Enzymatic hydroxylation of unactivated primary carbons is generally associated with the use of molecular oxygen as co-substrate for monooxygenases. However, in anaerobic cholesterol-degrading bacteria such as *Sterolibacterium denitrificans* the primary carbon of the isoprenoid side chain is oxidised to a carboxylate in the absence of oxygen. Here, we identify an enzymatic reaction sequence comprising two molybdenum-dependent hydroxylases and one ATP-dependent dehydratase that accomplish the hydroxylation of unactivated primary C26 methyl group of cholesterol with water: (i) hydroxylation of C25 to a tertiary alcohol, (ii) ATP-dependent dehydration to an alkene via a phosphorylated intermediate, (iii) hydroxylation of C26 to an allylic alcohol that is subsequently oxidised to the carboxylate. The three-step enzymatic reaction cascade divides the high activation energy barrier of primary C–H bond cleavage into three biologically feasible steps. This finding expands our knowledge of biological C–H activations beyond canonical oxygenase-dependent reactions.
The selective oxidation of unactivated C–H bonds at alkyl functionalities to alcohols is of great importance for a plethora of synthetic processes. Enzymatic solutions for these challenging reactions have continuously motivated organic chemists for developing bioinspired strategies. Biocatalytic C–H bond activations of alkanes via hydroxylation have generally been associated with metal-dependent monoxygenases, peroxides or peroxogenases. As an example, the well-studied cytochrome P450 monoxygenases reduce the dioxygen co-substrate to the formal oxidation state of a hydroxyl radical, which allows for the hydroxylation of unactivated primary carbons to alcohols. While cytochrome P450 enzymes require an auxiliary electron donor system, peroxides and peroxidases depend on a balanced supply with its reactive co-substrate.

The use of peroxides or peroxogenases is not an option for C–H bond oxidations in anaerobic hydrocarbon-degrading microorganisms, for which a few oxygen-independent enzymatic solutions for C–H bond hydroxylation at activated positions have been discovered in the past decades. Prototypical examples are the water-dependent hydroxylations of the benzylic carbons of p-cresol, p-cymene, and ethylbenzene, as well as of the allylic carbon of limonene. Here, C–H bond activation proceeds via hydride transfer to FAD or Mo(VI) = O cofactors (Fig. 1a–d). In all these reactions the resulting carbocation intermediates are stabilised by the possibility of forming multiple resonance structures. However, the water-dependent hydroxylation of unactivated primary carbons of alkanes or isoprenoids has not been reported so far in enzyme catalysis.

The ubiquitous, biologically active steroids are composed of a tetracyclic steran system and an isoprenoid side chain resulting in prenoids. However, the water-dependent hydroxylation at activated positions has not been reported so far in enzyme catalysis.

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In the denitrifying β-proteobacterial model organism Sterolibacterium denitrificans Chol-1S, anaerobic cholesterol degradation is initiated by the periplasmic isomerisation/dehydrogenation of ring A to cholest-4-en-3-one (CEO) by the AcmA gene product, that may be further dehydrogenated to cholesterol-1,4-dien-3-one (CEO) by AcmB. Both intermediates serve as substrate for steroid C25 dehydrogenases (S25DH) that hydroxylates tertiary C25 in the presence of cytochrome c and water to 25-OH-CEO/CDO in the presence of cytochrome c or an artificial electron acceptor. Periplasmic S25DH belongs to the DMSO reductase family of Mo-cofactor containing enzymes and is composed of the catalytic α-subunit harbouring the Mo-cofactor and the FeS-clusters/heme b containing electron transferring β- and γ-subunits. This enzyme is proposed to abstract a hydride from tertiary C25 to the Mo(VI) = O species yielding the Mo(IV)–OH intermediate. The latter transfers the hydroxyl group then back to the assumed tertiary carboxylation intermediate; electrons are transferred to cytochrome c. The genome of S. denitrificans contains eight homologous genes putatively encoding catalytic α-subunits of S25DH-like enzymes, four of which (S25DH1-4) have been heterologously produced together with the corresponding β- and γ-subunits and an essential chaperone. They specifically hydroxylate side chains of pathogens. In aerobic cholesterol degrading bacteria, side chain oxidation is initiated by the hydroxylation of the unactivated primary C26 (or equivalent C27) to a primary alcohol by cytochrome P450 enzymes that is further oxidised to a C26-carboxylate. The isoprenoid side chain of the latter is then converted to acetyl-CoA and propionyl-CoA units via modified β-oxidation. In denitrifying bacteria, oxygen-independent cholesterol degradation also proceeds via β-oxidation of the 26-carboxylate intermediate involving highly similar enzymes.

However, the oxidation of the primary, unactivated C26 to the 26-carboxylate must proceed in an oxygen-independent manner.

In the denitrifying β-proteobacterial model organism Sterolibacterium denitrificans Chol-1S, anaerobic cholesterol degradation is initiated by the periplasmic isomerisation/dehydrogenation of ring A to cholest-4-en-3-one (CEO) by the AcmA gene product, that may be further dehydrogenated to cholesterol-1,4-dien-3-one (CEO) by AcmB. Both intermediates serve as substrate for steroid C25 dehydrogenases (S25DH) that hydroxylates tertiary C25 in the presence of cytochrome c and water to 25-OH-CEO/CDO in the presence of cytochrome c or an artificial electron acceptor. Periplasmic S25DH belongs to the DMSO reductase family of Mo-cofactor containing enzymes and is composed of the catalytic α-subunit harbouring the Mo-cofactor and the FeS-clusters/heme b containing electron transferring β- and γ-subunits. This enzyme is proposed to abstract a hydride from tertiary C25 to the Mo(VI) = O species yielding the Mo(IV)–OH intermediate. The latter transfers the hydroxyl group then back to the assumed tertiary carboxylation intermediate; electrons are transferred to cytochrome c. The genome of S. denitrificans contains eight homologous genes putatively encoding catalytic α-subunits of S25DH-like enzymes, four of which (S25DH1-4) have been heterologously produced together with the corresponding β- and γ-subunits and an essential chaperone. They specifically hydroxylate side chains of

Fig. 1 Enzymatic water-dependent alkyl hydroxylations. a p-Cresol methyl hydroxylase. b Limonene dehydrogenase. c Ethylbenzene dehydrogenase. d p-Cymene dehydrogenase. e Initial steps of anaerobic cholesterol degradation. The enzyme cascade involved in the proposed conversion of 25-OH-CDO (25-OH-cholesta-1,4-diene-3-one) to CDO-26-carboxylate (cholesta-1,4-diene-3-one-26-carboxylate) via a 26-OH-CDO (26-OH-cholesta-1,4-diene-3-one) intermediate was studied in this work.
cholesterol and its analogues to tertiary alcohols. The ratio-
na for the initial formation of the tertiary alcohol 25-OH-CDO,
that cannot be directly further oxidised, during anaerobic cho-
lesterol catabolism has remained enigmatic. Though previous
high resolution mass spectrometry (HRMS)-based analyses sug-
gested the primary alcohol 26-OH-CDO as an intermediate,
the underlying conversion of a tertiary alcohol to a primary
alcohol involves an unknown enzymology.

Here, we aim to identify the enzymatic steps involved in the
water-dependent oxidation of a primary carbon atom during
anaerobic cholesterol degradation. We demonstrate that the for-
mation of a tertiary alcohol at the cholesterol side chain initiates a
three-step enzymatic reaction cascade finally resulting in the
oxidation of unactivated C26 to a carboxylate.

Results

Enzymatic dehydration of 25-OH-cholesta-1,4-diene-3-one. To
elucidate the enzymology responsible for production of CDO-26-
oate, we synthesised 25-OH-CDO from CEO using overproduced
S25DH and AcmB. Cell-free extracts of \textit{S. denitrificans} grown
with cholesterol under denitrifying condition were anoxically
reacted with 25-OH-CDO in the presence of a multitude of
natural and artificial electron acceptors (e.g., NAD$[P]^+$, K$_3$[Fe
(CN)$_6$, or 2,6-dichlorophenolindophenol at 0.5 mM, respectively).
In no case, conversion of 25-OH-CDO to a product was observed.

In further trials, electron acceptors were omitted in the reaction
assays and a number of potential co-substrates were tested. In the
presence of ATP and MgCl$_2$ (5 mM each), the time-dependent
conversion of 25-OH-CDO to a minor, more polar intermediate
was observed using ultra-fast performance liquid chromato-
graphy (UPLC) analyses, which was readily further converted to a
second less polar product, (Fig. 2). After ultracentrifugation of
cell-free extracts this conversion was found to occur only in the
soluble protein fraction. UPLC-HRMS analysis of the
 did not allow
a clear assignment to a molecular mass, whereas analysis of
compound 3 revealed a [M + H]$^+$ ion with $m/z = 399.3274 \pm 1.1$; for MS data of all steroid intermediates analysed
in this work, see Supplementary Table 1). The product 3 was
purified by preparative HPLC and identified by $^1$H, $^{13}$C, and 2D
NMR analyses as desmost-1,4-diene-3-one (DDO) containing an
allylic $\Delta^{24}$ double bond (Supplementary Figs. 1–3). Thus, the
hydroxyl functionality introduced by S25DH was subsequently
eliminated to an alkene by an ATP-dependent 25-OH-CDO
dehydration activity.

We tested whether product 2 represents a phosphorylated
intermediate during the ATP-dependent dehydration of 25-OH-
CDO. For this purpose we chemically synthesised a 25-phospho-
CDO standard from enzymatically prepared 25-OH-CDO. We
opted for a P(III)-amidite approach due to the steric hindrance of
the tertiary OH, using a bis fluorenylmethyl (Fm) protected

![Fig. 2 Oxygen-independent conversion of 25-OH-cholesta-1,4-diene-3-one (1). a Time-, ATP-, and MgCl$_2$-dependent conversion of 0.5 mM 25-OH-CDO in soluble cell extracts of \textit{S. denitrificans}. b ATP-independent conversion of chemically synthesised 25-phospho-CDO. c Proposed reaction sequence of ATP-dependent dehydration of 25-OH-CDO 1 via 25-phospho-CDO 2 to desmost-1,4-diene-3-one (DDO, 3). Source data are provided as a Source Data file.](https://doi.org/10.1038/s41467-020-17675-7)
P-amidite\textsuperscript{33} that indeed facilitated the reaction. After oxidation and removal of the Fm groups with 1,8-diazabicyclo[5.4.0]undec-7-en (DBU), the product was obtained in a one-flask operation without elimination of the labile phosphate in 39% yield after purification (for MS and NMR analyses, see Supplementary Figs. 4 and 5). When 25-phospho-CDO (2) was incubated with soluble \textit{S. denitrificans} extracts, the ATP-independent conversion into DDO (3) was observed (Fig. 2b). The kinetic parameters of 25-OH- and 25-phospho-CDO conversions were $V_{\text{max}} = 11.7 \pm 0.6 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{K}_{\text{m}} = 41 \pm 8 \mu M$ for 25-OH-CDO, and $V_{\text{max}} = 23.2 \pm 1 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{K}_{\text{m}} = 227 \pm 32 \mu M$ for 25-phospho-CDO (mean values $\pm$ standard deviations of enzyme activity measurements at various substrate concentrations, for the data points used see Supplementary Fig. 6a, b). In summary, the results indicate that kinase and phosphate elimination partial activities are involved in the conversion of 25-OH-CDO (1) to DDO (3) via 25-phospho-CDO (2) (Fig. 2c).

For the determination of cofactor specificity and stoichiometry, the ATP-dependent 25-OH-CDO dehydration activity was enriched via ammonium sulphate precipitation (activity retained in the suspended 50% saturation pellet). During formation of DDO from 25-OH-CDO no other nucleoside triphosphate than ATP was accepted as co-substrate; Mg\textsuperscript{2+} could substitute for Mn\textsuperscript{2+} albeit at only 5% activity. UPLC analyses of adenosine nucleotides revealed that 1.2 $\pm$ 0.1 mol ATP were hydrolysed to 0.9 $\pm$ 0.1 mol ADP and 0.3 $\pm$ 0.1 mol AMP per mol 25-OH-CDO consumed (mean value $\pm$ standard deviation in three independent determinations); ATP hydrolysis was negligible in the absence of the steroid.

**Oxidation of the allylic methyl group.** For analysing the further conversion of DDO, we enzymatically synthesised DDO from 25-OH-CDO using cell-free extracts from \textit{S. denitrificans}. Strictly depending on $K_{\text{a}}[\text{Fe(CN)}_{6}]$ as electron acceptor, cell-free extracts of \textit{S. denitrificans} converted DDO to product 4 that was subsequently converted to 5 in assays performed anaerobically (Fig. 3).

Compounds 4 and 5 were analysed by UPLC-HRMS and $^1\text{H}$, $^{13}\text{C}$, and 2D NMR spectroscopy. The products were identified as 26-OH-DDO 4 and the corresponding C26-aldehyde DDO-26-al 5. The enzyme involved was identified as a Mo-dependent hydroxylase. Source data are provided as a Source Data file.

**Heterologous production of desmost-1,4-dien-3-one (DDO) hydroxylase.** In addition to the previously investigated S25DH\textsubscript{1-4} isoenzymes, the genome of \textit{S. denitrificans} contains four gene clusters encoding the putative $\alpha\beta\gamma$-subunits of related Mo-dependent dehydrogenases (DH\textsubscript{5,7}). Among these, the $\alpha$-subunits DH\textsubscript{5,7} form a phylogenetic subcluster with amino acid sequence identities $>$80%; that of DH\textsubscript{8} is rather distantly related (Fig. 4a).

To test whether one of the DH\textsubscript{5,7} enzymes may be involved in the water-dependent hydroxylation of DDO, we heterologously expressed the genes potentially encoding the active site $\alpha$-$\beta$-$\gamma$-subunits of related Mo-dependent dehydrogenases (DH\textsubscript{5,7}) and an assumed $\delta$-chaperone in the $\beta$-proteobacterium \textit{Thauera aromatica} as described for S25DH\textsubscript{1-4}.\textsuperscript{30}

Using cell-free extracts from \textit{T. aromatica} producing DH\textsubscript{5,7}, only with DH\textsubscript{5} we observed the $K_{\text{a}}[\text{Fe(CN)}_{6}]$ dependent conversion of DDO to 26-OH-DDO; we therefore refer this enzyme to S26DH\textsubscript{1}. Heterologously produced S26DH\textsubscript{1} was enriched from cell-free extracts by three chromatographic steps under anaerobic conditions using a modified protocol established for S25DH\textsubscript{1}.\textsuperscript{30}

SDS–PAGE analysis of enriched S26DH\textsubscript{1} revealed four major protein bands (Fig. 4b) that were excised, tryptically digested, and analysed by electrospray ionisation quadrupole time-of-flight...
mass spectrometry (ESI-Q-TOF-MS). The masses of the ions obtained from tryptic peptides confirmed the presence of the αβγ
γβα-subunits of S26DH1 from S. denitricans, those obtained from the band eluting at 43 kDa were assigned to a co-purified 6-oxocyclohex-1-ene-1-carboxyl-CoA hydrolase from T. aromatica; the latter cofactor-free enzyme plays a role in the catabolic benzoyl-CoA degradation pathway and is unlikely to be associated with steroid hydroxylation.

Amino acid sequence similarities of S26DH1 with characterised S25DHs suggested the presence of one Mo cofactor, four [4Fe-4S] clusters, one [3Fe-4S] cluster, and a heme b. The metal content of S26DH1 determined by colorimetric procedures was 0.81 ± 0.03 mol Mo, 19.7 ± 0.4 mol Fe, and 0.89 ± 0.01 mol heme b per mol S26DH1, respectively (mean value ± standard deviation in three different enzyme preparations). These values are in good agreement with the expected cofactor content and with the values determined for other S25DHs.

Unexpectedly, enriched S26DH1 catalysed the hydroxylation of DDO to 26-OH-DDO and the subsequent dehydrogenation of the latter to DDO-26-al with K3[Fe(CN)_6] as electron acceptor (Supplementary Fig. 13). As a control, T. aromatica extracts containing the plasmid without the S26DH1 genes did not catalyse such a reaction. Both partial reactions of S26DH1 dehydrogenase followed Michaelis–Menten kinetics with $V_{\text{max}} = 219 \pm 11 \text{ nmol min}^{-1} \text{mg}^{-1}/K_m = 123 \pm 25 \mu M$ for DDO hydroxylation, and $V_{\text{max}} = 73 \pm 3 \text{ nmol min}^{-1} \text{mg}^{-1}/K_m = 83 \pm 12 \mu M$ for 26-OH-DDO dehydrogenation (mean values ± standard deviations of enzyme activity measurements at various substrate concentrations, for the data points used see Supplementary Fig. 6c, d). No further oxidation to a carboxylate was observed by enriched S26DH1.

**Oxidation of 26-OH-DDO to DDO-26-carboxylate.** Enriched S26DH1 used K3[Fe(CN)_6] but not NAD$^+$ as electron acceptor for the oxidation of the allylic alcohol 4 to the aldehyde 5, which is in agreement with its proposed periplasmic location (see Discussion). We tested whether this activity is relevant for the cholesterol catabolic pathway or whether an additional cytoplasmic alcohol dehydrogenase is involved. Using NAD$^+$ as electron acceptor, extracts from S. denitricans grown with cholesterol showed virtually no conversion of 26-OH-DDO 4 to DDO-26-al 5 (<0.1 nmol min$^{-1}$ mg$^{-1}$), whereas it served as acceptor for the ready oxidation of DDO-26-al 5 to DDO-26-carboxylate (5 ± 0.2 nmol min$^{-1}$ mg$^{-1}$) (mean value ± standard deviation in three replicates). In this assay, formation of an additional product was observed that after ESI-Q-TOF-MS analyses was assigned to 26-carboxylate. The latter reaction is not observed with S26DH1 containing the plasmid without the S26DH1 genes.

**Fig. 4 Phylogeny and enrichment of recombinant S26DH1 from S. denitricans.** a Phylogenetic tree of S25DH-like Mo-enzymes of the DMSO reductase family. $A.$ = *Aromatoleum*; $D.$ = *Desulfococcus*; α-subunits of CmDH = p-cymene dehydrogenase; EbDH = ethylbenzene dehydrogenase; NarG = nitrate reductase. The previously assigned DH6 is now referred to as S26DH1 (marked in red). b SDS-PAGE of S26DH1 heterologously produced in *T. aromatica*; the latter cofactor-free enzyme plays a role in the catabolic benzoyl-CoA degradation pathway and is unlikely to be associated with steroid hydroxylation. c Cluster of genes encoding the αβγ-subunits and the gene encoding the δ-chaperone of S26DH1 (accession numbers: γβα-subunits, WP_154715926-8; δ-chaperone, WP_154716737). Source data are provided as a Source Data file.
Indeed, the conversion of DDO-26-al to DDO-26-dicarboxylate (Supplementary Fig. 15a); in control experiments no such conversions were observed in E. coli wild-type without the plasmid expressing WP_154716401. In the presence of MgATP and CoA, the DDO-26-carboxylate formed was further converted to DDO-26-CoA by the previously characterised AMP-forming C26-carboxylate CoA ligase activity25, thereby initiating β-oxidation of the isoprenoid side chain (Supplementary Fig. 15b).

Discussion

In this work we identified an enzymatic reaction sequence that allows for the oxidation of an unactivated primary carbon to a carboxylate using water as only hydroxylating agent. In this enzyme cascade the high activation energy barrier for C–H bond hydroxylation of a primary carbon is divided into three individual steps, each of which being energetically and mechanistically plausible under cellular conditions. The C–H bond dissociation energies for carboxylations and hydroxylations relevant for the hydroxylation of tertiary C25 in cholesterol and allylic C26 in desmosterol are 142 kJ mol\(^{-1}\) and 117 kJ mol\(^{-1}\) lower than that for the primary C26 of cholesterol, respectively (based on gas phase calculations for the isopentane and 2-methyl-2-butenyl analogues)35. Thus, the rationale for the initial formation of a tertiary alcohol during anaerobic cholesterol catabolism28,29 is to enable the ATP-dependent dehydration to a trisubstituted alkene that is crucial for the subsequent water-dependent hydroxylation of the allylic carbon via a relatively stable allylic carbocation intermediate. The major challenge of oxygen-independent primary carbon activation represents the dehydration of the tertiary alcohol that is achieved by coupling to exergonic ATP hydrolysis (\(\Delta G^\circ = -50\) kJ mol\(^{-1}\)). The phosphate eliminated from the phospho-ester intermediate is a much better leaving group than water. A related reaction is known from isoprenoid biosynthesis via the mevalonate pathway; (R)-mevalonate-5-diphosphate is ATP-dependently decarboxylated to isopentenyl diphosphate36,37. Here, phosphate elimination from an assumed phosphorylated intermediate is accompanied by decarboxylation, which additionally drives the elimination reaction forward (Supplementary Fig. 16). ATP-dependent dehydration has also been reported for the recycling of spontaneously formed NAD(P)H hydrates38, however it is unclear whether it proceeds via a phosphorylated intermediate.

While a reaction mechanism via tertiary and allylic carbocation intermediates is plausible for the two Mo-dependent hydroxylases S25DH/S26DH, it remains uncertain whether this is the case for phosphate elimination from 25-phospho-DDO. If the phosphate elimination would proceed via the identical tertiary carbocation intermediate to the carbocation is much faster than a competing deprotonation at C24 to an alkene by a putative base.

The reaction cascade identified in this work allows in principle for any enzymatic water-dependent hydroxylation at a primary carbon next to a tertiary one. The genome of S. denitrificans contains two further copies of S26DH-like enzymes and they may represent isozymes specifically involved in the hydroxylation of allylic methyl groups of intermediates during catabolism of steroids with modified isoprenoid side chains such as β-sitosterol or ergosterol. Hence, a pair of specific Mo-dependent S25DH and S26DH appears to be required for the hydroxylation of primary carbons at the individual isoprenoid chains. The reaction cascade may also be involved in the degradation of non-steroidal isoprenoids or tertiary alcohols. E.g., the anaerobic degradation of the fuel oxygenate methyl tert-butyl ether, an environmental pollutant of global concern, has frequently been described at anoxic environments39,40. However, the anoxic degradation of the tert-butanol intermediate is unknown but could in principle be accomplished via ATP-dependent dehydrogenation to isobutene, analogous to the described pathway.

Cholesterol degradation in anaerobic bacteria is initiated in the periplasm by AcmA, S25DH1, and probably AcmB dependent conversion into 25-OH-DDO/25-OH-DDO (oxygenation to the diene in ring A may also occur later in the pathway). However, subsequent alkene formation is ATP-dependent and therefore has to occur in the cytoplasm. In contrast, subsequent C26 hydroxylation and oxidation to the C26 aldehyde will again take place in the periplasm as evidenced by the N-terminal twin-arginine translocation (TAT) sequence present in S25DH and S26DH (Supplementary Table S3). Thus, initial steps of anaerobic cholesterol degradation involve enzymes alternatively accessing their substrates from the periplasm and the cytoplasm (Fig. 5). Though flip-flop of cholesterol between the two leaflets of biological membranes is generally considered fast with rate constants in the 10^{-4}–10^{-1} range41–43, it is 5.5-fold faster for 25-OH-cholesterol vs cholesterol43. Thus, initial side-chain-hydroxylation facilitates the accessibility of cholesterol-derived intermediates from both sides of the cytoplasmic membrane, which appears to be crucial for the reaction cascade involved in water-dependent primary carbon oxidation.

Methods

Chemicals and bacterial strains. The chemicals used were of analytic grade. Sterolibacterium denitrificans Chol-15 (DSMZ 13999) and Thauera aromatica K172 (DSMZ 6984) were obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). E. coli BL21 (DE3) and E. coli S2 were obtained from New England Biolabs (Frankfurt, Germany).

Synthesis of non-phosphorylated steroidal compounds. 25-Hydroxy-cholesta-1,4-diene-3-one (25-OH-DDO) was enzymatically synthesised from the commercially available cholest-4-en-3-one (CDO) using AcmA and S25DH1, from S. denitrificans after heterologous production in E. coli BL21 (DE3) and T. aromatica K172, respectively39. The enzyme assays contained 1–2 mM CDO, 50 mM Tris/HCl buffer (pH 7.5), 6% (w/v) 2-hydroxypropyl-β-cyclohexetrin (HPCD), 5 mM K3[Fe(CN)6], 2% (v/v) volume cell-free extracts from E. coli BL21 producing AcmA, and 4% (v/v) volume cell-free extracts from T. aromatica K172 producing S25DH. For synthesis of desmost-1,4-diene-3-one (DDO), soluble proteins from Sterolibacterium denitrificans (S. denitrificans) were used anaerobically as follows: 1–2 mM 25-OH-DDO, 50 mM Tris/HCl buffer (pH 7.8), 6% (w/v) HPCD, 5 mM ATP, 5 mM MgCl2, and 5% (v/v) volume (cell free) of S. denitrificans. 26-OH-ΑDDO and desmost-1,4-diene-3-one-26-al (DDO-26-al) were produced as follows: 1.1 mM DDO, 50 mM Tris/HCl (pH 7.0), 6% (w/v) HPCD, 5 mM K3[Fe(CN)6], and 5% (v/v) volume (cell free) of S. denitrificans. The enzymatic assays were performed on the 100–1000 µL scale at 30 °C for at least 16 hours. Products were extracted in 2 volumes ethyl acetate, evaporated, and purified by preparative HPLC. For columns and solvents used, see the description of enzyme assays with individual steroids. Samples were lyophilised and solved in 2-propanol before use.

Synthesis of 25-phospho-cholesta-1,4-diene-3-one. Bis(9H-fluoren-9-yl) diisopropylphosphoramide (90%, 29.0 mg, 50.0 µmol, 2.0 eq.) was evaporated with dry acetone (2 mL) and dissolved in dry N,N-dimethylformamide (1 mL). 25-OH-DDO (10 mg, 25 µmol, 1 eq.) and 4,5-dicyanoimidazole (3.9 mg, 30 µmol, 2 eq.) were added and the solution was stirred for 30 min. The solution was cooled to 0 °C and was treated with chloroperoxybenzoic acid (77%, 6.7 mg, 50 µmol, 2 eq.) was added. After stirring for 15 min, 1,8-diazabicyclo[5.4.0]undec-7-ene (20 µL, 125 µmol, 5 eq.) was added and the mixture was stirred for another 15 min. Subsequently, the solution was removed under reduced pressure and the crude mixture was purified by preparative HPLC. Solvent A: 10 mM NH4OAc in water, Solvent B: 10 mM NH4OAc in methanol, 0–1 min isocratic 100%, 8–9 min gradient to 100%, 9–35 min isocratic 100% B) to obtain the desired phosphate ammonium salt (5 mg, 9.74 µmol, 39%) as a colourless solid. The product was analysed by 1H, 13C, 31P(1H) NMR, and ESI-HRMS. The spectra are shown in the Supplementary Figs. S2 and S3. 1H NMR (400 MHz, methanol-d4) δ = 6.7–7.2 (d, J = 10.1 Hz, 1H), 6.23 (dd, J = 10.1, 2.0 Hz, 1H), 6.08 (t, J = 1.7 Hz, 1H), 2.64 – 2.55 (m, 1H), 2.45 – 2.38 (m, 1H), 2.11 (dt, J = 13.1, 3.7 Hz, 1H), 2.07 – 1.99 (m, 1H), 1.95 – 1.85 (m, 1H), 1.82 – 1.70 (m, 4H), 1.66 – 1.50 (m, 4H), 1.43 (s, 6H),
**Fig. 5 Proposed subcellular localisation of initial anaerobic cholesterol degradation steps.** For easier presentation, reduction of Δ24 double bond is omitted. The dotted arrows indicate that hydrophobic side chain needs to be bound by S25DH1 and S26DH1. The assignment of S25DH1 and S26DH1 to the periplasm are based on their TAT sequence, that of S25 dehydratase and aldehyde dehydrogenase (ALDH) to the cytoplasm on their cytoplasmic co-substrates. Hydroxylation at C25 and C26 abolish polarity of CDO/DDO and improves access from both sides of the cytoplasmic membrane.

Enzyme assays. (1) Preliminary tests for the conversion of 25-OH-CDO with cell-free extracts (0.4–0.6 mg mL⁻¹) of *S. denitrificans* were performed in 50 mM MOPS/KOH buffer (pH 7.0), 0.5 mM 25-hydroxy-cholesta-1,4-dien-3-one (25-OH-CDO) or 25-hydroxy-cholest-4-en-3-one (25-OH-CEO), 2 mM electron acceptor (K₂[Fe(CN)₆]), phenazine methosulfate, 2,6-dichlorophenolindophenol, methylene blue, NADP⁺, or NAD⁺ or 2 mM electron donor (titanium(III) citrate, sodium dithionite, benzyl viologen, methyl viologen, NADPH, or NADH) and 6% (v/v) HPCD at 30 °C under aerobic conditions. (2) ATP-dependent dehydration of 25-OH-CDO to DDO was determined at 30 °C in the presence of 50 mM Tris/HCl buffer (pH 7.8), 0.2 mM 25-OH-cholesta-1,4-diene-3-one (10–25 mM concentrated in 2-propanol), 5 mM ATP, 5 mM MgCl₂, and 6% (v/v) HPCD. For determination of kinetic parameters and stoichiometry of ATP hydrolysis, soluble proteins from *S. denitrificans* were subjected to (NH₄)₂SO₄ precipitation. At 40% saturation, the activity remained in the supernatant, whereas at 50% the enzyme was found in the precipitate. Enzyme assays were stopped by adding 5 volumes 2-propanol. After twofold-centrifugation, the supernatant was applied to an Acquity H-class UPLC system (Waters) for analysis. An Acquity UPLC CSH C18 (1.7 µm, 2.1 × 100 mm) column was used to separate the steroid compounds using 10 mM aqueous NH₄OAc and an increasing acetonitrile gradient. For the ATP/ADP stoichiometry measurements, the enzymatic assays were stopped using two (v/v) volumes ethyl acetate. The organic solvent was diluted in 5 (v/v) volumes 2-propanol and centrifuged twice (8,000 × g) and loaded onto an Acquity H-class UPLC system. The aqueous phase (20 µL) was acidified using 5 µL 0.25 M HCl and centrifuged twice for 20 min at 4 °C. The supernatant was neutralised using 20 (v/v) volumes MOPS/KOH buffer (pH 7.0). To determine the ATP consumption, an Acquity UPLC HSS T3 (1.8 µm, 2.1 × 100 mm) column was used isocratically with a 10 mM potassium phosphate buffer (pH 6.5).  

(3) Conversion of DDO to 26-OH-DDO and DDO-26-al was performed in cell-free extracts (0.4–0.6 mg mL⁻¹) of *S. denitrificans* or enriched S26DH (0.05–0.1 mg mL⁻¹) using 50 mM MOPS/KOH buffer (pH 7.0) in the presence of 0.2 mM DDO (added from 10–25 mM stock in 2-propanol), 5 mM K₂[Fe(CN)₆], and 6% (v/v) HPCD at 30 °C under shaking (300 rpm) in an anaerobic glove box (O₂ levels < 2 ppm). Assays were stopped as described above.  

(4) Conversion of DDO-26-al to DDO-26-carboxylate was carried out in 50 mM MOPS/KOH buffer (pH 7.0) using 0.2 mM DDO-26-al (10–25 mM stock in 2-propanol), 5 mM NAD⁺, and 6% (v/v) HPCD using cell-free extract (0.4–0.6 mg mL⁻¹) of *S. denitrificans* or cell-free extracts (1.5 mg mL⁻¹) of *E. coli* BL21 producing C26-ALDH at 30 °C under shaking (300 rpm). Assays were stopped as described above.  

Heterologous production of S26DHs in *T. aromatica*. (1) Using primers suitable for T4 ligation (Supplementary Table S4), the resulting 1.8-kb DNA fragment that was SpeI/XbaI double digested was used as a donor fragment for *T. aromatica*. Cells were cultivated under denitrifying conditions (30 °C) in phosphate-buffered medium with benzoate, supplemented with 100 µg mL⁻¹ ampicillin and 20 µg mL⁻¹ anhydrotetracycline. Cells were harvested in the late exponential phase (16 hours induction) and used for further experiments.
Heterologous production of DDO-26-al dehydrogenase (C26-ALDH) in E. coli BL21. The C26-ALDH gene (WP_154716401) was amplified using the primers listed in Supplementary Table S4. The resulting 1.5-kb DNA Fragment was digested by NheI/NcoI and cloned into pASK-IBA15plus and transformed into E. coli BL21. Induction of C26-ALDH was carried out as described for the AcmB gene.

Enrichment of S26DH, after heterologous production in T. aromaticum K172. All steps were performed under anaerobic conditions in an anaerobic glove box (95% N2, 5% H2, by vol.; O2 < 2 ppm). The buffers and reagents used were degassed using two volumes (v/v) of buffer A (20 mM Tris/HCl pH 7.0, 0.05 mM DTE, 0.02% (v/v) Tween 20, 500 mM KCl) from 50 mM to 150 mM KCl. Active fractions were concentrated (30-kDa cut-off membrane), diluted in 10 volumes of buffer C (20 mM TRIS/MES pH 6.0, 0.5 mM DTE, 0.01% (v/v) Tween 20), and applied to a Reactive Red 120 column (40 mL; GE Healthcare) at 5 mL min\(^{-1}\). Elution of active fraction was carried out by increasing the gradient of buffer D (20 mM Tris/HCl pH 8.0, 0.05 mM DTE, 0.01% (v/v) Tween 20) from pH 7 to pH 8. The active protein fraction was concentrated (30-kDa cut-off membrane), and applied to a Superdex 200 column (320 mL) at 2 mL min\(^{-1}\) that was equilibrated with buffer E (20 mM Tris/HCl pH 7.5, 150 mM KCl). The protein eluted in a single peak and was used for further experiments. Uncropped and unprocessed SDS-polyacrylamide gels of enriched enzyme are available in the accompanying Source Data File.

Spectrophotometric determination of Mo, Fe and heme b. Iron was determined with o-phenanthroline using a modified protocol as described\(^{45}\). Enriched proteins (5 µL) were added to 245 µL H2O and acidified with 7.5 µL 25% HCl, mixed and incubated at 80 °C for 10 min. The supernatant was used for enzyme enrichment. The soluble proteins were anaerobically conducted complete reduction of the enzyme with sodium dithionite.

Protein identification by mass spectrometry of peptides. Proteins were identified by excising the bands of interest from SDS-PAGE gels. After in-gel digestion with trypsin, the resulting peptides were separated by UPLC and identified using a Synapt G2-Si high-resolution mass spectrometry (HRMS) electrospray ionisation quadrupole time-of-flight (ESI-Q-TOF) mass spectrometry system (Waters); the system has been described in detail previously\(^{46}\).

LC-ESI-MS analyses of steroid compounds. Metabolites were analysed by an Acquity UPLC system (Waters) using an Acquity UPLC CSH C18 (1.7 µm, 2.1 x 100 mm) column coupled to a Synapt G2-Si HRMS ESI-Q-TOF device (Waters). For separation, an aqueous 10 mM NH4OAc/acetonitrile gradient was applied to a DEAE-Sepharose column (75 mL; GE Healthcare) at 7.5 mL min\(^{-1}\). Elution of active fraction was monitored by mass spectrometry of peptides with o-phenantroline using a modified protocol as described\(^{45}\). Enriched proteins were incubated at 80 °C for 10 min. The absorbance was measured in the organic phase (lipids).

Heterologous production of DDO-26-al dehydrogenase (C26-ALDH) in E. coli BL21. The C26-ALDH gene was amplified using the primers listed in Supplementary Table S4. The resulting 1.5-kb DNA Fragment was digested by NheI/NcoI and cloned into pASK-IBA15plus and transformed into E. coli BL21. Induction of C26-ALDH was carried out as described for the AcmB gene.
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Author contributions

CJ performed all enzyme assays, carried out UPLC and MS analyses, analysed data, heterologously produced and characterised enzymes, prepared figures and contributed to the writing of the manuscript; S.F. conducted NMR analyses, D.B. and H.I. chemically synthesised and analysed a 25-phospho-desmost-14,14-diene-3-one standard, and contributed to the writing of the paper; M.M. analysed NMR data and contributed to the design of the study; M.B., designed the study, analysed data and wrote the paper. All authors discussed the results and commented on the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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