Supplementary Information

In vitro reconstruction of tetronate RK-682 biosynthesis

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### Supplementary Results

#### Table 1. Features of orfs in the RK-682 biosynthetic gene cluster.

| Gene | Amino acids | Sizes of proteins including His-tag* expressed in this study (Da) | Proposed function |
|------|-------------|---------------------------------------------------------------|-------------------|
| orf1 | 381         |                                                               | putative dipeptidyl aminopeptidase |
| rkA  | 598         | 67,124                                                        | palmitoyl-CoA synthetase            |
| rkB  | 104         | 13,444 (holo form)                                            | discrete ACP                   |
| rkC  | 1006        | 108,446 (holo form)                                          | type I PKS (KS-ATa-ACP)          |
| rkD  | 343         | 38,057                                                        | 3-oxoacyl-(acyl carrier protein) synthase III (FabH) |
| rkE  | 641         | 72,317                                                        | FkbH-like glyceryl-S-ACP synthase |
| rkF  | 79          | 11,153 (holo form)                                            | discrete ACP                   |
| rkG  | 267         |                                                               | type II thioesterase             |
| rkH  | 531         |                                                               | antibiotic efflux protein        |
| rkl  | 229         |                                                               | TetR-family transcriptional regulator |
| orf1*| 283         |                                                               | putative β-lactamase domain-containing protein |

* The molecular mass of each protein was calculated assuming removal of the N-terminal methionine residue.
Table 2. Mass spectrometric analysis of production of glyceryl-S-ACP.

| Substrates | Products       |          |          |
|------------|----------------|----------|----------|
|            | ACP            | holo-ACP | glyceryl-S-ACP |
| FkbH analogue | RkB 100%     | ND       |           |
| RkE        | Tmn7a 80%      | 20%      |           |
| Tmn16      | RkB 100%      | ND       |           |
|            | Tmn7a 5%      | 95%      |           |

Percentages are relative to total amount of ACP species present. ND, the predicted mass was not detected by LC-MS.
Figure 1. Schematic representation of in-frame deletion of *rkD* mediated by construct pYH230 (a) and confirmation by Southern blot (b), and LC-MS analysis of RK-682 standard, wild-type, ΔrkD mutant and complementation strains (Supplementary Methods) (c). The numbers between *Age*I sites, and also marked by open or solid arrows in Southern blot (b) represent the expected size of the fragments in wild-type and *rkD* mutant chromosomal DNA, respectively, after digestion with *Age*I and hybridization to the probe (a 568 bp PCR product amplified from pYH230 by oligonucleotide primers rkD-CP1 and rkD-CP2, shown in the solid quadrangle). Peaks (due to complexation with divalent metal ions) in (c) identified as RK-682 are indicated with asterisks.
Figure 2. Schematic representation of the RK-682 biosynthetic gene cluster and of the plasmids (Supplementary Methods) used for heterologous expression in *Saccharopolyspora erythraea* JCB2.
Figure 3. LC-MS detection of RK-682 in culture extracts from wild-type strain (a), heterologous expression strains JCB2::pYH281 with PactII-ORF4 promoter (b) and JCB2::pYH282 with PermE* promoter (c). Peaks identified as RK-682 are indicated with asterisks. Strains were grown and analyzed under the same conditions (Supplementary Methods).
Figure 4. LC-MSn spectra of RK-682 in culture extracts of heterologous expression strain JCB2::pYH273 (Supplementary Methods). Peaks identified as RK-682 are indicated with asterisks.
Figure 5. In-frame deletion of \textit{rkH}. (a) Deletion was mediated by construct pYH280 and (b) confirmation was by Southern blot analysis. The numbers between \textit{Pvu}I or \textit{Kpn}I sites and also marked by open or solid arrows in the Southern blot represent the expected size of the fragments in wild-type and \textit{rkH} mutant chromosomal DNA, respectively, after digestion with \textit{Pvu}I or \textit{Kpn}I and hybridization to the probe (a 537 bp PCR product amplified by primers \textit{rkH-CP1} and \textit{rkH-CP2}, shown in the solid quadrangle).
Figure 6. LC-MS detection of RK-682 in culture extracts from RK-682 producer wild-type strain (a) and ΔrkH mutant (b). Peaks identified as RK-682 are indicated with asterisks. (c) Strains 88-682 (wild-type) and ΔrkH (mutant) were cultured on TWM plates for 6 days (Supplementary Methods).
Figure 7. In-frame deletion of rkG. (a) Deletion was mediated by construct pYH279; (b) PCR-sequencing confirmation of gene disruption of rkG by in-frame deletion; LC-MS analysis of culture extracts of RK-682 producer wild-type strain (c) and ΔrkG mutant (d). The numbers in (a) and also marked by open or solid arrows in (b) represent the expected size of the fragments in wild-type and ΔrkG mutant chromosomal DNA, respectively, after PCR with primers rkG-CP1 and rkG-CP2. Peaks identified as RK-682 are indicated with asterisks.
Figure 8. LC-MS detection of RK-682 in culture extracts from wild-type strain (a), heterologous expression strains JCB2::pYH285 (b) and JCB2::pYH284 (c). Peaks identified as RK-682 are indicated with asterisks. Strains were grown and analyzed under the same conditions (Supplementary Methods).
Figure 9. LC-MS evidence for formation of glyceryl-S-RkF. (a) LC-MS analysis of reaction mixture containing RkE, holo-RkF, D-3-phosphoglycerate (D-3-PG), D-3-phosphoglyceric acid phosphokinase (D-PGK), ATP and MgCl₂; (b) corresponds to glyceryl-S-RkF (11,241 Da), while the peak in (a) corresponds to holo-RkF (11,153 Da) that was used as a control. Additional peaks at M+178 Da represent adventitious gluconylation of N-terminal His6-tag during expression of the recombinant protein in *E. coli*. 
Figure 10. LC-MS evidence for in vitro formation of palmitoyl-S-RkB. The assay mixture (a) contained RkA, holo-RkB, palmitic acid, CoA, ATP and MgCl₂, while the assay (b) without RkA was used as control. The peaks representing holo-RkB (13,442 Da) and palmitoyl-S-RkB (13,681 Da) are indicated by vertical dashed lines.
Figure 11. In-frame deletion of rkA. (a) construct pYH214 used for the deletion; (b) confirmation of the deletion by Southern blot analysis. The numbers between PvuII sites, and also marked by open or solid arrows in the Southern blot, represent the expected size of the fragments in wild-type and ΔrkA mutant chromosomal DNA, respectively, after digestion with PvuII and hybridization to the probe (a 501 bp PCR product amplified by primers rkA-CP1 and rkA-CP2, shown in the solid quadrangle).
Figure 12. Polyketide chain elongation catalyzed by RkC. Supplementation of the incubation mixture (RkC multienzyme, palmitoyl-S-RkB and malonyl-CoA) with recombinant holo-ACP(RkC) domain leads to accumulation of 3-oxo-stearoyl-S-ACP(RkC).
**Figure 13.** LC-MS evidence for *in vitro* formation of 3-oxo-stearoyl-S-ACP(RkC). The assay mixture contained (a) holo-RkC, holo-ACP(RkC), palmitoyl-S-RkB, malonyl CoA and the reducing agent tris(2-carboxyethyl)phosphine hydrochloride (TCEP); (b) as in (a) but without RkA as a control. The deconvoluted spectra corresponding to peak A (holo-ACP(RkC), 14,901 Da) and peak B (3-oxo-stearoyl-S-ACP(RkC), 15,180 Da) are also shown.
Figure 14. Sequence alignments of FabH-like protein from ChlM in chlorothricin, KijB in kijanimicin, TcaD4 in tetrocarcin, Tmn15 in tetronomycin and RkD from RK-682 biosynthetic gene cluster. The conserved active site cysteine residues are indicated with asterisk.
**Supplementary Methods**

**Table 3. Bacterial strains and plasmids used in this study**

| Strain/Plasmid          | Characteristics                                      | Reference |
|-------------------------|------------------------------------------------------|-----------|
| *E. coli*               |                                                      |           |
| DH10B                   | Host for general cloning                             | Invitrogen|
| BL21(DE3)               | Host for protein expression                          | Invitrogen|
| XL1-Blue MR             | Host for constructing the gene library               | Stratagene|
| ET12567(pUZ8002)        | Donor strain for conjugation between *E. coli* and *Streptomyces* | 1         |
| *Streptomyces sp.*      |                                                      |           |
| 88-682                  | RK-682 producing wild-type strain                    | 2         |
| ΔrkA                    | *rkA* gene in-frame deletion mutant, RK682 production reduced, 2000-fold | This work |
| ΔrkD                    | *rkD* gene in-frame deletion mutant, RK682 non-producing | This work |
| ΔrkH                    | *rkH* gene in-frame deletion mutant, RK682 non-producing | This work |
| ΔrkG                    | *rkG* gene in-frame deletion mutant, RK682 production reduced by 4-fold | This work |
| ΔrkD::pYH217            | ΔrkD complementation strain with PermE* promoter     | This work |
| Strain            | Description                                                                 | Reference |
|-------------------|------------------------------------------------------------------------------|-----------|
| ΔrkD::pYH218      | ΔrkD complementation strain with PactII-ORF4 promoter                         | This work |
| Saccharopolyspora | RK-682 heterologous expression host JCB2 strain selected from JC2, erythromycin non-producing strain | 3         |
| erythraea JCB2    | JCB2 integrated with a heterologous expression construct containing contiguous \( rkB-rkE \) under the control of the PactII-ORF4 promoter | This work |
| JCB2::pYH264      | JCB2 integrated with a heterologous expression construct containing contiguous \( rkA-rkE \) under the control of the PactII-ORF4 promoter | This work |
| JCB2::pYH266      | JCB2 integrated with a heterologous expression construct containing contiguous \( rkA-rkE \) and tmn7a under the control of two independent PactII-ORF4 promoters | This work |
| JCB2::pYH281      | JCB2 integrated with a heterologous expression construct containing contiguous \( rkA-rkF \) under the control of the PactII-ORF4 promoter | This work |
|                | Description                                                                 | Source   |
|----------------|-----------------------------------------------------------------------------|----------|
| JCB2::pYH282   | JCB2 integrated with a heterologous expression construct containing \( \text{rkA-rkG} \) under the control of the \( \text{PactII-ORF4} \) promoter | This work |
| JCB2::pYH284   | JCB2 integrated with a heterologous expression construct containing \( \text{rkA-rkF} \) and \( \text{rkH-rkl} \) under the control of two independent \( \text{PactII-ORF4} \) promoters | This work |
| JCB2::pYH285   | JCB2 integrated with a heterologous expression construct containing \( \text{rkA-rkG} \) and \( \text{rkH-rkl} \) under the control of two independent \( \text{PactII-ORF4} \) promoters | This work |

**Plasmid**

| Name           | Description                                                                 | Source   |
|----------------|-----------------------------------------------------------------------------|----------|
| SuperCos 1     | Vector for constructing genomic library                                     | Stratagene |
| pSHG397        | \( \text{E. coli} \) subcloning vector                                       | 4        |
| pJTU412        | \( \text{E. coli-Streptomyces} \) shuttle vector                           | 5,6      |
| pYH7           | \( \text{E. coli-Streptomyces} \) shuttle vector                           | 5-7      |
| pYH214         | \( \text{rkA} \) gene disruption construct in which a 1626 bp internal fragment of \( \text{rkA} \) was deleted in-frame | This work |
| pYH230         | \( \text{rkD} \) gene disruption construct in which a 999 bp internal fragment of \( \text{rkD} \) was deleted in-frame | This work |
| Construct | Description | Source |
|-----------|-------------|--------|
| pYH217    | ΔrkD complementation construct containing \(rkD\) under the control of the \(PermE^*\) promoter | This work |
| pYH218    | ΔrkD complementation construct containing \(rkD\) under the control of the \(PactII-ORF4\) promoter | This work |
| pYH279    | \(rkG\) gene disruption construct in which a 762 bp internal fragment of \(rkG\) was deleted in-frame | This work |
| pYH280    | \(rkH\) gene disruption construct in which a 1,560 bp internal fragment of \(rkH\) was deleted in-frame | This work |
| pCJR124   | Site-specific integrative vector with \(PactII-ORF4\) promoter | 8 |
| pIB139    | Site-specific integrative vector with \(PermE^*\) promoter | 8,9 |
| pYH264    | A heterologous expression construct containing contiguous \(rkB-rkE\) under the control of the \(PactII-ORF4\) promoter | This work |
| pYH266    | A heterologous expression construct containing contiguous \(rkA-rkE\) under | This work |
the control of the *Pactll-ORF4* promoter

pYH273  A heterologous expression construct  This work

containing contiguous *rkA-rkE* and

*tmn7a* under the control of two

independent *Pactll-ORF4* promoters

pYH281  A heterologous expression construct  This work

containing contiguous *rkA-rkF* under the

control of the *Pactll-ORF4* promoter

pYH282  A heterologous expression construct  This work

containing contiguous *rkA-rkG* under

the control of the *Pactll-ORF4* promoter

pYH284  A heterologous expression construct  This work

containing contiguous *rkA-rkF* and

*rkH-rkl* under the control of two

independent *Pactll-ORF4* promoters

pYH285  A heterologous expression construct  This work

containing *rkA-rkG* and *rkH-rkl* under

the control of two independent

*Pactll-ORF4* promoters

pET28a(+)  *E. coli* protein expression vector  Novagen

pET29b(+)  *E. coli* protein expression vector  Novagen

pYH94  Tmn16  protein expression construct  10
with N-terminal His-tag based on pET28a(+)

| Protein | Description | Source |
|---------|-------------|--------|
| pYH225  | RkA protein expression construct | This work |
|         | N-terminal His-tag based on pET28a(+) |
| pYH226  | RkB protein expression construct | This work |
|         | N-terminal His-tag based on pET28a(+) |
| pYH228  | RkD protein expression construct | This work |
|         | N-terminal His-tag based on pET28a(+) |
| pYH229  | RkE protein expression construct | This work |
|         | N-terminal His-tag based on pET28a(+) |
| pYH252  | RkC protein expression construct | This work |
|         | C-terminal His-tag based on pET29b(+) |
| pYH258  | ACP(RkC) protein expression construct | This work |
|         | N-terminal His-tag based on pET28a(+) |
| pYH277  | RkF protein expression construct | This work |
|         | N-terminal His-tag based on pET28a(+) |

*Escherichia coli* DH10B and BL21(DE3) were purchased from Invitrogen. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs. Synthetic oligonucleotides were purchased from Invitrogen. Polymerase chain reactions were carried out by using Pfu DNA polymerase.
from Strategene. Bacterial strains and plasmids used during this study are summarized in Table 3. Isolation of chromosomal and plasmid DNA was performed by standard protocols\textsuperscript{11,12}. The plasmids pET28a(+) and pET29b(+) were purchased from Novagen. His-Bind nickel chelate chromatography resin was obtained from Novagen. All chemicals and materials used for assays were purchased from Sigma-Aldrich. NMR spectra were recorded at 300 K using Bruker DPX-400, Avance 400 QNP and Avance 500 spectrometers. For $^{1}$H- and $^{13}$C NMR the chemical shifts are reported relative to the solvent signal (CDCl$_3$ δ$_H$ 7.26, CDCl$_3$ δ$_C$ 77.0). For $^{31}$P NMR spectra H$_3$PO$_4$ (85% in D$_2$O) was used as external standard. TLC was performed on glass sheets pre-coated with silica gel 60 (HF254, E. Merck) and spots were visualized by UV, charring with permanganate dip, or exposure to iodine vapour. Flash column chromatography was carried out with silica gel 60 (0.04-0.63 mm, Merck) using a stepwise solvent polarity gradient. Solvents were purchased dry and stored over molecular sieves. Gel filtration was carried out on PD MiniTrap\textsuperscript{TM} G-10 columns (GE Healthcare). ESI-MS analyses of the CoA derivatives was performed using an Agilent HP1100 HPLC coupled to a Finnigan MAT LCQ mass spectrometer fitted with an ESI source. The mass spectrometer was run in positive ionization mode, scanning from $m/z$ 150 to 1,800 and the collision energy was set to 25%. A solvent composition of 30% H$_2$O, 70% MeCN, 0.1% trifluoracetic acid (TFA) was used. HPLC-ESI-HRMS analysis was performed on a Thermo Electron LTQ-Orbitrap. Samples were injected onto a Dionex
Acclaim C18 PepMap100 column (100 Å, 150 mm×1.0 mm, 3 μm), eluting with a gradient of 20 mM ammonium acetate (A) and MeOH (B) at a flow rate of 50 μl min⁻¹ (starting with 80% B for 5 min, increasing to 100% B in 20 min and maintaining at 100% B for 4 min). The mass spectrometer was run in positive or negative ionization mode, scanning from m/z 100 to 1,800, with the FTMS analyser resolution set at 60 K.

**Construction and screening of genomic cosmid library:**

The genomic DNA of *Streptomyces* sp. 88-682 wild type was partially digested with *Bam*HI, dephosphorylated by alkaline phosphatase SAP and ligated to the prepared SuperCos 1 (Stratagene) vector without fractionation. *E. coli* XL1-Blue MR strain and Gigapack III XL packaging extract (Stratagene) were used for library construction according to the manufacturer's instructions. About 2,000 colonies were screened by colony-hybridization with a 1,926 bp *Nde*I-*Eco*RI DIG-labeled probe containing a complete *tmn16* gene recovered from pYH94. Three overlapping positive cosmids were obtained by restriction enzyme analysis.

**Sequencing and annotation of the RK-682 biosynthetic gene cluster:**

One of the overlapping positive cosmids (cosmid 6.3) was sequenced by shotgun sequencing of a subclone library, consisting of 1.5-2.0 kb fragments (obtained through partial digestion with *Sau*3AI) cloned into pHSG397. DNA
sequencing was carried out with an ABI Prism 3700 DNA Analyzer automated sequencer (Applied Biosystems). The raw sequence data were processed and assembled with the phred/phrap/consed software package (www.phrap.org). The annotation analysis of the sequence data (8 times coverage) was performed through database comparison with the BLAST search tools on the server of the National Center for Biotechnology Information, Bethesda, Maryland (http://www.ncbi.nlm.nih.gov).

**Culture conditions:**

*Streptomyces* sp. RK-682 wild type and mutants were grown in TSBY liquid medium (3% tryptone soy broth, 10.3% sucrose, 0.5% yeast extract) for isolation of chromosomal DNA, and TWM medium (0.5% glucose, 1% sucrose, 0.5% tryptone, 0.25% yeast extract, 0.0036% EDTA, 2% agar, pH 7.1) for RK-682 production. For liquid culture, the strains were grown at 28°C and 200 rpm on a rotary incubator and harvested after 2 days, and for solid culture, the strains were grown at 28°C for 6 days. *E. coli* strains were cultured in 2×TY medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) at 37°C with the appropriate antibiotic selection at a final concentration of 100 $\mu$g ml$^{-1}$ carbenicillin, 50 $\mu$g ml$^{-1}$ apramycin or 25 $\mu$g ml$^{-1}$ chloramphenicol.

**Gene disruption, complementation and heterologous expression:**
The constructs used in this study for gene disruption, complementation and heterologous expression are summarized in the Supplementary Table 3. The constructs were introduced into *Streptomyces* sp. 88-682 by conjugation using donor strain ET12657/pUZ8002 on 20ml of SFM plates (2% mannitol, 2% soya flour, 2% agar). After incubating at 30°C for 8 hours, exconjugants were selected with 50 μg ml\(^{-1}\) apramycin or 25 μg ml\(^{-1}\) thiostrepton and 25 μg ml\(^{-1}\) nalidixic acid. Single colonies from this plate were transferred to a TWM plate containing 50 μg ml\(^{-1}\) apramycin or 25 μg ml\(^{-1}\) thiostrepton to double check for antibiotic resistance. To screen for mutants in which a double cross-over event had occurred, single colonies from this plate were patched onto TWM plates and also onto TWM plates containing 50 μg ml\(^{-1}\) apramycin or 25 μg ml\(^{-1}\) thiostrepton, respectively. Candidate colonies with the correct phenotype (Apr\(^{S}\) or Thio\(^{S}\)) were used for further identification by Southern blot analysis, PCR and sequencing. For complementation, the constructs pYH217 and pYH218 were introduced into ΔrkD mutant, respectively, by conjugation through the procedure described above, and selected with 50 μg ml\(^{-1}\) apramycin and 25 μg ml\(^{-1}\) nalidixic acid. Candidate colonies with the correct phenotype (Apr\(^{R}\)) were confirmed by PCR and sequencing. For heterologous expression, the constructs were introduced into *Saccharopolyspora erythraea* JCB2 by conjugation through the procedure described above, and selected with 50 μg ml\(^{-1}\) apramycin and 25 μg ml\(^{-1}\) nalidixic acid. Candidate colonies with the correct phenotype (Apr\(^{R}\)) were confirmed by PCR and sequencing.
Production, isolation and analysis of RK-682:

To assess RK-682 production, each plate containing 10 ml TWM medium was inoculated with the same amount of seed culture and grown as described above. After cultivation for 6 days, the whole plate culture was extracted with 40 ml ethyl acetate. The organic phase was evaporated to dryness under reduced pressure using a Speed-Vac. The residue was redissolved in 800 μl methanol and clarified by centrifugation, then subjected to liquid chromatography-mass spectrometry (LC-MS). LC-MS analysis was carried out on a Thermo Finnigan LTQ mass spectrometer using positive ion mode electrospray ionization. The LTQ was coupled to an Agilent 1,200 LC fitted with a Prodigy C18 column (5 μm, 4.6×250 mm, Phenomenex) equilibrated with 20 mM ammonium acetate and methanol. Samples were eluted using a gradient of 80 to 95% methanol over 20 min at a flow rate of 1 ml min⁻¹. The mass spectrometer was set to full scan (from 100 to 1,500 m/z), MS² (369.3 m/z), MS³ (351.3 m/z) and MS⁴ (333.3 m/z) modes with normalized collision energy of 20%. The quantification of RK-682 was achieved by using calibrated standards obtained from BIOMOL International LP.

Construction of gene disruption plasmids:

To disrupt rkA by in-frame deletion, a 6,070 bp Sphl fragment that contains rkA from cosmid 6.3 was cloned into the unique Sphl site of pJTU412,
an *E. coli*-Streptomyces shuttle vector derived from pHZ1358\textsuperscript{5,6}, to create pYH213. The resultant plasmid was digested with SacI and re-ligated itself to produce pYH214, in which a 1,626 bp fragment between two SacI sites in *rkA* had been deleted in-frame. To verify the in-frame deletion in the gene disruption construct and mutant, the following pair of primers was used:

RK682A-CP1: 5’-CCG AAG CTG CGA CGT GCA C-3’, RK682A-CP2: 5’-CAC CGA GTC GAT GCC GTA GTC-3’.

To disrupt *rkD* by in-frame deletion, two fragments used for homologous recombination were amplified from cosmid 6.3 by PCR using the following primers *rkD*-L1: 5’-GGG \underline{AGA TCT} GGC GTC CGT GGT GGC CGG G-3’ (*Bgl*II site is underlined), and *rkD*-L2: 5’-GAC \underline{GGT ACC} GGG TGA CGG ACT CAT GTG CGC GG-3’ (*Kpn*I site is underlined), *rkD*-R1: 5’-TCC \underline{GGT ACC} GCC CTG GAG CGC GCA TGA C-3’ (*Kpn*I site is underlined) and *rkD*-R2: 5’-GGA AAA \underline{GCT TGG} TGC GCA GGG TGA GTT GGG-3’ (*Hind*III site is underlined).

After digestion with appropriate restriction enzymes, these two fragments were cloned into the *Streptomyces*-*E. coli* shuttle vector pJTU412\textsuperscript{5,6} treated with *Bgl*II and *Hind*III and via three-way ligation used to create construct pYH230.

To verify the in-frame deletion in construct and mutant, a pair of primers was designed as follows for PCR and sequencing, *rkD*-CP1: 5’-ACG GGG TGT CCN AGG T-3’ and *rkD*-CP2: 5’-TGG ACT GGC CGG TGA GTT GGG-3’.

To disrupt *rkG* by in-frame deletion, two fragments used for homologous recombination were amplified from cosmid 6.3 by PCR using the following
primers rkG-L1: 5’-GCC CAT ATG CCC GTG GTG ACC GTC GC-3’ (*NdeI* site is underlined), rkG-L2: 5’-GCC CTG CAG GCC GGC CGT GCC-3’ (*PstI* site is underlined), rkG-R1: 5’-GCG CTG CAG GCC CCC GCG TGA C-3’ (*PstI* site is underlined) and rkG-R2: 5’-CGT AAG CTG GGT CAC CGA CCC CGG-3’ (*HindIII* site is underlined). After digestion with appropriate restriction enzymes, these two fragments were cloned into *Streptomyces-E. coli* shuttle vector *pYH7*<sup>5-7</sup>, treated with *NdeI* and *HindIII*, and via three-way ligation used to create construct *pYH279*. To verify the in-frame deletion in construct and mutant, a pair of primers was designed as follows for PCR and sequencing, rkG-CP1: 5’-CGC CAC CGT CGT CTT CC-3’ and rkG-CP2: 5’-GCA ACC CGC TGG TCT TCT G-3’.

To disrupt *rkH* by in-frame deletion, two fragments used for homologous recombination were amplified from cosmid 6.3 by PCR using the following primers rkH-L1: 5’-GAA CAT ATG CAG GGT CTT GGG GCC-3’ (*NdeI* site is underlined), rkH-L2: 5’-GTC GAA TTC TCC TCG GAG GTC TGA G-3’ (*EcoRI* site is underlined), rkH-R1: 5’-GCG GAA TTC GAG CTG AGT GGT CAC GG-3’ (*EcoRI* site is underlined) and rkH-R2: 5’-GGT AAG CTT GCG GAT CGC GCC GGG-3’ (*HindIII* site is underlined). After digestion with appropriate restriction enzymes, these two fragments were cloned into *Streptomyces-E. coli* shuttle vector *pYH7*<sup>5-7</sup> treated with *NdeI* and *HindIII* and by three-way ligation used to create construct *pYH280*. To verify the in-frame deletion in construct and mutant, a pair of primers was designed as follows for PCR and
sequencing, rkH-CP1: 5’-TTG CGC CAG CGG CGG TAG AT-3’ and rkH-CP2: 5’-TTC GGA GGT TCC CGC AGG GTG T-3’.

**Construction of protein expression plasmids:**

Amplification of *rkA* was accomplished with primers RK682A-EP1: 5’-GAA CAT ATG CAC CAG ACG TTG ACC GAG G-3’ (*Nde*I site is underlined) and RK682A-EP2: 5’-CGG GAA TTC TCA GCG GCC GGG GGC G-3’ (*EcoRI* site is underlined). The PCR product was cloned into similarly digested pET28a(+) to yield a construct pYH225 with an N-terminal hexahistidine tag. The constructs pYH226, pYH228, pYH229, pYH258 and pYH277, all with N-terminal hexahistidine tags, were generated in an analogous manner by using primers RK682B-EP1: 5’-TAC CAT ATG TCC ATC GCC CCC GAC ACC-3’ (*Nde*I site is underlined) and RK682B-EP2: 5’-GAC GAA TTC GGG TGC GCT CAC GAG G-3’ (*EcoRI* site is underlined) for cloning the *rkB*; RK682D-EP1: 5’-GCG CAT ATG AGT CCG TCA CCC GCC C-3’ (*Nde*I site is underlined) and RK682D-EP2: 5’-ATG GAA TTC ATG GTC AGT TCT CCT C-3’ (*EcoRI* site is underlined) for cloning *rkD*; RK682E-EP1: 5’-GCG CAT ATG ACC ACC ATC ACG GC-3’ (*Nde*I site is underlined) and RK682E-EP2: 5’-GTG GAA TTC ATG GTC AGT TCT CCT C-3’ (*EcoRI* site is underlined) for cloning *rkE*; . RkC-ACP-EPN: 5’-AGC CAT ATG GCC TGG CGC TTC GC-3’ (*Nde*I site is underlined) and RK682C-EP2: 5’-TGA CG AAT TCA TGT GCG CGG TCC TTC CG-3’ (*EcoRI* site is underlined) for cloning ACP domain of
RkC; RK682F-EP1: 5’-CTG CAT ATG ACC GCC ACC GCC ATC G-3’ (NdeI site is underlined) and RK682F-EP2: 5’-GGG GAA TTC ACG CGC GCA CCA CCT C-3’ (EcoRI site is underlined) for cloning the rkF; The PCR product of rkC by primers RkC-EPN: 5’-CGC CAT ATG AGC GCA CCC GAA CCG G-3’ (NdeI site is underlined) and RkC-EPC: 5’-TGA GAA TTC CAT GTG CGC GGT CCT TCC-3’ (EcoRI site is underlined) was cloned into similarly-digested pET29b(+) to yield a construct pYH252 with a C-terminal hexahistidine tag. The construction of genes encoding Tmn16 and Tmn7a has been previously described⁹.

**Construction of heterologous expression plasmids:**

To construct a heterologous expression plasmid containing contiguous genes \(rkB-rkE\), a PCR product was obtained by using primers RK682B-EP1 and RK682E-EP2, then cloned into the integrative vector pCJR124 (previously treated with NdeI and EcoRI) to yield pYH264, in which \(rkB-rkE\) were under the control of the \(P_{actII-ORF4}\) promoter.

To construct a heterologous expression plasmid containing contiguous genes \(rkA-rkE\), a PCR product was obtained by using primers RK682A-EP1 and RK682G-EP2, then cloned into integrative vector pCJR124 (previously treated with NdeI and EcoRI) to yield pYH266, in which \(rkA-rkE\) were under the control of the \(P_{actII-ORF4}\) promoter.
To construct a heterologous co-expression plasmid containing contiguous genes \textit{rkA-rkE} and \textit{tmn7a}, the \textit{tmn7a} was first cloned into the \textit{NdeI-EcoRI} sites of pCJR124 by PCR using primers Tmn7a-EP1: 5’-CAC CAT ATG ACG ACC ATC GAC GAT CTT G-3’ (\textit{NdeI} site is underlined) and Tmn7a-EP2: 5’-AGC GAA TTC TTA TCA CGC CGC GAC CGC CGC C-3’ (\textit{EcoRI} site is underlined). The PCR product of \textit{tmn7a} with promoter \textit{PactII-ORF4} amplified with primers Pact-tmn7a-P1: 5’-TAC GCT AGC TAG TCC ACT GCC TCT C-3’ (\textit{NheI} site is underlined) and Pact-tmn7a-P2: 5’-GAC GCT AGC CTA TGA ATT CTT ATC AC-3’ (\textit{NheI} site is underlined) was then cloned into the unique \textit{NheI} site of pYH266 to yield pYH273, in which \textit{rkA-rkE} and \textit{tmn7a} were under the control of two independent \textit{PactII-ORF4} promoters.

To construct a heterologous expression plasmid containing contiguous genes \textit{rkA-rkF}, a PCR product was obtained by using primers RK682A-EP1 and RK682F-EP2, then cloned into the integrative vector pCJR124 (previously treated with \textit{NdeI} and \textit{EcoRI}) to yield pYH281, in which \textit{rkA-rkF} were under the control of the \textit{PactII-ORF4} promoter.

To construct a heterologous expression plasmid containing contiguous genes \textit{rkA-rkG}, a PCR product was obtained by using primers RK682A-EP1 and RK682G-EP2, then cloned into the integrative vector pCJR124 (previously treated with \textit{NdeI} and \textit{EcoRI}) to yield pYH282, in which \textit{rkA-rkG} were under the control of the \textit{PactII-ORF4} promoter.
To construct a heterologous co-expression plasmid containing contiguous genes $rkA-rkF$ and $rkH-rkl$, $rkH-rkl$ was first cloned into the Ndel-EcoRI sites of pCJR124 by PCR using primers $rkH-I$-P1: 5’-CAT CAT ATG ACC ACT CAG CTC GAA CC-3’ (Ndel site is underlined) and $rkH-I$-P2: 5’-CGG GAA TTC TAC CGG GGT GCG GGG-3’ (EcoRI site is underlined). The PCR product of $rkH-rkl$ with the promoter $PactII-ORF4$ was amplified with primers Nhel-P1: 5’-TAC GCT AGC TAG TCC ACT GCC TC-3’ (Nhel site is underlined) and Nhel-P2: 5’-CGC GCT AGC CGA TTC ATT AAT GC-3’ (Nhel site is underlined) was then cloned into the unique Nhel site of pYH281 to yield pYH284, in which $rkA-rkF$ and $rkH-rkl$ were under the control of two independent $PactII-ORF4$ promoters.

To construct a heterologous co-expression plasmid containing contiguous genes $rkA-rkG$ and $rkH-rkl$, the PCR product of $rkH-rkl$ with promoter $PactII-ORF4$ was amplified with primers Nhel-P1 and Nhel-P2 and was cloned into the unique Nhel site of pYH282 to yield pYH285, in which $rkA-rkG$ and $rkH-rkl$ were under the control of two independent $PactII-ORF4$ promoters.

**Construction of complementation plasmids:**

The entire $rkD$ gene was cloned as an Ndel-EcoRI fragment from RkD expression plasmid pYH228 and placed under the control of the $PermE^*$ promoter in the integrative vector pIB139 and $PactII-ORF4$ promoter in the integrative vector pCJR124, respectively, produced pYH217 and pYH218.
Expression and purification of proteins:

For expression and purification of RkA, apo-ACP(RkC), RkD and RkE, the expression plasmids pYH225, pYH258, pYH228 and pYH229 were introduced, respectively, into *E. coli* strain BL21(DE3) and grown in 2×TY medium supplemented with kanamycin (50 μg ml⁻¹). Each litre of culture was inoculated with 10 ml overnight starter culture. The cultures were grown at 20°C to an A₆₀₀ of 0.3-0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to 0.1 mM, and incubation was continued at 18°C for an additional 18 h. Cells were harvested by centrifugation (10 min at 5,000×g), resuspended in lysis buffer (20 mM Tris-HCl buffer pH 7.9 containing 50 mM NaCl and 5 mM imidazole), and lysed by sonication. The lysate was clarified by centrifugation (30 min at 18,000 g). His-Bind resin (2 ml slurry per 1 liter of culture) was added to the clarified lysate and was washed with five column volumes of lysis buffer. Bound proteins were then eluted with a step gradient of increasing imidazole concentrations (50, 100, 200 and 300 mM in lysis buffer). The proteins were further purified by gel filtration on an ÄKTA Explorer FPLC system fitted with a HiLoad 16/60 Superdex×200 Prep Grade column. The isocratic mobile phase contained 100 mM potassium phosphate buffer, pH 7.2. Fractions containing the target protein, as judged by LC-MS analysis, were pooled and concentrated, and stored at -80°C in 100 mM potassium phosphate buffer, pH 7.2 containing 10% glycerol. For expression and purification of holo-RkB,
holo-RkC, holo-ACP(RkC) and holo-RkF, an auxiliary plasmid pSUMtaA containing a 4'-phosphopantetheinyl transferase sfp gene\textsuperscript{13} was co-transferred with the expression plasmids pYH226, pYH252, pYH258 and pYH277, respectively, into \textit{E. coli} strain BL21(DE3) and additional chloromphenicol (25 μg ml\textsuperscript{-1}) was added in the culture. Also, an extra heat shock (42°C for 20 min) before induction was applied when expressing holo-RkC. Tmn7a and Tmn16 were expressed and purified as previously described\textsuperscript{9}.

**Production of glyceryl-S-RkF:**

The reaction mixture (50 μl) contained 5 μM RkE, 5 units D-3-phosphoglyceric phosphokinase (D-PGK), 1 mM D-3-phosphoglyceric acid (D-3-PG), 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 1 mM adenosine triphosphate (ATP) and 10 mM MgCl\textsubscript{2}. After incubation at 28°C for 30 min, 50 μM holo-RkF was added and incubation was continued at 28°C for 1 h.

**Production of palmitoyl-S-RkB:**

The reaction mixture (50 μl) contained 5 μM RkA, 0.5 mM sodium palmitate, 1 mM CoA, 1 mM ATP and 10 mM MgCl\textsubscript{2}. After incubation at 28°C for 30 min, 50 μM holo-RkB was added and incubation was continued at 28°C for 1 h.

**Production of 3-oxo-stearoyl-S-RkC:**
The reaction mixture (50 μl) containing 3 μM palmitoyl-S-RkB, 3 μM holo-RkC, 0.5 mM malonyl-CoA and 1 mM TCEP were incubated at 28°C for 1 h, together with 12 μM holo-ACP(RkC).

**Production of RK-682 in vitro:**

The reaction mixture (1 ml) containing 24 μM glyceryl-S-RkF, 14 μM RkD, 5 mM MgCl₂, 1 mM TCEP and about 26 μM 3-oxo-stearoyl-S-RkC created from above assay was incubated at 28°C for 2 h, then extracted with 1 ml ethyl acetate and dried in vacuo. Finally, the residue was dissolved in 100 μl methanol and analyzed by LC-MS.

Alternatively, 500 μl of a reaction mixture containing 50 μM glyceryl-S-RkF, 50 μM apo-ACP(RkC), 3.7 μM 4'-phosphopantetheinyl transferase Sfp, 500 μM 3-oxo-stearoyl-S-CoA thioester, 10 μM RkD, 5 mM MgCl₂ and 1 mM TCEP was incubated at 28°C for 2 h, then extracted with 1 ml ethyl acetate and dried in vacuo. Finally, the residue was dissolved in 100 μl methanol and analyzed by LC-MS.

**Analysis of protein samples from in vitro assays:**

The proteins from an assay mixture were loaded onto a Jupiter C4 column (300 Å, 250×2.0 mm, Phenomenex) and eluted with a gradient of 5-25% acetonitrile/0.1% trifluoroacetic acid over 5 min, then 25-75% acetonitrile/0.1% TFA over 40 min at a flow rate of 0.3 ml min⁻¹. The mass spectrometer
was operated in the positive ion mode, scanning from 600 to 2,000 m/z. The mass spectrometric data were processed and deconvoluted using the Bioworks software (Thermo Finnigan).

**Synthesis of 3-oxo-stearoyl-S-CoA thioester** (see Scheme 1)

5-(1-hydroxyhexadecylidene)-2,2-dimethyl-1,3-dioxane-4,6-dione (3) 1 g (3.9 mmol, 1 equiv.) palmitic acid, 1.77 g (8.6 mmol, 2.2 equiv.) N,N´-dicyclohexylcarbodiimide, and 1.05 g (8.6 mmol, 2.2 equiv.) 4-(dimethylamino)pyridine were dissolved in 15 ml dry CH₂Cl₂ and stirred at room temperature. A solution of 843 mg (5.8 mmol, 1.5 equiv.) Meldrum’s acid in 10 ml dry CH₂Cl₂ was added and stirring was continued for 7 h. Diethyl ether was added and the precipitated N,N´-dicyclohexylurea was removed by filtration. The filtrate was washed three times with an equal volume of HOAc/H₂O (1:10). The organic layer was dried over Na₂SO₄ and the solvent was removed under reduced pressure. The product was purified by flash chromatography on silica gel (petroleum ether to petroleum ether:EtOAc (2:1)). After drying in vacuo, 1.37 g (3.57 mmol) of a white solid 3 were obtained (92% yield).

TLC (petroleum ether:EtOAc, 2:1 (v/v)): Rₐ = 0.48; ¹H NMR (400 MHz, CDCl₃) δ 0.86 (t, J = 7.0 Hz, 3 H), 1.20 – 1.33 (m, 22 H), 1.38 (tt, J₁ = 8.0 Hz, J₂ = 7.0 Hz,
2 H), 1.68 (tt, $J_1 = 8.0$ Hz, $J_2 = 7.5$ Hz, 2 H), 1.71 (s, 6 H), 3.05 (dd, $J_1 = 8.0$ Hz, $J_2 = 7.5$ Hz, 2 H), 15.29 (s, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 14.1, 22.6, 26.1, (26.8; 2 C), (29.2, 29.3, 29.4, 29.5, 29.6; 10 C), 31.9, 35.7, 91.2, 104.7, 170.5, 198.3 (2 C); MS (ESI, $m/z$): 381.26 (9) [M-H$^-$], 279.31 (100) [M-$C_4H_7O_3$]$^-$, calc.: 381.27 (Supplementary Fig. 15).

3-nitrobenzyl 3-oxo-octadecanoate (4) A mixture of 300 mg (0.79 mmol, 1 equiv.) of 3 and 466 μl (3.92 mmol, 5 equiv.) 3-nitrobenzylalcohol in 10 ml toluene was heated under reflux at 80°C for 18 h. The solvent was removed under reduced pressure. The residue was taken up in a minimal amount of CH$_2$Cl$_2$ and the product was precipitated by addition of MeOH. The suspension was filtered, and the residue was washed three times with a mixture of MeOH/H$_2$O (5:1). The solid was dried in vacuo. 290 mg (0.67 mmol) of a white solid 3 were obtained (85% yield).

TLC (petroleum ether:EtOAc, 10:1 (v/v)): $R_f = 0.25$; $^1$H NMR (400 MHz, CDCl$_3$) δ 0.87 (t, $J = 7.1$ Hz, 3 H), 1.22 – 1.33 (m, 24 H), 1.58 (tt, $J_1 = 7.3$ Hz, $J_2 = 7.1$ Hz, 2 H), 2.51 (t, $J = 7.5$ Hz, 2 H), 3.53 (s, 2 H), 5.26 (s, 2 H), 7.55 (dd, $J_1 = 8.0$ Hz, $J_2 = 7.5$ Hz, 1 H), 7.69 (d, $J = 7.5$ Hz, 1 H), 8.18 (d, $J_1 = 8.0$ Hz, $J_2 = 2.0$ Hz, 1 H), 8.22 (d, $J = 2.0$ Hz, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 14.1, 22.6, 23.4, (29.0, 29.3, 29.4, 29.5, 29.6, 29.9; 10 C), 31.9, 43.2, 48.9, 65.5, 122.3, 122.8, 129.6, 133.9, 137.5, 166.8, 202.4; MS (ESI, $m/z$): 432.27 (22) [M-H$^-$], 388.33 (13) [M-CO$_2$]$^-$, 279.32 (100) [M-$C_7H_7O_3$]$^-$, calc.: 432.28 (Supplementary Fig. 16).
3-nitrobenzyl 2-(2-pentadecyl-1,3-dioxolan-2-yl)acetate (5) A mixture of 40 mg (0.092 mmol, 1 equiv.) 4, 20 µg (9 nmol, 0.001 equiv.) p-toluenesulfonic acid monohydrate, and 10.0 µl (185 µmol, 2 equiv.) ethylene glycol in 4 ml cyclohexane was heated under reflux to 120°C using a Dean-Stark apparatus. After 5 h, the resulting solution was washed three times with an equal volume of 1 M NaHCO₃. The organic layer was dried over Na₂SO₄ and the solvent was removed under reduced pressure. The products were purified by flash chromatography on silica gel (petroleum ether:EtOAc (15:1 to 5:1)). After drying in vacuo, 26 mg (54.5 µmol) of a white solid 5 were obtained (59% overall yield).

5: TLC (petroleum ether:EtOAc, 5:1 (v/v)): Rₜ = 0.45; ¹H NMR (400 MHz, CDCl₃) δ 0.87 (t, J = 6.9 Hz, 3 H), 1.20 – 1.32 (m, 24 H), 1.37 (tt, J₁ = 7.5 Hz, J₂ = 7.0 Hz, 2 H), 1.74 – 1.79 (m, 2 H), 2.74 (s, 2 H), 3.95 – 3.98 (m, 4 H), 5.22 (s, 2 H), 7.53 (dd, J₁ = 8.2 Hz, J₂ = 7.6 Hz, 1 H), 7.68 (d, J = 7.6 Hz, J = 1.8 Hz, 1 H), 8.16 (d, J₁ = 8.5 Hz, J₂ = 2.3 Hz, 1 H), 8.25 (m, 1 H); ¹³C NMR (100 MHz, CDCl₃) δ 14.1, 22.6, 23.5, (29.3, 29.5, 29.6; 10 C), 31.9, 37.8, 42.5, 65.1, (64.7; 2 H), 109.4, 122.7, 123.0 129.5, 133.7, 138.2, 148.4, 169.1; MS (ESI, m/z): 495.18 (100), 478.24 (7) [M+H]⁺, 448.36 (21) [M-CO]⁺ [M-C₇H₇O₃]⁺, calc.: 478.32 (Supplementary Fig. 17).
2-(2-pentadecyl-1,3-dioxolan-2-yl)acetic acid (6) 26 mg of 5 (54.5 µmol) were dissolved in 2 ml of NaOH (1 M)/EtOH (1:4) and heated under reflux at 80°C for 16 h. The bulk of the EtOH was removed under reduced pressure and the pH was adjusted to 5 by addition of HOAc. The turbid solution was quickly extracted three times with two volumes of CH$_2$Cl$_2$. The crude product was purified by flash chromatography on silica gel (petroleum ether:EtOAc (4:1) to MeOH). After drying in vacuo, 12 mg (34.9 µmol) of a white solid 6 were obtained (64% overall yield).

TLC (petroleum ether:EtOAc, 1:1): $R_f = 0.08$; $^1$H NMR (400 MHz, MeOD:CDCl$_3$ (1:4)) $\delta$ 0.88 (t, $J = 6.7$ Hz, 3 H), 1.20 – 1.33 (m, 24 H), 1.33 – 1.44 (m, 2 H), 1.75 – 1.81 (m, 2 H), 2.70 (s, 2 H), 4.01 (ddd, $J_1 = 12.0$ Hz, $J_2 = 4.1$ Hz, $J_3 = 2.0$ Hz, 2 H), 4.02 (ddd, $J_1 = 11.7$ Hz, $J_2 = 3.8$ Hz, $J_3 = 1.8$ Hz, 2 H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 14.1, 22.7, 23.5, (29.3, 29.5, 29.6, 29.7; 10 H), 31.9, 37.6, 42.3, 65.1, 109.3; MS (ESI, m/z): 341.29 (50) [M-H$^-$], 253.31 (100) [M-C$_3$H$_4$O$_3$]$^+$, calc.: 341.27 (Supplementary Fig. 18).

3-oxo-stearoyl-S-CoA thioester (8) 4.6 mg (28.29 µmol, 2.2 equiv.) 1,1´-carbonyldiimidazole and 9.4 mg (25.46 µmol, 2.0 equiv.) 6 were dissolved in 1 ml dry THF under nitrogen. The reaction mixture was left, with occasional shaking, for 1 h. THF was removed in a stream of N$_2$. A solution of 10 mg (12.73 µmol, 1 equiv.) of coenzyme A trilithium salt in 1.5 ml THF/H$_2$O (2:1) was added and the solution was left, with occasional shaking, for 17 h. THF
was removed in a stream of N₂ and the sample was acidified with HCl (1 M). The remaining suspension was extracted three times with an equal volume of diethyl ether. Analysis of the crude product showed complete conversion of HSCoA into the thioester 2-(2-pentadecyl-1,3-dioxolan-2-yl)acetic-S-CoA (7). To 1 μmol of 7 were added a solution of 1 mg p-toluenesulfonic acid in 200 μl THF/acetone (1:1) and 50 μl HCl (1 M). After two days the pH of the solution was adjusted to about 3 by addition of NaHCO₃ (0.1 M, pH 7.3) and HOAc. The solution was extracted three times with an equal volume of diethyl ether. The sample was subjected to gel filtration (HOAc:H₂O (1:10)). ESI-MS analysis of the appropriate fractions showed the desired product 8 in high purity, contaminated with only a small amount of 7. The sample was freeze dried and used directly for the enzyme assays.

7: MS (ESI, m/z): 1,092.23 (100) [M+H]⁺ (Supplementary Fig. 19). 8: MS (ESI, m/z): 1,048.12 (100) [M+H]⁺; ³¹P NMR (162 MHz, H₃PO₄) δ 0.37, -10.5, -11.0 (Supplementary Fig. 20).
Figure 15. $^1$H NMR (a), $^{13}$C NMR (b) and ESI-MS (c) spectra of compound 5-(1-hydroxyhexadecylidene)-2,2-dimethyl-1,3-dioxane-4,6-dione (3). Spectra were recorded in CDCl$_3$. The peak at 381.26 in (c) corresponds to [M-H]$^-$ (calc.: 381.27) and the peak at 279.31 corresponds to decarboxylation and loss of acetone. The mass spectrometer was run in negative ion detection mode.
Figure 16. $^{1}$H NMR (a), $^{13}$C NMR (b) and ESI-MS (c) spectra of compound 3-nitrobenzyl 3-oxooctadecanoate (4). Spectra were recorded in CDCl$_3$. The peak at 432.27 in (c) corresponds to [M-H]$^-$ (calc.: 432.28) and the peaks at 388.33 and 279.32 correspond to decarboxylation and loss of nitrobenzyl alcohol group, respectively. The mass spectrometer was run in negative ion detection mode.
Figure 17. $^1$H NMR (a) and $^{13}$C NMR (b) spectra of compound 3-nitrobenzyl 2-(2-pentadecyl-1,3-dioxolan-2-yl)acetate (5). Spectra were recorded in CDCl$_3$. The peak at 478.24 in (c) corresponds to [M+H]$^+$ (calc.: 478.32) and the peaks at 495.18 and 448.36 correspond to the ammonium adduct and the loss of CO, respectively. The mass spectrometer was run in positive ion detection mode.
Figure 18. $^1$H NMR (a), $^{13}$C NMR (b) and ESI-MS (c) spectra of compound 2-(2-pentadecyl-1,3-dioxolan-2-yl)acetic acid (6). Spectra were recorded in MeOD/CDCl$_3$ (1:4). The peak at 341.29 in (c) corresponds to [M-H]$^-$ (calc.: 341.27) and the peak at 253.31 corresponds to decarboxylation and loss of C$_2$H$_4$O from the dioxolane group. The mass spectrometer was run in negative ion detection mode.
Figure 19. ESI-MS spectrum of compound 2-(2-pentadecyl-1,3-dioxolan-2-yl)acetic acid-S-CoA (7). The peak at 1,092.23 corresponds to [M+H]⁺ (calc.: 1,092.39). The mass spectrometer was run in positive ion detection mode.
Figure 20. ESI-MS spectrum of compound 3-oxo-stearoyl-S-CoA (8). The peak at 1,048.12 corresponds to [M+H]+ (calc.: 1,048.36). The mass spectrometer was run in positive ion detection mode.
Scheme 1. Synthesis of 3-oxo-stearoyl-S-CoA thioester. Reagents and conditions: (a) Meldrum’s acid, \( N,N’ \)-dicyclohexylcarbodiimide, 4-(dimethylamino)pyridine, \( \text{CH}_2\text{Cl}_2 \), 7 h, room temperature. (b) 3-nitrobenzyl alcohol, toluene, 18 h, 80°C. (c) ethylene glycol, \( p \)-toluenesulfonic acid monohydrate, cyclohexane, 5 h, 120°C. (d) \( \text{NaOH} \) (1 M)/EtOH (1:4), 16 h, 80°C. (e) I) 1,1’-carbonyldiimidazole, THF, 1 h, room temperature; II) coenzyme A (CoA), \( \text{H}_2\text{O}/\text{THF} \) (1:2), 17 h, room temperature. (f) \( p \)-toluenesulfonic acid monohydrate, THF/aceton (1:1), HCl (1 M), 2 d, room temperature.
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