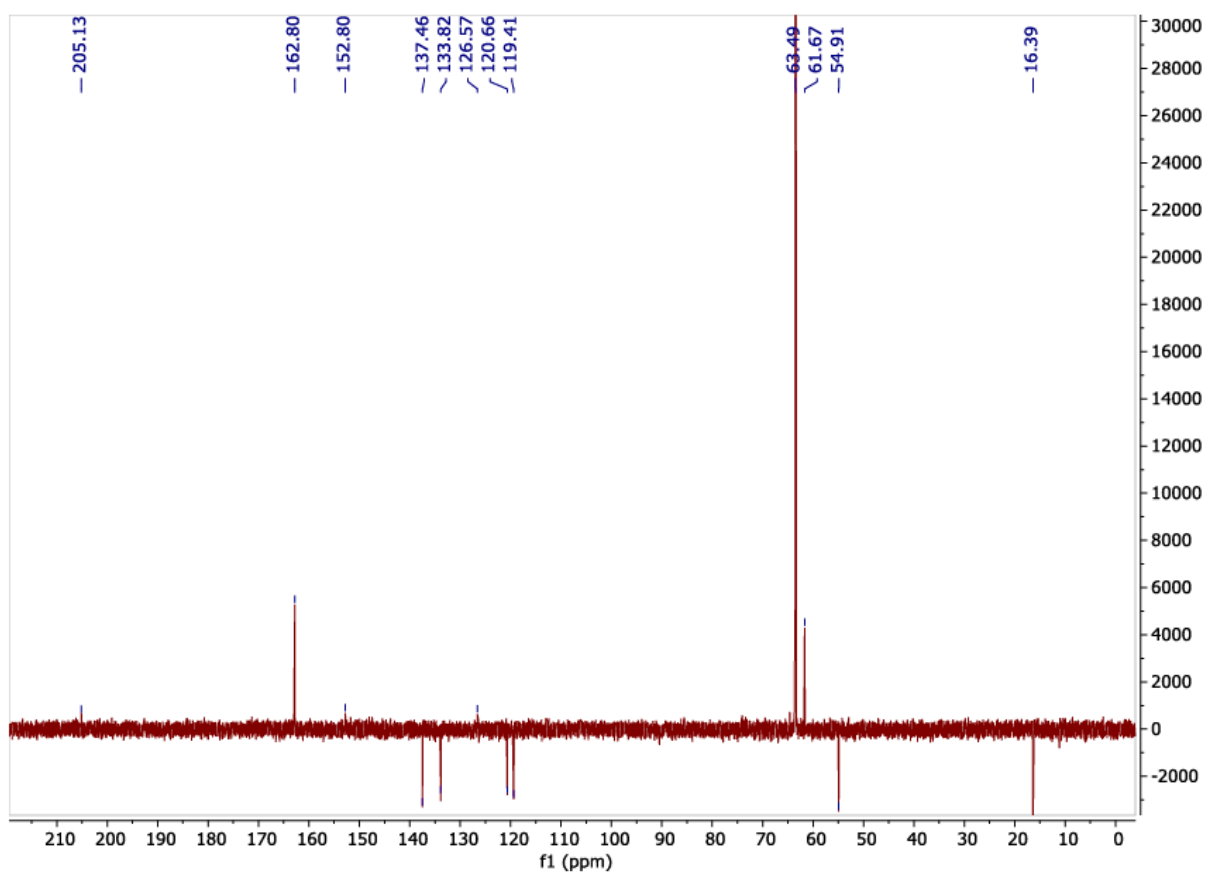
Benzyl 2-(2'-nitrobenzoyl) propionate (**1b**) ¹H-NMR (above) and ¹³C-NMR (below) in CDCl₃





2-(2'-aminobenzoyl) propionate (2-ABP) (**1c**) ^1H -NMR (above) and ^{13}C -NMR (below) in D_2O (25 mM NH_4HCO_3 , pH 8.25)

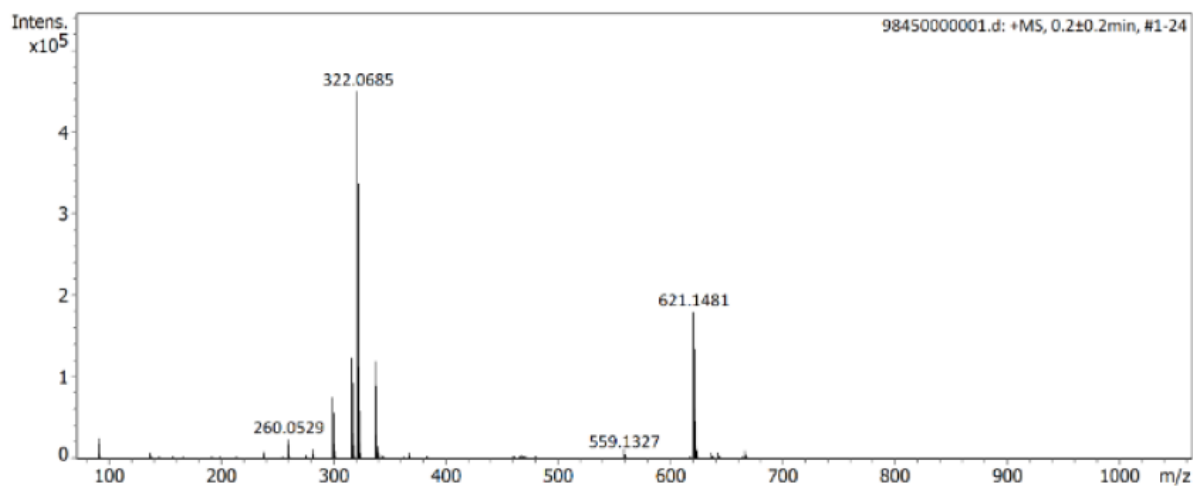


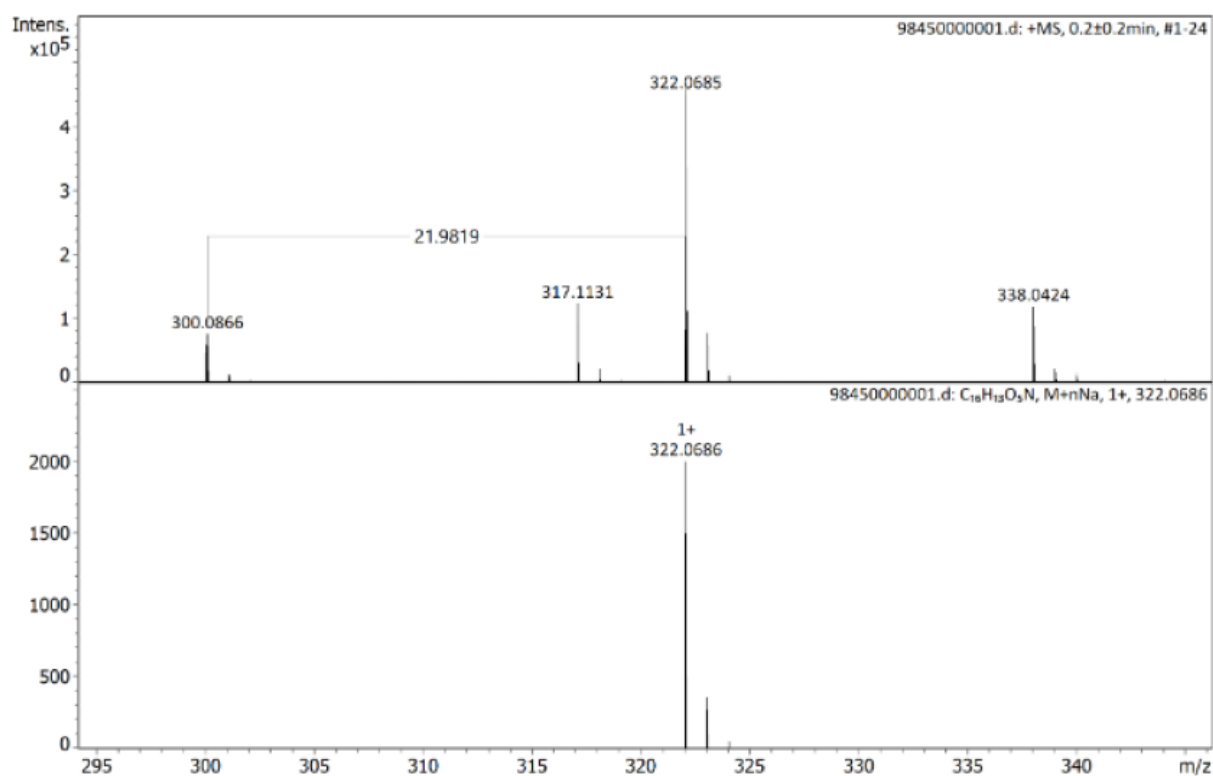


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Mass spectra of small molecules

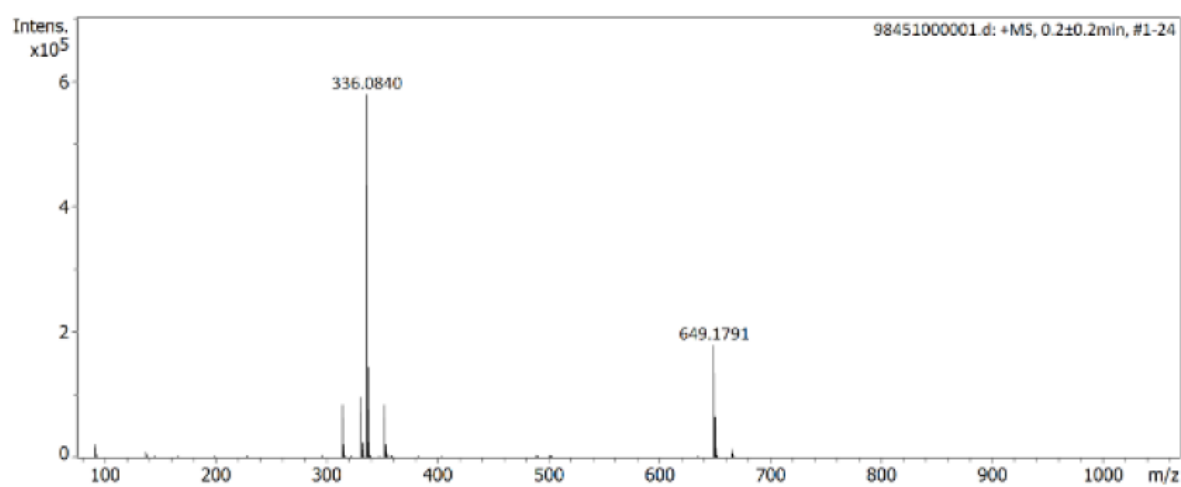
Benzyl 2-nitrobenzoyl acetate (**1a**)

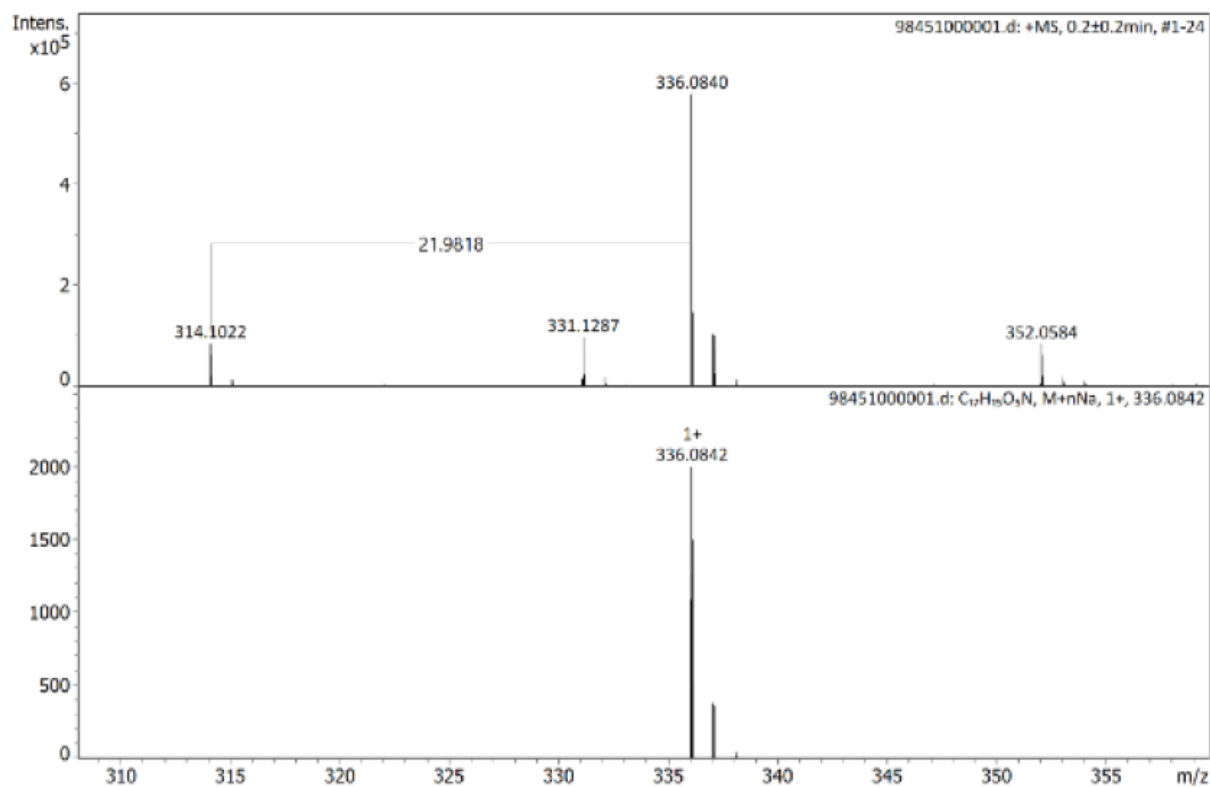




12

Benzyl 2-(2'-nitrobenzoyl) propionate (**1b**)

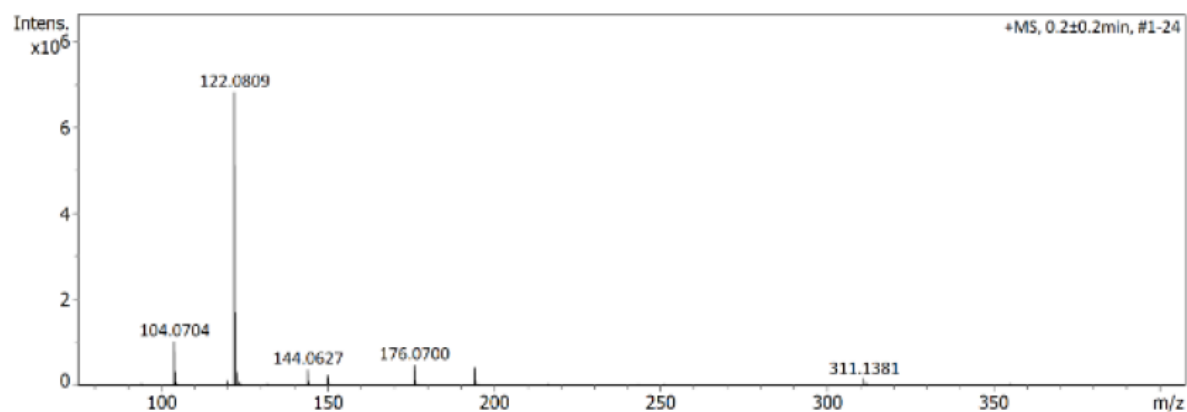


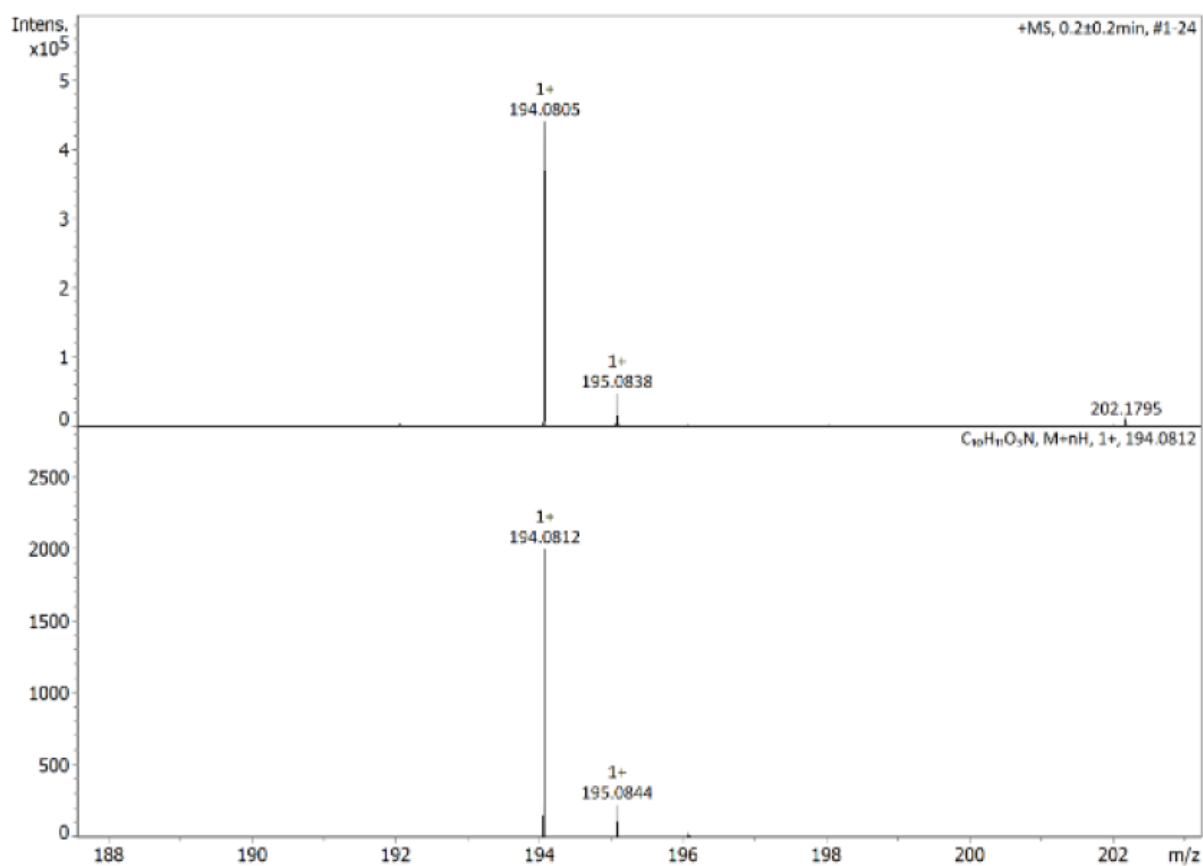


13

Synthetic 2-(2'-aminobenzoyl) propionate (2-ABP) (**1c**)

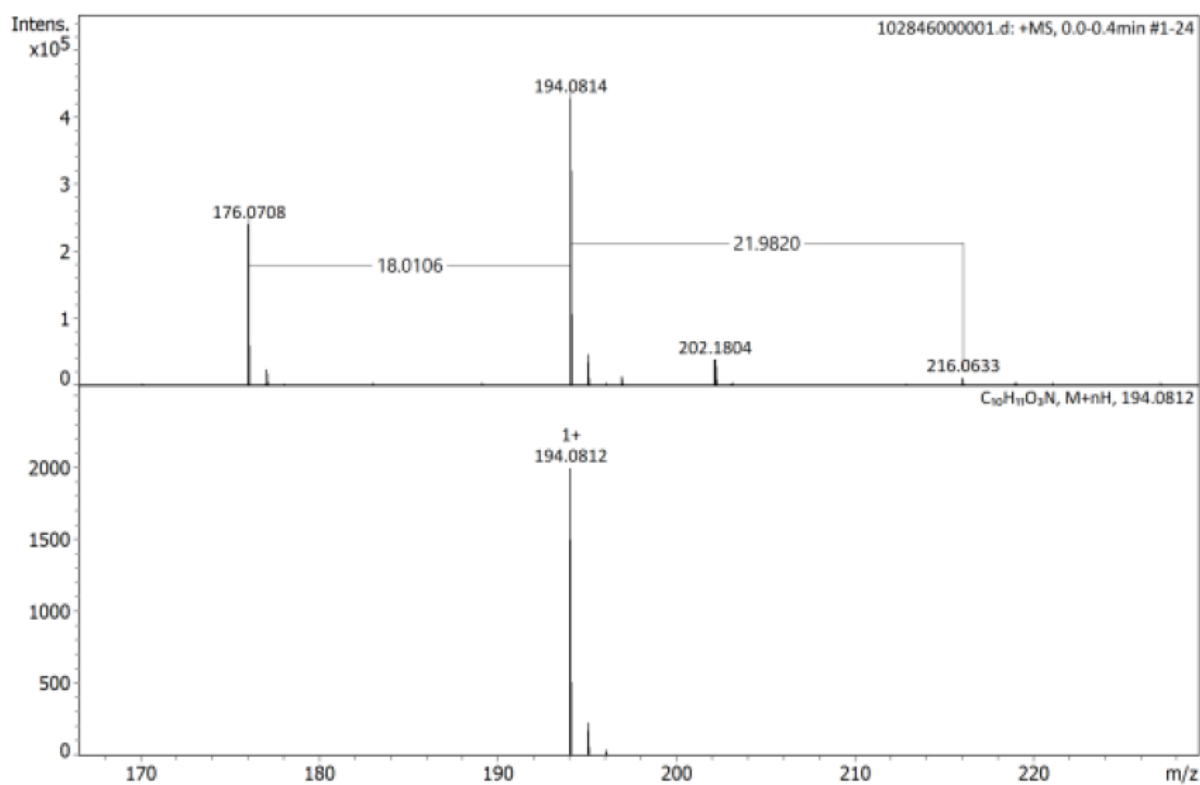
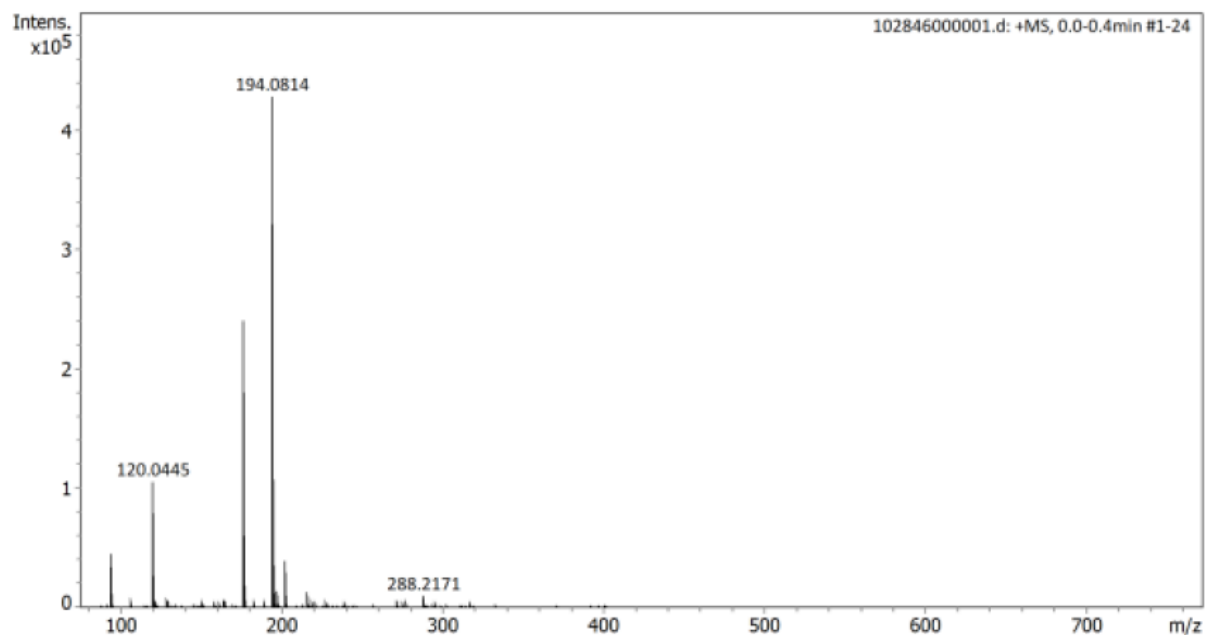
Note: sample solvent system is 93% 10 mM ammonium bicarbonate (pH 8.7) and 7% acetonitrile





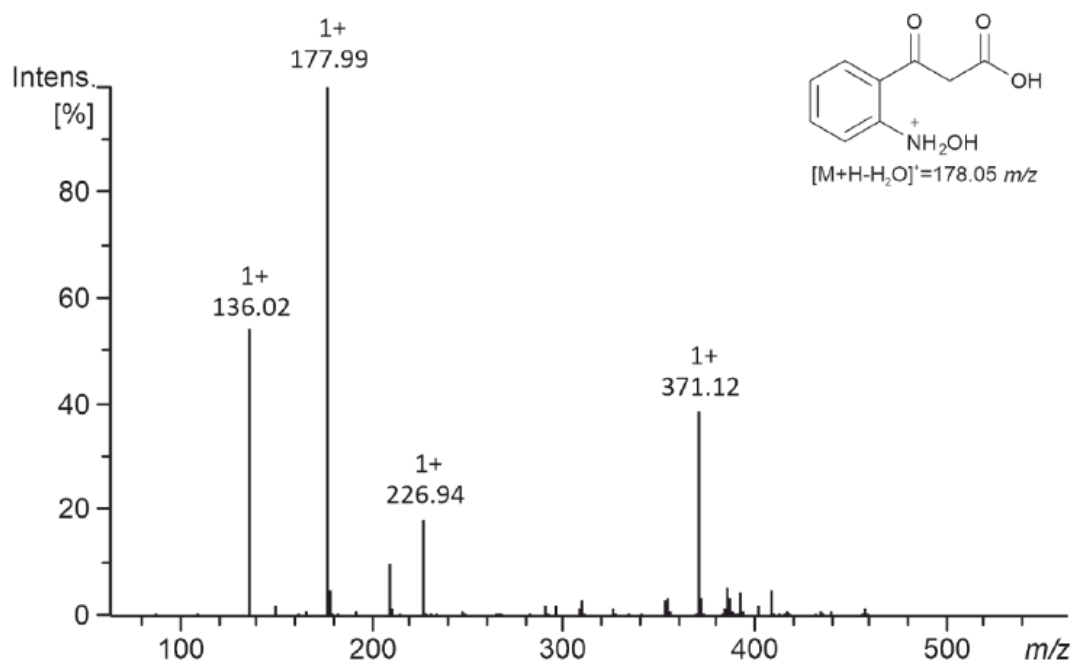
Enzymatic 2-(2'-aminobenzoyl) propionate (2-ABP) (**1c**)

Note: sample solvent system is 93% 10 mM ammonium bicarbonate (pH 8.7) and 7% acetonitrile



Low Resolution MS spectrum of enzymatically produced 3-(2-(hydroxylamino)phenyl)-3-oxopropanoic acid (2-hydroxylaminobenzoylacetate, 2-HABA)

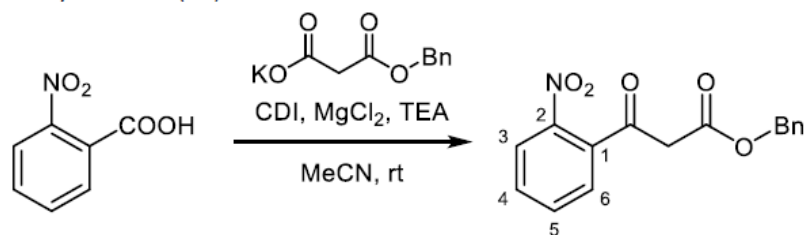
Note: sample solvent system is 95% 10 mM ammonium bicarbonate (pH 8.7) and 5% acetonitrile



Supplemental experimental procedures

Synthesis

Benzyl 2-nitrobenzoyl acetate (**1a**)



Compound **1a** was synthesized based on the procedure described by Šenica *et. al.*^[S1].

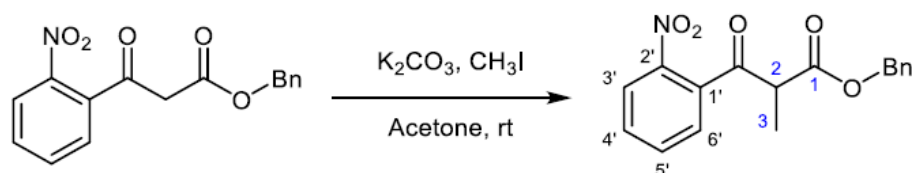
2-nitrobenzoic acid (845.0 mg, 5.06 mmol) was dissolved in acetonitrile (10 mL) and carbonyldiimidazole (CDI) (901.9 mg, 5.56 mmol) was added. The resulted mixture was stirred for 1 h at room temperature. Benzyl potassium malonate (1.76 g, 7.58 mmol) and MgCl_2 (519.9 mg, 5.46 mmol) were dissolved in acetonitrile (25 mL) and stirred for 30 min at room temperature. Triethylamine (TEA) (881.6 μL , 6.32 mmol) was added to this mixture and stirred for 30 min at room temperature. The activated 2-nitrobenzoic acid was added dropwise to the formed slurry and stirred for 22 h at room temperature. The majority of acetonitrile was evaporated and the residue was diluted with ethyl acetate, washed with sat. KHSO_4 , sat. NaHCO_3 , and brine solution. The organic phase was dried over MgSO_4 , filtered, and concentrated *in vacuo*. The residue was purified by silica column chromatography (ethyl acetate/petroleum ether 1:2, R_f = 0.42). Product **1a** was obtained as a slightly yellow oil (583.0 mg, 1.95 mmol, 38.6 %, mixture of keto- and enol- tautomers (7:1)).

$^1\text{H-NMR}$ (CDCl_3 , 400 MHz) δ (mixture of keto- and enol-tautomers (7:1), ppm): 3.92 (s, 2H, $\text{CO-CH}_2\text{-CO}$), 5.13 (s, 2H, $\text{O-CH}_2\text{-C}_6\text{H}_5$), 5.26 (s, 0.30H, $\text{O-CH}_2\text{-C}_6\text{H}_5$ (enol-form)), 5.47 (s, 0.14H, COH=CH-CO (enol-form)), 7.28-7.40 (m, 5.48H, $\text{O-CH}_2\text{-C}_6\text{H}_5$, both forms), 7.42 (dd, 1H, J = 7.6, 1.6 Hz, H-6), 7.54-7.63 (m, 1.40H, H-4, both forms), 7.67 (td, 1H, J = 7.5, 1.3 Hz, H-5), 7.88 (dd, 0.14H, J = 7.8, 1.5 Hz, H-3 (enol-form)), 8.12 (dd, 1H, J = 8.2, 1.3 Hz, H-3), 12.21 (s, 0.11H, COH (enol-form)).

$^{13}\text{C-NMR}$ (CDCl_3 , 101 MHz) δ (ppm): 49.18 ($\text{CO-CH}_2\text{-CO}$), 66.63 ($\text{O-CH}_2\text{-C}_6\text{H}_5$, (enol-form)), 67.51 ($\text{O-CH}_2\text{-C}_6\text{H}_5$), 92.06 (COH=CH-CO (enol-form), visible in HSQC-NMR), 124.40 (C-3), 124.57 (C-3 (enol-form), visible in HSQC-NMR), 128.31 (C-6), (128.55, 128.62, 128.67, 128.78, 128.82) (tertiary carbons of C_6H_5 , both forms), 131.03 (C-4), 134.59 (C-5), 135.22 (quaternary carbon of C_6H_5), 136.87 (C-2), 166.53 (ester), 194.44 (C1-CO).

ESI-HRMS: m/z = 300.0866 $[\text{M}+\text{H}]^+$, calc. for $\text{C}_{16}\text{H}_{13}\text{O}_5\text{N} + \text{H}^+ = 300.0867$.

Benzyl 2-(2'-nitrobenzoyl) propionate (**1b**)



Compound **1b** was synthesized based on the procedure described by Kitahara *et. al.*^[S2].

Benzyl 2-nitrobenzoyl acetate (**1a**) (162.5 mg, 543.0 μmol) was dissolved in anhydrous acetone (2 mL) under nitrogen atmosphere. K_2CO_3 (67.5 mg, 488.7 μmol) was added to the mixture and stirred at room temperature for 10 min. Subsequently, iodomethane (37.2 μL , 597.3 μmol) was added dropwise and the resulting mixture was refluxed for 4 h. After the reaction was completed, the mixture was cooled to room temperature and diethyl ether was added (10 mL). The mixture was filtered and organic solvent evaporated *in vacuo*. The residue was purified portionwise by prep. RP-HPLC (A: H_2O , B: MeOH; 5% B to 100% B) (SPOT PREP Liquid Chromatography by Armen Instrument; Dr. Maisch

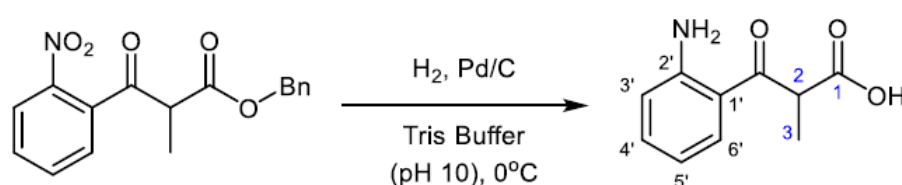
column ReproSil 100 C18 250 x 20 mm, 10µm). Product **1b** was obtained as a slightly yellow oil (112.5 mg, 359.1 µmol, 66.1 %).

¹H-NMR (CDCl₃, 400 MHz) δ (mixture of keto- and enol-tautomers (12:1), ppm): 1.59 (d, 3H, *J* = 7.1 Hz, H-3), 1.66 (s, 0.23H, H-3 (enol-form)), 4.00 (q, 1H, *J* = 7.1 Hz, H-2), 5.07 (s, 2H, O-CH₂-C₆H₅), 5.29 (s, 0.16H, O-CH₂-C₆H₅ (enol-form)), (7.20-7.25 (m, 2H), 7.30-7.36 (m, 3H)) (O-CH₂-C₆H₅), 7.27 (m, 1H, H-6'), 7.38-7.43 (m, 0.40H, O-CH₂-C₆H₅ (enol-form)), 7.46 (dd, 0.09H, *J* = 7.6, 1.5 Hz, H-4' (enol-form)), 7.56 (m, 2H, H-4' and H-5'), 7.68 (m, 0.09H, H-5' (enol-form)), 8.07-8.13 (m, 1H, H-3'), 12.67 (s, 0.7H, COH (enol-form)).

¹³C-NMR (CDCl₃, 101 MHz) δ (ppm): 13.52 (C-3), 53.20 (C-2), 67.44 (O-CH₂-C₆H₅), 124.39 (C-3'), 128.26 (C-6'), (128.64, 128.66, 128.67, 128.75, 128.79) (tertiary carbons of C₆H₅, both forms), 130.72 (C-4'), 134.41 (C-5'), 135.20 (quaternary carbon of C₆H₅), 137.02 (C-2'), 169.82 (C-1), 197.76 (C1'-CO).

ESI-HRMS: *m/z* = 314.1022 [M+H]⁺, calc. for C₁₇H₁₅O₅N + H⁺ = 314.1023.

2-(2'-aminobenzoyl) propionate (2-ABP) (**1c**)



Compound **1c** was synthesized based on a procedure described by Drees *et al.*^[53]. Compound **1b** (3.0 mg, 9.58 µmol) was dissolved in 200 µL acetonitrile, followed by addition of 1.8 mL of aqueous tris buffer (100 mM, pH 10.0). The mixture was transferred to a gas tight glass vessel together with palladium on activated charcoal (1 mg) and a magnetic stirrer. Subsequently, the vessel was placed under a constant stream of hydrogen gas with an overpressure of 0.5 bar and stirred on ice for 30 min. The resulting mixture was centrifuged (10000 x *g*, 5 min, 4 °C) and filtered through a 0.45 µm nylon syringe filter. Compound **1c** was purified by semi-preparative RP-HPLC (A: 10 mM ammonium bicarbonate pH 8.7 (adjusted with ammonia), B: acetonitrile; isocratic 7 % B over 7 min, followed by 5 min 80 % B and 5 min 7 % B at a constant flow rate of 2.2 mL min⁻¹; Agilent 1100 series; Eurospher II 5 µm C18 100 Å LC column 250 x 5 mm, Knauer). Fractions containing compound **1c** were pooled, frozen in liquid nitrogen and lyophilized. Product **1c** was obtained as a slightly yellow powder, in a mixture with tris and NH₄HCO₃ salts. In order to prevent the compound's degradation, it has to be obtained and characterized under basic conditions (pH > 8.0).

¹H-NMR (D₂O (25 mM NH₄HCO₃, pH 8.25) 600 MHz) δ (ppm): 1.37 (d, 3H, *J* = 7.0 Hz, H-3), 4.34 (q, 1H, *J* = 7.0 Hz, H-2), 6.81 (ddd, 1H, *J* = 8.2, 7.0, 1.2 Hz, H-5'), 6.88 (dd, 1H, *J* = 8.3, 1.2 Hz, H-3'), 7.40 (ddd, 1H, *J* = 8.4, 7.0, 1.5 Hz, H-4'), 7.93 (dd, 1H, *J* = 8.2, 1.5 Hz, H-6'); (1.99, 7.28, 7.34, 7.53, 8.01—enol-tautomer in very low concentration); 3.64 (CH₂-groups of tris).

¹³C-NMR (D₂O (25 mM NH₄HCO₃, pH 8.25) 151 MHz) δ (ppm): 16.39 (C-3), 54.91 (C-2), 61.67 (quaternary carbon of tris), 63.49 (CH₂-groups of tris), 119.41 (C-5'), 120.66 (C-3'), 126.57 (C-1'), 133.82 (C-6'), 137.46 (C-4'), 152.80 (C-2'), 162.80 (NH₄HCO₃), 182.42 (C-1, visible in HSQC-NMR), 205.13 (C1'-CO).

ESI-HRMS: *m/z* = 194.0805 [M+H]⁺, calc. for C₁₀H₁₁O₃N + H⁺ = 194.0812.

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3 Discussion and Conclusion

The aim of this thesis was to expand our understanding of bacterial quinolone biosynthesis, discover new classes of these 2-alkyl-4-quinolones (AQs), and get hints into their biological roles in pathogenic bacteria.

Within the first project presented (Publication 1), we could combine organic synthesis, microbiology, and analytical chemistry to identify a new class of AQs produced by human pathogen *Pseudomonas aeruginosa*. For the first time, we have synthesized 2'-hydroxy- and 2'-oxo-AQs and their *N*-oxides, using a new synthesis strategy, as the classical quinolone cyclization methods failed. By using LC-MS/MS techniques we could demonstrate that 2'-OH-NQ and 2'-OH-NQNO are produced naturally by *P. aeruginosa* PA14 and PAO1, whereas the 2'-oxo counterparts were not detected. Remarkably, 2'-OH-NQ occurs at levels similar to those of NQ — one of the main quinolones of *P. aeruginosa*. By collecting bacterial supernatants every 3 h of incubation time, we showed that 2'-OH-NQ and 2'-OH-NQNO production is time-dependent, peaking at 12 h.

Our fatty acids feeding experiments revealed that respective exogenous fatty acids can substantially increase 2'-OH-NQ and 2'-OH-NQNO generation in *P. aeruginosa*. Specifically, the addition of 50 μ M (final concentration) of β -hydroxydecanoic acid to cultures of *P. aeruginosa* PAO1 resulted in a 5.8-fold and 5.5-fold rise in the production of 2'-OH-NQ and 2'-OH-NQNO, respectively. However, supplying even higher concentrations of β -hydroxydecanoic acid (up to 200 μ M final) did not result in further boosts in the production of these hydroxylated quinolones. To explore the potential direct integration of β -hydroxydecanoic acid into quinolone biosynthesis, we conducted another feeding experiment in *P. aeruginosa* PAO1 using 100 μ M of the deuterated analog of β -hydroxydecanoic acid. Through LC-MS/MS analysis of the $[M+1\text{Da}+H]^+$ signal for 2'-OH-NQ and 2'-OH-NQNO, we observed a 4.2-fold and 4.6-fold increase in the production of the parent mass + 1 Da, respectively, in comparison to the DMSO control. This observation suggests that hydroxylated fatty acids are used as direct substrates for the quinolone biosynthesis.

Interestingly, feeding β -oxodecanoic acid to cultures of *P. aeruginosa* did not result in the production of respective 2'-oxo-AQs. This fact might be explained by the enzymes' kinetics and substrate preferences. Most likely the β -oxodecanoic acid is

processed further in fatty acid biosynthesis/ β -oxidation processes before it could be introduced into AQs biosynthesis by PqsBC enzyme.

Our investigations of the biological role of newly discovered 2'-OH-NQ and 2'-OH-NQNO in *P. aeruginosa* showed that they do not exhibit antibacterial effects against *Staphylococcus aureus* (MRSA) USA300 – one of the main competitors of *P. aeruginosa*. We further proceeded to examine their role in bacteria-host interaction. Notably, none of the new quinolones showed cytotoxicity up to 500 nM. We noticed a strong impact on the generation of the pro-inflammatory interleukin-8 in Caco-2 cells at just 100 nM when exposed to 2'-OH-NQ and 2'-OH-NQNO and their 2'-oxo counterparts. The highest response was observed for 2'-OH-NQ, being close to the response induced by 10 nM LPS (positive control). Remarkably, none of the respective saturated quinolones (NQ and NQNO) demonstrated any stimulation of interleukin-8 production even at concentrations as high as 500 nM. These findings show how *P. aeruginosa* can fine-tune the biological effects of produced AQs by minimum changes in their fatty side chains.

Our second project (Publication 2) could uncover even more biological functions of the multi-functional PQS (*Pseudomonas* quinolone signal) of *P. aeruginosa*. Although PQS is primarily recognized as a quorum sensing molecule in *P. aeruginosa*, it is also involved in outer membrane vesicles formation, iron trapping, and bacteria-bacteria interaction. By using HRMS analysis we could finally validate the formation of PQS-complexes like $\text{MoO}_2(\text{PQS})_2$, $\text{Al}(\text{PQS})_2^+$, and $\text{Al}(\text{PQS})_3$. Our discovery that PQS acts as a trap for Al^{3+} , MoO_4^{3-} , and VO_4^{3-} alongside Fe^{3+} indicates a vital role of PQS in the metal ion balance of *P. aeruginosa* that extends beyond just ferric iron.

Within the third project (Publication 3), we could finally complete our understanding of the 3-methyl-2-alkyl-4-quinolone (MAQ) biosynthesis pathway in quinolone-producing species of the *Burkholderia* genus (Figure 12). While AQ biosynthesis in *P. aeruginosa* was clarified much earlier, our current work has finally solved the mystery regarding the substrates accepted for methylation by HmqG and oxidation by HmqL within MAQ biosynthesis in *Burkholderia* spp.. We've revealed methylation as a highly effective initial stage in biosynthesis, where 2-ABA gets methylated into 2-ABP, which further results in the production of methylated quinolones. We could show that HmqL oxidizes 2-ABA, but not its methylated product, 2-ABP, which results in AQNO generation. Remarkably, 2-HABA (oxidized product of 2-ABA) can also be

accepted by HmqG as a substrate for methylation, thus allowing the formation of MAQNOs.

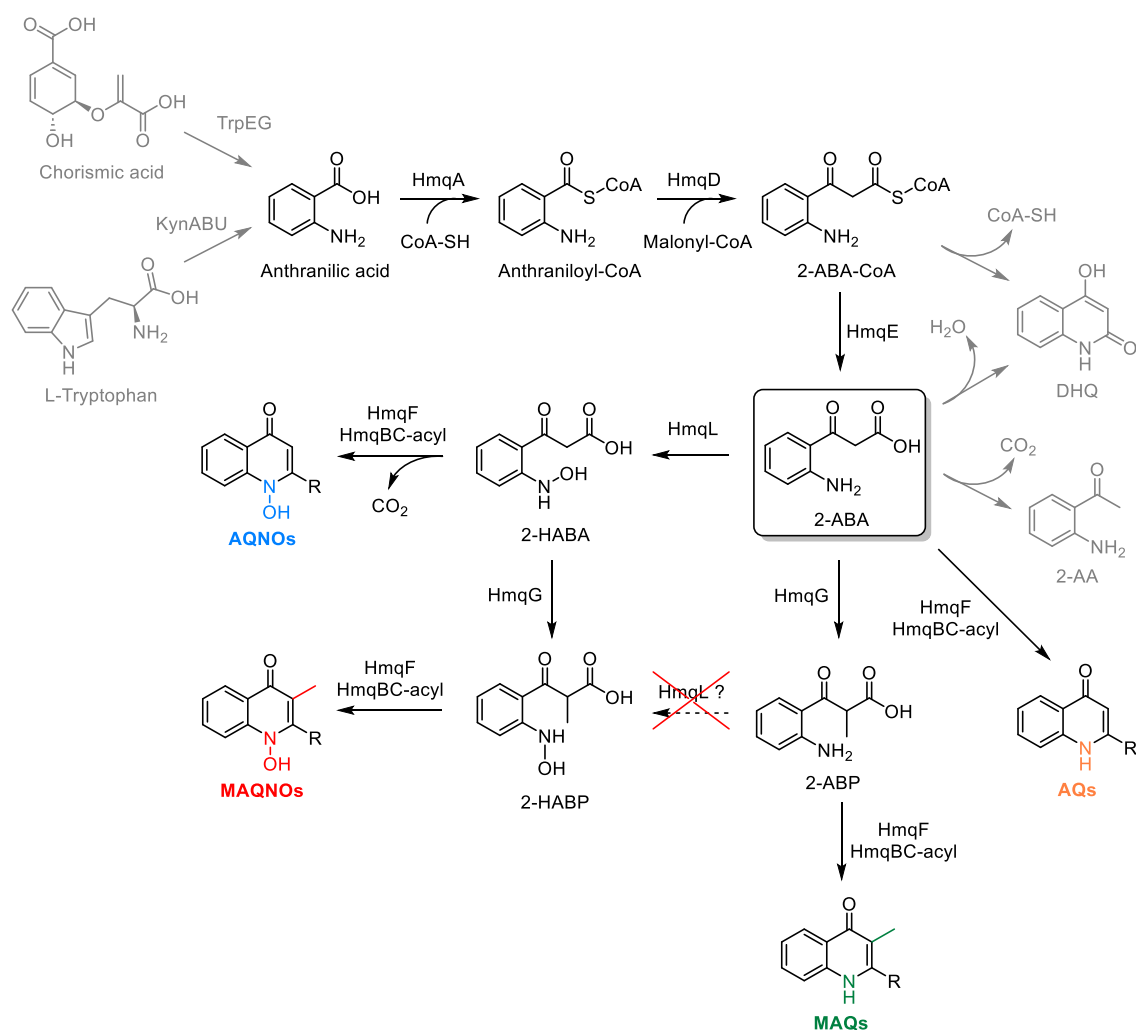


Figure 12. Completed scheme of the quinolone biosynthesis in *B. thailandensis*: 2-alkyl-4-quinolones (AQs) (orange), 2-alkyl-4-quinolone *N*-oxides (AQNOs) (blue), 3-methyl-2-alkyl-4-quinolones (MAQs) (green), and 3-methyl-2-alkyl-4-quinolone *N*-oxides (MAQNOs) (red).

Additionally, we were puzzled by the predominant production of *trans*- Δ^2 -unsaturated MAQ(NO)s by *Burkholderia*, compared to the favored *trans*- Δ^1 -unsaturation in AQ(NO) of *P. aeruginosa*. Our work could demonstrate that HmqBC not only shows a preference for 2-ABP over 2-ABA but also regulates the attached acyl component, accepting 3-decenoic and decanoic acids, but not 2-decenoic acid, which would result in quinolones with *trans*- Δ^1 -unsaturation. These findings can finally allow us to draw MAQ(NO) biosynthetic pathway in *B. thailandensis* without question marks (Figure 12).

4 Literature

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