Ribosomally synthesized and post-translationally modified peptides (RiPPs) are structurally complex natural products with diverse bioactivities. Here we report discovery of a RiPP, kintamdin, for which the structure is determined through spectroscopy, spectrometry and genomic analysis to feature a bis-thioether macrocyclic ring and a β-enamino acid residue. Biosynthetic investigation demonstrated that its pathway relies on four dedicated proteins: phosphotransferase KinD, Lyase KinC, kinase homolog KinH and flavoprotein KinI, which share low homologues to enzymes known in other RiPP biosynthesis. During the posttranslational modifications, KinCD is responsible for the formation of the characteristic dehydroamino acid residues including the β-enamino acid residue, followed by oxidative decarboxylation on the C-terminal Cys and subsequent cyclization to provide the bis-thioether ring moiety mediated by coordinated action of KinH and KinI. Finally, conserved genomic investigation allows further identification of two kintamdin-like peptides among the kin-like BGCs, suggesting the occurrence of RiPPs from actinobacteria.

Cyclopeptides are a subset of secondary metabolites that can be of ribosomal or non-ribosomal origin. While non-ribosomal peptides (NRPs) have long been studied, the chemical diversity of peptides from ribosomal origin, now called ribosomally synthesised and post-translationally modified peptides (RiPPs), have only been recognised relatively recently and across all three domains of life during the last decade, as a result of recent advances in genome sequencing technology1. It is now known that the majority of RiPPs are synthesized from precursor peptides, typically ranging from 20 to 110 amino acid residues in length, encoded by a structural gene1.

Although the RiPPs exploit only the 20 proteinogenic amino acids, extensive post-translational modifications (PTMs) increase their structural diversity. Dehydroalanine (Dha) and (Z)-dehydrobutyrine ((Z)-Dhb) residues are commonly found in the RiPPs. Dha and (Z)-Dhb residues in RiPPs are derived from serine/cysteine and threonine respectively3. In some cases, D-amino acid residues, such as D-Ala, are introduced in lanthipeptides by an F420H2-dependent reductase4. The structural complexity of RiPPs extends to a range of cyclic motifs, including noncanonical thioether amino acid residues, such as lantionine (Lan), labionin (Lab)5, 2-aminovinly-cysteine (AviCys), and...
AviCys-labionin (Avionin, a decarboxylated analogue of Lab) (Fig. 1a), which are enzymatically installed post-translationally. Among these cyclic residues, Lan residues are well-understood in the biosynthesis of lanthipeptides (Fig. 1b). The enzymes responsible for the Lan moiety catalyse the dehydration of either Ser or Thr residues to generate Dha or Dhb, respectively, followed by the intramolecular Michael-like addition of the side chain of Cys to Dha or Dhb to form the Lan or methyl-Lan motif, respectively. When a second Dha motif is involved in the class III lanthipeptide NAI-112 (Fig. 1a), the triamino triacid Lab residues can be formed (Fig. 1b). However, the factors that determine Lan or Lab formation are not yet understood.

Both AviCys and Avionin residues are found in several RiPPs including lanthipeptides, thioamitides, lipolanthines, lanthidins and linaridins (Fig. 1a). In the case of AviCys residues in lanthipeptides, the PTMs normally involve the dehydration of the upstream Ser or Thr residue of a precursor peptide, followed by oxidative decarboxylation of C-terminal Cys and subsequent Michael addition of the resultant thioenol nucleophile onto the preceding Dha or Dhb residue to provide AviCys or AviMeCys residue, respectively. In this pathway, the formation of the reactive thioenol species from the C-terminal Cys is catalysed by the flavoprotein LanD belonging to the family of flavin-dependent cysteine decarboxylases (HFCDs) (Fig. 1c). However, this is determined.

Fig. 1 | Known thioether and β-amino acid residues-associated structures in RiPPs and proposed mechanisms. a Selected RiPPs containing a thioether residue (red), mechanistic comparison in the formation of thioether residue Lan/Lab (b) and AviCys/Avionin (c). b For Lan (top) and Lab (bottom) moieties in lanthipeptides, the factors determine Lan/Lab formation are not yet understood. c The enzymes responsible for the formation of AviCys/AviMeCys residues are either LanD homologues in NAI-112 (Fig. 1a), the coordinated action of HFCD-like TvaEs, and inactive kinase homologue TvaFs in thioviridamides 2. The enzymes responsible for AviCys (top) and Avionin (bottom) residues in 3, 4, 5 and 6 have not yet been determined. d Example of a splicease reaction to provide α-keto-β-amino acid residue. Stereochemistry at the preceding Leu α-carbon has not yet been defined. e Example of a SAM-dependent methyltransferase OVs2, to catalyse a rearrangement of Asp residue to l-isoAsp in Class I lanthipeptides. Other non-proteinogenic amino acid residues modified by PTMs are colour coded. Dhb (2,3-dehydrobutyrine), deep green a-Ile (allo-isoleucine), pink Dha (dehydroalanine), light blue, D-Ala (D-alanine) orange.
not the case for thioviridamidine (TVa) 2 (Fig. 1a), a thioamidite RIPP, in Streptomyces sp. NRRL S-87. Recent biochemical analysis demonstrated that the formation of AviCys residue in TVa relies on the coordination of two-component enzyme complex, the LanD-like flavoprotein TvaE_{S-87} and an inactive kinase-like TvaE_{S-87}. TvaE_{S-87} and TvaF_{S-87} function together by binding tightly with the leader peptide (LP) sequence of the precursor peptide to catalyse the decarboxylation of the C-terminal Cys residue, followed by subsequent addition to provide the corresponding AviCys moiety (Fig. 1b). Interestingly, while LxmX and CaoI, in the pathways of the class VI lantibiotic lexapeptide 3, and the lanthidin cacaoidin 4 (Fig. 1a), respectively, appear to be TvaE_{S-87} homologues, LxmK and CaoI are HFCD-like decarboxylases, suggesting that the AviCys moieties in these RPPs may also result from the action of two-component enzyme complexes. AviCys residues are also present in a subclass of lanthionate RPPs, such as cypemycin 5 (Fig. 1a). Biochemical analyses indicated that no AviCys is formed when an assay of the recombinant HFCD-like CypDp together with a synthetic core peptide (CP) mimic and necessary cofactors was performed, raising an unanswered question of whether an as-yet unidentiﬁed enzyme partner is needed to coordinate the decarboxylation and cyclization. In the case of the Avionin formation in the lipopantin RPP microvinon 6 from the Microbacterium arborescens, another LP sequence of the precursor peptide is post-translationally modiﬁed by oxidative decarboxylation of the C-terminal Cys and subsequent cyclization to yield avionin (Fig. 1c). Similar to Lan and Lab, the factors that determine the Avionin formation has remained to be determined.

Unlike NRPs which contain a range of β-amino acids, most RPPs discovered so far only contains α-amino acid residues. However, recent studies indicated that β-amino acid residues can be formed in the RPP pathways. This can be exemplified by a recent conserved genomic survey which discovered the unprecedented function of orphan radical SAM enzymes (PlpX-type splicesases) encoded in several bacterial RPP biosynthetic gene clusters (BGCs). Biochemical analysis demonstrated that PlpX splicesase, together with its partner protein pair, PlpY, in the plp BGC from the cyanobacterium Pleurocapsa catalyses a unique peptide backbone slicing reaction to remove all atoms of a C-terminal Tyr unit except the amide carbonyl in the precursor peptide PlpA 3. This generates an α-keto-β-amino acid residue, indicating that the corresponding mature RIPP metabolite contains β-amino acid residues (Fig. 1d). However, the structure of this RIPP associated with the plp BGC has not been determined. A second example was found that l-isosapartate, a β-amino acid residue, can be formed in Class I lanthipeptide pathways (Fig. 1e). In vitro reconstitution indicated that the S-adenosyl-L-methionine (SAM)-dependent O-methyltransferase, Ovh\textsubscript{S\textsubscript{A}}, encoded in the ovt gene cluster from Streptomyces olivaceus NRRL B-3009, catalyses the re-arrangement of a highly conserved aspartate to l-isosapartate residue (Fig. 1e).

Here we report a structural and biosynthetic investigation of a macrocyclic peptide that does not fall into any of the known categories of RPPs (Fig. 2a). It is produced by the soil isolate, Streptomyces sp. RK44 and bears an unprecedented chemical motif, bis-thioether crosslink (methyl-ami-no-bithionin (Mabi)), and a rare β-enamine acid ((Z)-3-amino-acrylic acid (Aaa)) or Δ^3-PAla residues, together with motifs that are observed in other classes of RPPs, such as β-Ala, N, N-dimethylated N-terminal lLe residues, Dha and Dhb residues (Fig. 2b). The identity of the minimal BGC (kin) coding the production of 7 is confirmed through heterologous expression and gene inactivation. Biosynthetic investigations indicate that the formation of dehydrasoroinoic acid, the featured β-enaminic acid and Mabi bicyclic residues relies on four enzymes: the Ser/Thr kinase KinE, the lysase KinC, the kinase homologue KinH and the HFCD-like KinI, all of which share low homologues with other enzymes in known RIPP biosynthetic pathways. The enzyme partner, KinD and KinC, are responsible for the formation of all dehydrasoroinoic acid residues in 7 in a likely processive mode starting from the N-terminal of the CP. Subsequent oxidative decarboxylation and cyclization on the resulting dehydrated CP decorate the bis-thioether ring system in the coordinated action of KinI and KinH (Fig. 2c). Furthermore, site-directed mutagenesis in the CP allows the identiﬁcation of key residues that play crucial roles on the PTM processes in the biosynthesis of 7. Finally, we provide evidence of the occurrence of two kintamin-like peptides among the kin-like BGCs in actinobacteria, suggesting a previously unnotiﬁed group of RPPs. Taken together, these results demonstrate that the recruitment of specialised classes of enzymes and CPs to biosynthetic clusters has likely increased the diversity of RIPP structures.

Results

Identification of kintamin 7 from Streptomyces sp RK44

Streptomyces sp. RK44 is a recent isolate from a soil sample collected at the Kintampo waterfall in the Bono East of Ghana in 2014. Metabolite proﬁling of the strain revealed the presence of a high molecular weight metabolite (2507 Da) in its natural product proﬁle under laboratory culture conditions. Large-scale fermentation (10 L), followed by chemical workup allowed the isolation of pure compound 7 (3 mg).

High-resolution electrospray ionisation mass spectrometry (HR-ESIMS) analysis gave a [M + 3H]^3+ ion at m/z of 836.7524, indicating a neutral monoisotopic mass of 2789 Da (Supplementary Fig. 1). Inspection of ^1H-NMR indicated the presence of numerous overlapping α proton signals of amino acids (δ\textsubscript{H} 3.4–4.6 ppm), suggesting that 7 is a peptidic natural product (Supplementary Fig. 2). Product 7 displays a pair of interesting ^1H-NMR signals (5.44 and 7.20 ppm, 8.4 Hz J-coupling) for a peptidyl natural product, suggesting the presence of a moiety containing a cis-configured alkene (Fig. 2b and Supplementary Fig. 2). Interpretation of 1- and 2D NMR spectra enabled identiﬁcation of the major fragment (19 mers) of the peptide (Supplementary Figs. 3–7). Along with different proteinogenic amino acids, modiﬁed residues such as dehydroalanine (Dha) and dehydrobutyrine (Dhb) were found in 7 (Supplementary Fig. S8–11). The unit containing cis-alkene was identiﬁed as an unsaturated β-enaminic acid, (Z)-3-amino-acrylic acid (Aaa), which to the best of our knowledge, has not been previously reported in any other RPPs (Supplementary Figs. 12–14). The sequence tag was also conﬁrmed through assignment of de novo analysis of MS\textsuperscript{3} spectra using tandem MS fragmentation and the sequential fragment ions (Supplementary Fig. 15 and Supplementary Table 1). Almost all of the mass shifts generated can be substituted with proteinogenic amino acids except mass shifts of 69 and 83 Da which were assigned to the non-proteinogenic amino acids, Dha and Dhb. It is noteworthy that Aaa (Δ^3-PAla), presumably a rearranged product of dehydrated serine, has the same molecular weight as Dha by MS analysis. Collectively, the NMR data combined with MS analysis allowed us to connect the major fragments of 7 in sequence although the N and C termini remained ambiguous.

Kintamin 7 featuring unusual chemical moieties

A genome mining strategy was then used to reﬁne the structure of 7. A BLAST search in the annotated RK44 genome in RAST servers using the sequence tag as a probe led to the identiﬁcation of a 174 bp open reading frame (orf) encoding the precursor peptide, KinA (Supplementary Fig. 16). With the amino acid (AA) sequence in hands, the molecular formula of 7 was established as C11H15N3O2S2 based on the HR-ESIMS analysis (observed [M + 3H]^3+ = 836.7524, calculated [M + 3H]^3+ = 836.7520, Δ = 0.518 ppm) (Supplementary Fig. 1). We then revisited the NMR spectra including the ^1H, ^13COSY, TOCSY, HSQC, HMBC and NOESY (Supplementary Figs. 17–27 and Supplementary Table 2). An N, N-dimethyl isouleucine was found to be present at the N-terminus of 7, a typical chemical feature of lexapeptide 3, cacaoidin 4 and linaridin family 14. Unfortunately, fragmentation within the C-terminal structure was not observed in MS\textsuperscript{2} and the ^31P ion was resistant to fragmentation in MS\textsuperscript{3} experiments. Therefore, HRMS and
isotope fine structure analysis was used to confirm the elemental formula of the \( y_6 \) ion as \([C_{26}H_{34}N_7O_8S_1]^+\) (Fig. 3b–d). However, inspection of the NMR spectra together with the sequence of the precursor peptide allowed us to locate an unprecedented ring system composed of a bis-thioether crosslink, MAbi, at the C-terminus of \( 7 \) (Supplementary Figs. 28–30 and Supplementary Table 2). This crosslink (MAbi) is unique in natural products and further highlights the unique structure of \( 7 \). Overall, the combination of these unusual chemical and structural features makes \( 7 \) unique among peptides in related families.

The primary structure of \( 7 \) (Fig. 3a and Supplementary Fig. 31) confirmed the dehydration of genetically encoded Thr-2, 3, 4, 6, 20, 22, and Ser 8. Subsequent decarboxylated Cys-27 at the C-terminal yields the reactive thioenol, followed by cyclization with Cys-11 and Dhb-22 to generate the bis-thioether crosslink. The Aaa-7 residue in \( 7 \) is derived from the rearranged dehydration of Ser-7. At the same time, it was shown that three Ser residues (Ser-13, 16 and 18) in the genomic sequence were present in the final structure as Ala residues. It is likely that these \( \epsilon \)-Ser residues are converted to \( \delta \)-Ala. Such a biochemical
precedent has been found in a few RPPs, suggesting that l-Ser undergoes dehydration, followed by subsequent reduction. To confirm those conversions and determine the absolute configurations for all the amino acids in 7, we performed advanced Marfey’s analysis (Supplementary Fig. 32 and Supplementary Table 3). The unmodified amino acid residues are t-configured, except the Cys-11 and Ala residues. Both l- and d-Ala are present in 7 and the relative peak area suggested a molecular ratio of two l-Ala’s to three d-Ala’s. This is consistent with the formation of d-Ala stereoisomer in 7 exclusively from the three genetically encoded serine residues (Supplementary Fig. 33).

The unusual fused macro cyclic MAbi ring system in 7 marks a structural motif for cyclic peptides with four uncharacterised chiral centres, Cys-11, α- carbon at aminooether 1,2 di thiol-27 (AED-27), and α- and β- carbons at Abu-22 (Fig. 3a). Under the reduced conditions using the previously established method, the MAbi residue remains intact, a similar observation also found that the Lan ring in cacaooid 4 resists reduction. Attempts to identify the derivatives of the crosslinked MAbi residue by advanced Marfey’s analysis were unsuccessful, preventing further analysis of using authentic samples to compare any possible synthetic mimics. One possible explanation is that the MAbi ring was degraded to as-yet-unknown fragments under the harsh conditions of Marfey’s derivatization.

Interestingly, interpretation of NOE correlations led identification of two unusual long-distance correlations from Aaa-7 (CH) to Abu(S2)-22 (Me) and from Leu-14 (Hβ) to AED-27 (Ha) with estimated distances of 1.8 – 5.0 Å based on previous reports (Fig. 3a, Supplementary Fig. 34–36). These long-distance NOE observations only arise from the correct configuration of 7, which could provide an experimental dataset in comparison with calculated diastereomers generated from computational modelling approaches that have been used in other structurally complex peptides. However, due to the high content of modified residues in these systems it is difficult to obtain accurate force fields and so less accurate potentials must be used. As such, we have employed electronic structure calculations at the density functional tight-binding level which avoid the need for force field parameter sets suitable for the compound under study. Distances between atom pairs identified by NMR NOESY correlations (Supplementary Fig. 34–36) were monitored during Ab-initio molecular dynamics (AIMD) simulations using the GFN2-xTB-MD method. Multiple structures containing different combinations of either the R or S configurations at the chiral carbons in the vicinity of the bis-thioether linkage were simulated. Of the structures of candidate isomers studied, three displayed one long-range monitor (Leu-14 (Hβ) to AED-27 (Ha)) satisfying the NOE-predicted distance range of 4–5 Å.
(Supplementary Table 4). Only one candidate (sterogenic centres: 11R, 22αS, 22βS, and 27S) satisfied both long-range monitors (Leu-14 (Hβ) to AED-27 (Hα) and Aaa-7 (CH) to Abu(S2)-22 (Me)), suggesting that this isomer is the only structure capable of generating the observed NOE signals (Fig. 3a, Supplementary Table 5, Supplementary Fig. 37).

Overall, 7 displays unusual chemical features which we named kintamdin. This name is associated with the Kintampo waterfall, the location where *Streptomyces* sp. RK44 was originally isolated. When tested for biological activity, 7 displayed an interesting level of cytotoxicity against cell lines for skin cancer (IC50 value of 2.4 ± 0.1 μM), and breast cancer (IC50 value of 0.6 ± 0.1 μM), when compared to the control molecule, staurosporine (Supplementary Table 6). However, 7 showed weak or no inhibitory activity against a range of bacterial strains (Supplementary Table 6), indicating that 7 possesses a considerable degree of biological selectivity.

The minimal BGC of 7

The structure of 7 motivated us to probe its biosynthetic origin in the producing strain, *Streptomyces* sp. RK44. Analysis of the surrounding genetic environment of *kinA* allowed identification of a candidate gene cluster (*kin*) (Fig. 4a and Supplementary Table 7). To validate the identity of the BGC, we carried out a TAR cloning strategy for heterogeneous expression. To this end, we modified the construction method of pathway-specific capture vectors in order to improve the capture efficiency of the BGC of interest from the genomic DNA of *Streptomyces* sp. RK44 as shown in Supplementary Fig. 38. One out of five clones after yeast transformation was identified to contain the correct length of the BGC in the construct. The construct pCAP03-kin2 was then transferred into various *Streptomyces* hosts via *E. coli-Streptomyces* conjugation. The production of 7 in *Streptomyces coelicolor* strain MI1523 was confirmed through HRMS analysis as well as MS2 fragmentation by comparing with an authentic peptide 7 reference (Fig. 4c, i–ii and xi and Supplementary Figs. 39–40).

In order to determine the boundary of the 7 BGC, a series of gene inactivation were carried out on pCAP03-kin2, followed by *E. coli-streptomyces* conjugation and fermentation. HRMS analyses of the extracts of these variants demonstrated that gene inactivation of orf(1) and orf(2) as well orf(7) at the boundaries of the cloned DNA fragment showed no perturbation to the production of 7 (Supplementary Fig. 41). Therefore, the minimal BGC directing the biosynthesis of 7 includes fifteen orfs, some of which the functions were assigned (Fig. 4a and Supplementary Table 7).

To assess the in vivo roles of these genes, we generated eight variants (Δ*kinC*, *H* and *O*) (Fig. 4c, traces iii–xi). Gene inactivation of *ΔkinE* resulted in significantly reduced production of 7 whilst knocking out *kinF* caused moderate reduction of the 7 production (Fig. 4c (iv–vi)), suggesting the presence of possible synergetic function between *KinE* and *KinF*, two putative metallopeptidases, to ensure the efficacy of the proteolytic activities, similar to proposed functions in the biosynthesis of ruminococcin C25.

The production of 7 was abolished in six other variants (Δ*kinC*, *D* and *H*–*O*), suggesting that these genes are essential for the 7 biosynthesis (Fig. 4c (iii, iv and viii–xii)). No intermediates were accumulated in the four variants of Δ*kinC*, Δ*kinD*, Δ*kinH* and Δ*kinI* (Fig. 4c (iii–iv, vii and x)). *KinI* is a putative F420H2-dependent reductase that may be responsible for the formation of α-Ala-E3 and -I8 in 7, as previously described for LanB214.9.15 and LxmB enzymes. *KinC* was initially annotated as a hypothetical protein with the length of 323 AA residues. However, structural modelling in Phyre2 Server17 suggested the C-terminus of *KinC* (143-229 AA residues) belongs to HopA1 superfamily including phosphothreonine lyases from various Gram-negative pathogens, such as OsPf from *Shigella flexneri*18, which irreversibly removes phosphate groups from phosphothreonine in the activation loop of mitogen-activated protein kinases in the host cells during the pathogen infections. HopA1-like proteins have been proposed to be responsible for the formation of Dha and Dhb residues in various RIPPs, such as class V lantipeptides29,30-32 and lantadins9. Blast search in NCBI suggested that both *KinD* and *KinH* are putative phosphotransferases, of which only part of *KinD* sequences (46-92 AA) show low homology (<34% AA identities) with *KinH*. Unlike *KinD*, which is likely to be an active kinase progressing the phosphorylation on Ser/Thr residues, *KinH* appears to be an inactive phosphotransferase homologue given the absence of the conserved residues in its sequence (Supplementary Fig. 42). Interestingly, recent biochemical investigation demonstrated that the participation of the inactive phosphotyrosyl phosphate homologue, TvaF5,57, is necessary in the formation of AviCys residue of *TVA* 2. Through protein-protein interaction, TvaF5,57 coordinates with TvaF5,57, a HFCD-like decarboxylase, to form a minimum AviCys synthetase complex for effective AviCys formation39-42. Similar to this case, it is likely that *KinH* may coordinate with *Kin* for decarboxylation on the C-terminal Cys-27 and subsequent cyclization to provide the bis-thioether ring system. However, detailed sequence analysis suggests that, while *KinL* displays low homologues (33% AA identity) with TvaF5,57, *KinC*, *KinD* and *KinH* share no significant homologues with the corresponding enzymes in the *TVA* pathway (Supplementary Table 8). Sequence similarity network (SSN) using the tools of the Enzyme Function Initiative (EFI) with *KinC* and *KinH* as query also suggested that both proteins form separate clusters with the corresponding enzymes in other RIPPs ( Supplementary Fig. 43). This may not be surprising because both *KinCD* and *KinH* are likely to catalyse different biotransformations, compared to other known RIPPs, to provide the unusual structural elements in kintamdin, such as Aaa and bis-thioether macrocycle residues.

Gene inactivation of *kinO*, encoding a putative methyltransferase, resulted in accumulation of a new metabolite 8 in the culture of the Δ*kinO* mutant (Fig. 4c (xii)). MS and MS2 fragmentation analysis demonstrated that 8 is the non-methylated 7 (Supplementary Fig. 44). Knocking out *kinI*, encoding a flavin-dependent decarboxylase, resulted in accumulation of another new metabolite 9 in the culture of Δ*kinI* variant (Fig. 4c (ix)). 9 is the N,N-dimethylated 27-mer linear peptide containing the intact cysteine residue at the C-terminus and eight dehydrated amino acid residues (1 × Dha, 1 × Aaa and 6 × Dhb) as well as three Ala residues derived from Dha, as evidenced in MS and tandem MS analysis ( Supplementary Fig. 45), suggesting that *KinI* may catalyse the decarboxylation on the C-terminal Cys-27 residue. To this end, overexpression of *kinO* and *kinI* in *E. coli* allowed purification of the recombinant proteins to near homogeneity, as observed in SDS Page analysis (Supplementary Fig. 46). An in vitro assay of a recombinant His6-*KinI* with 8 in the presence of S-adenosyl-L-methionine (SAM, 1 mM) was performed. The production of 7 was confirmed by LC-MS analysis using the authentic sample as comparison (Supplementary Fig. 47), confirming that *KinO* is responsible for the N-terminal Ile dimethylation. An assay of incubating His6-*KinI* with 9 and other necessary cofactors was performed but no new product was observed, suggesting that 9 is not the bona fide substrate of *KinI*.

*KinC* and *KinD* catalyse the formation of dehydroamino acid residues in 7

Combined with our heterologous expression, gene disruption and bioinformatic analysis, it was speculated that *KinD* and *KinC* may act as a kinase and a phosphoSer/Thr lyase, respectively, on the CP of *KinA*, to provide dehydroamino acid and Aaa-7 residues in the early stage of the 7 biosynthesis. To this end, attempts to overexpress recombinant proteins, *KinC* and *KinD*, as well as fusion proteins of *KinC* and *KinD* in *E. coli* were carried out. Unfortunately, no detectable traces of soluble recombinant proteins with or without the inclusion of chaperon proteins were observed, preventing further in vitro assays.
We developed two co-expression systems of KinAD and KinACD with attempts to produce SUMO-fused modified linear precursor peptides, His6-SUMO-KinA(D) and His6-SUMO-KinA(CD), respectively. After purification of the recombinant proteins of interest via immobilised metal affinity chromatography (IMAC), we then adapted the Gluc endoprotease cleavage method to release the leader peptide (LP) and SUMO tag for our MS analysis (Fig. 5a). Indeed, UPLC-QTOF-MS analysis of the Gluc-treated product from co-expression of KinAD gave two dominant ions species with m/z values of 730.3133 ([M + 2H]2+) and 907.9186 ([M + 2H]2+), respectively, corresponding to two fragments of the CP. The first fragment has a +80 Da increase, an addition of one phosphate group, compared to the predicted corresponding fragment of unmodified peptide from Ser(-5) to Glu-9 (Fig. 5b). Detailed MS2 fragmentation analysis revealed that the phosphate group is located at Thr-2 residue of the CP (Supplementary Fig. 48). The second fragment corresponds to the unmodified 18-mer CP from Ile-10 to Cys-27 that was hydrolysed between Ser-13 and Leu-14 residues, probably due to unspecific hydrolysis, and two cysteine residues (Cys-11 and Cys-27) are oxidised to form a disulphide bridge as evidenced in MS and MS2 fragmentation analyses (Supplementary Fig. 49). Therefore, the corresponding intact compound is likely to be phosphokintamdin, strongly indicating that KinD catalyses the phosphorylation reaction on one amino acid residue in a consecutive fashion at a time starting from the N-terminal Thr-2 residue.

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**Fig. 4** | Identification of the minimal BGC of 7 through bioinformatics analysis, heterologous expression and genetic inactivation experiments. a The minimal BGC of 7 with colour-coded individual genes of proposed functions. b Extracted ion chromatogram (EIC) traces of the ions corresponding to peptide 7 and the biosynthetic intermediates, 8 and 9, in different Streptomyces coelicolor M1152 variants. (i) a variant harbouring an empty plasmid, pCAP03; (ii) a variant harbouring the construct with the kin BGC; (iii) A knockout variant ΔkinC; (iv) A knockout variant ΔkinD; (v) A knockout variant ΔkinE; (vi) A knockout variant ΔkinF; (vii) A knockout variant ΔkinH; (ix) A knockout variant ΔkinJ; (x) A knockout variant ΔkinO; (vi) and (xi). The isolated 7 as the standard.
The presence of KinI and KinH is necessary for MAbi moiety

Nature Communications | (2022) 13:5044

in vitro assays. We then used co-expression systems of kinACDH, kinACDI, and kinACDHI to investigate the roles of KinI and KinI. MS analysis of the Gluc-treated His6-SUMO-KinA(CD) from the corresponding co-expression system only gave the ion which is identical to 11 (Supplementary Fig. 51), the sequence of which was confirmed by tandem MS analysis (Supplementary Fig. 51). We also expressed kinACD with kin in E. coli, to examine the activity of KinI for Cys-27 oxidative decarboxylation. Analysing the resulting product profile in MS and tandem MS spectra again revealed the only presence of the ion corresponding to 11 (Supplementary Fig. 52), suggesting that, although the presence of the leader peptide, KinI alone is not sufficient for the oxidative decarboxylation and subsequent cyclization. No thionol or its derivatives, such as aldehyde, alcohol and thiol species, were observed in our case, different to the observations in previous studies of thioamides. Finally, addition of kinH into the kinCDI E. coli co-expression system was performed. Analysing the product profile indicated the presence of an ion with an \( m/z \) value of 1458.1789 (\([M + 2H]^2+\)) (Fig. 5d). Tandem MS analysis indicated that the corresponding peptide 12 shared the same sequence as kindamidin 7 except the presence of three Dha residues at positions 13, 16 and 18 in 12, strongly indicating the
presence of MAbi (Supplementary Fig. 53). Clearly, the incorporation of the kinase homologue KinH and the HFCD-like decarboxylase Kinl together with KinCD facilitates the MAbi formation, suggesting a coordinated action of KinH and Kinl. A similar observation was also reported in the recent investigation of AviCys formation in the TVA biosynthesis that the AviCys formation is dependent on the coordination of the kinase homologue TvaE87 and the decarboxylase TvaF87.

Site-directed mutagenesis to determine key residues in the CP

Interestingly, the N-terminal of the CP is enriched with four Thr (Thr-2, 3, 4 and 6) residues that are converted into corresponding Dha moieties. It is known that peptides containing dehydroamino acid residues tend to be 2.0-ß-helix, meaning that the helix is 2 residues per turn and stabilised by H-bonds encompassing 5-membered pseudo-cycles between NH and the carbonyl of the same amino acid residue (Supplementary Fig. 54). As a result, dehydrated amino acid residues stretches are arrange in an extended conformation. It was hypothesised that individual Dha residue or the synergetic impact of these four Dha residues in the CP could affect the catalytic activity of Kin and/or KinC to provide the Aaa-7 formation. To probe this, a series of alanine scanning mutagenesis was performed, generating six different His6-SUMO-KinA variants, KinAT2A, KinAT3A, KinAT4A, KinAT6A, KinAS8A, as well as KinAT3A in which the corresponding peptide has a +14 Da increase compared to the corresponding ion of MAbi. To confirm this, we changed Cys-11 to Ala to provide the variant, His6-SUMO-KinAC11A. To our surprise, no ions corresponding to either intact linear dehydrated or cyclic peptides were found. Analysing the resulting product profile, however, revealed two KinAC11A related peptide fragments. The first one was characterised to be the Ala-11 containing mutated C-terminal fragment (Ile-10 to Cys-27 with 5 dehydroamino acid residues (Dha-13, 16, 18 and Dhb-20, 22)), which was believed to arise from the Gluc-cleavage between Glu-9 and Ile-10 of the corresponding dehydrated product (Supplementary Fig. 64). A similar result was observed in our kinetic gene co-expression system. The second one was the N-terminal fragment (Ser-1 to Dhb-6-NH₂ with 4 dehydroamino acid residues (Dhb-2, 3, 4, 6)) (Supplementary Fig. 64), suggesting that the resulting N-terminal fragment (Ser-1 to Glu-9) after Gluc treatment was unstable and tends to undergo spontaneous cleavage between Dhb-6-CONH₂ and the cis-alkene moiety (presumably due to immediate cleavages during the MS analysis). It is likely that substitution of Cys-11 to Ala allowed Gluc to access the cleavage site between Glu-9 and Ile-9 even though the CP was modified. Clearly, the intact PTM-processed peptide from this variant is the fully dehydrated peptide (Fig. 6d).

Interestingly, no peptidyl fragment containing AviMeCys was found, suggesting that no decarboxylation on Cys-27 occurred in the KinAC11A variant, even in the presence of both KinH and Kinl.

Taken together, the above analyses strongly indicated that the synergetic presence of the enriched dehydroamino acid residues in the N-terminal of CP plays an important role in the formation of Aaa-7 residue and KinCD also has capacity of converting Thr residue into methyl-Aaa residue. Changing Cys-11 to Ala completely abolished the cyclization but not the KinCD-catalysed dehydration processing, strongly indicating that Cys-11 is key to the coordinated action by KinH and Kinl.

Prevalence of the kin cluster

The structure of C and the unique sequences of KinA led us to further address the occurrence of its homologues in bacterial genomes. Searches with KinA as a query in the NCBI database provided the occurrence of its homologues in actinobacteria. Comparisons among these sequences revealed the occurrence of kin-like orfs which encode AA sequences with the conserved signature motif of S-S/T-X-X-C-X₂₇-S-S/T-X-X-C (5'-S-S/CII-T22-C27 in the of 7) (Supplementary Fig. 65). Similar to the CP of KinA, these putative precursor peptides contain enriched Thr residues (3–5) in the corresponding CPs.

The occurrence of the kin-like orfs always coincides with the presence, in close proximity, of genes with homology to seven genes in the kin BGC, namely kinC-T and H1 in Streptomyces, and rare actinomycetes (i.e. Actinopluspora, Nocardioides, thermophilic Microbispora) (Supplementary Fig. 66). While some of these identified BGCs encode putative methyltransferases, others lack the encoded methyltransferases, suggesting the presence of demethylated kintamdin-like RIPPs. One of these BGCs, encoding two putative precursor peptides, was identified in Streptomyces kurzsanovii NCIMB21278 available in our laboratory (Supplementary Fig. 67). Screening of S. kurzsanovii in various culture broth conditions resulted in the identification of two ions with m/z values of 1025.1783 and 1009.5079 ([M + 3H]+) as observed in our MS analysis, respectively. The sequences of these peptides confirmed by MS² fragmentation analysis were correlated to two predicted ones (Supplementary Figs. 68 and 69, respectively), strongly suggesting the presence of two demethylated kintamdin-like peptides.

Given invariant co-occurrence of homologous genes of kinC, kinD, kinH and kinI, as well as structural uniqueness among RIPPs, we propose that kintamdin 7 is the founding member of a previously undescribed family of RIPPs, which we named the ‘ß-bithionins’.

Discussion

RIPPs are a group of structurally complex naturally occurring metabolites. Although exploring only 20 proteinogenic amino acids, their biosynthetic pathways recruit many PTM enzymes to install diversified structural features in RIPPs including Lan/Lab/Avi cyclic systems that are unique in RIPPs. Kintamdin 7 contains an MAbi crosslink motif.
**Fig. 6** | Schematic representation of results from amino-acid substitution experiments in the CP of KinA, designed to probe the key residues in the biosynthesis of 7. 

**a** Individual amino-acid substitution of Thr-2 or -3 or -4 or -6→Ala and Ser-8→Ala had no impact on the dehydration reaction. 

**b** Mutation of all of the first four Thr residues (Thr-2, 3, 4 and 6) to Ala partially impede Aaa formation. In some cases, Ser-7 residues were retained. 

**c** Substitution of Ser-7→Thr resulted in identification of fully dehydrated peptide containing methyl-Aaa-7 (mAaa) residue. 

**d** Substitution of Cys-11→Ala only resulted in the fully dehydrated peptide. No cyclization occurred. The AA residues subjected to site directed mutagenesis were highlighted in light green colour. Dha: light blue; Dhb: deep green; Aaa: blue.
which, to our knowledge, is unique in natural products. Furthermore, an unusual β-amino acid, (Z)-3-amino-acrylic acid (Aaa(ΔβAla)), was identified at the N-terminal of 7. β-Amino acid residues were largely thought to be a hallmark of NRP synthesis. Recent biochemical characterisations, however, indicated that β-amino acid residues do occur on peptides synthesized by ribosome11,12. The Aaa (ΔβAla) residue is rarely in the natural product inventory. Only one cyclopeptide metabolite containing the Aaa residue has been discovered from a marine gut fungus, Aspergillus flavigus, from Litia gimagea thus far.26 However, the bio-origin of this cyclopeptide has remained elusive.

Combining structural elucidation, bioinformatics, genetic and biochemical analyses, a biosynthetic pathway of kintamidin 7 is proposed as shown in Fig. 7. KinCD is responsible for Ser/Thr dehydration to install dehydroamino acid moieties in 7. It is highly likely that the enzyme partner of KinD and KinC is processive in the phosphorylation and elimination of one amino acid residue at a time, starting from N-terminus of the CP (Fig. 7). This is not the case for the TVA biosynthesis where the phosphorylation and elimination are likely to initiate from the C-terminal Thr residue in the corresponding CP.31,32

After processing the dehydration on the first four Thr residues in the CP of KinA, the modified CP is likely to be further extended. Such synergetic conformation changes are likely to play an important role of KinD-catalysed phosphorylation (Fig. 7). Changing all of these four Thr residues to Ala resulted in less phosphorylation efficiency on Ser-7 residue in the proceeding peptide as observed in our mutagenesis experiments, leading to accumulation of the partially dehydrated peptide (Fig. 6c). The stretch proceeding CP is also likely to affect KinC activity, leading to the abstraction of the NH hydrogen of the amide between Dhb-6 and phosphoSer-7 instead of the phosphoSer-7 Co-H, followed by a nucleophilic attack at the β-carbon of phosphoSer-7 to yield an aziridine intermediate (Fig. 7a). Future structural biology of KinC and KinD will shed light on the underlying mechanisms of KinD-catalysed phosphorylation and KinC-mediated elimination and rearrangement. Interestingly, such activation and aziridination are akin to the Mitsunobu-like reaction used for the conversion of α-Thr residue in peptides into an aziridine motif. In this reaction, triphenylphosphine (Ph3P) combines with diethylazodicarboxylate to form oxygenphosphonium intermediate at the β-oxygen of α-Thr, which undergoes an intramolecular cyclization attacked by the deprotonated amide-NH group to generate an aziridine ring (Supplementary Fig. 7k,l,m). Subsequently, KinC could further catalyse a ring opening on the aziridine-containing intermediate to provide Aaa residue. A similar ring-opening transformation was also found in the conversion of a strained tricyclic aziridino compound into a β-enamino acid under basic conditions.27 When Ser-7 was mutated to Thr, KinCD was able to proceed this residue to produce methyl-Ala, which is comparable to the chemical activation and aziridination on the Thr residue.32 (Supplementary Fig. 7j).

After six more rounds of dehydration reactions to provide the fully dehydrated peptide, the kinase homologue KinH coordinates with the HFCD-like decarboxylase KinI to catalyse Cys oxidative decarboxylation and subsequent Michael-type conjugate additions among the resultant thioenol moiety, Dha-22 and Cys-11 to give the MAbi ring system (Fig. 7). The presence of KinH is crucial for the decarboxylation activity of KinI. No compounds containing thioenol moiety or its derivatives were observed in our co-expression system of kinIACDI where kinH was absent. However, when kinH was added into this aforementioned co-expression system, the MAbi moiety was formed in the resulting peptide. Furthermore, the presence of Cys-11 residue in KinA is key to the biosynthesis of 7. Changing Cys-11 to Ala appeared to have detrimental effects on the activities of KinH. No cyclic peptide was observed (Fig. 6d).

KinJ, a putative F420H2-dependent reductase, could catalyse three stereospecific reductions on the Dha-13, -16 and -18 residues of the resultant cyclic peptide to yield three D-Ala moieties (Fig. 7). No apparent intermediate was observed in the culture broth of the ΔkinI variant, suggesting the instability of the cyclic peptide in the cells of Streptomyces heterologous system. However, once D-Ala-13, -16 and -18 residues are formed, the resulting modified precursor peptide containing D-Ala residues could confer resistance to cellular protease hydrolysis, similar to the observation in the lantibiotic lacticin 3147.30 After the byplic modified KinI is formed, the leader peptide can be removed by KinEF peptides to provide 8 (Fig. 4b (ii)), followed by KinO catalyses N-terminal dimethylation to generate the mature kintamidin 7 (Fig. 7). The proteases, KinE and KinF, the reductase KinJ and the methyltransferase KinO display considerable substrate promiscuity. When kinI was inactivated to provide the ΔkinI variant, three Dha residues (Dha-13, 16 and 18) in the linear dehydrated precursor peptide could be further reduced to the corresponding α-Ala residues. Subsequent removal of the leader peptide and dimethylation provide the shunt intermediate 9 (Fig. 4b (ii)).

In conclusion, we have discovered and characterised kintamidin 7, a RiPP containing unusual chemical features through a combination of chemical workup, tandem MS analysis and structural elucidation assisted by computational modelling studies. Natural product 7 contains the rare β-amino acid, (Z)-3-amino-acrylic acid (Aaa or ΔβAla), and an unprecedented β-thioether macroyclic crosslink (Mabi) motif. The minimal Kin BGC of 7 contains a unique set of biosynthetic enzymes, the kinase KinD, the lyase KinC, the HFCD-like decarboxylase KinI and the kinase homologue KinH, that share low homologues with known enzymes in other RiPP pathways. Gene co-expression in E. coli indicated that KinCD enzymes are responsible for introducing dehydroamino acid residues including Aaa-7 residue, and KinI together with KinH catalyses oxidative decarboxylation on the C-terminal Cys-27 and subsequent cyclization to provide the β-thioether Mabi formation. Site directed mutagenesis on kinA demonstrated that the synergetic impact of the first four Thr residues (Thr-2, 3, 4 and 6) in the CP play crucial roles on the formation of Aaa residue. Mutation on Cys-II to Ala in the CP completely abolished the decarboxylic cyclization reaction. Conserved genomic analysis also allowed identification of the occurrence of two kintamdin-like peptides among the Kin-like BGCs, which appears to be widespread in actinobacteria.

Methods

General chemicals, reagents and analytical methods

1H-NMR spectra were obtained on a Bruker AVANCE III HD 400 MHz (Ascend T 9.4 Tesla, UK) with Prodigy TCTM cryoprobe at 298 K in CD3OD and DMSO-d6 (Goss Scientific, Massachusetts, MA, USA). Chemical shifts are reported in parts per million (ppm), relative to the solvent signals. 13C NMR spectra were obtained with proton decoupling on the same NMR spectrometer and are reported in ppm with TMS for internal standard. Multiplicity is defined as: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet, br = broad, or combinations of the above. Coupling constants (J) are reported in Hertz. High-resolution mass spectra (HRMS) for chemical workup and molecular networking were obtained on either a 12 T Solarix 2XR FT-ICR MS (Bruker Daltonics) via direct infusion or an LTQ Orbitrap Thermo Scientific MS system coupled to a Thermo Instrument HPLC system (Accela PDA detector, Accela PDA autosampler, and Accela pump). The injected samples were chromatographically separated by a C18 (Sunfire 150 × 46 mm) column. The gradient elution for separation was CH3CN/H2O with 0.1% trifluoroacetic acid (TFA) (from 0 to 100% for 30 min, flow rate, 1.0 mL/min, UV detection max 340 nm).

Structure elucidation of 7

Detail analysis of 1H, 13C, COSY, gHSQC, HSQC-TOCSY, gHMBC and ROESY were collected using Bruker Topspin 3 and processed using Mestrenova 12.0 for elucidating the structure of kintamidin (Supplementary Figs. 1–7). 1H spectra indicated the usual fingerprint of peptides exhibiting multiple exchangeable protons (6, 7.6–10.4 ppm),
numerous overlapping α protons of amino acids (δ_H 3.4–4.6 ppm), various alkyls (δ_H 0.8–3.3 ppm), aromatic (δ_H 6.9–7.6 ppm) sidechains. Additionally, there were nine olefinic protons (δ_H 5.3–7.2 ppm) and two chemically equivalent protons (δ_H 2.89 ppm) assigned to a N,N-dimethyl group found in the spectrum of 7. Analysis of COSY, HSQC and HSQC-TOCSY spectra enabled the assignment of various proteinogenic amino acids such as Trp (×1), Val (×3), Ile (×1), Glu (×1), Asp (×1), Gly (×1), Ser (×1), Ala (×5). Also observed were 7 dehydrated amino acids (DHAAs) such as Dha (×1), and Dhb (×5). It also accounted for one MAbi linkage. Structural configurations of Dhb at positions 2, 3, 4, 6, 7, 8, 9.

**Fig. 7 | A biosynthetic model of kintamin 7 with the proposed formation of Aaa-7 (Δ^2ZβAla) (a) and MAbi moieties.** The modified amino-acid residues by PTMs are colour coded. Aaa-7: blue, Dhb: green, Dha: light blue, D-Ala: orange, MAbi: black. LP leader peptide, CP core peptide.

in vivo ΔkinO variant
and 20 have been deduced as Z based on observed NOE correlation between the Me(y) group and its corresponding NH (Supplementary Fig. 2).

FT-ICR mass spectrometry
Peptide samples were ionized by electrospray (nESI) at a typical concentration of 5 μM. MS analyses were performed on a Solarix FT-ICR instrument equipped with an Infinity ICR cell and a 12 T magnet (Bruker Daltonics, Bremen, Germany). For intact mass analysis, spectra were acquired between m/z 500 and 5000, to yield a broadband 1 or 2 MW time-domain transient. Ion accumulation was set to between 50 and 200 ms, and typically each spectrum was the sum of 50 acquisitions.

Peptide charge state were selected for MS/MS and isolated using CASI (continuous accumulation of selected ions) with a quadrupole width of 2–5 m/z. Fragmentation was performed using both collision-induced dissociation in the collision cell and electron capture dissociation (ECD) using a heated hollow dispenser cathode in the ICR cell. For ECD, typical cathode conditions were bias voltage 1.5 V, lens voltage 15 V, and a pulse length of between 10 and 30 ms. MS2 spectra were recorded between m/z 300 and 5000 and were the sum of 100 acquisitions.

For CID, typical conditions were positive voltage 15 V, and a pulse length of between 10 and 30 ms. MS2 spectra were acquired between m/z 500 and 5000, to yield a broadband 1 or 2 MW time-domain transient. Pseudo MS3 experiments were performed using Bruker DataAnalysis 5.1 (Bruker Daltonics) and, if required, monoisotopic masses were determined using the sophisticated numerical annotation procedure (SNAP; Bruker Daltonics).

Gene co-expression in E. coli
The genes encoding KinA, KinD, KinC, KinH and KinI were synthesized by Genescript and inserted into E. coli Duet expression vectors to yield pCDFDuet-kinA-kinD, pET-kinC, pETDuet-kinC-kinH and pRSFDuet-kinH. In the case of generating modified fusion proteins, His6-SUMO-KinA, the kinA gene was fused with a small ubiquitin-related modifier (SUMO) gene using In Fusion® HD Cloning kit with primers Sumo_for and Sumo_Arev (Supplementary Table 11). E. coli BL21 (DE3) cells (New England Biolabs, catalogue number: C2527H) harbouring the plasmids or cultured in LB medium (300 mL) containing streptomycin (50 mg/L) or with or without ampicillin (50 mg/L) or kanamycin (50 mg/L) at 37 °C. These co-transformed plasmids include (1) pCDFDuet-80° CW time-domain transient. Ion accumulation was set to between 50 and 200 ms, and typically each spectrum was the sum of 50 acquisitions.

Purification of modified His6-SUMO-KinA and its variants from E. coli
The cell pellets of co-expression cultures were resuspended in buffer A (50 mM Tris-HCl, 0.3 M NaCl, 10 mM imidazole, pH 8.0), followed by lysis with an ultrasonic processor. After centrifugation to remove cell debris in the lysate, (3000 × g, 4 °C for 15 min), the resultant supernatant was filtered through a 0.45 μm filter prior to purification with HIS-Select® Nickel Affinity Gel (Millipore). The resin was washed with 15 mL of buffer A, followed by two times of washing with buffer B (50 mM Tris-HCl, 0.3 M NaCl, 200 mM imidazole, pH 8.0). The recombinant protein was eluted with 10 mL of buffer C containing imidazole (200 mM) and concentrated to 0.5 mL using an Amicon Ultra-15 Centrifugal Filter Unit (Millipore). The purified protein was stored at −80 °C in storage buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% (w/v) glycerol, 1 mM DTT).

Proteolytic cleavage of modified His6-SUMO-KinA or its variants by GluC protease
The modified His6-SUMO-KinAs were diluted to a concentration of 2 mg/mL with 50 mM HEPES buffer (pH 7.5). To the peptide solution (90 μL), GluC protease (Sigma Aldrich) (10 μL, 2 mg/mL) was added. The mixture was incubated for 12–16 h, followed by quenching with trifluoroacetic acid (TFA, 0.1%) prior UPLC-HRMS analysis.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Data supporting the findings of this work are available within the paper and its Supplementary Information files. A reporting summary for this Article is available as a Supplementary Information file. The datasets generated and analysed during the current study are available from the corresponding author upon request. GenBank accession numbers used are reported in Supplementary Tables 7, 8 and 12.

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Author contributions
S.W., S.L., Z.L., K.W. and Y.G. performed genetic, site-directed mutagenesis and in vitro assays. Y.Y. and H.D. performed genome mining and bioinformatic analyses. J.T., Q.F., D.J.C., R.G., H.D. determined chemical structures. B.F.M. performed computational simulations. S.W., S.L., Q.F., B.F.M., J.T., K.K., D.J.C., L.T. and H.D. analysed the data. B.F.M., K.K., J.T., and H.D. wrote the paper.

Competing interests
The authors declare no competing interests.

Additional information
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