Short-chain aurachin D derivatives are selective inhibitors of \textit{E. coli} cytochrome \textit{bd-I} and \textit{bd-II} oxidases

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Cytochrome \textit{bd}-type oxidases play a crucial role for survival of pathogenic bacteria during infection and proliferation. This role and the fact that there are no homologues in the mitochondrial respiratory chain qualify cytochrome \textit{bd} as a potential antimicrobial target. However, few \textit{bd} oxidase selective inhibitors have been described so far. In this report, inhibitory effects of Aurachin C (AurC-type) and new Aurachin D (AurD-type) derivatives on oxygen reductase activity of isolated terminal \textit{bd-I}, \textit{bd-II} and \textit{bo} oxidases from \textit{Escherichia coli} were potentiometrically measured using a Clark-type electrode. We synthesized long- (C10, decyl or longer) and short-chain (C4, butyl to C8, octyl) AurD-type compounds and tested this set of molecules towards their selectivity and potency. We confirmed strong inhibition of all three terminal oxidases for AurC-type compounds, whereas the 4(1H)-quinolone scaffold of AurD-type compounds mainly inhibits \textit{bd}-type oxidases. We assessed a direct effect of chain length on inhibition activity with highest potency and selectivity observed for heptyl AurD-type derivatives. While Aurachin C and Aurachin D are widely considered as selective inhibitors for terminal oxidases, their structure–activity relationship is incompletely understood. This work fills this gap and illustrates how structural differences of Aurachin derivatives determine inhibitory potency and selectivity for \textit{bd}-type oxidases of \textit{E. coli}.

Aurachins are isoprenoid quinoline alkaloids that have first been isolated from myxobacteria over 30 years ago\textsuperscript{1}. These natural compounds have been sub-classified according to their structures (A-H, E, RE, SS). Previous studies have provided insights into the biosynthesis of aurachins and new methods of chemical semi- or full synthesis of aurachins have been described\textsuperscript{2–7}. Studies on biological activity of these compounds demonstrated antiplasmodial, antifungal and antibacterial effects of aurachins. Antibacterial activity was mainly observed for gram-positive strains, like \textit{B. subtilis} with reported minimum inhibitory concentration values ranging from 0.15 µg/ml for AurC/D to 5 µg/ml for AurA. Treatment with aurachins showed only weak inhibitory effect on gram-negative species\textsuperscript{1}. Interestingly, experiments with the multidrug efflux transporter deficient TolC strain of \textit{Escherichia coli} (\textit{E. coli}) showed a moderate antibacterial effect of AurD (farnesyl) and two derivative variants with geranyl and prenyl isoprenoid side chain. These results raised hopes that this group of compounds may serve as a new antibiotic class in the future\textsuperscript{4}. In contrast, studies on human U-2 OS osteosarcoma cells revealed that the mitochondrial membrane potential was reduced to 50–80% upon treatment with AurD and some, but not all tested, derivatives. Noticeable cytotoxicity on murine fibroblast, human carcinoma cell lines and vero cells (L929; HCT-116; K562) has been described in the same study\textsuperscript{4}. It is conceivable that cytotoxicity is a result of an inhibitory effect on the mitochondrial NADH-ubiquinone oxidoreductase (Complex I)\textsuperscript{1,2,4}. Thus, this kind of long-chain aurachins are not yet ready to be used as antibiotics. At the same time, the use of quinol-like species offer the possibility to gain deeper insights into the mechanism of action of respiratory quinol: \textit{O}_2 oxidoreductases.

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E. coli encodes for three terminal membrane-integrated oxygen reductases: cytochromes bd-1, bd-2 and bo.

All of these terminal oxidases contribute to the generation of an electrochemical proton gradient (proton motive force) across the cytoplasmic membrane which is the driving force for ATP synthesis of and secondary active transport. This proton gradient is generated by either pumping proton across the membrane by cytochrome bo, or vectorial charge separation via the cytochrome bd oxidase. The terminal oxidases of E. coli oxidize quinols in the cytoplasmic membrane and reduce oxygen to water as part of the aerobic respiration. E. coli is a facultative anaerobic organism that is able to adapt to different environmental oxygen conditions. In highly oxygenated environments cytochrome bo oxidase is the predominantly active terminal oxidase31. Cytochrome bo, belongs to the heme-copper containing oxidase (HCO) superfamily with homologues that are widely distributed among bacteria and shares structural homology to mitochondrial oxidases. However, no eukaryotic homologues exist for the members of the cytochrome bd family. Cytochrome bd-1 and bd-2 are two additional terminal oxidases of E. coli with high oxygen affinity. At lower oxygen concentrations the efficiency of cytochrome bo, decreases due to its lower oxygen affinity. To enable aerobic growth under microaerobic conditions, oxygen reduction is shifted from cytochrome bo, to the bd-1 and bd-2 oxidases12-14. For E. coli it was shown that content and ratio of primarily three different quinol species adapt to environmental conditions. Ubiquinol is the main species under aerobic growth conditions. Menaquinol and demethylmenaquinol are found to be mainly present under microaerobic or anaerobic conditions17-18. Consequently, aurachins have been used in functional and structural studies of bd-type oxidases, due to their selectivity and good activity19-20. Interaction studies of decyl-aurachin D with cytochrome bd from Azotobacter vinelandii revealed that decyl-aurachin D acts in the vicinity of heme b-558 and prevents the oxidation of substrate quinols and the resulting electron transfer to heme b-558. Early studies with monoclonal antibodies identified the Q-loop as the plausible ubiquinol binding site19. Studies involving microaerobic or anaerobic conditions15,16. Consequently, aurachins have been used in functional and structural under aerobic growth conditions. Menaquinol and demethylmenaquinol are found to be mainly present under aerobic growth conditions. Menaquinol and demethylmenaquinol are found to be mainly present under aerobic growth conditions.

Results and discussion

We synthesized a new set of short-chain AurD-type compounds as derivatives of the previously characterized natural source inhibitor AurD. AurD has a similar molecular scaffold as the substrate quinol species of quinol oxidases: demethylmenaquinol-8 (DMK-8) and menaquinol-8 (MK-8). In E. coli DMK-8 and MK-8 are present under microaerobic and anaerobic conditions. However, ubiquinols (UQ) are the main species in an aerobic environment21. In contrast to UQ species that contain a benzoquinone framework, MK-8 possesses a naphthalene 1,4-dione ring with a methyl-group and an eight-unit isoprene unit side chain. The main difference between MK-8 and aurachins, as indicated in Fig. 1, is the molecular scaffold. Aurachins consist of quinolone heterocycles with an N–OH (AurC) or N–I (AurD) group at position 1. Originally, AurC and AurD possess a methyl and an isoprenoid side chain. This structural similarity inspired us to synthesize and test a set of aurachin analogues to the inhibitor20. Hydrogen/deuterium exchange mass spectrometry revealed that AD3-11 reduces flexibility of the N-terminal Q-loop25. However, no other interactions were found for AD3-11, implying that no secondary binding site other than the Q-loop is present in bd-1 for this type of compounds.

The biological activity of naturally occurring Aurachins C, D1,2,4,22, RE5, SS6 and P23 have been described previously. However, there are no systematic studies of the inhibitory potentials of short-chain AurD analogues on neither cytochrome bd oxidase, nor on cytochrome bo, oxidase from E. coli. We expand the variety of inhibitors by introducing new side chains to the aurachin scaffold to obtain a more comprehensive understanding of potential cytochrome bd oxidase inhibitors. Functional assays on purified protein with a diverse set of quinolones enabled us to identify structural features that determine selectivity and potency.

Synthesis.

The 4-quinolone scaffold of aurachins forms the basis of numerous natural products and drugs. Therefore, several synthetic methods can be found in the literature that offer a way for construction of such substituted heterocycles. An example is the nickel catalyzed cycloaddition of isatoic anhydride through addition of reactive alkyne derivatives25. However, this method is more suitable for symmetrical alkynes due to the difficult purification of delivered regioisomers in case of unsymmetrical alkynes. In 2020, Liu et al. reported a metal free synthetic strategy that combined Michael addition and Smiles rearrangement generating 1,2,3-trisubstituted 4-quinolones26. The feasibility of this base-mediated sequential protocol is limited to the use of N-arylated sulfonamide. In contrast, Dejon et al. presented an efficient possibility based on Conrad-Limpach cyclization of several isoprenoid side chain substituted enamine derivatives27. By employing this simplified procedure, an ensemble of 3-alkyl substituted 2-methyl-4-quinolones with different aliphatic side chain length were successfully synthesized. Since not every desired allylic bromide was commercially available, we started with the generation of appropriate allylic alcohols that was performed by following a method previously described in the study of Camps et al.28. In this synthesis strategy, the allylic alcohol derivative firstly undergoes a conversion to the corresponding mesylate that reacted with Lithium bromide yielding the allylic bromide. Subsequently, ethyl acetoacetate was selectively substituted with previously obtained allylic bromides at position R2 by using sodium hydride (NaH) in tetrahydrofuran (THF). In a next step, the ceric ammonium nitrate (CAN) catalyzed
and given in SI Table S1. All tested AurC-type compounds have a strong inhibitory effect on cytochromes and to assess the selectivity for bd-type oxidases we tested the inhibitory effect via potentiometric set-up, we kept the compound at a high molar excess compared to the protein. Figures 3 and 4 illustrate the determination of oxygen reduction using purified protein samples and ubiquinol-1 as electron donor. In this screening results for cytochromes inhibit only bd-type oxidases. Our inhibitor design is based on two scaffolds where AurD-type compounds are expected to inhibit only bd branch enzymes and AurC-type compounds are non-selective on bd and HCO branch/terminal oxidases. Green: bd-oxidases; Turquoise: bo3-oxidase; Blue: Dehydrogenases.

To determine inhibition of our test set compounds and to assess the selectivity for bd-type oxidases we tested the inhibitory effect via potentiometric determination of oxygen reduction using purified protein samples and ubiquinol-1 as electron donor. In this set-up, we kept the compound at a high molar excess compared to the protein. Figures 3 and 4 illustrate the screening results for cytochromes bd-I, bd-II and bo3, respectively. A comparative overview of all compounds is given in SI Table S1. All tested AurC-type compounds have a strong inhibitory effect on cytochromes bd-I, bd-II and bo3, resulting in residual activity < 3% at 250 µM concentration. Despite a strong inhibitory effect, none of the tested AurC-type compounds selectively inhibits a certain terminal oxidase (Fig. 3A). Among these compounds, AC2-11 has the strongest inhibitory effect on all three oxidases. These derivatives show a higher inhibitory activity compared to the reference inhibitor HQNO. HQNO is a non-selective ubiquinol oxidase inhibitor that acts on cytochromes bo3 and bd at low micromolar concentrations. For AurC and its analogues it was reported that replacement of N–OH with an N–H group decreases the inhibitory potential only for cytochrome bo3, keeping a strong inhibitory effect on cytochrome bd17. Correspondingly, we observed a clear inhibition of cytochromes bd-I and bd-II with all AurD-type compounds. Regarding the long-chain derivatives AC4-11, AC2-11 and AD3-11, inhibition of cytochromes bd-I and bd-II and bo3 highlight the importance of the residue at the nitrogen group at position 1 for enzyme selectivity. AD3-11 reduces activity of both bd-type oxidases but is less active on cytochrome bo3, (Fig. 3B). However, AD3-11 shows the highest inhibition potential of cytochrome bd-I. In case of cytochrome bo3, an inhibitory effect of only ~ 50% at 250 µM was reached with AD3-11. The AurD-type compound AD0-11 marks an exception, as we observed a comparatively low residual activity of 23% for cytochrome bo3 in presence of this compound. Comparison with AD3-11, that possesses an additional propyl side chain, indicates that elongation of the hydrocarbon side chain at position R1 reduces the inhibitory potential.

Figure 1. Schematic representation of the two terminal oxidase branches of E. coli composed of the proton pumping (HCO) type cytochrome bo3 oxidase and two bd-type oxidases. All three enzymes oxidize membrane quinols (QH2) and transfer electrons to their respective active site for the reduction of molecular oxygen to water. The resulting quinones (Q) are reduced by transfer of two electrons in the enzymatic action of dehydrogenases. Our inhibitor design is based on two scaffolds where AurD-type compounds are expected to inhibit only bd branch enzymes and AurC-type compounds are non-selective on bd and HCO branch/terminal oxidases. Green: bd-oxidases; Turquoise: bo3-oxidase; Blue: Dehydrogenases.

Oxygen reductase assay to determine inhibition activity. To determine inhibition of our test set compounds and to assess the selectivity for bd-type oxidases we tested the inhibitory effect via potentiometric determination of oxygen reduction using purified protein samples and ubiquinol-1 as electron donor. In this set-up, we kept the compound at a high molar excess compared to the protein. Figures 3 and 4 illustrate the screening results for cytochromes bd-I, bd-II and bo3, respectively. A comparative overview of all compounds is given in SI Table S1. All tested AurC-type compounds have a strong inhibitory effect on cytochromes bd-I, bd-II and bo3, resulting in residual activity < 3% at 250 µM concentration. Despite a strong inhibitory effect, none of the tested AurC-type compounds selectively inhibits a certain terminal oxidase (Fig. 3A). Among these compounds, AC2-11 has the strongest inhibitory effect on all three oxidases. These derivatives show a higher inhibitory activity compared to the reference inhibitor HQNO. HQNO is a non-selective ubiquinol oxidase inhibitor that acts on cytochromes bo3 and bd at low micromolar concentrations. For AurC and its analogues it was reported that replacement of N–OH with an N–H group decreases the inhibitory potential only for cytochrome bo3, keeping a strong inhibitory effect on cytochrome bd17. Correspondingly, we observed a clear inhibition of cytochromes bd-I and bd-II with all AurD-type compounds. Regarding the long-chain derivatives AC4-11, AC2-11 and AD3-11, inhibition of cytochromes bd-I and bd-II and bo3 highlight the importance of the residue at the nitrogen group at position 1 for enzyme selectivity. AD3-11 reduces activity of both bd-type oxidases but is less active on cytochrome bo3, (Fig. 3B). However, AD3-11 shows the highest inhibition potential of cytochrome bd-I. In case of cytochrome bo3, an inhibitory effect of only ~ 50% at 250 µM was reached with AD3-11. The AurD-type compound AD0-11 marks an exception, as we observed a comparatively low residual activity of 23% for cytochrome bo3 in presence of this compound. Comparison with AD3-11, that possesses an additional propyl side chain, indicates that elongation of the hydrocarbon side chain at position R1 reduces the inhibitory potential.
for cytochrome bo₃. Regarding the cyclic derivatives, 7-MTHA causes only a minor inhibition of cytochrome bd-I. This effect could be due to the methoxy group in position 7 that introduces an additional polarity to the molecule. Steric hindrance as a consequence of the third ring structure is less likely, because AD5-cyclo is bulkier and reduces activity < 10% of cytochromes bd-I and bd-II. All tested short-chain AurD-type compounds clearly showed inhibition of cytochromes bd-I and bd-II resulting in residual activities for bd-I of < 10% and bd-II of < 2% at 250 µM compound concentrations (Fig. 4). The new AurD-type compounds reduce cytochromes bd-I and bd-II activities in a higher extend compared to HQNO. Interestingly, increasing heptyl to octyl resulted in a higher residual activity of cytochrome bo₃. This observation suggests a higher selectivity for cytochromes bd-I and bd-II. AD7-1, AD7-1* and AD7-2 have a similar inhibitory effect compared to AurD on both bd-type oxidases (Fig. 4B). However, for cytochrome bo₃ we determined 30% residual activity in the presence of 250 µM AurD. Hence, AurD is the least selective inhibitor regarding the HCO-type bo₃ oxidase within our test set. We observed a decreased inhibitory effect for compounds without a second chain at position R₂. The small AurD-type compound 2-MQ lacks the second aliphatic residue and is one of the least active compounds. In presence of 2-MQ and AD0-11 cytochrome bd-I possesses more than 30% residual activity, indicating that the methyl side chain at R¹ is important for the inhibitory potential (Fig. 3B). In order to find new cytochrome bd selective inhibitors, these findings motivated us to concentrate on short-chain compounds that do not inhibit cytochrome bo₃ as strongly as the bd oxidases. We investigated cytochrome bd-I in further experiments since we found a higher inhibition effect, compared to cytochrome bd-II. To facilitate a ranking of our test set we determined Kᵢ apparent (Kᵢ,app) as described earlier⁹,30. The Kᵢ,app represents the inhibitor concentration at which the residual oxygen reductase activity of the terminal oxidase is 50%. These Kᵢ,app values of AurD-type compounds with respect to cytochrome bd-I are summarized in Table 1. As expected, 2-MQ, 7-MTHA and AD0-11 which already had minor inhibitory effects on cytochrome bd-I were also the candidates with Kᵢ,app values in the higher micromolar range (Figs. 3, 4). This finding supports our hypothesis that the methyl group at R¹ is important for inhibition of bd-I oxidase. Reduced inhibitory activity of 7-MTHA could be traced to a different molecule shape because of the third ring structure or the additional hydrophilicity obtained by the methoxy group. For AD4-1, AD5-1 and AD5-cyclo Kᵢ,app was

**Figure 2.** Overview of test compounds. General synthesis route to generate substituted 2-methyl-4-quinolones (AurD-type). (a) Allyl alcohol (1.0 equiv.), Mesyl chloride (1.3 equiv.), NEt₃ (2.0 equiv.), LiBr (4.0 equiv.), anh. THF, − 40°C, 0 °C, 1 h, 88%. (b) Allyl bromide (1.1 equiv.), NaH (1.1 equiv.), Ethyl acetoacetate (1.0 equiv.), anh. THF, 0°C-RT, 12 h, 54%. (c) 2-Allylacetooctetate (10.0 equiv.), CAN (0.5 equiv.), Aniline (10.0 equiv.), anh. EtOH, RT, 2 h, 23%. (d) Enamine (1.0 equiv.), diphenyl ether, 250 °C-RT, 1 h, 33%. Reaction yields correspond to the average of all synthesized derivatives. [a] from¹⁷ [b] 2-methylquinolin-4-ol (2-MQ) and 7-methoxy-1,3,4,10-tetrahydro-9(2H)-acridinone (7-MTHA) are commercially available. *Indicates double bond in position C1 of R¹.
found to be in the medium micromolar range. All three compounds showed good selective inhibition at high concentration for cytochromes bd-I and bd-II (Figs. 3, 4). It is conceivable that the short aliphatic side chains are not able to allow sufficient interactions with the enzyme to result in a higher inhibitory effect. Additionally, as cyclic compounds AD5-cyclo and 7-MTHA showed minor inhibitory effects, it is plausible that aliphatic or isoprene-like side chains are favorable. For AD5-cyclo it is plausible that the cycloheptene group is not as suitable to interact with cytochrome bd-I as aliphatic or isoprene-like side chains. We suggest that the higher flexibility of aliphatic and isoprene-like side chains enables more intense interactions with the binding region and consequently results in stronger inhibition of cytochrome bd-I.

Regarding the short-chain compounds, we observed that with increasing chain length from butyl to heptyl $K_i^{app}$ decreases. For AD7-2, $K_i^{app}$ is 45-fold lower compared to AD4-1 (120 nM vs. 5.4 µM). With respect to AurD, our synthesized short-chain AurD-type compounds result in higher $K_i^{app}$ values for cytochrome bd-I. However, our new set of compounds appears to have a higher selectivity towards cytochrome bd-I than AurD (Fig. 4). We determined $K_i^{app}$ in the nanomolar range (0.1–1 µM) for AD6-1, AD7-1, AD7-1*, AD7-2, AD8-1 and AD3-11.

Figure 3. (A) Screening results for cytochromes bd-I (light-grey), bd-II (dark-grey) and bo$_3$ (white) in presence of AurC-type compounds and HQNO. (B) Screening results for cytochromes bd-I, bd-II and bo$_3$ in presence of cyclic and long-chain AurD-type compounds. Inhibition assay using test compounds at 250 µM in presence of 200 µM ubiquinone-1 and 5 mM dithiothreitol. Inhibitory activities were calculated from oxygen consumption rates at RT. Reference activity (100%) of each oxidase was determined in presence of DMSO to exclude secondary effects of the solvent. Data are given as mean ± S.E.M. (n = 3) ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, NS$p > 0.05$.

Figure 4. (A) Screening results for cytochromes bd-I (light-grey), bd-II (dark-grey) and bo$_3$ (white) for short-chain AurD-type compounds. (B) Screening results for selected AurD-type compounds with decreasing residual activity for cytochrome bo$_3$ from left to right. Inhibition assay using test compounds at 250 µM in presence of 200 µM ubiquinone-1 and 5 mM dithiothreitol. Oxygen reduction activity was calculated from oxygen consumption rates at RT. Reference activity (100%) of each oxidase was determined in presence of DMSO to exclude secondary effects of the solvent. Presented data are mean ± S.E.M. (n = 3) ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, NS$p > 0.05$. 
For AD7-1 and AD7-2 we determined lowest $K_{i,app}$ values in the nanomolar range. Compared to AD7-2, AD8-1 resulted in a sixfold increased $K_{i,app}$ value (120 nM vs. 660 nM). AD3-11 differs from AD0-11 by the presence of a propyl residue at position $R_1$. This change results in a 188-fold higher inhibitory potential of AD3-11 for cytochrome $bd-I$, compared to AD0-11.

Because we were able to identify AD7-1 as a good selective inhibitor for cytochrome $bd-I$, we used this scaffold for further modifications. We explored how introduction of a double bond or elongation of $R_2$ affects inhibitory activity (Fig. 4B). Introducing a double bond within the side chain at $R_1$ did not significantly affect the inhibitory potential for cytochrome $bd-I$ (AD7-1 vs. AD7-1*). Elongation of the residue in position $R_2$ (AD7-1 vs. AD7-2) or introduction of a double bond within the side chain at $R_1$ (AD7-1 vs. AD7-1*) resulted in a higher inhibition of cytochrome $bo_3$.

For further characterization, we determined $K_{i,app}$ for cytochrome $bo_3$. Because of the low inhibitory effect on cytochrome $bo_3$ by most of the tested compounds, $K_{i,app}$ determination was only possible for a part of the test set compounds. A summary of $K_{i,app}$ for cytochromes $bd-I$ and $bo_3$ is given in Table 2. Among the AurC-type compounds we found AC2-11 to be the most potent inhibitor for all tested oxidases. Moreover, our results imply that the butyl side chain in AC4-11 results in lower inhibition of the cytochrome $bd-I$ activity. Miyoshi et al. described AC1-10 and AC1-11 as the most potent competitive inhibitors for cytochromes $bd$ and $bo_3$ [17]. In case of AC2-11, $K_{i,app}$ for cytochromes $bo_3$ (46 nM) and $bd$ (440 nM) have been determined spectrophotometrically [17]. Their final protein concentration was 30 to 200 times lower compared to our assay conditions and a preincubation period of protein and compound was implemented [17]. Thus, we can confirm the non-selective inhibition of AC2-11 and a high potency of the long-chain AurC-type compounds in inhibition of cytochrome $bd-I$ oxygen reduction activity.

Regarding the AurD-type compounds, we identified AD7-1 and AD7-1* as potent inhibitors for cytochrome $bd-I$ while causing no cytochrome $bo_3$ inhibition in the low nanomolar to micromolar range (Fig. S5). For AurD we found a difference of three orders of magnitude for $K_{i,app}-bo_3$ vs. $K_{i,app}-bd-I$ (<10 nM vs. 18 nM) (Fig. S5; Table 2). For AD3-11 we determined a ratio of around 300. Therefore, it is noteworthy that the short-chain AurD-type compounds AD7-1 and AD7-1* share a similar selectivity for $bd-I$ as AurD. Further we can confirm the inhibitory activity of AurC-type compounds in a nanomolar range on both different types $bd$ and $bo_3$ terminal oxidases in E. coli.

### Table 1. $K_{i,app}$ values of selected AurD-type compounds acting as inhibitors of cytochrome $bd-I$ from E. coli at a final enzyme concentration of 30 nM.

| Compound | $K_{i,app}$ $bd-I$ | Concentration range |
|----------|-------------------|---------------------|
| 2-MQ     | >250 µM           | > 50 µM             |
| 7-MTHA   | >250 µM           | > 50 µM             |
| AD0-11   | 56.6 ± 5.9 µM     | 1–10 µM             |
| AD4-1    | 5.4 ± 0.7 µM      | 0.1–1 µM            |
| AD5-cyclo| 4.0 ± 0.6 µM      | 1–10 µM             |
| AD5-1    | 0.81 ± 0.04 µM    | 0.1–1 µM            |
| AD8-1    | 0.66 ± 0.06 µM    | 0.1–1 µM            |
| AD7-1*   | 0.39 ± 0.04 µM    | 0.1–1 µM            |
| AD3-11   | 0.30 ± 0.04 µM    | 0.1–1 µM            |
| AD6-1    | 0.28 ± 0.04 µM    | 0.1–1 µM            |
| AD7-1    | 0.13 ± 0.02 µM    | 0.1–1 µM            |
| AD7-2    | 0.12 ± 0.02 µM    | 0.1–1 µM            |
| AurD     | 0.018 ± 0.002 µM  | > 0.1 µM            |

### Table 2. $K_{i,app}$ for $bd-I$ and $bo_3$ oxidoreductases for selected test compounds at a final enzyme concentration of 30 nM. [a] No data available for $bo_3$ [b] previous data from [17].

| Compound | $K_{i,app}$ $bd-I$ | $K_{i,app}$ $bo_3$ |
|----------|--------------------|---------------------|
| AD7-1    | 0.13 ± 0.02 µM     | > 250 µM            |
| AD7-1*   | 0.40 ± 0.04 µM     | > 250 µM            |
| AD3-11   | 0.30 ± 0.04 µM     | > 250 µM            |
| AurD     | 0.018 ± 0.002 µM   | > 250 µM            |
| HQNO     | 3.6 ± 0.3 µM       | 2.6 ± 0.4 µM        |
| AC2-11   | 0.034 ± 0.006 µM (0.44 µM) | 0.051 ± 0.006 µM (0.064 µM) |
| AC1-10[a] | 0.002 ± 0.011 µM (0.28 µM) | (0.013 µM) |
| AC4-11[a] | 0.106 ± 0.013 µM (0.34 µM) | (0.144 µM) |

For AD7-1 and AD7-2 we determined lowest $K_{i,app}$ values in the nanomolar range. Compared to AD7-2, AD8-1 resulted in a sixfold increased $K_{i,app}$ value (120 nM vs. 660 nM). AD3-11 differs from AD0-11 by the presence of a propyl residue at position $R_1$. This change results in a 188-fold higher inhibitory potential of AD3-11 for cytochrome $bd-I$, compared to AD0-11. Because we were able to identify AD7-1 as a good selective inhibitor for cytochrome $bd-I$, we used this scaffold for further modifications. We explored how introduction of a double bond or elongation of $R_2$ affects inhibitory activity (Fig. 4B). Introducing a double bond within the side chain at $R_1$ did not significantly affect the inhibitory potential for cytochrome $bd-I$ (AD7-1 vs. AD7-1*). Elongation of the residue in position $R_2$ (AD7-1 vs. AD7-2) or introduction of a double bond within the side chain at $R_1$ (AD7-1 vs. AD7-1*) resulted in a higher inhibition of cytochrome $bo_3$. For further characterization, we determined $K_{i,app}$ for cytochrome $bo_3$. Because of the low inhibitory effect on cytochrome $bo_3$ by most of the tested compounds, $K_{i,app}$ determination was only possible for a part of the test set compounds. A summary of $K_{i,app}$ for cytochromes $bd-I$ and $bo_3$ is given in Table 2. Among the AurC-type compounds we found AC2-11 to be the most potent inhibitor for all tested oxidases. Moreover, our results imply that the butyl side chain in AC4-11 results in lower inhibition of the cytochrome $bd-I$ activity. Miyoshi et al. described AC1-10 and AC1-11 as the most potent competitive inhibitors for cytochromes $bd$ and $bo_3$ [17]. In case of AC2-11, $K_{i,app}$ for cytochromes $bo_3$ (46 nM) and $bd$ (440 nM) have been determined spectrophotometrically [17]. Their final protein concentration was 30 to 200 times lower compared to our assay conditions and a preincubation period of protein and compound was implemented [17]. Thus, we can confirm the non-selective inhibition of AC2-11 and a high potency of the long-chain AurC-type compounds in inhibition of cytochrome $bd-I$ oxygen reduction activity.

Regarding the AurD-type compounds, we identified AD7-1 and AD7-1* as potent inhibitors for cytochrome $bd-I$ while causing no cytochrome $bo_3$ inhibition in the low nanomolar to micromolar range (Fig. S5). For AurD we found a difference of three orders of magnitude for $K_{i,app}-bo_3$ vs. $K_{i,app}-bd-I$ (< 10 nM vs. 18 nM) (Fig. S5; Table 2). For AD3-11 we determined a ratio of around 300. Therefore, it is noteworthy that the short-chain AurD-type compounds AD7-1 and AD7-1* share a similar selectivity for $bd-I$ as AurD. Further we can confirm the inhibitory activity of AurC-type compounds in a nanomolar range on both different types $bd$ and $bo_3$ terminal oxidases in E. coli.
Short- and long-chain AurD-type compounds are reported to be efficient inhibitors of the isolated cytochrome bc₁ complex (complex III) from beef heart mitochondria. Similar to our findings, an increase of the inhibitory potential with increasing alkyl chain length was reported. A maximum inhibitory activity was determined for 2-undecylquinolone (AD11-0, pIC₅₀ 6.66). Here, the undecyl chain in position R₁ is switched with R₀, compared to our AD0-11. A further increase of the alkyl chain length (resp. position R₁ in Fig. 2) results in reduced inhibition. Our results show a similar trend, as the inhibition of cytochrome bd-I reached a maximum for AD7-1 and decreased for AD8-1. Moreover, addition of a 3-methyl group at position R₂ further increases the potency of inhibition and results in the highest inhibition by 3-undecyl-3-methylquinolone (AD11-3, pIC₅₀ 7.70). Elongation of the 2-alkyl chain decreases the inhibition. A parabolic dependence of inhibition on the chain length was observed. Our results confirm an increase of inhibition by the long-chain aurachins from AD0-11 to AD3-11 for all tested oxidases. Furthermore, cytochrome bo₃ was the least sensitive. Further, we could identify a similar parabolic dependence of inhibitory potency of the chain length for our compounds, as the inhibitory activity on cytochrome bd-I was the highest for 2-heptyl-3-methyl-quinolone (AD7-1).

Conclusions
Specific protein inhibitors can be of use not only as antibiotically active compounds, but also as binding ligands to enable deeper investigations of protein mechanisms. As deeper investigations of the respiratory chain from E. coli contribute knowledge to a broad spectrum of bacteria, introducing specific inhibitors in structural investigations can open new perspectives. Earlier studies shed light on the binding site and the modes of cytochrome bd inhibition. However, no structure with bound aurachin has been published yet, due to lack of a defined conformation or increased flexibility of the quinol binding site. To clarify the nature of aurachin bd interactions, research about structure–function relationship is urgently required. Here, we investigated the selectivity and potency of AurC- and AurD-type compounds on the oxygen reduction activity of all three terminal oxidases of E. coli (cytochromes bd-I, bd-II and bo₃). We confirmed that three AurC-type N-oxide-quinolones have a similar inhibitory potency in a nanomolar range on cytochromes bd-I and bo₃. Two short-chain AurD-type compounds AD7-1 and AD7-1* are highly selective towards cytochrome bd-I with similar Kᵢapp values as the natural compound AurD. In addition, we provide evidence that inhibitory activity increases with increasing chain length at position R₀ of the 2-methyl-4-quinolones backbone. AD7-1 (2-heptyl-3-methyl-quinolone) combines properties of high inhibitory potency and selectivity for cytochrome bd-I. From the set of characterized inhibitors, AD7-1 qualifies as the best candidate to be subjected to downstream inhibition trials on a physiological level.

Material and methods
Chemicals. The long-chain AurD-type compounds AD3-11, AD0-11 and AD4-1 and AurC-type compounds AC1-10, AC2-11 and AC4-11 used in this study were synthesized as described previously. 2-MQ and 7-MTHA were purchased from Sigma.

Production of cytochrome bd-I and bd-II from E. coli. Cytochrome bd oxidase from E. coli was produced in E. coli C43 (DE3) Δ bo₃ (CLY) cells (kindly provided by Prof. Robert B. Gennis, University of Illinois) transformed with pET17b-yclABX-StrepII plasmid, carrying carbenicillin/kanamycin resistance (bd-I) or the pET17b-appCBX-StrepII plasmid carrying carbenicillin/chloramphenicol resistance (bd-II). A 1 ml of 50% glycerol stock was added to 50 ml LB with corresponding antibiotics (50 µg/ml carbenicillin—carb; 50 µg/ml kanamycin—kan; 25 µg/ml chloramphenicol—cam) and incubated at 175 rpm at 37 °C for 8 h. Preculture was transferred to 1 L LB-carb-kan/cam to grow overnight. 2.5 L LB-kan/cam was inoculated with 70 ml overnight culture supplemented with 0.025 mM IPTG to start basal production from the beginning. After reaching OD₆₀₀ 0.7 bd-I/bd-II oxidation production was started by adding IPTG to a final concentration of 0.25 mM. 4 h incubation at 37 °C followed by 16 h at 30 °C. Cell harvesting was carried out by centrifugation with Avanti J-26XP at 4 °C at 8000g. Cell disruption via microfluidizer for four cycles at 80 psi in 50 mM sodium phosphate buffer (NaPi) pH 8.0 and 100 mM NaCl supplemented with 1 mM MgCl₂, recombinant DNase I (Sigma) and protease inhibitor Aprotinin (Boehringer). Low-velocity centrifugation at 5000g at 4 °C for 30 min before high-velocity centrifugation of the supernatant at 220,000g for 90 min. Membrane pellets were resuspended in 50 mM NaPi (pH 8.0), 100 mM NaCl containing buffer and stored at –80 °C.

Streptactin purification of E. coli bd-I and bd-II oxidase. Isolated membranes were solubilized in 50 mM NaPi (pH 8.0), 100 mM NaCl with 1% n-dodecyl β-D-maltoside (β-DDM) to the mass ratio of 1:5 detergent:membrane protein at 3 °C for 40 min on an orbital shaker followed by removal of unsolubilized material by 70,000g for 30 min. Avidin was added to the filtrated supernatant to a final concentration of 0.2 mg/ml. Affinity chromatography was done via peristaltic pump with prepacked 5 ml StrepTrap HP column (GE Healthcare) equilibrated with 20 mM NaPi (pH 8.0), 100 mM NaCl, 0.02% DDM at a flow rate of 3 ml/min. Washing was carried out with the same buffer for 15CV. For elution this buffer was supplemented with 10 mM desthiobiotin (IBA Lifesciences). Sample dialysis with Slide-A-Lyzer (CutOff 10 K) dialysis cassettes (Thermo Fisher Scientific) in 4L 50 mM NaPi (pH 8.0), 100 mM NaCl with 1% β-DDM overnight. Presence of the E. coli bd oxidase and purity of the product was analyzed by SDS-page and native page gel electrophoresis.

Production and Ni–NTA purification of cytochrome bo₃ from E. coli. Strain GO195 transformed with pIRHisA plasmid was a kind gift from by Prof. Robert B. Gennis, University of Illinois. For bo₃—oxidase production a protocol from 19 was used. Purification protocol was modified according to 18 and described in detail in 34.
Oxygen reductase activity measurements. Oxygen reductase activity was measured as oxygen consumption rate of purified protein by OX-MR Clark-type oxygen electrode linked to a PA 2000 picoammeter and to the ADC-216 AD-converter. Data recording with SensorTrace Basic 2.1 software (all Unisense, Denmark). Measurements were performed at RT in stirred 2 ml—glass vials with a total reaction volume of 600 µl. Oxygen consumption was initiated by adding 30 nM (bd-I, bo) or 120 nM (bd-II) of the respective enzyme to the equilibrated mixture containing 20 mM NaPi (pH 8.0), 50 mM NaCl, 0.02% DDM, 5 mM Dithiothreitol (DTT) and 200 µM Ubiquinone-1 (2,3-Dimethoxy-5-methyl-6-(3-methyl-2-buten-1-yl)-1,4-benzoquinone). A 10 min equilibration period was implemented prior to enzyme addition. Inhibition experiments were performed with 250 µM of the respective compound from 20 mM stock solution in DMSO before equilibration. Data analysis and visualization was carried out using OriginLab Pro 9.5 (Additive GmbH, Germany). Data analysis included an unpaired, two-sided t-test of two samples to check that given residual activities were sufficiently different between proteins (at significance levels p < 0.05, 0.01 or 0.001). HQNO (2-heptyl-4-quinolinol 1-oxide) was purchased from biomol GmbH, Hamburg. Determination of apparent K_i was performed under the same conditions but with a range of inhibitor concentrations from 250 µM to 0.2 nM to test protein/inhibitor ratio from 1/0.0067 up to 1/8333 for cytochromes bd-I and bo (tested at 30 nM). The K_i value was adopted as the EC50 from the sigmoidal DoseRespFit. Further information can be found in Supporting Information Figures S2 to S5.

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Author contributions
M.R. and S.S. designed and planned experiments. M.R., T.N.G., L.F.W., K.F.H. and S.N. carried out experiments and analyzed the data. M.R., T.N.G. and L.F.W. produced and purified protein. I.E., H.M. and H.N. synthesized and provided inhibitors. H.G.G. provided cytochrome bd-II plasmid. H.X. provided cytochrome bo3 membranes. M.R. and I.E. wrote the manuscript in consultation with J.S., D.B., H.M., H.S. and S.S. H.S. and S.S. supervised the project. All authors reviewed the manuscript.

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Competing interests
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