

Supplementary materials

Cultivation protocols

Cultivation and growth conditions of Methanopyrus kandleri and Methanothermobacter marburgensis

Methanopyrus kandleri and *Methanothermobacter marburgensis* were grown in closed batch cultivation mode under anaerobic conditions with a mixture of molecular hydrogen (H_2) and carbon dioxide (CO_2) containing 20 vol.-% CO_2 in H_2 (H_2/CO_2 , 4:1) at an overpressure of 2 bar. Both methanogens were grown in 117 mL serum flasks with a crimped-on rubber stopper (pretreated by boiling ten times for 30 min in fresh ddH₂O; 20 mm, butyl rubber, CLS-3409-14, Chemglass Life Sciences, USA) as reported before [1]. *M. kandleri* was grown at 97 °C shaking at 120 revolutions per minute (rpm) in an water bath (GFL Shaking and Heating Water Bath 1083, Lauda Dr. R. Wobser GmbH & Co. KG, Lauda-Königshofen, Germany) filled with ROTITHERM® fluid, while *M. marburgensis* was grown in a water bath at 65 °C shaking at 120 rpm (GFL Shaking and Heating Water Bath 1083, Lauda Dr. R. Wobser GmbH & Co. KG, Lauda-Königshofen, Germany).

Methanopyrus kandleri growth medium

M. kandleri was grown in DSMZ 511 media containing (per 1 L ultrapure, i.e., 18 MΩ water, further referred to as MQ water) 0.25 g NH_4Cl , 0.087 g K_2HPO_4 , 0.09 g KH_2PO_4 , 11.80 g $NaCl$, 1.75 g $MgSO_4 \cdot 7H_2O$, 4.5 g $MgCl_2 \cdot 6H_2O$, 0.81 g Na_2SO_4 , 0.78 g $CaCl_2 \cdot 2H_2O$, 0.3 g KCl , 10 mL TE solution, 2 mL $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ solution (0.1% w/v), 2 mL $(NH_4)_2Ni(SO_4)_2$ solution (0.1% w/v), 2 mL $Na_2WO_4 \cdot 2H_2O$ solution (0.1% w/v), 10 mL Marine TE, 0.5 mL Na-resazurin solution (0.1% w/v), 1 g $NaHCO_3$, and 10 mL vitamin solution. Fifty mL of medium was then added to 117 mL serum bottles, closed with rubber stoppers (see above) and sealed with crimp caps (20 mm aluminum, Ochs Laborbedarf, Bovenden, Germany). The medium was flushed with 20 vol.-% CO_2 in N_2 (N_2/CO_2 , 4:1). The pH was adjusted to 6.5 using HCl and the bottles were autoclaved. After the bottles cooled down, 0.2 mL of $Na_2S \cdot 9H_2O$ (0.5 mol L⁻¹) were added per bottle and the medium was flushed with H_2/CO_2 (4:1). The media was stored in the dark at room temperature.

The TE solution contained (per 1 L of MQ water) 1.5 g nitrilotriacetic acid, 3 g $MgSO_4 \cdot 7H_2O$, 585 mg $MnCl_2 \cdot 4H_2O$, 1 g $NaCl$, 100 mg $FeSO_4 \cdot 7H_2O$, 180 mg $CoSO_4 \cdot 7H_2O$, 100 mg $CaCl_2 \cdot 2H_2O$, 180 mg $ZnSO_4 \cdot 7H_2O$, 6 mg $CuSO_4$, 20 mg $KAl(SO_4)_2 \cdot 12H_2O$, 10 mg H_3BO_3 , 10 mg $Na_2MoO_4 \cdot 2H_2O$, 30 mg $NiCl_2 \cdot 6H_2O$, 0.3 mg $Na_2SeO_3 \cdot 5H_2O$, and 0.4 mg $NaWO_4 \cdot 2H_2O$. The TE solution was autoclaved.

The marine TE solution contained (per 1L of MQ water) 4 g $NaBr$, 1.8 g $SrCl_2 \cdot 6H_2O$, 0.529 g H_3BO_3 , 1 g KI , 100 mg Na-silicate, 0.183 mg NaF , 100 mg KNO_3 , and 0.128 mg Na_2HPO_4 . The marine TE solution was autoclaved.

The vitamin solution contained (per 1 L of MQ water) 2 mg biotin, 2 mg folic acid, 10 mg pyridoxine-HCl, 5 mg thiamine-HCl- $2H_2O$, 5 mg riboflavin, 5 mg nicotinic acid, 5 mg D-Ca-pantothenate, 0.1 mg vitamin B12, 5 mg p-aminobenzoic acid, and 5 mg lipoic acid. The vitamin solution was filter-sterilized and kept at 4 °C in the dark.

Methanothermobacter marburgensis growth medium

M. marburgensis was grown in minimal medium containing 2.1 g NH_4Cl , 6.8 g KH_2PO_4 , 3.6 g Na_2CO_3 , 10 mL of 100× TE solution (TES3) in 1000 mL of ddH₂O. Fifty mL of medium was then added to 117 mL serum bottles, closed with rubber stoppers (see above) and sealed with crimp caps as above. The medium was flushed with N_2/CO_2 and the bottles were autoclaved. After anaerobization and autoclaving the medium [1], 0.2 mL of $Na_2S \cdot 9H_2O$ (0.5 mol L⁻¹) were added per bottle and the medium was flushed with H_2/CO_2 (4:1).

The TES3 solution contained 9 g Titriplex I, to which 800 mL ddH₂O was added and pH adjusted to 6.5 with 5 mol L⁻¹ NaOH solution, then other components were added, namely 4 g $MgCl_2 \cdot 6H_2O$, 1 g

FeCl₂·4H₂O, 20 mg CoCl₂·6H₂O, 120 mg NiCl₂·6H₂O, and 20 mg NaMoO₄·2H₂O. The volume was made up to 1000 mL and the pH was adjusted to 7.0 with 1 mol L⁻¹ NaOH. The TES3 was autoclaved.

Haloferax volcanii growth conditions and medium

H. volcanii was grown as described previously [2] in the medium containing 3 mol L⁻¹ NaCl, 150 mmol L⁻¹ MgSO₄, 50 mmol L⁻¹ KCl, 3 mmol L⁻¹ CaCl₂·H₂O, 10 nmol L⁻¹ MnCl₂, 25 mmol L⁻¹ Tris-HCl pH 7.2, 0.5% (w/v) tryptone, 0.3% (w/v) yeast extract, and 0.02% (w/v) histidine. The medium was filter sterilized prior to inoculation. The organism was cultivated at 37 °C in an air incubator 170 L ZWYR-2102C (LABWIT Scientific, Burwood East, VIC, 3151, Australia) upon constant orbital shaking at 180 rpm.

Cultivation and growth conditions of Saccharolobus solfataricus and Sulfolobus acidocaldarius

S. solfataricus and *S. acidocaldarius* were cultivated in 200 mL long neck bottles containing 50 mL of the cultivation medium at 73 °C upon orbital shaking (100 rpm) in a water bath filled with ROTITHERM® fluid as described before [3]. The cultivation (Brock) medium was prepared from defined stock solutions: Brock solution I (100×) contained 130 g L⁻¹ (NH₄)₂SO₄, 25 g L⁻¹ MgSO₄·7H₂O, 2 g L⁻¹ FeCl₃·6H₂O, and 3 mL 50% H₂SO₄. Brock solution II (200×) contained 56 g L⁻¹ KH₂PO₄ supplemented with trace element stock solutions (10 g L⁻¹ each of MnCl₂, ZnSO₄, CuCl₂, VOSO₄, Na₂MoO₄, CoSO₄, Na₂B₄O₇) in the following volumes: 36 mL MnCl₂, 4.4 mL ZnSO₄, 1 mL CuCl₂, 600 µL VOSO₄, 600 µL Na₂MoO₄, 200 µL CoSO₄, and 90 µL Na₂B₄O₇. After autoclaving, the pH was adjusted to 5.0 with 50% (v/v) H₂SO₄. Brock solution III (1000×) consisted of 14 g L⁻¹ CaCl₂·2H₂O. Additional supplements included 20% (w/v) D(+) sucrose and 10% (w/v) tryptone. All stock solutions were prepared separately, sterilized by filtration where appropriate, and stored at 4 °C.

For culture preparation, 50 mL of Brock medium was assembled as follows: 40 mL sterile water, 500 µL Brock solution I, 250 µL Brock solution II, 50 µL Brock solution III, 500 µL 20% sucrose, and 500 µL 10% tryptone. The volume was adjusted to 50 mL with sterile MQ water.

Cultivation and growth conditions of Metallosphaera sedula

M. sedula was cultivated under the same cultivation conditions as described above for *S. solfataricus* and *S. acidocaldarius*. However, DSMZ medium 88 was used for cultivation of *M. sedula* and contained per L: 1.30 g (NH₄)₂SO₄, 0.28 g KH₂PO₄, 0.25 g MgSO₄·7H₂O, 0.07g CaCl₂·2H₂O, 0.02 g FeCl₃·6H₂O and 10 mL of 100× Allen's trace element solution.

Allen's trace element solution contained (per L): 180 mg MnCl₂·4H₂O, 450 mg Na₂B₄O₇·10H₂O, 22 mg ZnSO₄·7H₂O, 5 mg CuCl₂·2H₂O, 3 mg Na₂MoO₄·2H₂O, 3 mg VOSO₄·2H₂O, 1 mg CoSO₄·7H₂O. The media was supplemented with 20 g L⁻¹ pyrite and subsequently titrated to a final pH of 2.0 with H₂SO₄ prior to autoclaving and inoculation.

Supplementary Tables

Table S1. Description of samples/isolates, cultivation temperatures, biomass and lipid yields, and presence of two enzymes, i.e. glycerol-1-phosphate dehydrogenase (G1P, EC 1.1.1.261) and glycerol-3-phosphate dehydrogenase (G3P, EC 1.1.5.3), as per GenBank. Gene accession numbers (GenBank) are quoted if available.

Sample/species	Collection accession number	Reference	Note	Cultivation temperature (°C)	Dry biomass (mg)	Total lipids (mg)	G1P	G3P
<i>Haloferax volcanii</i>	WR340	[2]		37	168.3	4.58	TVT93737.1	TVT95752.1
<i>Metallosphaera sedula</i>	DSM 8348	[3]		73	60.1	2.15	AKV81900.1	AKV80190.1
<i>Methanopyrus kandleri</i> 1	AV19	[4]		97	14.7	0.76	WP_011019598.1	not found
<i>Methanopyrus kandleri</i> 2	AV19	[4]	Dark and slimy pellet	97	34.5	1.38	WP_011019598.1	not found
<i>Methanothermobacter marburgensis</i>	DSM 2133	[5]		65	37.0	1.62	WP_013295808.1	not found
<i>Saccharolobus solfataricus</i> 1		[6]	1 st generation from cryostock	73	37.3	1.08	QPG51023.1	QPG49855.1
<i>Saccharolobus solfataricus</i> 2	DSM 1616	[6]	2 nd generation from cryostock	73	32.8	0.84	QPG51023.1	QPG49855.1
<i>Sulfolobus acidocaldarius</i>	DSM 639	[6]		73	22.0	1.13	ALU31773.1	ALU32401.1

Table S2. Specific rotation of archaeol isomers obtained from the commercial standard. For chromatogram please see Fig. 4.

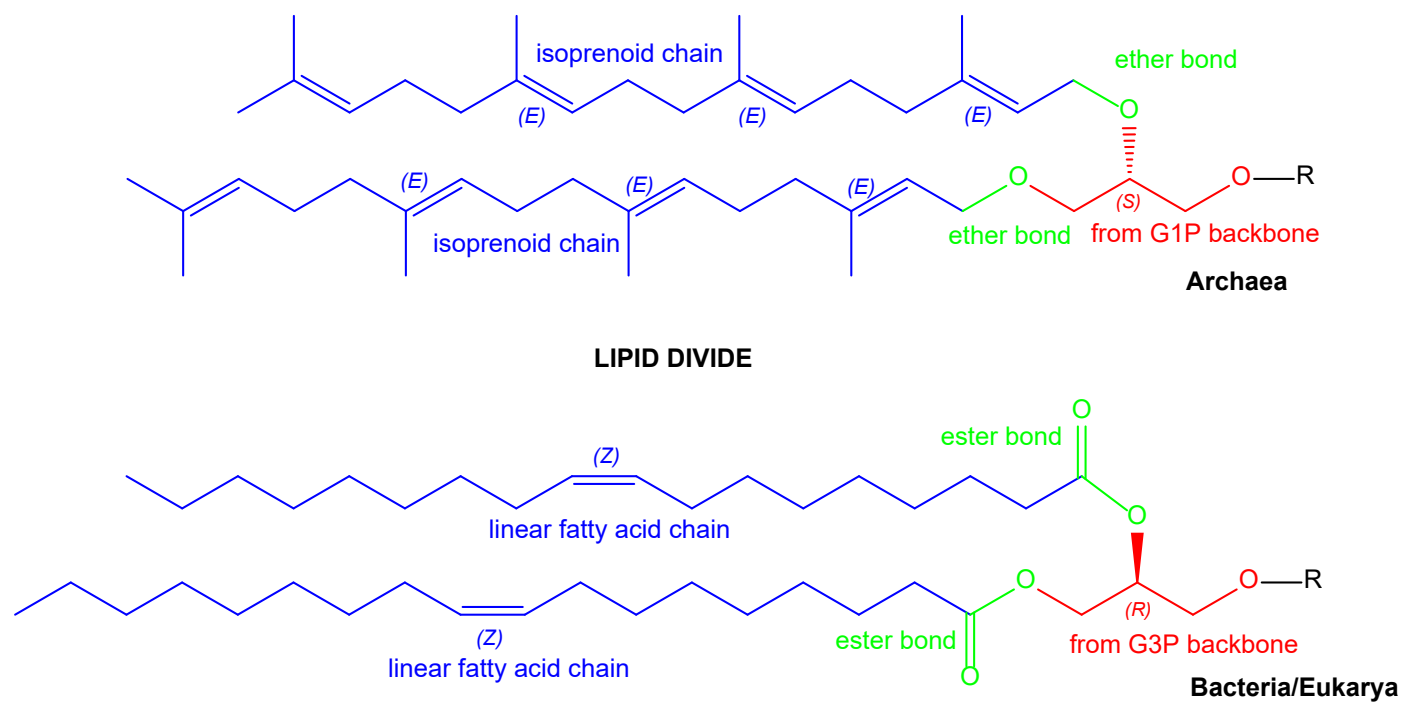
Archaeol	% of all isomers	$[\alpha]_D$	tR (min)
(<i>S</i>)-2,3-bis(((3 <i>S</i> ,7 <i>R</i> ,11 <i>R</i>)-3,7,11,15-tetramethylhexadecyl)oxy)propan-1-ol	1.1	- ^a	31.11
(<i>S</i>)-2,3-bis(((3 <i>R</i> ,7 <i>R</i> ,11 <i>R</i>)-3,7,11,15-tetramethylhexadecyl)oxy)propan-1-ol	10.7	-8.06 ^b	31.67
(<i>R</i>)-2,3-bis(((3 <i>S</i> ,7 <i>R</i> ,11 <i>R</i>)-3,7,11,15-tetramethylhexadecyl)oxy)propan-1-ol	4.3	7.19	32.04
(<i>R</i>)-2,3-bis(((3 <i>R</i> ,7 <i>R</i> ,11 <i>R</i>)-3,7,11,15-tetramethylhexadecyl)oxy)propan-1-ol	83.9	8.15 ^c	32.66

^a not measured due to low yield

^b a value $[\alpha]_D -7,1^\circ$ was published for (*S*)-2,3-bis(((3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyl)oxy)propan-1-ol [7].

^c various authors have published the following $[\alpha]_D$ for (*R*)-2,3-bis(((3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyl)oxy)propan-1-ol, i.e., +7,1° [8], +8,5° [9], +9.68 [10], or +8,4° [11].

Supplementary Figures



R = polar head including a phosphoric acid ester and carbohydrate, choline, ethanolamine, etc.

Fig. S1. Structures of core lipids of Archaea and Bacteria/Eukarya, defined as lipids lacking a polar head group including a phosphoric acid ester.

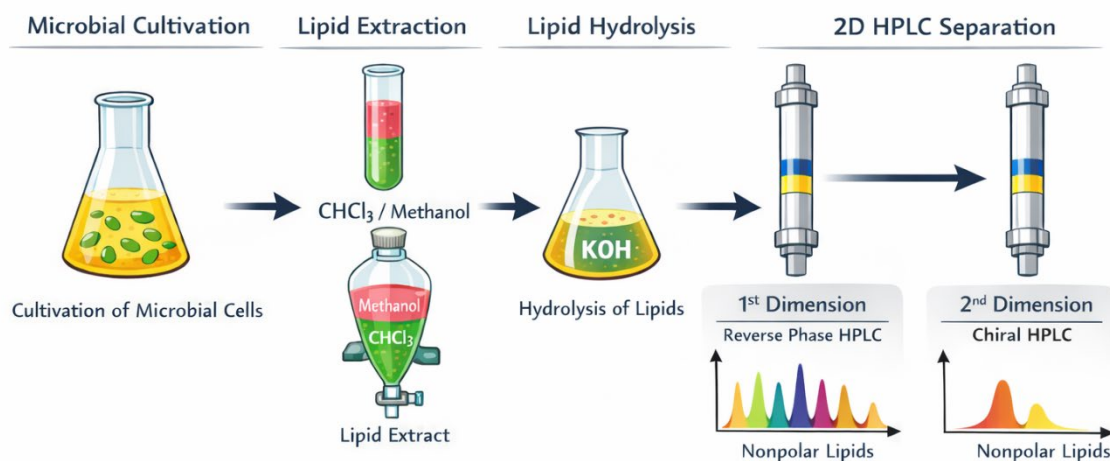


Fig. S2. Step-by-step lipid analysis process. Microbial lipids were extracted from cultivated cells using a chloroform/methanol-based protocol and subsequently hydrolyzed under alkaline conditions to obtain core lipids. The hydrolyzed lipid fraction was analyzed using two-dimensional HPLC, combining reverse-phase separation with chiral HPLC. This approach enabled efficient separation and characterization of nonpolar lipids, including stereochemical resolution. The image was created in BioRender.com.

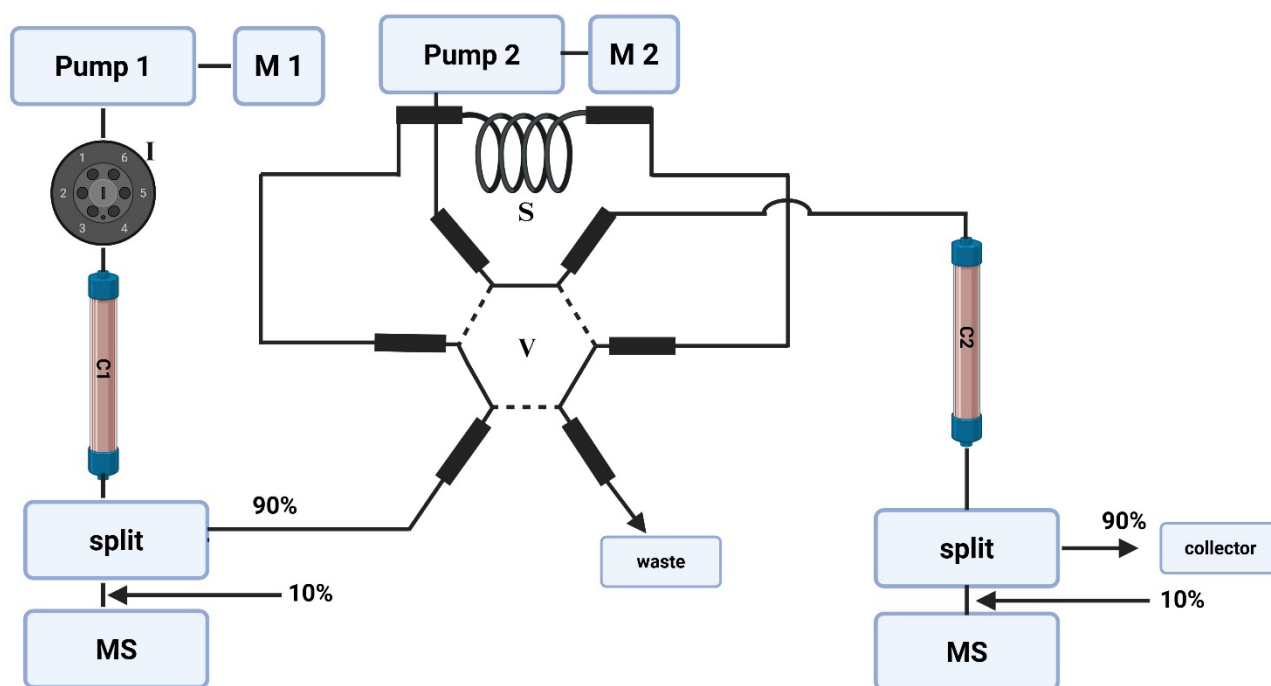


Fig. S3. A schematic diagram of the automatic column-switching HPLC system used in this study is shown. The solid and dotted lines indicate the switch positions of valve A and B, respectively. The abbreviations used are as follows: C1, three Luna Omega columns (C18); C2, two Astec Cyclobond™ I 2000 DMP chiral LC columns (3,5-dimethyl-phenyl-carbamate modified β -cyclodextrin); I, injector, S; sample loop (200 μ L); M1, ACN with 5 mmol L⁻¹ AcNH₄ and iPrOH with 5 mmol L⁻¹ AcNH₄ (gradient); M2, hexane and iPrOH (97:3 v:v with 10 mmol L⁻¹ AcNH₄). The image was created in BioRender.com.

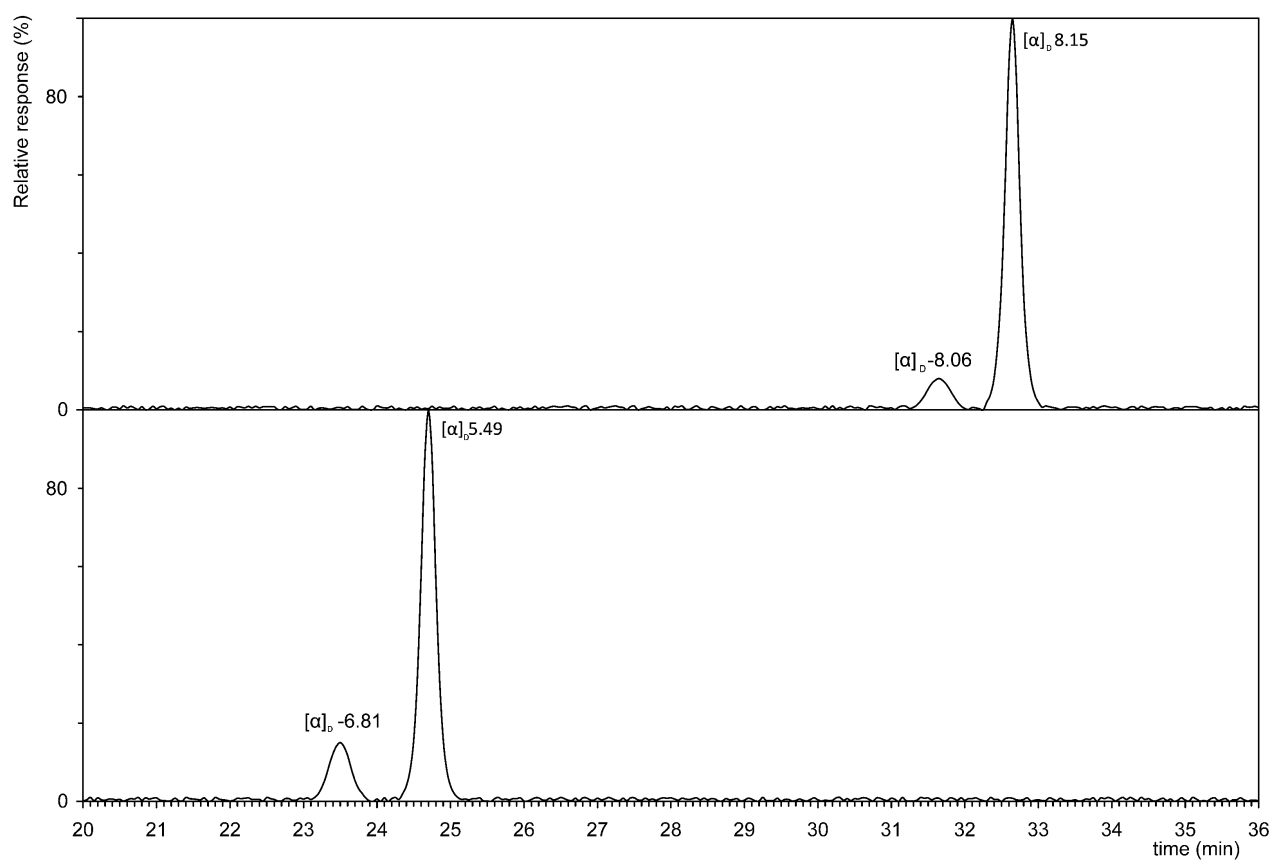
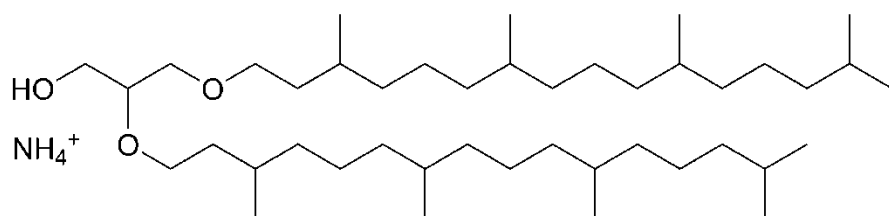


Fig. S4. Chiral chromatography of archaeols (tR 31.67 min and tR 32.66 min) and extended archaeols (tR 23.52 min and tR 24.71 min) including specific rotation ($[\alpha]_D$) values of all four isomers.

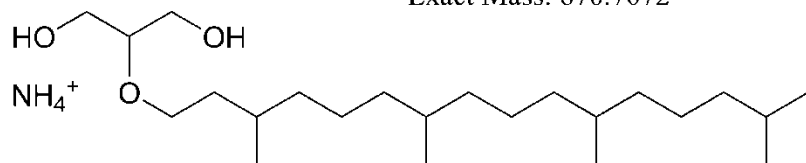
<i>Haloferax</i> /1-348	1	-----MFDKSTWIKLPNRVLOGHGVLDLSSAAVDLYL-SGDPFLVTSPPDRIAGDRVRDQFDG-----ITSISLDRASFES	72
<i>Metallosphaera</i> /1-351	1	-----MEIHEHI IELPKKVYVNGILSKLRDYLQNLN-LEPVLVVTGPNVRKIV----IDEVAKGLNEIGKIEFIEVLDSSIDE	75
<i>Methanopyrus</i> /1-353	1	-----MSVPKKRMQLPREVVVGSNVLPVVKLLRSVGVDPGVAVFSGRRTMKIAGNEVADHLEEAGY---QTSFVIVKSGTGDD	77
<i>Methanothermobacter</i> /1-347	1	-----MDPRKIQLPREIHTGPGVIEDTGRICSDLRF-EGRVMVVTGPTLKIAGETAIESLQDAGF---EVDQVTVGDMATMAS	74
<i>Saccharolobus</i> /1-351	1	-----MNVKEHVISLPRRVFVGHDIYDISIYFSQLGV-TTPFLIVTGTYTKKIADKVIENLPKDA---KYEVEIDSATLDD	75
<i>Sulfolobus</i> /1-356	1	MLYKTVETKEHI INLPKHYYTGIGLDNFRNYLQTLNL-PQPLVITGPIVHQEIFSKRIEHEIKDF---KYEVIIVNKSDDLSE	80
<i>Haloferax</i> /1-348	73	VERVVDAAAREADAGYLIALGGGKPIDVAKMASDRLDCCFISVPTAASHDGI VSGRSSIPEGDTRHSVAADPPLAVVADTELLAEA	157
<i>Metallosphaera</i> /1-351	76	VNRVEEKAKQLNPKFVIIGIGGKTIIDVAKYVAYKLVNFIISVPTAPSHDGI TSPFASIKGLGKPVSVKAKMPYAI IADINVLSA	160
<i>Methanopyrus</i> /1-353	78	VKKALEALDEIDADVPAVGGGKVIDVAKVASVRRGIPFISVPTSASHDGI ASPFASIRREGRPYSEPAQAPLAILADIEVIREA	162
<i>Methanothermobacter</i> /1-347	75	VAQVQDGLN--GTSLVIGVGGGKVIDVAKMAATLEGVHFISVPTAASHDGI ASPRASIRNREGTASLEASSPIGVIADETVIRKA	157
<i>Saccharolobus</i> /1-351	76	VYMVEEVKIRISPSLLIGIGGKVIDVTKYAAFRNSLEFVS IPTSPSHDGI TSPFASIKGLGKPVSVKAKEPLAI IADIEILSLS	160
<i>Sulfolobus</i> /1-356	81	AEKVEDIARQKGKIIIGVGGGKVIDIAKFTAYKIDREFIS IPTSPSHDGI TSPFAAIKGLGKPIGKAKEPLAI ISDEIILASA	165
★			
<i>Haloferax</i> /1-348	158	PWELTTAGCADIISNYTAVKDWELAHRLKNVEYSEYAGALSQMTAEMLVGSADSIKPGLEESAWIVTKALVSSGVAMSIAGSSRP	242
<i>Metallosphaera</i> /1-351	161	PRRLINSGIGDTIGKLVAVRDWKLASKLTGEYYGDYTASLALSAXHALSCTRIIHRDLKYSVSLLEALISSGVAMGMAGSTRP	245
<i>Methanopyrus</i> /1-353	163	PERLIRAGVGDVVSNTAVKDWRLAHRLRNEPYSEYASSLSLMAARIVMKNAPKIGKLLPEGIKKLVLQALISGGVAMSIAGSSRP	247
<i>Methanothermobacter</i> /1-347	158	PFRLLASGCADIISNYTAIMDKLHRLNERYSESAAALSLMTAKMI IKSADAIKEGLEESARLAVKSLISSGIVISAGSSRP	242
<i>Saccharolobus</i> /1-351	161	PRRLINAGIGDTIGKIIAVRDWKLAAKLGEYYGDYTASLALMSAKHAFQCTKI INKDIKYGVRLMEALISSGVAMGMAGSTRP	245
<i>Sulfolobus</i> /1-356	166	PRRLINAGIGDTLGKITA VRDWRHLAKLRGEYYGDYTASLALMSARHALSCTKI INKDIRAGVRVLTEALISSGVAMGMAGSTRP	250
X			
<i>Haloferax</i> /1-348	243	ASGAELHFSHQLDRIAPGSALHGHQVGVGSIIMTEYFHSRGARGEWTDIRDALSTMGAPTADELGIIDETII EALTTAHEIR-DRY	326
<i>Metallosphaera</i> /1-351	246	ASGSEHLFAHVDLQPNALHGLVGMGSIIMAYIHG---INWREIRNALDRIGAPTTAKQLGIPNDVIIKALTIAHTIRPERY	327
<i>Methanopyrus</i> /1-353	248	CSGSEHLFSHALDVIAEPALHGEQCGVGTIIMEYHLG---GNWREIRETLETAGAPTTAEDLGVSDDEEII EALCRAHKIRPDY	329
<i>Methanothermobacter</i> /1-347	243	ASGSEHKFSHALDMVAEPALHGEQCGVGTIMMMHLG---GDWRFRIDALARINAPTTAAELGIDPEYII EALTVAHTIRKERY	324
<i>Saccharolobus</i> /1-351	246	ASGSEHLFAHVELIHPEGILHGLVGLGTIIMAYIHG---INWKIIRNRLKKIGFPVKAKDLGLSDEEVIKALTIAHTIRPERY	327
<i>Sulfolobus</i> /1-356	251	ASGSEHLFAHAIELLYPNLGLHGLVALGTIIMAYIHG---INWRRIRRAMKKIGLFPVTSKQIGIPDEGII KALTIAHSIRPERY	332
X X			
<i>Haloferax</i> /1-348	327	TILGN-GVSEEAIEAATITGVI-	348
<i>Metallosphaera</i> /1-351	328	TILGDRGLTWASAEKVARDTGVI	351
<i>Methanopyrus</i> /1-353	330	TILGDKGLTREAAERAAETGVIQ	353
<i>Methanothermobacter</i> /1-347	325	TILGDRGLTREAAERLAKITEVI-	347
<i>Saccharolobus</i> /1-351	328	TILGDRGLTWSSAEKIARVTKIID	351
<i>Sulfolobus</i> /1-356	333	TILGDRGLTWESAEKIARETGVL	356

Fig. S5. Multiple sequence alignment of identified glycerol-1-phosphate dehydrogenases from the organisms used in this study. Grey-highlighted amino acids indicate conserved residues. Squared residues represent the Rossmann fold, which facilitates NADH/NADPH coenzyme binding. X-marked residues indicate Zn²⁺-binding sites, while star-marked residues denote substrate-binding positions.



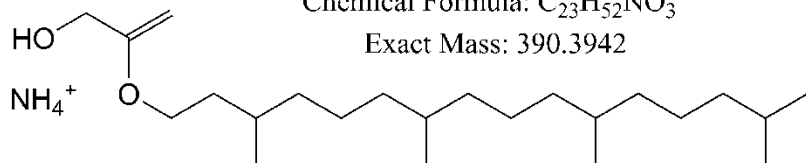
Chemical Formula: $C_{43}H_{92}NO_3^+$

Exact Mass: 670.7072



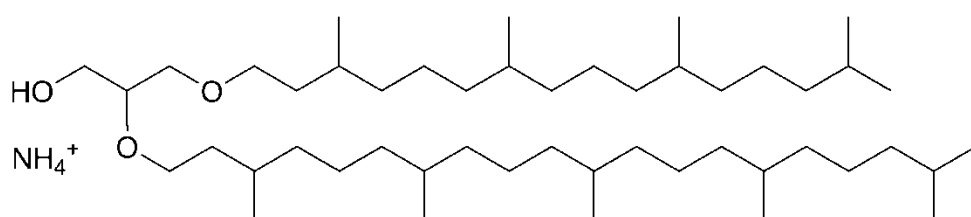
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Exact Mass: 390.3942



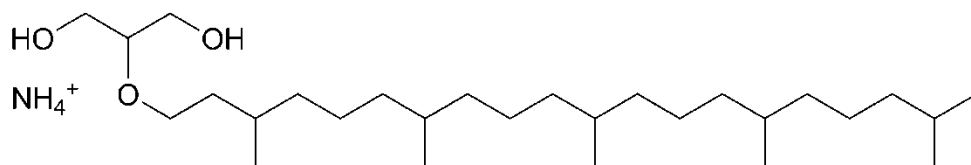
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Exact Mass: 372.3836



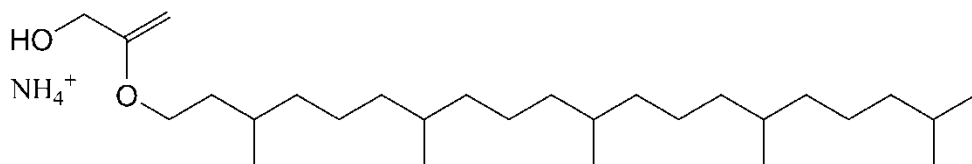
Chemical Formula: $C_{48}H_{102}NO_3^+$

Exact Mass: 740.7854



Chemical Formula: $C_{28}H_{62}NO_3^+$

Exact Mass: 460.4724



Chemical Formula: $C_{28}H_{60}NO_2^+$

Exact Mass: 442.4619

Fig. S6. Predicted structure of archaeol (C20-C20-AR, uppermost row) and its fragment ions, rows 2 and 3 from the top, quoting exact masses and summary chemical formulas. The lower half shows extended archaeol (C25-C20-AR, 4th row from the top) and its fragment ions quoting the same information as above.

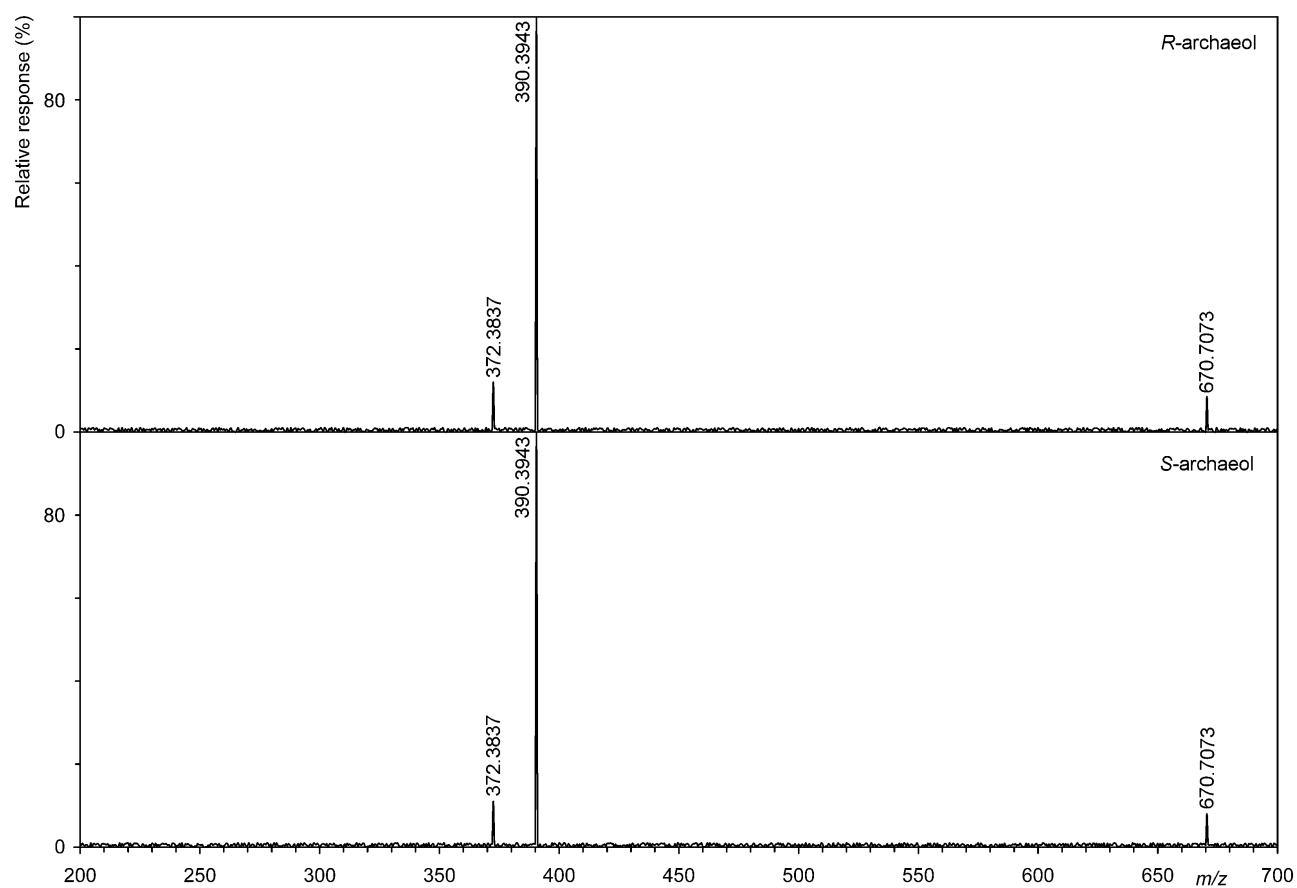


Fig. S7. Tandem MS of *R*-archaeol and *S*-archaeol.

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