Structure of a modular polyketide synthase

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Polyketide natural products constitute a broad class of compounds with diverse structural features and biological activities. Their biosynthetic machinery, represented by type I polyketide synthases (PKSs), has an architecture in which successive modules catalyse two-carbon linear extensions and keto-group processing reactions on intermediates covalently tethered to carrier domains. Here we used electron cryo-microscopy to determine sub-nanometre-resolution three-dimensional reconstructions of a full-length PKS module from the bacterium Streptomyces venezuelae that revealed an unexpectedly different architecture compared to the homologous dimeric mammalian fatty acid synthase. A single reaction chamber provides access to all catalytic sites for the intramodule carrier domain. In contrast, the carrier from the preceding module uses a separate entrance outside the reaction chamber to deliver the upstream polyketide intermediate for subsequent extension and modification. This study reveals for the first time, to our knowledge, the structural basis for both intramodule and intermodule substrate transfer in polyketide synthases, and establishes a new model for molecular dissection of these multifunctional enzyme systems.

Type I modular polyketide synthases are the enigmatic assembly lines for the synthesis of chemically diverse polyketide natural products that form the basis for nearly one-third of pharmaceuticals. Accordingly, it is essential to dissect the architecture of the PKS module and to elucidate the molecular basis for selectivity and catalysis to enable rational bioengineering efforts for the creation of high-value chemicals and novel drugs.

Type I PKS modules act sequentially in polyketide chain elongation, processing and termination (Fig. 1). Each module contains acyl carrier protein (ACP), ketosynthase (KS) and acyltransferase (AT) domains that extend the linear sequence of an intermediate by two carbon atoms. The KS catalyses carbon–carbon bond formation between the immediate from the upstream module and the acyl-ACP. In addition, modules may also contain domains that successively modify the β-keto group to a hydroxyl (ketoreductase (KR)), a double bond (dehydratase (DH)), or a single bond (enoylreductase (ER)). The ACP employs a phosphopantetheine (Ppant) arm and thioester bond to tether polyketide intermediates and building blocks, and transfers them to respective catalytic domains for loading, extension and keto-group processing or modification. Through direct fusion or a non-covalent docking interaction, the ACP also transfers the fully processed intermediate to the KS in the subsequent module for further extension or to the thioesterase (TE) in the final module for release through hydrolysis or cyclization.

Modular PKSs are thought to share a common ancestor with the mammalian fatty acid synthase (FAS). However, to achieve perfect fidelity in product formation, the sequential modular PKSs have evolved to be highly ordered in structure and function. The FAS on the other hand acts iteratively within a single multifunctional protein with no inherent ability to deviate from its role to construct saturated fatty acid chains. Whereas the FAS accesses a single ACP, the PKS must ensure that the many ACPs within a multimodule assembly line deliver their respective polyketide intermediates with absolute fidelity to the appropriate modules and catalytic domains. Furthermore, PKSs have evolved through insertion or deletion of catalytic domains to vary extensively in the series of reactions performed within a single module.

Currently, no high-resolution structure is available for any PKS module, although crystal structures have been reported for various excised PKS catalytic domains, KS–AT di-domains and docking domains. Despite the important insights from these studies, the working model for the PKS module is based on a crystal structure of the porcine FAS, which has several limitations due to differences in the oligomeric state and structure of individual catalytic domains as well as substantial differences in linker regions. Thus, obtaining high-resolution information on the overall structure and organization of a complete PKS module is central for understanding polyketide extension, processing and diversification.

Electron cryo-microscopy of PikAIII

To investigate the structure of a full-length type I PKS module, we applied single-particle electron cryo-microscopy (cryo-EM) to visualize pikromycin PKS module 5 (PikAIII) from Streptomyces venezuelae (Fig. 1). The monomeric polypeptide, PikAIII, bearing the KS5–AT5–KR5–ACP5 domain architecture (Fig. 1), can be produced in pure, stable form (Extended Data Fig. 1a) and characterized by established biochemical assays.

Cryo-EM three-dimensional reconstructions of PikAIII were obtained in different physiological states. In conditions in which more than one PikAIII conformation was observed, we followed a multiple-reference classification approach to separate cryo-EM projections and calculate independent three-dimensional maps of each conformer. On the basis of micrograph quality control and inclusion of several thousands of cryo-EM images, we obtained final reconstructions with indicated resolutions of 7.3–9.5 Å (Extended Data Figs 1–4). The three-dimensional maps revealed secondary structures within PikAIII and allowed precise rigid body fitting of the homologous structures of the KS, AT, KR and ACP domains from 6-deoxyerythronolide B synthase (DEBS), leading to highly accurate pseudo-atomic-resolution structures of PikAIII.

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Thus, here we describe the first structural snapshots—to our knowledge—of a full-length PKS module in different functional states.

**Overall structure of the PKS module**

The cryo-EM structure of the 328 kilodalton (kDa) dimeric PikAIII with the intramodule ACPs, bearing a Ppant arm (holo-PikAIII) (Extended Data Figs 1b, c, 5a–d) reveals an arch-shaped symmetric dimer, with the homodimeric KS, at the dome supported on either side through a post (Fig. 2a–c). The ACP domains, which fit unambiguously into their corresponding densities (Fig. 2d), are found to populate two distinct positions in a 1:1 ratio, one near KR, and one near AT (Fig. 2 and Extended Data Fig. 4). In both conformers the remaining domains display identical configurations. Bottom-up liquid chromatography/Fourier transform ion cyclotron resonance mass spectrometry (LC/FT-ICR MS) established that the Ppant arm was present at a high level (Extended Data Fig. 5a–d), suggesting that the distinct ACP locations are not attributable to the presence or absence of this post-translational modification. In addition, multiple-reference-supervised classification tests could not detect a statistically significant fraction of holo-PikAIII dimers with the two ACP domains in non-equivalent positions, that is, one near the AT and the other near the KR, providing evidence for concerted ACP movement.

The KS–AT configuration in the complete PikAIII module resembles an earlier proposed PKS model but is strikingly different from the configuration of the excised KS–AT di-domains (Extended Data Fig. 6a) and the mammalian FAS (Fig. 2e). In full-length PikAIII, each AT is rotated by \( \sim 120^\circ \) relative to its position in the FAS and excised KS–ATs, and forms an extensive interface with KS (Extended Data Fig. 7a). In this arrangement, AT and the following KR domain reside below KS, creating a single ACP reaction chamber in the centre of the PikAIII dimer, with the AT and KR active sites facing the chamber. The characteristic long and narrow shape of the PikAIII reaction chamber is probably crucial for excluding ACPs of other modules, thus providing a mechanism to maintain biosynthetic fidelity.

The three-dimensional maps of PikAIII reveal weak density for the post-ACP dimerization helices (Extended Data Fig. 6b, c) and no density for the interdomain connecting loops, either because of insufficient resolution, disorder, or a combination of both. Nevertheless, linker lengths and distance limitations indicate that each AT–KR post includes domains of the same chain, while each AT interfaces with the KS of the opposite polypeptide (Fig. 1 and Extended Data Fig. 6d). The linker from KR to ACP (43 amino acids) is long enough to facilitate ACP interaction with the KS of either subunit, suggesting that other types of steric constraints may limit each ACP to interact with the KS domain of the opposite monomer, as reported for a DEBS module. Only the KS–AT linker (15 amino acids) is too short, requiring an additional eight amino acids to span the shortest distance between a KS carboxy terminus and an AT amino terminus (70 Å). This observation suggests that some amino acids at the AT N terminus of excised KS–ATs may be part of the KS–AT linker in the full module. While the resolution of the EM maps does not allow us to confirm this hypothesis, several lines of evidence indicate that the KS–AT interaction is associated with structural rearrangements at the domain interface. First, this is the only region of domain clashes between the crystal structures rigidly docked in the
three-dimensional maps, and structural rearrangements must occur to alleviate these clashes (Extended Data Fig. 7a, b). Furthermore, the position of AT5 relative to KS5 changes according to the substrate condition of PikAIII, indicating a dynamic KS5–AT5 interface that adapts to different states during the PikAIII catalytic cycle (see accompanying paper31).

Mutagenesis of PikAIII domain interfaces

To probe the KS5–AT5 interaction and the associated conformational changes, we generated KS5 and AT5 mutants in PikAIII–TE (PikAIII fused to the TE domain of PikAIV)24,25,32 and assessed their ability to generate 10-deoxymethynolide (10-dml) (Extended Data Fig. 8a, b), which is a natural macrolactone product of the Pik PKS24,25. PikAIII AT5 Lys 490 and KS5 Asp 352 are near one another within the KS5–AT5 clash zone whereas Arg 525 is outside the clash zone (Extended Data Fig. 7b). Mutagenesis at these sites highlighted a critical role for Arg 525, as also observed in the DEBS system33. The results suggest that conformational changes within AT5 accompany its interaction with KS5, whereby Arg 525 is probably crucial to the domain interface while Lys 490 is outside the interaction zone. Furthermore, these AT5 amino acids are near the KS5 active-site entrance in full-length PikAIII, but far away in the excised KS–AT5 structures, providing further validation of the cryo-EM structure.

Linker distance limitations suggest that each AT interacts with the KS of the opposite chain but with the KR of the same monomer (Extended Data Fig. 6d). The AT–KR conformation is incompatible with anchoring of the post-AT linker (residues 891–903) to the side of a KS monomer (Extended Data Fig. 6a), as observed in crystal structures of excised KS–AT di-domains and mammalian FAS6,7,21,22, consistent with the lack of any corresponding density in the cryo-EM maps. To confirm this finding, we mutated conserved Phe 897 and Trp 903, which make extensive KS contacts in the excised KS–AT crystal structures (Extended Data Fig. 8b). PikAIII W903A and F897A exhibited 7% and 130% of wild-type activity, respectively, suggesting that Phe 897 does not tether the post-AT5 linker in the position found in the crystal structures, and that Trp 903 may have some other important functional role. We probed the AT5–KR5 interface (Extended Data Fig. 7c) with three single-site substitutions, E766R and E768R in AT5 and G929E in KR5. The AT5 mutations reduced activity (77%, E766R; 45%, E768R) compared to wild type, while the KR5 mutation had no effect, indicating that the observed AT5–KR5 interface may not be crucial for PikAIII function (Extended Data Fig. 8b).

KS5 active-site entrance for PikAII ACP4

To examine how the upstream ACP4 domain (PikAII module 4; Fig. 1) docks and transfers the pentaketide intermediate to PikAIII KS5, we created a PikAIII lacking ACP5 to allow visualization of the upstream ACP4 without the added complexity of the intramodule ACP. Unexpectedly, we found that a PikAIII lacking ACP5 and its corresponding...
structures (right) of the cryo-EM map of pentaketide–ACP 4–PikAIII(C209A/
for holo-PikAIII, but with KR5 domains rotated by 165° about each leg of the
PikAIII arch (Extended Data Fig. 7d). Thus, the orientation of the
KR5 domains with respect to the AT5 domains appears to be strongly
influenced by the presence of the intramodule ACPs.

We next examined a PikAIII(ΔACP5) variant bearing ACP4 fused
with a flexible linker to the N-terminal docking domain of KS5. Fusion
of the ACP4 was necessary owing to the weak affinity of docking domains
(dissociation constant (Kd) of 5 μM). The KS5 active-site Cys 209 was
substituted with an alanine (ACP4–PikAIII(C209A/ΔACP5)) to pre-
vent intermediate transfer from a pentaketide-loaded ACP4 (Extended
Data Fig. 5e, f). Bottom-up LC/FT-ICR MS confirmed pentaketide loading
onto the ACP4–PikAIII(C209A/ΔACP5) fusion protein (Extended
Data Figs 5g–l, 9c, d). The structure of pentaketide–ACP4–PikAIII(C209A/
ΔACP5) at 8.6 Å revealed ACP4 bound on the top of KS, completely
outside the intramodule ACP5 chamber (Fig. 3a). The structure of the
DEBS ACP5 was accurately docked in its corresponding density near
the entrance of the KS5 active-site channel, revealing that ACP2 Ser 3605
is near the KS5 catalytic Cys 209 (Fig. 3b).

To probe the interaction between upstream ACP and downstream
KS, we mutagenized the interaction region of PikAIV (module 6) KS6.
The well-established PikAIII/PikAIV35–36 assay generates two products,
which are formed by transfer of the β-hydroxyhexaketide from PikAIII
ACP5 to the PikAIV KS6 (Fig. 4a and Extended Data Fig. 8c). Therefore,
formation of nbl requires a functional ACP5–KS6 interaction while forma-
tion of 10-dml does not34. PikAIV KS6 residues Arg 147 and Arg 320 are
involved in the KS5 active-site entrance, suggesting the existence of a second
entrance at the bottom of KS5. Indeed, we find that the KS domains of
modular PKSs have a previously unrecognized active-site entrance, which
faces the central ACP5 chamber of PikAIII (Fig. 4b) and is surrounded by a
loop (163–174 in PikAIII) with low sequence conservation and high
flexibility in the KS5–AT crystal structures6,7,21. To confirm the functional
role of the channel, we sought to obstruct it by substituting tryptophan
for amino acids surrounding the channel entrance. Consistent with our
hypothesis, the single substitutions S164W, N241W and D243W showed
4%, 12% and 6% of wild-type activity, respectively (Extended Data Fig. 8b).
Taken together, the MM–PikAIII structure, mutagenesis results and
sequence alignments across many type I modular PKSs reveal a new
active-site entrance that faces into the KS5–AT catalytic chamber. The
entrance is used by the intramodule ACP to transfer substrates to

KS5 active-site entrance for PikAIII ACP5

Next, we probed how the intramodule ACP5 engages the KS5 active site
when it carries the ACP5-bound methylimononyl (MM) extender unit for
elongation. To prepare MM-loaded ACP5, we incubated holo-PikAIII
with MM–CoA and confirmed the presence of MM–ACP5 and MM–
AT5 (Extended Data Fig. 5m–s) by bottom-up LC/FT-ICR MS. The
cryo-EM structure of MM–PikAIII at 7.3 Å resolution (Fig. 4a) showed
the same overall PikAIII conformation. However, MM–ACP5 interacts
with a loop (residues 83–92) at the bottom of the KS5 within the ACP5
chamber, further indicating that ACPs localize according to the teth-
ered substrate. Despite the overall high resolution of the MM–PikAIII
cryo-EM map, the ACP5 does not fit its corresponding density with the
remarkable accuracy we found in the other states, suggesting consid-
erable flexibility. The lack of ACP positional rigidity is expected, consid-
ering the flexibility of loops at this site in the KS5–AT crystal structures6,7,21.

Nevertheless, the conformation that provides the highest cross-correlation
between the ACP5 model and the EM density is consistent with previous
ACP mutagenesis data35,36 and places the ACP5 Ser 1438 below the KS5
active site at a distance of 25 Å (Extended Data Fig. 7e). To confirm this
interaction, we produced single-site loop substitutions, which abolished
macrolactone formation (R91A) or decreased it (D87A, 30% reduction)
(Extended Data Fig. 8b).

The ACP5 location in MM–PikAIII is far from the previously estab-
lished KS5 active-site entrance, suggesting the existence of a second
entrance at the bottom of KS5. Indeed, we find that the KS domains of
modular PKSs have a previously unrecognized active-site entrance, which
faces the central ACP5 chamber of PikAIII (Fig. 4b) and is surrounded by a
loop (163–174 in PikAIII) with low sequence conservation and high
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role of the channel, we sought to obstruct it by substituting tryptophan
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hypothesis, the single substitutions S164W, N241W and D243W showed
4%, 12% and 6% of wild-type activity, respectively (Extended Data Fig. 8b).
Taken together, the MM–PikAIII structure, mutagenesis results and
sequence alignments across many type I modular PKSs reveal a new
active-site entrance that faces into the KS5–AT catalytic chamber. The
entrance is used by the intramodule ACP to transfer substrates to
and from the KS. In contrast, FAS I and II, as well as type II and III PKSs, lack this second entrance. Instead, the bottom channel is closed by well-ordered loops and the channel harbouring the KS catalytic cysteine has only one entrance.

Discussion

The PikAIII structure, strikingly different from the more open architecture of mammalian FAS with its two ACP chambers, reveals nature’s remarkable design of a dimeric type I PKS module with a single chamber that sequesters the active-site entrances so that they are readily accessible to the intramodule ACP and unlikely to encounter incorrect substrates delivered by other near-neighbour ACPs. This architecture facilitates efficient substrate channeling by providing separate entrances to the KS active site for the ACP carrying an intermediate from the upstream module and for the intramodule ACP that carries the next extender unit and, subsequently, the elongated intermediate (Figs 3c, 4c). The newly identified intramodule ACP entrance on the KS is almost certainly inaccessible to the upstream ACP, which is docked to the KS coiled coil. It is less certain whether the intramodule ACP can reach the KS entrance for the ACP carrying an intermediate from the upstream module and for the intramodule ACP that carries the next extender unit and, subsequently, the elongated intermediate (Figs 3c, 4c).

The structure of docking domains in modular polyketide synthases. The structure of docking domains in modular polyketide synthases.

METHODS SUMMARY

PikAIII variants for cryo-EM analysis were purified by Ni-affinity chromatography followed by duplicate gel filtration steps. To trap PikAIII in different states, the purified proteins were incubated with substrate, and adsorbed on holey carbon grids followed by vitrification. Bottom-up LC/FT-ICR MS confirmed substrate loading in each of the PikAIII states. All specimens were imaged on a Tecnai F20 transmission electron microscope (FEI) equipped with a field emission gun operated at 120 kV. Images were recorded at a magnification of ×66,964 on a Gatan US4000 CCD camera with defocus values ranging from −1.5 to −3.5 μm. EMAN routines were used for interactive particle selection, generation of initial models, and three-dimensional map refinement. Crystal structures of domains were independently fit in the EM maps as rigid bodies. Functional assays that require intermediate processing by PikAIII detected TE-offloaded products from PikAIII–TE or PikAIII/PikAIV.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Figure 4 Interaction of intramodule ACP5 with KS5 in MM-PikAIII.

a, Orthogonal views of solid rendering (left) and transparent representation with modelled structures (right) of the MM–PikAIII cryo-EM map at 7.3 Å resolution. The ACP (orange) has shifted ~20 Å relative to its position in holo-PikAIII conformer II. b, KS active-site channels. Internal cavity analysis (purple surface) depicts channels to the active site from both the side and bottom entrances. c, Cartoon representation of MM–PikAIII. AT (green, yellow active site) loading of the MM building block (red) onto the intramodule ACP (orange, yellow serine) positions the carrier domain at the bottom entrance of KS (blue, yellow active site) for decarboxylative condensation, remote from the KR domain (purple, yellow active site).
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Author Contributions S.D. carried out cryo-EM data collection and processing. J.R.W. produced PikAIII variants and conducted enzyme assays. G.R.C. assisted with cryo-EM image processing. W.A.H., A.R.H.N. and K.H. carried out mass-spectrometry analysis. D.A.H. synthesized the thiophenol-activated pentaketide and pentaketide–CoA subunit. D.H.S. and G.S. analysed the data and interpreted results. K.H., D.H.S., J.L.S. and G.S. designed research. S.D., W.A.H., D.H.S., J.L.S. and G.S. wrote the manuscript.

Author Information Cryo-EM maps have been deposited in the Electron Microscopy Data Bank under accession numbers EMD-5647 (holo-PikAIII distortion), EMD-5648 (holo-PikAIII conformation II), EMD-5649 (PikAIII(ACP)), EMD-5651 (pentaketide–ACP–PikAIII(C209A)(ACP)), EMD-5653 (MM–PikAIII) and EMD-5662 (holo-ACP–PikAIII(C209A)(ACP)). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to G.S. (skiniot@umich.edu).
METHODS
Design of expression constructs. Construction of expression plasmids for PikAIII (pPikAIII) and PikAIV (pPikAIV) was described previously. All PikAIII constructs were ligated into a PET2b expression plasmid and the PikAIV construct was ligated into a PET2b expression plasmid (Novagen). The expression plasmid for PikAIII lacking the ACP (pPikAIII(ΔACP)) was constructed with overlap PCR. Two PikAIII fragments were amplified from pPikAIII: (1) starts upstream of a natural Fsel site and ends at the ACP N-terminus; and (2) starts at the ACP C-terminus and ends at the PikAIII C-terminus. These fragments, which contained complementary ends, were put into a second PCR to amplify a fragment lacking the ACP. This fragment was digested with Fsel and HindIII and inserted into pPikAIII digested with the same enzymes to produce pPikAIIIC209A(ACP). Primers for construction of PikAIII (ACP) (AACP) were as follows (bold font indicates overhangs used for ligation into vectors and italics indicate complementary overhangs used for overlap PCR). PikAIII-FseI, ACGGACGTACTGCCGGTCCGG; PikAIII-D87A, TGGCACACCGGCTACACGGGCCCTCCCAGCAG. This fusion was digested with NdeI and MfeI and inserted into pPikAIII(ΔACP), in which the ACP was loaded with pentaketide, 6 µM apo-APC5–PikAIII(ΔACP). From the first gel filtration column was incubated with 100 µM pentaketide–CoA, 10 µM SVP (a non-specific phosphopantetheinylation transferase) 10 µM MgCl2 for 2 h at 30 °C in buffer A, pH 7.4, and re-purified with a second HiPrep 16/60 Sephacryl S300 HR column equilibrated with 30 mM HEPES pH 6.8, 300 mM NaCl, 10% glycerol (buffer A, pH 6.8). The peak fraction was collected from the second gel filtration, dialyzed into 50 mM HEPES pH 6.8, 100 mM NaCl.

Substrate loading of PikAIII constructs. Proteins were dialyzed into 50 mM HEPES pH 7.4, 100 mM NaCl before substrate loading. The holo-PikAIII and PikAIII(ACP) samples were not incubated with substrate. For the MM–PikAIII sample, 1 µM holo-PikAIII was incubated with 500 mM MM–CoA for 30 min at room temperature. To prepare ACP5–PikAIII(290A/ΔACP), in which the ACP was loaded with pentaketide, 6 µM apo-APC5–PikAIII(290A/ΔACP) from the first gel filtration column was incubated with 100 µM pentaketide–CoA, 10 µM SVP (a non-specific phosphopantetheinylation transferase) 10 µM MgCl2 for 2 h at 30 °C in buffer A, pH 7.4, and re-purified with a second HiPrep 16/60 Sephacryl S300 HR column equilibrated with 50 mM HEPES pH 6.8, 300 mM NaCl, 10% glycerol (buffer A, pH 6.8). The peak fraction was collected from the second gel filtration, dialyzed into 50 mM HEPES pH 6.8, 100 mM NaCl.

Mass-spectrometric analysis of active-site occupancy. Bottom-up LC/FT-ICR MS was used to confirm the presence or absence of substrate in each domain of holo-PikAIII–MM, MM–PikAIII and ACP5–PikAIII(290A/ΔACP). Twenty-five micro-liters of 2 µM holo-PikAIII, 25 µl 2 mM MM–PikAIII, 25 µl 2 µM pentaketide–ACP5–PikAIII(290A/ΔACP), and 25 µl 2 µM holo-ACP5–PikAIII(290A/ΔACP) were diluted with 20 µl of 250 mM ammonium bicarbonate pH 8.0. Trypsin in 50 mM acetic acid was added in an enzyme-substrate ratio of 1:10. Proteolysis was allowed to proceed for 15 min at 37 °C followed by addition of formic acid (pH 4). Samples were stored at −20 °C until analysis.

Forty-five micro-liters of sample were injected onto a Synergi Hydro C18 hydrophilically end-capped 1×150 mm column with 4 µm particles (Phenomenex). A gradient was generated on an Agilent 1100 HPLC. The gradient was as follows (with isocratic elution between 40 and 50 min): 0 (98,2), 20 (70,30), 40 (50,50), 50 (50,50), 55 (30,70), 70 (2,98). Values are provided as time (%A, %B) over a total run time of 90 min. Flow was at 50 μl/min and was diverted for the first 5 min of the run. HPLC solvent A was 0.1% formic acid (ThermoFisher Scientific) in HPLC-grade water (ThermoFisher Scientific), and solvent B was 0.1% formic acid in acetonitrile (ThermoFisher Scientific). The LC was coupled to a quadrupole FTICR-MS (SolariX with 7T magnet, Bruker Daltonics). Data were gathered from m/z = 200–2000 in positive-ion mode. Electrospray was conducted at 4,500 V with four scans per spectrum and a 256k transient. External ion accumulation in a hexapole was 0.2 s and there was 1 ICR fill before excitation and detection. External calibration used HP-mix (Agilent). PikAIII peptide products were detected over three samples in separate runs.

Mass spectrometric analysis of the pentaketide–ACP5–PikAIII(290A/ΔACP) unexpectedly detected apo-ACP in the pentaketide-loaded sample. Therefore, we used a second method to directly quantitate the level of apo-ACP5–PikAIII(290A/ΔACP), which can be loaded with pentaketide from CoA (CoA 547, New England Biolabs) (Extended Data Fig. 9c, d). Ten micromolar apo-ACP5–PikAIII(290A/ΔACP) or 10 µM pentaketide-loaded apo-ACP5–PikAIII(290A/ΔACP) (loading as described earlier) was incubated for 2 h with 20 µM SVP, 100 µM CoA 547 (New England Biolabs), 10 mM MgCl2, 100 mM Tris pH 8 at 30 °C in 15 µl reaction volumes. Reactions were quenched with the addition of 30 µM EDTA and diluted to 75 µl with dH2O. Fifty microlitres of each reaction were injected onto a PLRP-S 4,000 Å column (8 µm, 50 x 2.1 mm) (Varian) with a flow rate of 0.3 ml min−1 and a protocol as follows: 5% solvent B (acetonitrile with 0.1% formic acid) for 2 min, 5–60% solvent B for 18 min, 60–100% solvent B for 5 min, and 100% solvent B for 3 min (solvent A, 0.1% formic acid in water). Levels of apo-ACP5–PikAIII(290A/ΔACP) were quantitated by comparison of peak areas monitored at 280 nm (total protein) and 550 nm (chromophore absorbance).

Sample preparation and cryo-EM imaging. Sample quality and homogeneity were evaluated by conventional negative-stain EM. For cryo-EM, 3 µl of PikAIII preparations under different conditions (described earlier) was adsorbed on glow-discharged Quantifoil R2/2 100 mesh grids followed by blotting and vitrification with a Vitrobot (FEI, Mark IV). All specimens were imaged using a Tecnai F20 transmission electron microscope (FEI) equipped with a field emission gun operated at 120 kV. Images were recorded at a magnification of ×66,964 in a Gatan US5000 CCD camera with exposures ranging from −1.5 to −3.5 µm (Extended Data Fig. 1c). All images were acquired using low-dose procedures with an estimated dose of ~20 electrons per Å2.

Image processing and three-dimensional reconstructions. PikAIII particle projections were interactively selected and excised using boxer (EMAN 1.9 software tool.
suites) or e2workflow.py (EMAN2 software suite)
4. During the CTF parameters for each
5. During the CTF determination process we selected only the images with a clear signal at resolutions
6. than 13 Å and whose sum of power spectra of excited particle projections did not reveal any drift or visible astigmatism. The CTF correction was applied to the selected particles using applyctf (EMAN 1.9 package).
7. During the initial stages of this project we obtained tilt-pair EM images (0/60°) of PikAIII embedded in negative stain. Reference-free classification and averaging revealed a particle architecture that was very different from the one of mammalian FAS,
8. by substituting the full data set of 45,183 projections into the threefold excess of methanol, vortexed, incubated for 15 min at
9. number of projections is provided in Extended Data Fig. 4. The final three-dimensional EM maps were sharpened using EMBFACTOR57,58 and subsequently low-pass filtered to their corresponding indicated resolution range.
10. The resolution of the MM–PikAIII reconstruction is 7.1 Å by the gold-standard FSC test, which is based on our smallest data set, the gold-standard FSC test indicates a resolution of 8.3 Å, about 0.5 Å beyond that of conventional FSC (Extended Data Fig. 4). For the pentakteïte–ACP–PikAIII(C209A/AACp) map, the gold-standard FSC test indicates a resolution of 8.9 Å, about 0.3 Å beyond that of conventional FSC (Extended Data Fig. 4). For the two conformations of holo-PikAIII, the gold-standard FSC test indicates resolutions of 9.3 Å and 9.7 Å, which are very similar to the values from the conventional FSC test (9.2 Å and 9.5 Å). Thus, the calculated indicated resolution of each reconstruction presented in this work is consistent with the results of gold-standard FSC procedures and does not indicate any detectable map over-refinement resulting in overestimation of resolution.
11. High-resolution phase randomization to determine any over-fitting.
12. In initial reconstructions of holo-PikAIII(C209A/AACp) with a tilt angle differential of 30° (+15°/-15°) using a Gatan K2 Summit on a Tecnai F20 transmission electron microscope (FEI) equipped with a field emission gun operated at 200 kV. Images were recorded at a nominal magnification of ×36,673, corresponding to a pixel size of 1.23 Å at the specimen level. The total dose of the first and second image was 22 e Å⁻² and 28 e Å⁻², respectively, and fractionated over 20 sub-frames acquired over 4 s exposures. The sub-frames of each exposure were aligned with the UCSF alignment program (provided by Y. Cheng and X. Li) and merged. Tilt-pair validation was processed with the corresponding routines of EMAN 2.07. One-hundred and two particle projection pairs were interactsively selected using e2RCTBoxer.py59, and CTF corrected using e2ctf.py. The tilt validation parameter plot (Extended Data Fig. 2c) was obtained with the program e2tiltvalidate.py and shows that the majority of tilt projection pairs cluster at the expected 30° tilt angle differential, thereby validating the three-dimensional map.
13. The crystal structures of the DEBS module 5 KS–AT di-domain (Protein Data Bank (PDB) accession 2HG4)7 and DEBS module 1 KR monomer (PDB accession 2FR0) 11, and the KR domain of DEBS module 2 KR monomer (PDB accession 2HSQ)63, were sharpened using EMBFACTOR57,58 and subsequently low-pass filtered to their corresponding indicated resolution range.
14. Multiple-reference-supervised classification. In initial reconstructions of holo-PikAIII we observed two densities corresponding to ACP, one close to KR, and one between KS and AT (Fig. 2). To separate the particle projections corresponding to each ACP state we employed multiple-reference-supervised classification.
15. To this end we docked the structures of KS, AT, KR and ACP in the corresponding densities and produced two initial models whose only difference was the ACP positioning. We proceeded with multiple reference-supervised classification using the full data set for each final reconstruction routine in EMAN (1.9)6,7 using the two above models as references. In this way the particle projections were classified into two categories according to their cross-correlation with re-projections of the two references of holo-PikAIII.
16. The number of particles in each category is provided in Extended Data Fig. 4. In the next step we used the two separate particle data sets to calculate the two independent three-dimensional reconstructions using the low-pass filtered EM map of MM–PikAIII as an initial reference volume and thus avoiding any reference bias.
17. Resolution calculations and refinement procedure validation. One set of resolution calculations was based on the conventional ‘even/odd’ test in EMAN, whereby each data set was split into two subsets, and each half was used for threefold reconstruction according to the orientation parameters established in the last round of refinement. The FSC plots for all three-dimensional reconstructions are provided in Extended Data Fig. 4. To test the validity of our reconstruction approach and also assess whether the obtained FSC values might be the result of over-refinement, we additionally ran gold-standard FSC tests for all the high-resolution PikAIII reconstructions. For these calculations we independently refined two half data sets of a condition against a 50 Å filtered EM map and compared the resulting two final independent maps by FSC (Extended Data Fig. 10). All resolution values reported below by conventional FSC measurements are according to the 0.5 criterion, and for gold-standard FSC tests are according to the 0.143 criterion.
18. The resolution calculations were performed using e2initialmodel.py (EMAN 1.9 suite), an initial model using class averages by e2initialmodel.py in EMAN2 (ref. 55), and a low-pass filtered model of the KS–AT di-domain crystal structure. Starting with these different models, and after several iterations of projection matching and three-dimensional reconstruction, the refinements consistently converged to the same overall low-resolution structures (Extended Data Fig. 2a). Re-projections of these volumes showed good agreement with class averages and raw particle projection from both cryo-EM and negative-stain PikAIII data (Extended Data Fig. 2b). Therefore, the structure from the sphere-like randomized initial reference volume was subsequently used for EMAN model-based projection matching and iterative angular refinement of the whole MM–PikAIII projection data set (~56,000 projections). Initial rounds of projection matching and three-dimensional reconstructions were performed with binned (2 × 2 pixels) projection images with a resulting pixel size of 4.48 Å on the specimen level. Non-binned images (pixel size 2.24 Å) and sub-pixel averaged were used in the later stages of refinement for calculation of high-resolution three-dimensional maps (Extended Data Fig. 3a). During refinement we progressively decreased the angular step to a final 2° until convergence. Thus the final map of MM–PikAIII was produced with an indicated resolution of 7.3 Å at the 0.5 level of Fourier shell correlation (FSC) (Extended Data Fig. 4). The three-dimensional map revealed distinct secondary structure features that are consistent with this resolution range. Importantly, the crystal structures of the DEBS module 5 KS–AT di-domain, and the DEBS module 1 KR11, could be rigidly docked with high precision into the MM–PikAIII map and revealed an excellent agreement with the features of the three-dimensional reconstruction (Fig. 4a). Cross-correlation values between the overall model and the three-dimensional maps are provided in Extended Data Fig. 3b. A 30 Å low-pass filtered map of MM–PikAIII was used as the initial model for iterative projection matching and three-dimensional reconstructions of holo-PikAIII and PikAIII(AACp). A 30 Å low-pass filtered map of PikAIII(AACp) was used as an initial model for three-dimensional reconstructions of holo-ACP–PikAIII(C209A/AACp) and pentakteïte–ACP–PikAIII(C209A/AACp). All refinements were executed based on the same overall protocol outlined earlier for MM–PikAIII and with final angular steps of 2°. The number of projection rejections for each final reconstruction shown is provided in Extended Data Fig. 4. The final three-dimensional EM maps were sharpened using EMBFACTOR57,58 and subsequently low-pass filtered to their corresponding indicated resolution range.
19. Enzyme assays. PikAIII–TE and PikAIIIPikAIV assays were described previously24,25. Either 1 µM PikAIII–TE or 1 µM PikAII and 1 µM PikAIV was added to a 100 µl reaction mixture containing 0.5 mM NADP⁺, 0.5 µM 1-glucose-6-phosphate dehydrogenase, 5 mM glucose-6-phosphate in 400 mM sodium phosphate pH 7.2, 20% glycerol, 5 mM NaCl. This mixture was incubated for 10 min at room temperature and the reaction was initiated by addition of 1 mM thiopeorin-activated pentakeïte, 8 mM 2- imidoylpropionyl, and 20 mM MM–N-acetyl cysteamine (NAC). After a 1 h incubation at room temperature, the reaction was quenched by addition of a threelfold excess of methanol, vortexed, incubated for 15 min at −20 °C, and centrifuged. The supernatant was analysed by reverse-phase HPLC on a Luna C18(2) (5 µm, 250 × 4.6 mm) column (Phenomenex) with a flow rate of 1.5 ml min⁻¹ and
by following this protocol: 5% solvent B (acetonitrile with 0.1% formic acid) for 1 min, 5–100% solvent B for 10 min, 100% solvent B for 4 min, and 5% solvent B for 2.5 min. Solvent A was 0.1% formic acid in water. Authentic standards confirmed the elution time of 10-dml and nbl. For the PikAIII–TE mutants, peak areas of 10-dml normalized to the values for wild-type PikAIII–TE were used to assess activity. For wild-type PikAIII and mutant PikAIV assays, activity was assessed from the peak areas of 10-dml and nbl normalized to the values for wild-type PikAIII and PikAIV. All reactions were completed in triplicate and the data are presented as average ± standard deviation.

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Extended Data Figure 1  |  PikAIll sample preparation and raw EM images.

**a**, SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gel of each purified form of PikAIll examined by cryo-EM. The numbers on the left indicate molecular weight in kDa. **b**, Raw EM image of holo-PikAIll particles embedded in negative stain. **c**, Raw cryo-EM image of holo-PikAIll particles. **d**, Boxed-out particle projections of holo-PikAIll.
Extended Data Figure 2 | PikAIII initial cryo-EM three-dimensional reconstructions. a, Generation of initial MM–PikAIII reconstructions using 3,600 particle projections and employing three different starting models. Top, generation of a MM–PikAIII reconstruction using a randomized Gaussian sphere from makeinitialmodel.py in EMAN2 (ref. 56). Middle, refinement of a starting model obtained by the EMAN2 initial-model-generation program e2initialmodel.py55. Bottom, refinement starting from a low-pass filtered model of the excised KS–AT di-domain crystal structure7. These three different starting models converged to similar structures that are also in agreement with reference-free class averages. b, Comparison of re-projections of a low-resolution cryo-EM three-dimensional map (iteration 28 of top panel in this figure) with reference-free two-dimensional class averages and raw particle projections from both cryo-EM and negative-stain MM–PikAIII data. c, Tilt-pair parameter plot of PikAIII(ΔACP5) with a tilt angle differential of 30° (+15°−15°). Black dots represent each particle pair’s tilt axis and tilt angle based on the cryo-EM map of PikAIII(ΔACP5). Most particle projection pairs cluster in a region (red circle) centred at 29.5° with a root mean squared deviation (r.m.s.d.) tilt angle of 8.3° and tilt axis of 90.23°.
Extended Data Figure 3 | Cryo-EM analysis of MM–PikAIII. a, Refinement of MM–PikAIII reconstruction: a three-dimensional model obtained \textit{ab initio} (see Extended Data Fig. 2) was used as a starting reference for initial projection matching of \(~29,000\) cryo-EM particle views to provide a map at 8.1 \(\AA\) resolution (middle), which was subsequently used for the refinement of the entire MM–PikAIII data set (56,292 projections) to obtain a final map at 7.3 \(\AA\) resolution. The panels on the bottom show vertical and horizontal cross-sections of the final MM–PikAIII cryo-EM map. b, Cross-correlation values between the overall pseudo-atomic-resolution model and the three-dimensional maps of various states of PikAIII.
Extended Data Figure 4 | Estimation of EM map resolution and assessment of over-refinement. Conventional Fourier shell correlation (FSC) curves (blue), gold-standard FSC (red) and high-resolution phase randomization tests (green) for PikAIII reconstructions. For conventional FSC calculations (full data-set refinement) we have used the conservative FSC $0.5$ criterion as a resolution indicator, whereas for gold-standard FSC calculations (half data-set independent refinements) we have used the FSC $0.143$ criterion, as previously applied. Consistently, the indicated resolution at FSC $0.5$ of the conventional FSC is in close agreement with FSC $0.143$ of the gold-standard FSC. The FSC calculations with phase randomized data show a sharp drop-off at the expected resolution level (10 Å, or 12 Å for holo-PikAIII) and a lack of noise refinement. Additionally, we measured the FSC between the average map of the two gold-standard half-maps and the conventional map (full data set) as implemented previously (see Extended Data Fig. 10). The agreement between the conventional and the average gold-standard map by this method is also fully consistent with conventional and gold-standard FSC calculations. The table inset summarizes the number of projections used for each reconstruction, the conventional and gold-standard FSC resolution indications, and the agreement by FSC between the average map of the two gold-standard half-maps and the conventional map.
Extended Data Figure 5 | Partial mass spectra of active-site PikAIII and ACP₅-PikAIII(C209A/ACP5) peptides from LC/FT-ICR MS of trypsin-digested proteins. a–d, ACP₅ active-site peptides in their apo (a, b) and holo (with phosphopantetheine (Ppant) (c, d) states at 2⁺ and 3⁺ charge states. On the basis of integrated peak abundances from multiple LC/MS runs, greater than 97% of the ACP₅ Ser 1438-containing peptides were modified with Ppant. e, f, Confirmation of the C209A mutation of the KS₅ active site. The mutated active-site peptide was detected in the 4⁺ (e) and 3⁺ (f) charge states. g–i, Example mass spectra of Ser 3605-containing active-site ACP₄-derived peptides following enzymatic loading of the pentaketide from pentaketide–CoA. Both apo- (g), holo- (with Ppant) (h) and pentaketide–ACP₄ (i) were detected. j–l, Example mass spectra of active-site ACP₄-derived peptides from a control experiment in which pentaketide–CoA was absent. The majority of the ACP₄ active-site peptides were detected in the apo and holo states, while a very small percentage (₁% of the ACP₄ active-site peptides was detected in the apo and holo states, while a very small percentage (<1%) contained the pentaketide intermediate. m–p, ACP₅ active-site peptides following incubation with MM–CoA. The MM building block was detected in high abundance on ACP₅ Ser 1438 (o, p) with some unloaded holo-protein as well (m, n). q–s, AT₅ active-site peptides following incubation with MM–CoA. The MM building block was detected on AT Ser 655.
Extended Data Figure 6 | PikAIII domain organization and connectivity.

a, Crystal structure of excised DEBS module 5 KS–AT di-domain. KS (blue, yellow active site) and AT (green with red active site) domains interact differently than in the full module (Fig. 2), and the post-AT linker (red) lies on the surface of the KS domain. 

b, Localization of post-ACP5 dimerization helices. Top, stereo view of holo-PikAIII conformer I with the density ascribed to the post-ACP5 dimerization helices (rendered in cyan) observed between the ACP5 domains (orange). Bottom, overview of localization and enlarged cut-out densities of post-ACP5 dimerization helices (cyan) in holo-PikAIII conformation I.

c, Stereo view of holo-PikAIII conformer II with the density ascribed to the post-ACP5 dimerization helices (rendered in cyan) observed between the ACP5 domains (orange).

d, Proposed connectivity of domains in PikAIII determined by distances between domain termini and linker lengths. The catalytic domains are coloured (green or blue) according to the assigned polypeptide chain. The AT interacts with the KS of the opposite monomer whereas the AT–KR interaction is within the monomer. Active-site locations are indicated in yellow.
**Extended Data Figure 7 | Domain interfaces in PikAIII.**

**a.** Stereo view of the docked crystal structures of KS (blue) and AT (green) in the holo-PikAIII cryo-EM map reveals an extensive interface. The red star marks the side entrance to the KS active site where the catalytic Cys 209 (yellow spheres) resides. **b.** The interface of KS (blue) and AT (green) is less than 20 Å from the KS active-site Cys 209 (spheres; blue C and yellow S). This is the only region of steric clashes between the KS and AT crystal structures rigidly docked in the three-dimensional maps (KS5 amino acids 350–357 clash with AT5 488–498 and 526–531). Asp 352 (spheres; blue C and red O) of the KS and Lys 490 and Arg 525 (spheres; green C and blue N) of the AT were substituted with Ala in PikAIII–TE. D352A and K490A, which are located in the clash zone, resulted in 0% and 50% activity, respectively, relative to wild-type PikAIII–TE. The R525A substitution abolished product formation even though this residue is outside the clash zone. The sensitivity of Arg 525 and insensitivity of Lys 490 to Ala substitution is consistent with a structural rearrangement at the KS–AT interface. **c.** The docked crystal structures of AT (green) and KR (purple) in the holo-PikAIII cryo-EM map. The interface is formed primarily by a loop of KR (residues 928–936) and an α-helix of AT (residues 760–775). **d.** The KR domain of PikAIII(ΔACP5) (right) is rotated by 165° compared to holo-PikAIII (left). **e.** View of the unobstructed path and proximity of Ser 1438 (red) and Cys 209 (yellow) in the docked structures of KS and ACP in the MM–PikAIII cryo-EM map.
Extended Data Figure 8 | PikAIII functional assays. 

**a**, Example high-performance liquid chromatography (HPLC) traces of the PikAIII–TE assay. The levels of 10-dml produced by wild-type (WT) PikAIII–TE (red trace), D352A PikAIII–TE (green), K490A PikAIII–TE (blue), R525A PikAIII–TE (orange), and a no enzyme control (yellow) are shown.

**b**, Activity of PikAIII–TE mutants. ND, not detectable. All reactions were completed in triplicate and the data are presented as average ± standard deviation.

| PikAIII–TE | % 10-dml produced |
|------------|-------------------|
| WT         | 100               |
| D87A       | 68 ± 5            |
| R91A       | ND                |
| S164W      | 4 ± 1             |
| N241W      | 12 ± 2            |
| D243W      | 6 ± 2             |
| D352A      | ND                |
| K490A      | 51 ± 3            |
| R525A      | ND                |
| F879A      | 130 ± 21          |
| W903A      | 7 ± 2             |
| E766R      | 77 ± 2            |
| E768R      | 45 ± 2            |
| G929E      | 124 ± 18          |

**c**, Example HPLC traces of the PikAIII/PikAIV assay. The levels of 10-dml and nbl produced by wild-type PikAIII/PikAIV (red trace), wild-type PikAIII with PikAIV(R147E) (green), wild-type PikAIII with PikAIV(R320E) (blue), and a no enzyme control (yellow) are shown.

**d**, Activity of PikAIV mutants. All reactions were completed in triplicate and the data are presented as average ± standard deviation.

| PikAIV | % 10-dml produced | % nbl produced | nbl:10-dml |
|--------|--------------------|----------------|------------|
| WT     | 100                | 100            | 4:1        |
| R147E  | 110 ± 17           | 83 ± 5         | 3:1        |
| H320E  | 107 ± 7            | 60 ± 4         | 2.6:1      |
| R320H  | 115 ± 8            | 80 ± 4         | 2.1:1      |
| R147E/R320H | 127 ± 10   | 44 ± 3         | 0.85:1     |

Note: The PikAIII/PikAIV assay involves the production of 10-dml and nbl, with the reaction data presented as average ± standard deviation.
Extended Data Figure 9 | Analysis of ACP-less PikAIII. a, Overlay of gel filtration chromatography elution profiles of PikAIII(ΔACP5) (blue) and PikAIII(Δ1403–1562) (red). PikAIII(ΔACP5) includes the post-ACP dimerization helices and elutes as a dimer whereas PikAIII(Δ1403–1562) lacks the dimerization helices and elutes as a monomer. The first peak in the red trace is apparently aggregated protein in the void volume of the S300 column. b, Solid rendering (left) and transparent representation with modelled structures (right) of the cryo-EM map of PikAIII(ΔACP5) at a resolution of 7.8 Å. c, Example HPLC traces of chromophore–CoA loading experiments. The blue trace (280 nm) indicates the level of protein and the red trace (550 nm) indicates the chromophore from CoA 547 (New England Biolabs). Incubation of apo-ACP4–PikAIII(C209A/ΔACP5) with SVP and CoA 547 indicates that 100% of the ACP4 was in the apo form, based on molar extinction coefficients for protein and chromophore. d, Incubation of pentaketide–ACP4–PikAIII(C209A/ΔACP5) with SVP and CoA 547 indicates that 80% of the ACP4 was loaded with pentaketide. e, Conventional FSC curve for the three-dimensional reconstruction of holo-ACP4–PikAIII(C209A/ΔACP5) (no pentaketide added). f, Orthogonal views of solid rendering (top) and transparent representations with modelled structures (bottom) of the cryo-EM three-dimensional reconstruction of holo-ACP4–PikAIII(C209A/ΔACP5) (no pentaketide added). No density for the upstream ACP4 was observed in the cryo-EM map even though densities corresponding to the N-terminal docking domains are clearly visible (compare with Fig. 3b). Fit into the three-dimensional maps shown in b and f are the structures of DEBS module 5 KS (blue; PDB accession 2HG4), DEBS module 5 AT (green; PDB accession 2HG4) and DEBS module 1 KR (purple; PDB accession 2FR0).
Extended Data Figure 10 | Cryo-EM map refinement and resolution validation scheme. The flow chart shows the overall three-dimensional reconstruction scheme and resolution calculation by conventional and gold-standard FSC procedures using MM–PikAIII as an example. The procedure was applied for every high-resolution three-dimensional reconstruction in this study. Besides the conventional full data set refinement (left), each data set was split into two separate half data sets, which were employed for two independent reconstructions using the 50 Å filtered EM map as an initial reference (right; gold-standard procedure). The final two gold half reconstructions were compared by FSC, and the indicated resolution by gold-standard FSC with the 0.143 criterion showed excellent agreement with the value indicated at the 0.5 level of the conventional FSC (Extended Data Fig. 4). In addition, the two gold-standard half-maps were averaged, and the resulting average gold-standard map was compared by FSC to the corresponding conventional map, again showing very good agreement at the same resolution range (Extended Data Fig. 4). These tests, along with the phase randomization tests (Extended Data Fig. 4), reveal the lack of over-refinement and accurate resolution values reported in this study.