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ORIGINAL ARTICLE

Identification of the incednine biosynthetic gene cluster: characterization of novel β-glutamate-β-decarboxylase IdnL3

Makoto Takaishi[1], Fumitaka Kudo[2] and Tadashi Eguchi[1]

A biosynthetic gene cluster for the 24-membered macrolactam antibiotic incednine was identified from the producer strain, *Streptomyces* sp. ML694-90F3. Among the putative incednine biosynthetic enzymes, a novel pyridoxal 5′-phosphate (PLP)-dependent β-glutamate-β-decarboxylase, IdnL3, was functionally characterized *in vitro* by demonstrating its (*S*)-3-aminobutyrate-forming activity with β-glutamate in the presence of PLP. Because (*S*)-3-aminobutyrate is known for the direct precursor of incednine, this enzyme supplies the unique β-amino acid starter unit. The identified gene cluster encodes five characteristic β-amino acid carrying enzymes, consisting of a pathway-specific ATP-dependent ligase, a discrete acyl carrier protein (ACP), β-aminoacyl-ACP β-amino group-protecting ATP-dependent ligase, dipeptidyl-ACP:PKS-loading ACP dipeptidyltransferase and a terminal amino acid peptidase, which are completely conserved in β-amino acid-containing macrolactam biosynthetic gene clusters. Overall, a plausible biosynthetic pathway for incednine was proposed.
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INTRODUCTION

Incednine is a 24-membered macrolactam antibiotic produced by *Streptomyces* sp. ML694-90F3 (Figure 1) and induces apoptosis even in the cells overexpressing antiapoptotic Bcl-xL/Bcl-2 with antitumor agent.[1] Recently, the structurally similar compound silvalactam was isolated from *Streptomyces* strain Tü 6392, and showed potent antiproliferative activity against various cancer lines.[2] Thus, incednine types of macrolactam glycosides are promising lead compounds for novel antitumor drugs. From a biosynthetic viewpoint, incednine is a hybrid molecule, with a β-amino acid and polyketide chain. Previous feeding experiments with stable isotopically labeled acetates and amino acids indicated that the amino acid starter is 3-aminobutyrate, derived from L-glutamate via β-glutamate.[3,4] Thus, it was proposed that L-glutamate is first converted to β-glutamate by glutamate-2,3-aminomutase, which is a member of the radical SAM superfamily of enzymes.[5] Next, β-glutamate appears to be decarboxylated to (*S*)-3-aminobutyrate, although such a β-glutamate-β-decarboxylase has never been recognized in nature.

Biosynthetic gene clusters for structurally related macrolactam antibiotics, including vicenistatin,[6] salinilactam,[7] BE-14106[8] and ML-449[9] (Figure 1), have been recently identified, showing that several conserved enzymes could be involved in common biosynthetic reactions for the construction of the amino acid starter units of polyketide synthases (PKSs). Furthermore, six vicenistatin biosynthetic enzymes were functionally characterized *in vitro*, revealing the involvement of a β-amino acid protection-deprotection strategy to carry a β-aminoacyl-acyl carrier protein (ACP).[10] In vicenistatin biosynthesis, (*2S*,*3S*)-3-methylaspartate is first activated by a unique ATP-dependent ligase and transferred onto a discrete ACP. The generated aminoacyl-ACP is then decarboxylated by a pyridoxal 5′-phosphate (PLP)-dependent enzyme to give 3-aminoisobutyryl-ACP. Interestingly, 3-aminoisobutyryl-ACP is acylated with alanine by another ATP-dependent ligase to afford a dipeptidyl-ACP, presumably to prevent spontaneous lactamization during the polyketide chain elongation process. The dipeptidyl-ACP is then selectively transferred onto a characteristic ACP-loading domain of vicenistatin-PKS. The terminal alanyl moiety is removed by a peptidase prior to macrocyclization by the thioesterase domain of PKS.[11] A similar scenario, except for the decarboxylation step, was presumed to be involved in incednine biosynthesis.

In the present study, we first identified the biosynthetic gene cluster for incednine to obtain the sequences of undiscovered 3-aminobutyrate biosynthetic enzymes, and then characterized a novel β-glutamate-β-decarboxylase, which converts β-glutamate to (*S*)-3-aminobutyrate as a unique β-amino acid starter unit of incednine PKS.

MATERIALS AND METHODS

All experimental details are described in Supplementary Information.

[1]Department of Chemistry and Materials Science, Tokyo Institute of Technology, Meguro-ku, Tokyo, Japan and [2]Department of Chemistry, Tokyo Institute of Technology, Meguro-ku, Tokyo, Japan
Correspondence: Professor T Eguchi, Department of Chemistry and Materials Science, Tokyo Institute of Technology, 2-12-1 O-okayama, Meguro-ku, Tokyo 152-8551, Japan.
E-mail: eguchi@cms.titech.ac.jp
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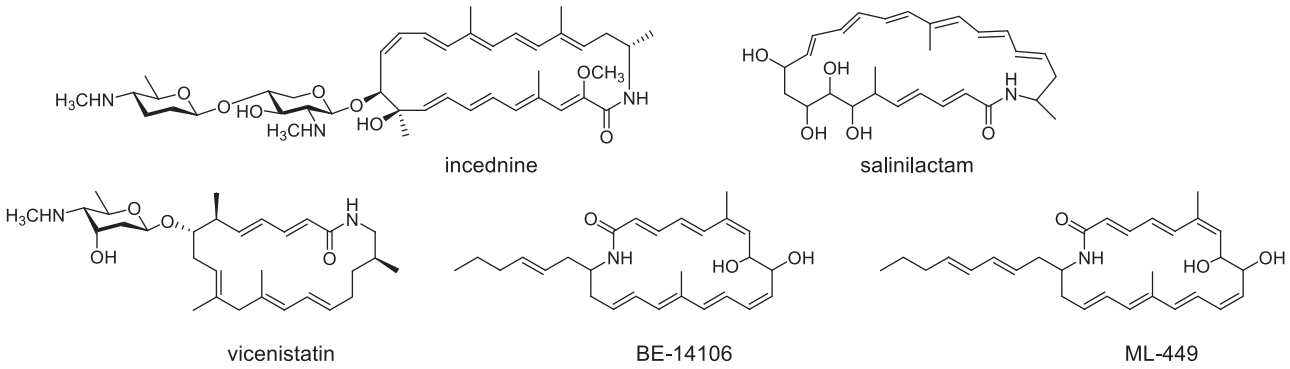

Figure 1 Incednine and structurally related macrolactams.

RESULTS AND DISCUSSION

Cloning of the incednine biosynthetic gene cluster

Based on the chemical structure and efficient incorporation of ^{13}C-labeled glycerol into the C1–C2 moiety of incednam (incednine aglycone), methoxymalonyl-ACP biosynthetic enzymes, which supply a unique extender unit of PKS,[12–18] seemed to be encoded in the incednine biosynthetic gene cluster. Among the methoxymalonyl-ACP biosynthetic enzymes, a sequentially highly conserved *fkbH* family gene was used as a probe to identify the incednine biosynthetic gene cluster (Supplementary Figure S1). In addition, an NDP-hexose-4,6-dehydratase (*4,6-DH*) gene was used as a probe, because *N*-demethylforosamine, a unique 2,6-dideoxysugar, appeared to be biosynthesized by common deoxyaminosugar biosynthetic enzymes, including *4,6-DH*.[19–21] As a result, only a single gene for each enzyme was cloned by PCR with the designed degenerate primer sets and chromosomal DNA template. Hybridization against a pOJ446-based cosmid library with the obtained specific genes, and subsequent chromosomal walking, gave a contiguous gene cluster containing both *fkbH* and *4,6-DH* gene homologs, which were presumably involved in incednine biosynthesis (Figure 2 and Table 1). The obtained DNA sequence was deposited in DDBJ (DNA Data Bank of Japan) under the accession number AB767280. The deduced products for each open reading frame (ORF) were compared with homologous sequences using the basic local alignment search tool. Seventy-five ORFs were identified within the sequenced region (~138 kb) and designated as an *idn* gene cluster. Although most of the ORFs appeared to be involved in incednine biosynthesis as discussed below, two unrelated gene clusters seemed to be inserted in this region of chromosomal DNA (*orf4–orf16* and *orf17–orf30* in Figure 2 and Table 1). Although biosynthetic gene clusters for natural products are usually clustered together in a region of chromosomal DNA, a few separated biosynthetic gene clusters, such as the ansamitocin and kedarcidin biosynthetic genes, have been reported recently.[15,22,23] Thus, the presently identified gene cluster appeared to be an example of such a cluster. Because gene disruption with the incednine producer strain was unsuccessful, we have not determined the precise boundaries for the incednine biosynthetic gene cluster. Therefore, only putative incednine biosynthetic genes (40 ORFs), determined by comparison with other structurally related macrolactam biosynthetic genes, are summarized in Table 1 with unrelated genes.

β-Amino acid starter-related genes

Two genes encoding putative ATP-dependent ligase (*idnL1* and *idnL7*), a gene encoding discrete ACP (*idnL6*), a gene encoding acyltransferase (*idnL2*) and a gene encoding peptidase (*idnL5*) were conserved in the *idn* gene cluster, as in the vicenistatin, salinilactam, BE-14106 and ML-449 gene clusters. IdnL1 showed similarity (29% identity, 43% similarity) to VinN, which specifically recognizes 3-methylaspartate and transfers it onto a discrete ACP VinL during vicenistatin biosynthesis.[10] Thus, IdnL1 was presumed to recognize (*S*)-3-aminobutyrate and transfer it onto the discrete ACP IdnL6. In addition, another ATP-dependent ligase, IdnL7, showed a high level of homology to VinM (57% identity, 68% similarity), suggesting that IdnL7 might recognize amino acids and transfer them onto 3-aminobutyryl-ACP at the β-position. This seemed to be the most striking feature in β-amino acid-containing macrolactam biosynthesis as reported in the vicenistatin biosynthetic machinery. As the next step, an acyltransferase, IdnL2, likely transfers the dipeptide moiety on the discrete ACP, IdnL6, to the loading ACP domain of IdnPKS1. The terminal amino acid residue on the elongated polyketide chain seemed to be removed by a peptidase, IdnL5, which showed high homology (61% identity) to a VinJ family peptidase.

idnL3 and *idnL4*, which are also conserved in the salinilactam biosynthetic gene cluster,[7] showed homology to a family of PLP-dependent aminotransferase genes and radical-SAM-PLP-dependent amino acid aminomutase genes,[5] respectively. As shown in our previous paper,[4] 3-aminobutyrate seems to be a common starter amino acid in the biosynthesis of incednine and salinilactam. Therefore, these gene products were presumed to be involved in 3-aminobutyrate formation from L-glutamate. A radical-SAM-PLP-dependent aminomutase, IdnL4, was presumed to catalyze the L-glutamate-2,3-aminomutase reaction to give β-glutamate, which is then decarboxylated to 3-aminobutyrate by a PLP-dependent enzyme, IdnL3.

PKS genes

The *idn* gene cluster was found to encode five large PKSs (IdnP1–P5), as shown in Figure 2 and Table 1. The number of the PKS modules (10 modules) agreed well with the length of the polyketide chain for incednam. Because IdnP1 contains a characteristic loading ACP domain at its N terminus, it was predicted to be the initiation PKS in incednine biosynthesis. On the other hand, IdnP5 contains a thioesterase domain at its C terminus, indicating it is the last PKS in the incednine PKS assembly line. The predicted substrate specificity of the acyltransferase (AT) domain (Supplementary Figure S2) suggested the order of PKS reactions as shown in Figure 2, and agreed well with the chemical structure of incednam, except for two AT domains (IdnAT9 and IdnAT10 in IdnP5). IdnAT9 should

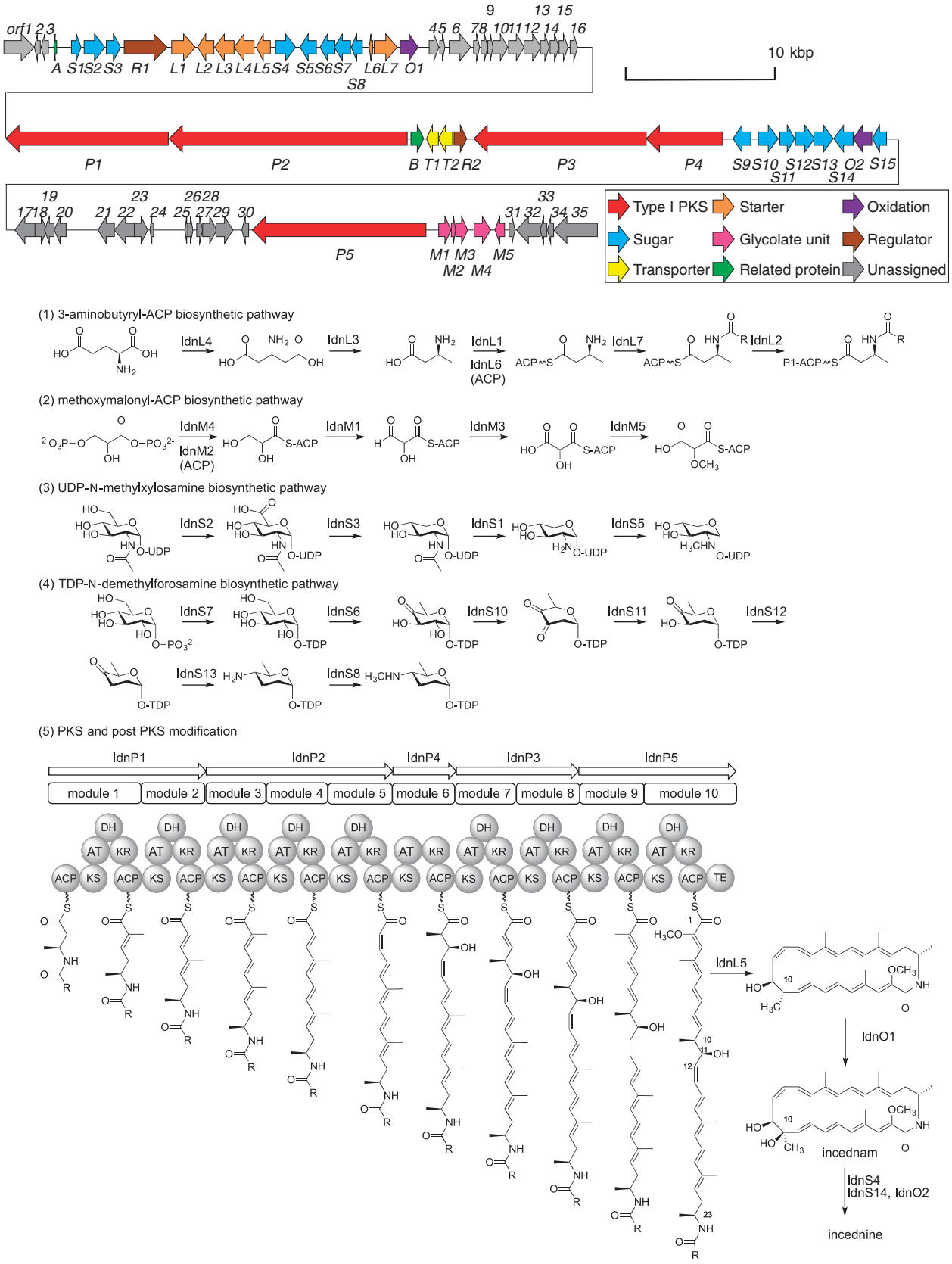

Figure 2 Incednine biosynthetic gene cluster and proposed biosynthetic pathway.

Table 1 Deduced functions of open reading frames (ORFs) in the incednine biosynthetic gene cluster (total 137 955 bp)

Gene	*Size*[a]	*Homolog (strain name, identity%/similarity%)*	*Predicted function in incednine biosynthesis*
orf1	>688	Superfamily I DNA and RNA helicase-like protein (*Streptomyces cattleya* NRRL 8057, 86/90)	Outside of gene cluster
orf2	131	Hypothetical protein (*Streptomyces pristinaespiralis* ATCC 25486, 78/85)	Outside of gene cluster
orf3	163	Hypothetical protein (*Streptomyces tsukubaensis* NRRL 18488, 83/87)	Outside of gene cluster
idnA	73	MbtH-like protein (*Rhodococcus equi* ATCC 33707, 81/93)	MbtH-like protein
idnS1	220	LmbE family protein (*Streptomyces* sp. AA4, 71/80)	GlcNAc/XylNAc deacetylase
idnS2	470	UDP-glucose/GDP-mannose dehydrogenase (*Streptomyces roseosporus* NRRL 15998, 72/80)	UDP-GlcNAc 6'-dehydrogenase
idnS3	318	NAD-dependent epimerase/dehydratase (*Streptomyces roseosporus* NRRL 15998, 77/85)	UDP-XylNAc synthase
idnR1	969	LuxR-family transcriptional regulator (*Streptomyces roseosporus* NRRL 15998, 54/66)	LuxR-family transcriptional regulator
idnL1	532	AMP-dependent synthetase and ligase (*Streptomyces roseosporus* NRRL 15998, 70/81)	ATP-dependent ligase (VinN family)
idnL2	369	malonyl-CoA:ACP transacylase (*Streptomyces roseosporus* NRRL 15998, 68/77)	Dipeptidyl-ACP:ACP transacylase (VinK family)
idnL3	428	(PLP)-dependent aspartate aminotransferase superfamily (*Streptomyces roseosporus* NRRL 15998, 75/81)	β-Glutamate β-decarboxylase
idnL4	453	Radical-SAM L-lysine 2,3-aminomutase (*Streptomyces roseosporus* NRRL 15998, 77/88)	L-Glutamate-2,3-aminomutase
idnL5	309	Proline iminopeptidase (*Streptomyces roseosporus* NRRL 15998, 74/83)	Terminal amino acid hydrolase (VinJ family)
idnS4	442	Glycosyltransferase (*Streptomyces roseosporus* NRRL 15998, 77/84)	Glycosyltransferase
idnS5	402	Methyltransferase (*Methylobacterium radiotolerans* JCM 2831, 39/51)	Methyltransferase (xylosamine moiety)
idnS6	324	dTDP-glucose 4,6-dehydratase (*Streptomyces halstedii*, VinB, 76/81)	dTDP-D-glucose 4,6-dehydratase
idnS7	355	Glucose-1-phosphate thymidylyltransferase (*Streptomyces halstedii*, VinA, 80/90)	Glucose-1-phosphate thymidylyltransferase
idnS8	245	*N*-methyltransferase (*Streptomyces halstedii*, VinG, 62/74)	TDP-forosamine-*N*-methyltransferase
idnL6	79	Acyl carrier protein (*Streptomyces halstedii*, VinL, 57/69)	Acyl carrier protein
idnL7	522	AMP-dependent synthetase and ligase (*Streptomyces roseosporus* NRRL 15998, 65/67)	ATP-dependent ligase (VinM family)
idnO1	401	Cytochrome P450 (*Streptomyces roseosporus* NRRL 15998, 82/89)	Cytochrome P450
orf4	211	Transcriptional regulator (*Streptomyces clavuligerus* ATCC 27064, 66/80)	Unrelated
orf5	114	Hypothetical protein	Unrelated
orf6	487	Hypothetical protein	Unrelated
orf7	134	Hypothetical protein	Unrelated
orf8	153	Putative hydrolase (*Streptomyces tsukubaensis* NRRL 18488, 52/67)	Unrelated
orf9	118	Hypothetical protein	Unrelated
orf10	340	Thymidylate synthase (*Streptomyces acidiscabies* 84-104, 72/79)	Unrelated
orf11	342	Nucleoside diphosphate kinase (*Streptomyces acidiscabies* 84-104, 70/77)	Unrelated
orf12	392	Hypothetical protein (*Streptomyces acidiscabies* 84-104, 76/85)	Unrelated
orf13	200	DNA polymerase III (*Streptomyces acidiscabies* 84-104, 71/84)	Unrelated
orf14	202	Hypothetical protein (*Streptomyces acidiscabies* 84-104, 50/63)	Unrelated
orf15	216	Oxidoreductase (*Streptomyces acidiscabies* 84-104, 68/77)	Unrelated
orf16	171	Hypothetical protein (*Streptomyces acidiscabies* 84-104, 44/59)	Unrelated
idnP1	3648	Type I polyketide synthase	PKS (LD-Mod1-Mod2) (ACP)–(KS-MMT-DH-KR-ACP)–(KS-MT-DH-KR-ACP)
idnP2	5343	Type I polyketide synthase	PKS (Mod3-Mod4-Mod5) (KS-MMT-DH-KR-ACP)–(KS-MT-DH-KR-ACP)–(KS-MT-DH-KR-ACP)
idnB	276	Thioesterase involved in nonribosomal peptide biosynthesis (*Streptomyces roseosporus* NRRL 15998, 61/75) (*Salinispora tropica* CNB-440, Strop_2763, 54/67) (*Amycolatopsis mediterranei* S699, RifR, 53/67)	Type II thioesterase
idnT1	265	ABC-type multidrug transport system (*Streptomyces roseosporus* NRRL 15998, 68/84)	ABC-type multidrug transporter
idnT2	318	ABC-type multidrug transport system (*Streptomyces roseosporus* NRRL 15998, 74/84) (*Streptomyces noursei* ATCC 11455, NysG, 28/45) (*Streptomyces noursei* ATCC 11455, NysH, 30/44)	ABC-type multidrug transporter
idnR2	282	Regulatory protein TetR (*Streptomyces tsukubaensis* NRRL 18488, 70/83)	Regulatory protein TetR
idnP3	3861	Type I polyketide synthase	PKS (Mod7-Mod8) (KS-MT-DH-KR-ACP)–(KS-MT-DH-KR-ACP)
idnP4	1723	Type I polyketide synthase	PKS (Mod6) (KS-MMT-KR-ACP)
idnS9	395	glycosyltransferase (*Streptomyces antibioticus*, 31/44)	Glycosyltransferase?
idnS10	451	NDP-hexose 2,3-dehydratase (*Streptomyces cyanogenus*, 55/67) (*Saccharopolyspora spinosa*, SpnO, 49/63)	NDP-hexose 2,3-dehydratase
idnS11	338	NDP-hexose-3-ketoreductase (*Streptomyces roseosporus* NRRL 15998, 68/74) (*Saccharopolyspora spinosa*, SpnN, 55/67)	NDP-hexose-3-ketoreductase
idnS12	444	NDP-hexose-3,4-dehydratase (*Streptomyces fradiae*, UrdQ, 73/84)	NDP-hexose-3,4-dehydratase

Table 1 (Continued)

Gene	*Size*[a]	*Homolog (strain name, identity%/similarity%)*	*Predicted function in incednine biosynthesis*
idnS13	431	Aminotransferase (*Streptomyces halstedii*, VinF, 54/67)	Aminotransferase
idnS14	425	Glycosyltransferase (*Streptomyces roseosporus* NRRL 15998, 61/76)	Glycosyltransferase
idnO2	423	Cytochrome P450 (*Streptomyces roseosporus* NRRL 15998, 47/58)	Glycosyltransferase auxiliary protein
idnS15	309	Glycosyltransferase (*Streptomyces antibioticus*, 35/47)	Glycosyltransferase?
orf17	444	Glutamate-1-semialdehyde aminotransferase (*Streptomyces griseus* subsp. griseus NBRC 13350, SGR_177, 79/88)	Unrelated
orf18	248	Dehydrogenase (*Streptomyces griseus* subsp. griseus NBRC 13350, SGR_178, 82/90)	Unrelated
orf19	216	hypothetical protein (*Streptomyces griseus* subsp. griseus NBRC 13350, SGR_179, 82/90)	Unrelated
orf20	269	Phytanoyl-CoA dioxygenase (*Streptomyces griseus* subsp. griseus NBRC 13350, SGR_180, 65/74)	Unrelated
orf21	367	Hypothetical protein (*Streptomyces tsukubaensis* NRRL 18488, 72/80)	Unrelated
orf22	500	Hypothetical protein (*Streptomyces davawensis* JCM 4913, 50/61)	Unrelated
orf23	244	Hypothetical protein (*Streptomyces tsukubaensis* NRRL 18488, 52/60)	Unrelated
orf24	85	Hypothetical protein (*Streptomyces tsukubaensis* NRRL 18488, 65/80)	Unrelated
orf25	80	Hypothetical protein (*Streptomyces tsukubaensis* NRRL 18488, 37/49)	Unrelated
orf26	121	Hypothetical protein (*Streptomyces zinciresistens* K42, 69/76)	Unrelated
orf27	128	Hypothetical protein (*Streptomyces* sp. SPB74, 43/54)	Unrelated
orf28	318	Hypothetical protein (*Streptomyces viridochromogenes* DSM 40736, 50/66)	Unrelated
orf29	398	*O*-methyltransferase (*Streptomyces* sp. Mg1, 49/63)	Unrelated
orf30	150	Secreted protein (*Streptomyces tsukubaensis* NRRL 18488, 85/91)	Unrelated
idnP5	3896	Type I polyketide synthase	PKS (Mod9-Mod10-TE) (KS-MMT-DH-KR-ACP)–(KS-meMT-DH-KR-ACP)-TE
idnM1	288	Glyceryl-ACP oxidoreductase (*Streptomyces himastatinicus* ATCC 53653, 66/76)	Glyceryl-ACP oxidoreductase
idnM2	98	Acyl carrier protein (*Streptomyces tsukubaensis* NRRL 18488, 54/68)	ACP for MeMal-ACP
idnM3	410	Acyl-CoA dehydrogenase (*Streptomyces acidiscabies* 84-104, 64/72)	Acyl-ACP dehydrogenase
idnM4	373	FkbH-like protein (*Streptomyces hygroscopicus* ATCC 53653, 71/82)	Glyceryl transferase
idnM5	220	*O*-methyltransferase (*Streptomyces hygroscopicus* ATCC 53653, 68/79)	*O*-methyltransferase
orf31	135	Hypothetical protein (Glyoxalase/bleomycin resistance protein/dioxygenase) (*Streptomyces sviceus* ATCC 29083, 55/75)	Outside of gene cluster
orf32	535	Spermidine synthase (*Streptomyces tsukubaensis* NRRL 18488, 89/94)	Outside of gene cluster
orf33	145	Hypothetical protein (*Streptomyces tsukubaensis* NRRL 18488, STSU_06448, 92/97)	Outside of gene cluster
orf34	138	Hypothetical protein (*Streptomyces tsukubaensis* NRRL 18488, STSU_06453, 61/71)	Outside of gene cluster
orf35	194	Hypothetical protein (*Streptomyces tsukubaensis* NRRL 18488, STSU_06458, 72/80)	Outside of gene cluster

[a]Size refers to the number of amino acids. The forty *orfs* (*idn*) were speculated to be required for incednine biosynthesis according to the proposed biosynthesis. Thus, *orf1–35* seemed not to be involved in the incednine biosynthesis.

recognize methylmalonyl-CoA based on the chemical structure of incednam. However, IdnAT9 is similar to a typical malonyltransferase domain (Supplementary Figure S2). IdnAT10 belongs to neither malonyltransferase domains nor methylmalonyltransferase domain. In general, methoxymalonyltransferase domain is similar to the other AT domains, and is hard to distinguish based only on sequence alignment.[24–26] From the chemical structure of incednam, IdnAT10 seems to recognize methoxymalonyl-ACP. The organization of the reductive domains, β-ketoreductase (KR) and dehydratase (DH), perfectly matched with the chemical structure of incednine. No enoylreductase domain was found in the identified PKSs. KR domains, IdnKR1-4 and IdnKR7-10, were predicted as B-type KR domains to give a (*3S*)-hydroxy configuration by β-keto reduction, and thus the following DH domains seemed to give a double bond with *trans* configuration (Supplementary Figure S3).[27–30] IdnKR5 and IdnKR6 were predicted to be A-type KR domains and gave opposite stereochemistry of the β-hydroxy group, which is converted to a *cis* double bond by the adjacent DH domain, if it exists. Overall, the identified PKS structures perfectly matched with the chemical structure of incednam, indicating that this gene cluster is responsible for incednine biosynthesis.

Biosynthetic genes for methoxymalonyl-ACP

The contiguous *idnM1*, *M2*, *M3*, *M4* and *M5* genes showed high homology to *fkbK*, *J*, *I*, *H* and *G*, respectively, whose products are involved in methoxymalonyl-ACP formation in FK-520 biosynthesis.[12,31] Thus, IdnM4 is likely to ligate phosphoglycerate onto a discrete ACP, IdnM2, to produce glyceryl-ACP,[32] which is then oxidized by IdnM1 and IdnM3 to give hydroxymalonyl-ACP.[33] IdnM5 seemed to catalyze the methylation to give methoxymalonyl-ACP as an extender unit of incednine PKS.

Deoxyaminosugar biosynthetic genes and glycosyltransferase genes

The *idnS8*, *S10*, *S11*, *S12* and *S13* gene products showed a high degree of similarity to the Spn enzymes involved in TDP-D-forosamine formation in spinosyn biosynthesis.[21,34–36] Thus, the same functions were predicted for these enzymes in the construction of NDP-*N*-demethyl-D-forosamine, which is a glycosyl donor in the post-PKS modification of incednam. Initially, TDP-4-keto-6-deoxy-D-glucose, which is a common biosynthetic intermediate in TDP-6-deoxysugar biosynthesis, is constructed by a TDP-D-glucose synthase (IdnS7) and 4,6-DH (IdnS6) from D-glucose-1-phosphate via TDP-D-glucose. A TDP-D-glucose-2,3-dehydratase (IdnS10) likely converts TDP-4-keto-

6-deoxy-D-glucose to TDP-3,4-diketo-2,6-dideoxy-D-glucose, which is then reduced by a TDP-D-glucose-3-ketoreductase (IdnS11). A putative 2Fe-2S cluster-containing enzyme (IdnS12) was predicted to catalyze the C-3 deoxygenation of TDP-4-keto-2,6-dideoxy-D-glucose with cellular reductase.[21] The resultant TDP-4-keto-2,3,6-trideoxy-D-glucose is transformed to TDP-4-amino-2,3,4,6-tetradeoxy-D-glucose by aminotransferase (IdnS13), followed by a *N*-methyltransferase IdnS8 to afford TDP-*N*-demethyl-D-forosamine.

IdnS2 showed similarity to UDP-*N*-acetyl-D-glucosamine (UDP-GlcNAc) 6-dehydrogenase from *Bacillus cytotoxicus* NVH 391–98 (35% identity, 50% similarity), which catalyzes the oxidation of UDP-GlcNAc to UDP-*N*-acetyl-D-glucosaminuronic acid (UDP-GlcNAcA) in UDP-*N*-acetyl-D-xylosamine (UDP-XylNAc) biosynthesis.[37] IdnS3 showed homology to a NAD-dependent UDP-XylNAc synthase (42% identity, 61% similarity). Thus, IdnS2 and IdnS3 are likely to be involved in UDP-XylNAc formation from UDP-GlcNAc. Deacetylation and *N*-methylation of UDP-XylNAc, to produce the *N*-methyl-D-xylosamine moiety, could be catalyzed by a putative *N*-acetyl-D-glucosamine deacetylase, IdnS1, and a putative methyltransferase, IdnS5.

In the *idn* gene cluster, four putative glycosyltransferases, IdnS4, 9, 14 and 15, are encoded and presumed to be involved in the attachment of sugars. Among these, IdnS4 and IdnS14 showed high similarity to the TDP-forosamyltransferase SpnP, involved in spinosyn biosynthesis, suggesting that either of these proteins could be responsible for the attachment of *N*-demethylforosamine. IdnS9 and IdnS15 lack many internal protein sequences conserved with the other known glycosyltransferases, as well as a crucial histidine residue that is critical for the deprotonation of the hydroxy group of the glycosyl acceptor molecules (Supplementary Figure S4).[38,39] Therefore, IdnS9 and IdnS15 appear to be inactive proteins, and IdnS4 and IdnS14 are predicted to be involved in the two glycosylation events, the attachment of *N*-methylxylosamine and *N*-demethylforosamine, although functional characterization of these glycosyltransferases is necessary to elucidate the precise biosynthetic role.

Cytochrome P450s and associated genes

Two putative cytochrome P450 type proteins, IdnO1 and IdnO2, are encoded in the *idn* cluster. IdnO2 lacks an active site Cys residue, which is conserved among cytochrome P450s for the coordination of heme iron,[40,41] indicating that IdnO2 does not function as an oxygenase. The *IdnO2* gene is adjacent to the glycosyltransferase *IdnS14* gene and might support its enzymatic activity as an auxiliary protein, similar to DesVIII with the DesVII glycosyltransferase during methymycin/picromycin biosynthesis, and AknT with the AknS glycosyltransferase in aclacinomycin biosynthesis.[42,43] In fact, IdnO2 shows similarity to AknT (37% identity, 50% similarity). A typical cytochrome P450, IdnO1, is predicted to catalyze a hydroxylation at C10.

IdnA is a small protein composed of 73 aa, and showed high similarity to the MbtH family proteins.[44] Homolog genes of *mbtHs* are often found in secondary metabolite biosynthetic gene clusters, encoding nonribosomal peptide synthetase and nonribosomal peptide synthetase-like enzymes, and affect the adenylation domain activity of nonribosomal peptide synthetase.[45] Thus, IdnA might support the enzymatic activity of two ATP-dependent ligases, IdnL1 and IdnL7, in the β-amino acid carrying system. IdnB showed high similarity to type II family thioesterases, which often associate with nonribosomal peptide synthetase- and PKS-containing gene clusters, and seemed to eliminate aberrant extender units loaded onto ACPs.[46–48] Thus, the same function was predicted for IdnB.

A LuxR-family regulatory protein, IdnR1, was presumed to work as a pathway-specific positive regulator, similar to TmcN in tautomycin biosynthesis by *Streptomyces* sp. CK4412,[49] and SamR0484 in stambomycin biosynthesis by *Streptomyces ambofaciens* ATCC 23877.[50] On the other hand, a putative TetR family regulatory protein, IdnR2, was predicted to act as a repressor.[51] A putative ABC transporter, IdnT2, showed similarity to nistatin transporters NysG and NysH (28 and 30% similarity, respectively) in *Streptomyces noursei* ATCC 11455,[52] suggesting that it is responsible for the efflux of incednine. Another putative ABC transporter gene, *idnT1*, is associated with *idnT2* in the *idn* gene cluster. As this set of ABC transporter genes are well associated with *Streptomyces* genomes, these ABC-type proteins are predicted to work cooperatively in incednine transport.

Functional characterization of β-glutamate-β-decarboxylase IdnL3

In the present study, a putative PLP-dependent enzyme, IdnL3, which was predicted to catalyze the decarboxylation of β-glutamate to give 3-aminobutyrate, was expressed in *Streptomyces lividans* TK-64, and the recombinant protein was purified by Ni-affinity chromatography (Supplementary Figure S1). The IdnL3 enzyme assay was performed with 1.0 μM of IdnL3, 1 mM of β-glutamate and 0.1 mM of PLP in 50 mM HEPES buffer (pH 7.0). Following incubation at 30 °C for 30 min, the reaction mixtures were treated with phenylisothiocyanate and the derived phenylisothiocyanate-amino acids were analyzed by HPLC. As a result, 3-aminobutyrate formation from β-glutamate by IdnL3 was clearly detected (Figure 3, Supplementary Figures S5, S6, and S7). Indeed, only (*S*)-3-aminobutyrate, which corresponds to the stereochemistry at C-23 of incednine, was formed by IdnL3 (Supplementary Figures S5, S8 and S9). This result indicated that the enzyme distinguishes the pro-chirality of β-glutamate. No other amino acids tested, including L-aspartate, L-glutamate, β-alanine and DL-3-aminobutyrate, produced new product in the HPLC analysis (data not shown). Therefore, IdnL3 strictly recognizes β-glutamate as its substrate. The steady-state kinetic parameters of IdnL3 were determined as $K_m = 0.21 \pm 0.1$ mM, $k_{cat} = 0.24 \pm 0.04\ s^{-1}$ and $k_{cat}/K_m = 1.1 \times 10^3\ s^{-1}\ M^{-1}$ (Supplementary Figure S12).

We propose the following reaction mechanism for this unique β-decarboxylase, IdnL3 (Figure 3). First, PLP-β-glutamate Schiff-base formation could induce decarboxylation to generate an enamine intermediate. Subsequent protonation then produces a PLP-3-aminobutyrate imine intermediate, which is then hydrolyzed to PLP and 3-aminobutyrate. A similar mechanism is proposed in the reaction of α-L-aspartate-β-decarboxylase, which converts α-L-aspartate to α-L-alanine and carbon dioxide.[53,54] However, IdnL3 is a sequentially different type of PLP-dependent enzyme to α-L-aspartate-β-decarboxylase. In homology search, Strop_2772 (69% identity to IdnL3) and some homologs are found, but these functions have never been characterized. Thus, its substrate recognition mechanism is an intriguing issue that remains to be solved.

In the incorporation of 2,2,4,4-deuterium-labeled β-glutamate into incednine, only the C-22 position was efficiently labeled.[4] Presumably, during rapid equilibrium between the enamine–imine intermediates in the β-glutamate-β-decarboxylation (Figure 3), deuterium atoms at the methylene group, which become the terminal methyl group of 3-aminobutyrate, would be exchanged with the protium atom of a water molecule at the active site of the enzyme. In the [1,2-$^{13}C_2$]acetate incorporation experiment, L-[4,5-$^{13}C_2$]glutamate that was expected to represent the majority of labeled glutamate via the tricarboxylic acid (TCA) cycle seemed to be first converted to β-glutamate by the putative L-glutamate-2,3-aminomutase IdnL4.

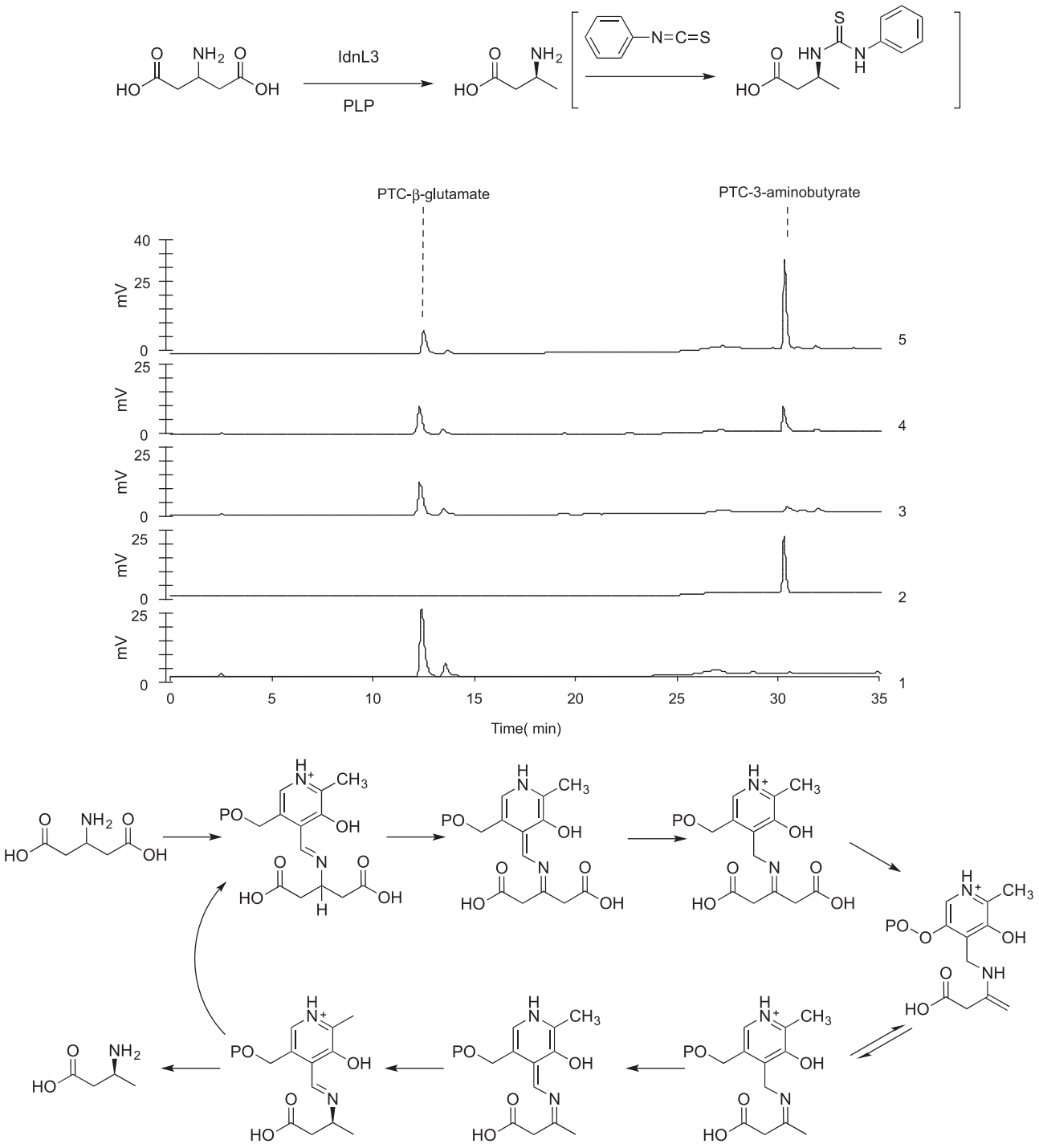

Figure 3 β-Glutamate-β-decarboxylase activity of IdnL3. IdnL3 (1.0 μM), β-glutamate (1 mM) and PLP (0.1 mM) were mixed in 50 mM HEPES buffer (pH 7.0) at 30 °C for 30 min (total volume 100 μl). An equal volume of CH_3CN was added to quench the reaction. Following phenylisothiocyanate treatment, the derivatives were analyzed by HPLC (254 nm). The *Y*-axis is intensity of analog-UV signal detected in HPLC system, which indicates relative abundance of substance absorbing 254 nm wavelength. (1) Authentic PTC-β-glutamate, (2) authentic-PTC-3-aminobutyrate, (3) IdnL3 was used after treatment in 100 °C water for 5 min as a negative control, (4) IdnL3 reaction product, (5) co-injection of IdnL3 reaction product and authentic PTC-3-aminobutyrate. A proposed reaction mechanism for β-glutamate-β-decarboxylation catalyzed by the PLP-dependent enzyme IdnL3 is shown.

Then, IdnL3 would recognize the pro-chirality of β-glutamate and generate (*S*)-3-[1,2-$^{13}C_2$]aminobutyrate, judging by the significant intact incorporation at C-21–C-22 of incednine.[3] However, a partial incorporation into the C-28 methyl group from [1,2-$^{13}C_2$]acetate could be explained by the reversibility of the L-glutamate-2,3-aminomutase reaction[5] to give another stereoisomer that leads to additional (*S*)-3-[4-^{13}C]aminobutyrate formation by IdnL3. Overall, the stereoselective deprotonation and protonation at the β-position of amino acid intermediates by IdnL3 are critical to determine the stereochemistry at C-23 of incednine.

We were able to identify the incednine biosynthetic gene cluster and propose the likely biosynthetic pathway, including two 3-aminobutyrate biosynthetic enzymes. Most strikingly, a new β-glutamate-β-decarboxylase, IdnL3, was successfully characterized *in vitro*. A highly homologous protein, Strop_2772, seems to have the same function as IdnL3 in salinilactam biosynthesis. In addition, a probable L-glutamate-2,3-aminomutase, Strop_2771 (69% identity to IdnL4), is also encoded in the salinilactam biosynthetic gene cluster, indicating that these two types of enzymes are responsible for 3-aminobutyrate formation to supply a pathway-specific β-amino acid for the PKS starter unit. Two ATP-dependent ligases, an ACP, an acyltransferase, and a peptidase, are also conserved in the *strop* gene cluster, suggesting that the same scenario as for the starter unit biosynthesis of incednine could be applicable for the salinilactam biosynthesis. Among these five starter-related enzymes, only a pathway-specific ATP-dependent ligase is sequentially quite different from the other types of macrolactam biosynthetic enzymes (Supplementary Figure S10). We are currently investigating this type of specific ATP-dependent ligase to gain further insight into the detailed recognition mechanism.

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1 Futamura, Y. *et al.* Discovery of incednine as a potent modulator of the anti-apoptotic function of Bcl-xL from microbial origin. *J. Am. Chem. Soc* **130,** 1822–1823 (2008).
2 Schulz, D. *et al.* Silvalactam, a 24-membered macrolactam antibiotic produced by *Streptomyces* sp. Tu 6392. *J. Antibiot.* **65,** 369–372 (2012).
3 Takaishi, M., Kudo, F. & Eguchi, T. Biosynthetic pathway of 24-membered macrolactam glycoside incednine. *Tetrahedron* **64,** 6651–6656 (2008).
4 Takaishi, M., Kudo, F. & Eguchi, T. A unique pathway for the 3-aminobutyrate starter unit from L-glutamate through b-glutamate during biosynthesis of the 24-membered macrolactam antibiotic, incednine. *Org. Lett.* **14,** 4591–4593 (2012).
5 Ruzicka, F. J. & Frey, P. A. Glutamate 2,3-aminomutase: a new member of the radical SAM superfamily of enzymes. *Biochim. Biophys. Acta* **1774,** 286–296 (2007).
6 Ogasawara, Y., Kakinuma, K. & Eguchi, T. Involvement of glutamate mutase in the biosynthesis of the unique starter unit of the macrolactam polyketide antibiotic vicenistatin. *J. Antibiot.* **58,** 468–472 (2005).
7 Udwary, D. W. *et al.* Genome sequencing reveals complex secondary metabolome in the marine actinomycete *Salinispora tropica*. *Proc. Natl. Acad. Sci. USA.* **104,** 10376–10381 (2007).
8 Jorgensen, H. *et al.* Biosynthesis of macrolactam BE-14106 involves two distinct PKS systems and amino acid processing enzymes for generation of the aminoacyl starter unit. *Chem. Biol.* **16,** 1109–1121 (2009).
9 Jorgensen, H. *et al.* Insights into the evolution of macrolactam biosynthesis through cloning and comparative analysis of the biosynthetic gene cluster for a novel macrocyclic lactam, ML-449. *Appl. Environ. Microbiol.* **76,** 283–293 (2010).
10 Shinohara, Y., Kudo, F. & Eguchi, T. A natural protecting group strategy to carry an amino acid starter unit in the biosynthesis of macrolactam polyketide antibiotics. *J. Am. Chem. Soc.* **133,** 18134–18137 (2011).
11 Kudo, F., Kitayama, T., Kakinuma, K. & Eguchi, T. Macrolactam formation catalyzed by the thioesterase domain of vicenistatin polyketide synthase. *Tetrahedron Lett.* **47,** 1529–1532 (2006).
12 Carroll, B. J. *et al.* Identification of a set of genes involved in the formation of the substrate for the incorporation of the unusual 'glycolate' chain extension unit in ansamitocin biosynthesis. *J. Am. Chem. Soc.* **124,** 4176–4177 (2002).
13 Haydock, S. F. *et al.* Organization of the biosynthetic gene cluster for the macrolide concanamycin A in *Streptomyces neyagawaensis* ATCC 27449. *Microbiology* **151,** 3161–3169 (2005).
14 Zhao, C. *et al*. Utilization of the methoxymalonyl-acyl carrier protein biosynthesis locus for cloning the oxazolomycin biosynthetic gene cluster from *Streptomyces albus* JA3453. *J. Bacteril.* **188,** 4142–4147 (2006).
15 Karki, S. *et al.* The methoxymalonyl-acyl carrier protein biosynthesis locus and the nearby gene with the b-ketoacyl synthase domain are involved in the biosynthesis of galbonolides in *Streptomyces galbus*, but these loci are separate from the modular polyketide synthase gene cluster. *FEMS Microbiol. Lett.* **310,** 69–75 (2010).
16 Chan, Y. A., Podevels, A. M., Kevany, B. M. & Thomas, M. G. Biosynthesis of polyketide synthase extender units. *Nat. Prod. Rep.* **26,** 90–114 (2009).
17 Haydock, S. F. *et al.* Divergent sequence motifs correlated with the substrate specificity of (methyl)malonyl-CoA:acyl carrier protein transacylase domains in modular polyketide synthases. *FEBS Lett.* **374,** 246–248 (1995).
18 Zhang, F. *et al.* Cloning and elucidation of the FR901464 gene cluster revealing a complex acyltransferase-less polyketide synthase using glycerate as starter units. *J. Am. Chem. Soc.* **133,** 2452–2462 (2011).
19 Nedal, A. & Zotchev, S. B. Biosynthesis of deoxyaminosugars in antibiotic-producing bacteria. *Appl. Microbiol. Biotechnol.* **64,** 7–15 (2004).
20 Trefzer, A., Salas, J. A. & Bechthold, A. Genes and enzymes involved in deoxysugar biosynthesis in bacteria. *Nat. Prod. Rep.* **16,** 283–299 (1999).
21 Hong, L., Zhao, Z., Melançon, C. E. III, Zhang, H. & Liu, H. W. In vitro characterization of the enzymes involved in TDP-D-forosamine biosynthesis in the spinosyn pathway of *Saccharopolyspora spinosa*. *J. Am. Chem. Soc.* **130,** 4954–4967 (2008).
22 Yu, T. W. *et al.* The biosynthetic gene cluster of the maytansinoid antitumor agent ansamitocin from *Actinosynnema pretiosum*. *Proc. Natl. Acad. Sci. USA.* **99,** 7968–7973 (2002).
23 Lohman, J. R. *et al.* Cloning and sequencing of the kedarcidin biosynthetic gene cluster from Streptoalloteichus sp. ATCC 53650 revealing new insights into biosynthesis of the enediyne family of antitumor antibiotics. *Mol. Biosyst.* **9,** 478–491 (2013).
24 Ansari, M. Z., Yadav, G., Gokhale, R. S. & Mohanty, D. NRPS-PKS: a knowledge-based resource for analysis of NRPS/PKS megasynthases. *Nucleic. Acids. Res.* **32,** W405–W413 (2004).
25 Tang, L., Yoon, Y. J., Choi, C. Y. & Hutchinson, C. R. Characterization of the enzymatic domains in the modular polyketide synthase involved in rifamycin B biosynthesis by *Amycolatopsis mediterranei*. *Gene* **216,** 255–265 (1998).
26 Del Vecchio, F. *et al.* Active-site residue, domain and module swaps in modular polyketide synthases. *J. Ind. Microbiol. Biotechnol.* **30,** 489–494 (2003).
27 Keatinge-Clay, A. T. A tylosin ketoreductase reveals how chirality is determined in polyketides. *Chem. Biol.* **14,** 898–908 (2007).
28 Keatinge-Clay, A. Crystal structure of the erythromycin polyketide synthase dehydratase. *J. Mol. Biol.* **384,** 941–953 (2008).
29 Keatinge-Clay, A. T. & Stroud, R. M. The structure of a ketoreductase determines the organization of the beta-carbon processing enzymes of modular polyketide synthases. *Structure* **14,** 737–748 (2006).
30 Caffrey, P. Conserved amino acid residues correlating with ketoreductase stereospecificity in modular polyketide synthases. *Chembiochem* **4,** 654–657 (2003).
31 Walton, L. J., Corre, C. & Challis, G. L. Mechanisms for incorporation of glycerol-derived precursors into polyketide metabolites. *J. Ind. Microbiol. Biotechnol.* **33,** 105–120 (2006).
32 Dorrestein, P. C. *et al.* The bifunctional glyceryl transferase/phosphatase OzmB belonging to the HAD superfamily that diverts 1,3-bisphosphoglycerate into polyketide biosynthesis. *J. Am. Chem. Soc.* **128,** 10386–10387 (2006).
33 Watanabe, K., Khosla, C., Stroud, R. M. & Tsai, S. C. Crystal structure of an Acyl-ACP dehydrogenase from the FK520 polyketide biosynthetic pathway: insights into extender unit biosynthesis. *J. Mol. Biol.* **334,** 435–444 (2003).
34 Waldron, C. *et al.* Cloning and analysis of the spinosad biosynthetic gene cluster of *Saccharopolyspora spinosa*. *Chem. Biol.* **8,** 487–499 (2001).
35 Hong, L., Zhao, Z. & Liu, H. W. Characterization of SpnQ from the spinosyn biosynthetic pathway of *Saccharopolyspora spinosa*: mechanistic and evolutionary implications for C-3 deoxygenation in deoxysugar biosynthesis. *J. Am. Chem. Soc.* **128,** 14262–14263 (2006).
36 Zhao, Z., Hong, L. & Liu, H. W. Characterization of protein encoded by *spnR* from the spinosyn gene cluster of *Saccharopolyspora spinosa*: mechanistic implications for forosamine biosynthesis. *J. Am. Chem. Soc.* **127,** 7692–7693 (2005).
37 Gu, X., Glushka, J., Lee, S. G. & Bar-Peled, M. Biosynthesis of a new UDP-sugar, UDP-2-acetamido-2-deoxyxylose, in the human pathogen *Bacillus cereus* subspecies cytotoxis NVH 391-98. *J. Biol. Chem.* **285,** 24825–24833 (2010).
38 Claesson, M., Siitonen, V., Dobritzsch, D., Metsa-Ketela, M. & Schneider, G. Crystal structure of the glycosyltransferase SnogD from the biosynthetic pathway of nogalamycin in *Streptomyces nogalater*. *FEBS J.* **279,** 3251–3263 (2012).
39 Isiorho, E. A., Liu, H. W. & Keatinge-Clay, A. T. Structural studies of the spinosyn rhamnosyltransferase, SpnG. *Biochemistry* **51,** 1213–1222 (2012).
40 Munro, A. W., Girvan, H. M. & McLean, K. J. Variations on a (t)heme–novel mechanisms, redox partners and catalytic functions in the cytochrome P450 superfamily. *Nat. Prod. Rep.* **24,** 585–609 (2007).
41 Denisov, I. G., Makris, T. M., Sligar, S. G. & Schlichting, I. Structure and chemistry of cytochrome P 450. *Chem. Rev.* **105,** 2253–2277 (2005).
42 Borisova, S. A. & Liu, H. W. Characterization of glycosyltransferase DesVII and its auxiliary partner protein DesVIII in the methymycin/picromycin biosynthetic pathway. *Biochemistry* **49,** 8071–8084 (2010).
43 Leimkuhler, C. *et al.* Characterization of rhodosaminyl transfer by the AknS/AknT glycosylation complex and its use in reconstituting the biosynthetic pathway of aclacinomycin A. *J. Am. Chem. Soc.* **129,** 10546–10550 (2007).
44 Baltz, R. H. Function of MbtH homologs in nonribosomal peptide biosynthesis and applications in secondary metabolite discovery. *J. Ind. Microbiol. Biotechnol.* **38,** 1747–1760 (2011).

45 Zhang, W., Heemstra, J. R. Jr, Walsh, C. T. & Imker, H. J. Activation of the pacidamycin PacL adenylation domain by MbtH-like proteins. *Biochemistry* **49,** 9946–9947 (2010).
46 Heathcote, M. L., Staunton, J. & Leadlay, P. F. Role of type II thioesterases: evidence for removal of short acyl chains produced by aberrant decarboxylation of chain extender units. *Chem. Biol.* **8,** 207–220 (2001).
47 Koglin, A. *et al.* Structural basis for the selectivity of the external thioesterase of the surfactin synthetase. *Nature* **454,** 907–911 (2008).
48 Claxton, H. B., Akey, D. L., Silver, M. K., Admiraal, S. J. & Smith, J. L. Structure and functional analysis of RifR, the type II thioesterase from the rifamycin biosynthetic pathway. *J. Biol. Chem.* **284,** 5021–5029 (2009).
49 Hur, Y. A., Choi, S. S., Sherman, D. H. & Kim, E. S. Identification of TmcN as a pathway-specific positive regulator of tautomycetin biosynthesis in *Streptomyces* sp. CK4412. *Microbiology* **154,** 2912–2919 (2008).
50 Laureti, L. *et al.* Identification of a bioactive 51-membered macrolide complex by activation of a silent polyketide synthase in *Streptomyces ambofaciens*. *Proc. Natl. Acad. Sci. USA.* **108,** 6258–6263 (2011).
51 Ramos, J. L. *et al.* The TetR family of transcriptional repressors. *Microbiol. Mol. Biol. Rev.* **69,** 326–356 (2005).
52 Sletta, H. *et al.* Nystatin biosynthesis and transport: *nysH* and *nysG* genes encoding a putative ABC transporter system in *Streptomyces noursei* ATCC 11455 are required for efficient conversion of 10-deoxynystatin to nystatin. *Antimicrob. Agents Chemother.* **49,** 4576–4583 (2005).
53 Toney, M. D. Controlling reaction specificity in pyridoxal phosphate enzymes. *Biochim. Biophys. Acta* **1814,** 1407–1418 (2011).
54 Lima, S. *et al.* The crystal structure of the *Pseudomonas dacunhae* aspartate-b-decarboxylase dodecamer reveals an unknown oligomeric assembly for a pyridoxal-5'-phosphate-dependent enzyme. *J. Mol. Biol.* **388,** 98–108 (2009).

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