Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2726297 C 2016/08/16

(11)(21) 2 726 297

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 2009/06/15
(87) Date publication PCT/PCT Publication Date: 2010/01/07
(45) Date de délivrance/Issue Date: 2016/08/16
(85) Entrée phase nationale/National Entry: 2010/11/29
(86) N° demande PCT/PCT Application No.: EP 2009/057336
(87) N° publication PCT/PCT Publication No.: 2010/000601
(30) Priorité/Priority: 2008/07/04 (EP08159743.7)

(51) Cl.Int./Int.Cl. *C07K 7/56* (2006.01), *C12N 15/52* (2006.01), *C12P 17/16* (2006.01)

(72) Inventeurs/Inventors:
MUELLER, ROLF, DE;
WENZEL, SILKE, DE;
GARCIA, RONALD, DE

(73) Propriétaire/Owner:
HELMHOLTZ-ZENTRUM FUER INFEKTIONSFORSCHUNG GMBH, DE

(74) Agent: HICKS & ASSOCIATES

(54) Titre : ENZYMES DE VOIES DE SYNTHESE POUR LA PRODUCTION D'ARGYRINES
(54) Title: SYNTHETIC PATHWAY ENZYMES FOR THE PRODUCTION OF ARGYRINS

(57) Abrégé/Abstract:
The invention provides the amino acid sequences comprised in or constituting the synthetic pathway enzymes participating in the production of Argyrins, as well as the nucleic acid sequences encoding the synthetic pathway enzymes participating in the production of Argyrins, as well as genetically manipulated micro-organisms containing nucleic acid sequences encoding the synthetic pathway enzymes for the production of Argyrins, e.g. for inserting one or more of these coding sequences, mutating in a targeted manner one or more of these nucleic acid sequences, in a wild type producer micro-organism or in a heterologous micro-organism, for the production of Argyrins.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 January 2010 (07.01.2010)

PCT

(10) International Publication Number
WO 2010/000601 A1

(51) International Patent Classification:
C07K 7/56 (2006.01) *C12N 5/06* (2006.01)
C12P 17/16 (2006.01) *C12N 15/52* (2006.01)

(21) International Application Number:
PCT/EP2009/057336

(22) International Filing Date:
15 June 2009 (15.06.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
08159743.7 4 July 2008 (04.07.2008) EP

(71) Applicants *(for all designated States except US)*: **UNIVERSITÄT DES SAARLANDES** [DE/DE]; Campus, 66123 Saarbrücken (DE). **HELMHOLTZ-ZENTRUM FÜR INFEKTIONSFORSCHUNG** [DE/DE]; Inhoffenstraße 7, 38124 Braunschweig (DE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **MÜLLER, Rolf** [DE/DE]; Kirkelerstr. 114, 66440 Blieskastel (DE). **WENZEL, Silke** [DE/DE]; Torgauer Str. 13, 34292 Ahnatal (DE). **GARCIA, Ronald** [DE/DE]; Gaußstr. 81, App. 37, 66123 Saarbrücken (DE).

(74) Agent: **TARUTTIS, Stefan**; Aegidientorplatz 2b, 30159 Hannover (DE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2010/000601 A1

(54) Title: SYNTHETIC PATHWAY ENZYMES FOR THE PRODUCTION OF ARGYRINS

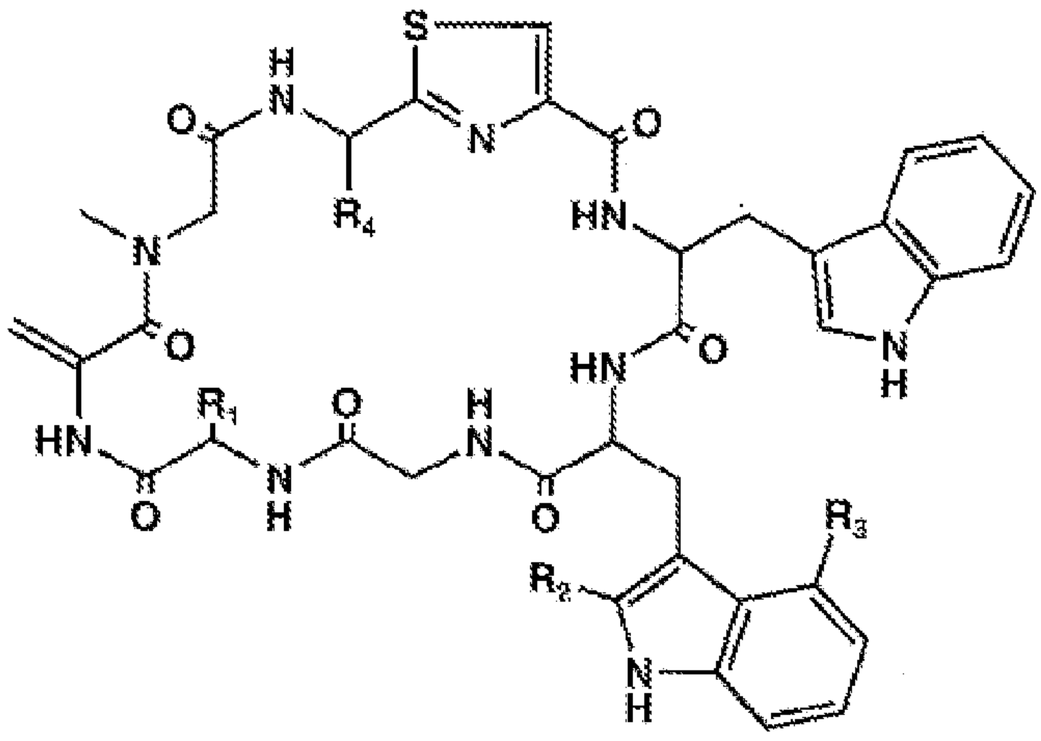
(57) Abstract: The invention provides the amino acid sequences comprised in or constituting the synthetic pathway enzymes participating in the production of Argyrins, as well as the nucleic acid sequences encoding the synthetic pathway enzymes participating in the production of Argyrins, as well as genetically manipulated micro-organisms containing nucleic acid sequences encoding the synthetic pathway enzymes for the production of Argyrins, e.g. for inserting one or more of these coding sequences, mutating in a targeted manner one or more of these nucleic acid sequences, in a wild type producer micro-organism or in a heterologous micro-organism, for the production of Argyrins.

Synthetic pathway enzymes for the production of Argyrins

The invention relates to nucleic acid sequences encoding synthetic pathway enzymes, which catalyze the production of Argyrins. Accordingly, the invention also relates to the synthetic pathway enzymes, to microorganisms expressing the synthetic pathway enzymes and to a method for production of Argyrins, making use of the synthetic pathway enzymes, preferably expressed in a micro-organism. The invention provides the proteins forming part of or constituting the non-ribosomal peptide synthetases (NRPS) having the activity to catalyse at least one conversion step in the synthesis of Argyrins, including the NRPS constituting the enzymes having the activity to catalyse the synthesis of pre-Argyrin, and additional enzymes having the activity which catalyse the conversion of pre-Argyrin to at least one derivative having the core structure I of Argyrin, including e.g. natural derivatives thereof comprising Argyrin A, Argyrin B, Argyrin C, Argyrin D, Argyrin E, Argyrin F, Argyrin G, and Argyrin H. Synthetic derivatives of Argyrin contain different substituents as R1, R2, R3, and R4 to common structure I.

The synthetic pathway enzymes catalyzing the synthesis of at least one Argyrin comprising the core structure I are encoded by nucleic acid sequences of the invention, containing the structural genes for the synthetic pathway enzymes.

Argyrins share the common core structure I:



(I),

wherein substituents to R1, R2, R3, and R4 can vary, giving e.g. rise to natural derivatives designated Argyrins A - H. Generally, R1 can be selected from an alkyl group, preferably methyl and ethyl, R2 preferably is hydrogen or methyl, R3 preferably is hydrogen or methoxy, and R4 preferably is selected from hydrogen, methyl and hydroxymethyl, as described in Vollbrecht et al. (Journal of Antibiotics 8, 715 – 721 (2002)) for Argyrins obtained from *Archangium gephyra*. In dependence on the pattern of substitution, natural Argyrins are designated as follows:

Argyrin A	**R_1= CH_3; R_2 = H; R_3 = OCH_3; R_4 = CH_3**
Argyrin B	**R_1 = C_2H_5; R_2 = H; R_3 = OCH_3; R_4 = CH_3**
Argyrin C	**R_1 = CH_3; R_2 = CH_3; R_3 = OCH_3; R_4 = CH_3**
Argyrin D	**R_1 = C_2H_5; R_2 = CH_3; R_3 = OCH_3; R_4 = CH_3**
Argyrin E	**R_1 = CH_3; R_2 = H; R_3 = H; R_4 = CH_3**
Argyrin F	**R_1 = CH_3; R_2 = H; R_3 = OCH_3; R_4 = CH_2OH**
Argyrin G	**R_1 = C_2H_5; R_2 = H; R_3 = OCH_3; R_4 = CH_2OH**
Argyrin H	**R_1 = CH_3; R_2 = H; R_3 = OCH_3; R_4 = H**

To-date, Argyrins are obtained from the natural producer organism *Archangium gephyra*, e.g. as a mixture of one or more of the above mentioned Argyrins, collectively referred to as

Argyrins A - H, e.g. by isolation from the fermentation broth, and purification by standard methods, e.g. using partition and chromatography.

The use of the original producer strain in production only allows to influence the production rate of Argyrins or the predominant synthesis of one specific Argyrin by altering culture conditions.

US 6833447 describes a nucleic acid sequence which encodes a nitrite reductase.

Sasse et al. in The Journal of Antibiotics 543-551 (2002) describe the production of the cell inhibiting compound termed Argyrin B in an *Archangium* strain. No nucleic acid sequence or amino acid sequences for synthetic pathway enzymes for the production of an Argyrin is given.

Rachid et al. in the Journal of Biotechnology 429-441 (2006) describe that Cytobacter fuscus is a producer of Argyrin. No nucleic acid sequence or amino acid sequences for synthetic pathway enzymes for the production of an Argyrin is given.

Objects of the invention

In view of the limited influence on the production of Argyrin in production methods using cultivation of a natural producer organism, it is an object of the present invention to provide for an alternative production method, and to provide the basis for manipulating the synthetic pathway for the production of Argyrins in micro-organisms, including producer strains and non-producer strains.

General description of the invention

The invention achieves the above-mentioned objects by providing the amino acid sequences comprised in or constituting the synthetic pathway enzymes participating in the production of Argyrins, as well as the nucleic acid sequences encoding the synthetic pathway enzymes participating in the production of Argyrins, as well as genetically manipulated micro-

organisms containing nucleic acid sequences encoding the synthetic pathway enzymes for the production of Argyrins, the use of nucleic acid sequences hybridizing to the nucleic acid sequences encoding synthetic pathway enzymes participating in the production of Argyrins, e.g. for inserting one or more of these coding sequences, mutating in a targeted manner one or more of these coding nucleic acid sequences, in a wild type producer micro-organism or in a heterologous micro-organism, for production of at least one Argyrin. The invention also comprises nucleic acid sequences having a homology of at least 90%, preferably of at least 95%, more preferably of at least 99% to the coding nucleic acid sequences and encoding synthetic pathway enzymes with a catalytic activity essentially corresponding to the catalytic activity of the coding sequences given below, or which have a nucleotide sequence reverse complementary to the coding sequences given below.

The terminology of the invention includes proteins, peptides, and enzymes in respect of catalytically active proteins for amino acid sequences, as well as oligonucleotides, e.g. DNA and/or RNA, also referred to as coding sequences or genes, for nucleic acid sequences, respectively, as equivalent terms. Unless indicated otherwise, nucleic acid sequences are given from 5′ to 3′, and amino acid sequences are given from N-terminus to C-terminus.

Accordingly, in one embodiment of the invention, a micro-organism is provided, which is genetically manipulated to contain nucleic acid sequences encoding synthetic pathway enzymes for the production of Argyrins, and a method for production of the Argyrins comprising the step of cultivating the genetically manipulated micro-organism. Preferably, the genetically manipulated heterologous micro-organism contains one or more expression cassettes containing the nucleic acid sequences encoding synthetic pathway enzymes for the production of Argyrins, which expression cassettes can be monocistronic or polycistronic.

In a second embodiment, the present invention provides the use of the nucleic acid sequences encoding synthetic pathway enzymes for the production of Argyrins for targeted mutation of these nucleic acid sequences encoding the synthetic pathway enzymes within natural producer strains of Argyrins, e.g. for site directed mutagenesis or for example for inserting one or more additional copies of the least one coding sequence within a monocistronic or polycistronic expression cassette, for altering the amino acid sequence encoded by the nucleic acid sequences of the invention, e.g. for changing the enzymatic activity of the synthetic pathway enzymes, or for inactivating one or more coding sequences encoding a synthetic pathway enzyme. The targeted inactivation of at least one coding sequence results in the change of the

synthetic products, e.g. for directing Argyrin synthesis to the preferred production of one or more of Argyrins A to H. Generally, mutations, e.g. insertions, deletions and base exchanges of coding sequences encoding the Argyrins´ synthetic pathway enzymes include the targeted mutation of the coding sequence, i.e. a mutation of the translated nucleic acid sections, as well as targeted mutation of the regulatory nucleic acid sections, e.g. of promoters and/or terminators. Mutations preferably cause for example the inactivation, alteration or increase of the catalytic activity of one or more enzymes, resulting in a change of the synthesis of Argyrins, e.g. in an increased Argyrin production or in the production of a different compound of Argyrins A to H when compared to the non-mutated strain.

In the alternative to or in addition to use of the synthetic pathway enzymes as expression products in genetically manipulated micro-organisms, the synthetic pathway enzymes can be expressed from the respective coding sequences and used for synthesis of Argyrins, e.g. in a production process for Argyrins using the synthetic pathway enzymes in a cell-free reaction composition, e.g. in solution or as immobilized enzymes, e.g. bound to the surface of a carrier. Accordingly, the invention provides the use of the amino acid sequences constituting at least one of the synthetic pathway enzymes for the cell-bound and/or cell-free conversion reaction of chemical compounds, e.g. of an Argyrin precursor compound, to an another precursor compound of Argyrin or at least one of the Argyrins.

Further, the invention also relates to the nucleic acid sequences encoding the synthetic pathway enzymes having activity to catalyse the synthesis of at least one Argyrin, the nucleic acid sequences being in substantially purified form, optionally contained in a synthetic nucleic acid construct suitable for genetic manipulation of at least one micro-organism.

Detailed description of the invention

The invention is described in further detail by way of examples and with reference to the figures, wherein

- Figure 1 schematically shows the arrangement of nucleic acid sequences encoding catalytically active amino acid sequences participating in the synthetic pathway of Argyrins,

- Figure 2 schematically shows the synthetic steps catalysed by amino acid sequences, i.e. enzymes translated from nucleic acids of Figure 1,
- Figure 3 A shows a schematic representation of the targeted mutation of genes encoding synthetic pathway enzymes,
- Figure 3 B shows a gel electrophoresis of PCR products confirming the mutation achieved according to Figure 3 A, and
- Figure 4 shows chromatogramms of HPLC of Argyrins synthesized, namely in A) by the non-mutated wild-type strain, and B) by a mutant obtained according to Figure 3 A.

The invention provides coding sequences, the translation products of which are synthetic pathway enzymes participating in the production of Argyrins, which coding sequences are contained in Seq ID No. 1.

Figure 1 gives a schematic overview of the arrangement of coding sequences which are contained in a wild-type Argyrin producer isolate that was identified as *Cystobacter* sp., termed strain SB-Cb004. Each nucleic acid sequence constituting an orf and encoding a catalytically active protein is indicated as an arrow, the arrow head designating the 3´-end.

From an analysis of catalytic domains encoded by the orfs identified, it is concluded that the genes designated arg2 and arg3 (black arrows) comprise catalytically active domains for synthesis of pre-Argyrin from amino acids, in co-operation with a radical SAM-domain protein encoded by arg1. Accordingly, genes arg2 and arg3, preferably in combination with arg3, encode the core enzymes for synthesis of pre-Argyrin. Adjacent the genes arg1, arg2, and arg3, there are located genes orf1, orf2, orf3, orf4, orf5, orf6, orf7, orf8, orf9, orf10, orf11, orf12, orf13, and orf14, (orfs1-14) which in Figure 1 are designated with their numbers only. Orfs1-14 encode enzymes having catalytic activities catalysing the synthesis of at least one of Argyrins A-H, e.g. from pre-Argyrin or another one of Argyrins A-H as a precursor. The following table gives the genes identified, which participate in the production of the least one Argyrin.

Table: Genes encoding amino acid sequences participating in the synthetic pathway of Argyrins and proposed catalytic activity of the encoded amino acid sequences

gene			encoded protein		
name	localization in Seq.-ID No. 1 (nt number)	GC [%]	size [aa]	amino acid sequence	proposed function (domain arrangement)
orf1	1608-4	66.3	534	Seq.-ID No. 2	ABC transporter
orf2	3615-1687	69.4	642	Seq.-ID No. 3	ABC transporter
orf3	5139-3661	71.1	492	Seq.-ID No. 4	ATP-dependent RNA helicase
orf4	7388-5274	64.3	704	Seq.-ID No. 5	elongation factor G
orf5	7710-8048	72.6	112	Seq.-ID No. 6	
orf6	8870-8043	71.7	275	Seq.-ID No. 7	pseudouridine synthase
orf7	9293-10282	69.2	329	Seq.-ID No. 8	
orf8	11057-10320	72.1	245	Seq.-ID No. 9	RNA methyltransferase
arg1	11545-13593	62.6	682	Seq.-ID No. 10	radical SAM domain protein
arg2	13706-24322	64.8	3538	Seq.-ID No. 11	NRPS loading module and modules 1-2 (A-PCP-E-C-A-PCP-C-A`-MT-A``-PCP)
arg3	24361-42201	66.0	5946	Seq.-ID No. 12	NRPS modules 3-7 (C-A-PCP-HC-A`-Ox-A``-PCP-C-A-PCP-C-A-PCP-C-A-PCP-TE)
arg4	42239-43249	63.7	336	Seq.-ID No. 13	*O*-methyl transferase
arg5	43309-44460	63.5	383	Seq.-ID No. 14	tryptophane 2,3-dioxygenase
orf9	45620-44706	62.7	304	Seq.-ID No. 15	
orf10	46507-45617	59.8	296	Seq.-ID No. 16	
orf11	47244-46504	85.9	246	Seq.-ID No. 17	N6-DNA methylase
orf12	47547-47975	67.8	142	Seq.-ID No. 18	
orf13	48288-49268	69.0	326	Seq.-ID No. 19	
orf14	49483-55209	69.7	1908	Seq.-ID No. 20	large extracellular alpha-helical protein
orf15	55212-55565	61.9	117	Seq.-ID No. 21	

nt = nucleotide; orf = open reading frame; aa = amino acid

From the above coding sequences, arg2 and arg3 are considered as essential for the production of Argyrins, e.g. for synthesis of pre-Argyrin, preferably in connection with one or both of arg4 and arg5, more preferably further in addition with a radical SAM domain protein, preferably encoded by arg1.

The nucleic acid sequences for all genes are contained in Seq.-ID No. 1, wherein the genes are located from 5′ to 3′ and from 3′ to 5′, as indicated in the sequence listing. Further, genes arg1 to arg5 are given in 5′ to 3′, as well as their translation products, i.e. the amino acid sequences of the enzymes Arg1 to Arg5.

Accordingly, the present invention in one aspect relates to isolated nucleic acid sequences encoding synthetic pathway enzymes for the production of the least one Argyrins, which nucleic acid sequences comprise at least coding sequences for Argyrin synthetic pathway enzymes, including or consisting of genes encoding enzymes Arg2 (Seq.-ID No. 11) and arg3 (Seq.-ID No. 12), preferably for enzyme Arg1 (Seq.-ID No. 10), and more preferably nucleic acids coding for at least one of enzymes encoded by at least one of orfs 1 – 14,
a heterologous micro-organism containing nucleic acid sequences encoding at least one Argyrin synthetic pathway enzyme, e.g. introduced into a micro-organism by genetic manipulation, preferably integrated into the genome of a heterologous host micro-organism or integrated by genetic manipulation into the genome of an Argyrin producer micro-organism,
nucleic acid molecules having a sequence complementary to at least one nucleic acid sequence encoding a synthetic pathway enzyme participating in the production of at least one Argyrin,
a nucleic acid molecule capable of hybridizing, especially under stringent conditions, to a nucleic acid molecule encoding at least one Argyrin synthetic pathway enzyme, especially to the sequence of arg2 and arg3, preferably in combination with arg1,
the translation products of which nucleic acid sequences are synthetic pathway enzymes for the production of Argyrins, and/or which translation products have the activity of at least one synthetic pathway enzyme in the production of Argyrins.

Further, the invention relates to micro-organisms containing nucleic acid sequences encoding at least one synthetic pathway enzyme for the production of at least one Argyrin, preferably nucleic acid sequences comprising arg1, arg2, arg3, arg4, more preferably additionally including arg5. Preferably, the micro-organisms are genetically manipulated to contain these nucleic acid sequences for use in the production of Argyrins, preferably for use in the production of pre-Argyrin.

Figure 2 depicts the synthesis of pre-Argyrin by synthetic pathway enzymes of the invention, wherein the following activities are identifiable in domains of enzymes: A = adenylation

domain, PCP = peptidyl carrier protein domain, C = condensation domain, HC = heterocyclization domain, E = epimerization domain, MT = methyl transferase domain, Ox = oxidation domain, and TE = thioesterase domain. However, the arrangement of domains shown in Figure 2 is arbitrary and does not necessarily reflect their arrangement in the enzyme.

The core biosynthetic genes are encoded by arg2 and arg3, which are preferably arranged in one common transcriptional unit with arg1, which encodes a radical SAM protein, and more preferably in combination with arg4 and arg5 which encode a *O*-methyl transferase and a tryptophane 2,3-dioxygenase. In accordance with the natural arrangement of arg2 and arg3 in one transcriptional unit, preferably in combination with arg1, it is preferred that in the nucleic acids of the invention, the coding sequences for arg2 and arg3 are arranged in one transcriptional unit, preferably in combination with arg1 within the same one transcriptional unit. Genes arg4 and arg5 can be contained in the same or a different transcriptional unit.

In detail, Figure 2 shows the assignment of catalytic domains as derived from the sequence of arg2, comprising the load-module, module 1 and module 2, as well as of arg3 comprising module 3, module 4, module 5, module 6, and module 7, which in co-operation catalyse step-wise synthesis of pre-Argyrin. Initially, the PCP-domain of the load-module, the coding sequence of which is contained in arg2, is charged with the initial alanine by the A domain. The synthesis of the Argyrin core structure I is obtainable by the combination of translation products of coding sequences comprising, preferably consisting of arg2, arg3, arg4, preferably including arg5, more preferably further including arg1.

As shown on the example of derivatisation of pre-Argyrine to Argyrin A, the derivatisation, i.e. introduction of substituents R1, R2, R3 and/or R4 to the Argyrin of core structure I is catalysed e.g. by the translation products of one or more of orfs1-15.

Analyses of the enzymes show that the translation product of arg1 (Arg1) catalyses the methylation of Argyrin A to form Argyrin B, that the translation product of arg5 (Arg5) catalyses the hydroxylation of the tryptophane ring, and that the translation product of arg4 (Arg4) catalyses the methylation of the OH-group of the tryptophane ring that was introduced by Arg5.

The catalytic activities of translation products of each of orfs1-15 and of arg1, arg4 and arg5 can be identified according to standard methods, e.g. by comparison of their amino acid sequences to known proteins, or preferably by analysis of reaction products generated in the presence of the translation products using defined substrates as precursor compounds for enzymatic catalysis. In the alternative, the catalytic activities of the translation products can be determined by generating mutant micro-organisms containing the genes encoding the enzymes for Argyrine synthesis, which micro-organisms are genetically manipulated to contain a non-functional copy of one or more of these genes replacing the functional gene copies, and analysing the resultant Argyrins synthesized by the micro-organism. For generating one or more non-functional genes, the respective gene copies in a wild-type Argyrin producer strain can be destroyed, e.g. by insertional site-directed mutagenesis as shown below, or a homologous or heterologous non-producer strain can be provided with the genes encoding the synthetic pathway enzymes but lacking one or more of these genes. Analysis of the resultant Argyrin production can be done by standard methods, e.g. by high-pressure liquid chromatography (HPLC), preferably coupled with a mass-spectrometer.

Example 1: Site-directed mutagenesis of an Argyrin producer and analysis of changes in synthesis of Argyrins

On the basis of nucleic acid sequences of genes encoding synthetic pathway enzymes for Argyrin synthesis, a first oligonucleotide fw1 (5'-CTCGATATCCCAGCGCAAGAGCT ATCG-3', Seq.-ID No. 12; the *Eco*RI restriction site is underlined), and a second oligonucleotide bw1 (5'-CTCGGATCCGGTCGGGAACCATGTACC-3', Seq.-ID No. 13, including a *Bam*HI restriction site, underlined) were constructed and used for amplification of a 1.1 kbp DNA fragment of *arg*3 by PCR (3 min at 95 °C, 30 cycles of 30 s at 95 °C, 50 s at 56 °C, 90 s at 72 °C). The fragment was isolated and ligated into the *Eco*RI and *Bam*HI restriction sites of an *E. coli – Cystobacter* shuttle vector pSUP carrying transposon sections and a kanamycin resistance gene, giving vector pArg, schematically shown in Figure 3A. For conjugational transfer of pArg1, methylation deficient *E. coli* SCS110 harbouring pArg1 and helper plasmid pRK600 for conjugation was grown in LB medium with kanamycin and chloramphenicol (pRK600) to 0.6 OD_{600}. *E. coli* cells were washed and combined with cells of *Cystobacter* cultured in 1 mL M medium under shaking at 30 °C for 30 min, collected and resuspended in M medium and plated on M agar containing 100 µg/mL kanamycin and 120 µg/mL tobramycin. Incubation was at 30 °C until transconjugants appeared, usually after about 3 to 4 days.

Upon conjugational transfer of the vector pArg1 into wild-type isolate Argyrin producer *Cystobacter* sp., integration of the vector into chromosomal DNA was confirmed by PCR on total DNA isolated from different transformants. An electrophoresis gel of PCR amplificates is shown in Figure 3B, namely for total DNA isolated from the wild-type (WT), transformant (Mut.), and negative control (*E. coli*) using primers fw1 and bw1 (indicated as fw1/bw1), primers fw1 and a reverse primer (pSup_B)specific for a section of the original shuttle vector, and primers bw1 and a reverse primer specific for a section of the original shuttle vector (pSup_E).

The analysis by gel electrophoresis shown in Figure 3B demonstrates that the vector was integrated in a site-directed manner within the genomic *arg*3.

For analysis of the effect of the inactivation of *arg*3 by insertional site-directed mutagenesis using the nucleotide sequences of the invention, the production of Argyrins was analysed for the wild-type and for the mutated *Cystobacter* sp. by HPLC. Production of Argyrins was by incubation in M medium in shake flasks in the presence of 2 % adsorber resin XAD for 4 days at 30 °C. Cells and adsorber resin were collected and extracted with methanol, the extract was concentrated 1:50 and analysed by HPLC-MS (reverse phase 125 x 2 mm, 3 µm particle size C18 column Nucleodur, Macherey-Nagel, using a 8 x 3 mm, 5 µm pre-column C18 with diode array detection at 200-600nm, followed by a HCTplus ion trap mass spectrometer, Bruker, positive and negative ionization detection at 100-1100 amu). HPLC was with a liner gradient 5 % B (0.1 % formic acid in water) at 2 min to 95 % B in A (0.1 % formic acid in acetonitrile) by 4 min at 0.4 mL/min. As shown in Figure 4 A, the wild-type culture produced Argyrin A (peak 5), Argyrin B (peak 6), Argyrin D (peak 7), and Argyrins E to H (peaks 1-4, respectively). In contrast to the wild-type, the mutated strain did not produce any of the Argyrins A, B, D-H, demonstrating the effect of this site-directed mutagenesis by the example of disruption of one gene in a site-directed manner by insertional mutagenesis, and the central role of the enzyme encoded by *arg*3 for Argyrin synthesis.

Example 2: Production of Argyrin using an original non-producer strain by expression of genes encoding the pathway enzymes for Argyrin synthesis

For demonstrating the synthesis of Argyrins from the genes encoding the synthetic pathway enzymes, a non-producer micro-organism was provided with the gene cluster comprising the complete synthetic pathway enzymes for Argyrin synthesis including arg1 to arg5 and,

optionally, orfs1-15. For transfer of the genes, Seq.-ID No. 1, which contains all of the genes, was transferred into the host organism of the genus myxobacteria, e.g. *Myxococcus xanthus* (described in Perlova et al., AEM 2006, 72, 7485-7494) by the method according to Pradella et al., *Arch. Microbiol.* 178, 484-492 (2002) using conjugational transfer from *E. coli*, preferably according to the genetic modification system using electroporation of myxobacteria in the presence of a carbohydrate as described in EP 1 619 241 A1.

Generally, production of Argyrins by heterologous expression of the nucleic acid sequences in a host micro-organism was monitored by analytical methods as described in Vollbrecht et al. (loc. cit.), preferably by chromatographic purification of an extract from the fermentation broth, with MS coupling and/or NMR of purified fractions. Using these analyses, the Argyrin derivates synthesized by the micro-organism were identified including changes in product spectra, e.g. indicating preferred or reduced synthesis of a specific Argyrin derivate in the heterologous expression host or in a natural producer micro-organism following genetic manipulation of the synthetic pathway genes.

Alternatively, using the method as e.g. described in Gross et al. (Chemistry and Biology 13, 1253-1264 (2006)), *Pseudomonas* spec. could be used for heterologous expression of the synthetic pathway enzymes of the invention, yielding synthesis of Argyrins. Further, the synthetic pathway enzymes could be expressed in *Pseudomonas putida* by adapting the method of Wenzel et al. (Chemistry and Biology 12, 349-356 (2005)), resulting in Argyrin synthesis.

Cultivation of micro-organisms and analysis of Argyrins was according to Example 1, optionally using SM medium containing 5 g/L asparagine, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 100 mM HEPES, 10 mg/L Fe-EDTA, 0.5 g/L $CaCl_2$, 0.06 g/L K_2HPO_4, 10 g/L maltose, pH 7.2, instead of M medium (1.0 % soy tryptone, 1.0 % maltose, 0.1 % $CaCl_2$, 0.1 % $MgSO_4 \cdot 7H_2O$, 50 mM HEPES and 8 mg/L Na-Fe-EDTA, adjusted to pH 7.2).

The wild-type strain without genetic modification did not produce any detectable amount of Argyrins, whereas the transformant produced pre-Argyrin, Argyrin A and Argyrin B, with detectable levels of Argyrins D-H.

The product spectrum of Argyrins could be altered by transformation with a nucleic acid containing at least arg1, arg2 and arg3 with one or more of arg4, arg5, and of orf 1 to orf 15.

Claims

1. Synthetic pathway enzymes for the production of Argyrins, comprising the amino acid sequences of Arg2 of Seq.-ID No. 8 and Arg3 of Seq.-ID No. 9.

2. The synthetic pathway enzymes according to claim 1, further comprising at least one of the group consisting of Arg1 of Seq.-ID No. 7, Arg4 of Seq.-ID No. 10 and Arg5 of Seq.-ID No. 11.

3. The synthetic pathway enzymes according to claim 1 or 2, further comprising at least one protein of the group of proteins encoded by orf1 of nucleotides 1608 to 4 of Seq.-ID No. 1, orf2 of nucleotides 3615 to 1687 of Seq.-ID No. 1, orf3 of nucleotides 5139 to 3661 of Seq.-ID No. 1, orf4 of nucleotides 7388 to 5274 of Seq.-ID No.1, orf5 of nucleotides 7710 to 8048 of Seq.-ID No.1, orf6 of nucleotides 8870 to 8043 of Seq.-ID No.1, orf7 of nucleotides 9293 to 10282 of Seq.-ID No.1, orf8 of nucleotides 11057 to 10320 of Seq.-ID No.1, orf9 of nucleotides 45620 to 44706 of Seq.-ID No.1, orf10 of nucleotides 46507 to 45617 of Seq.-ID No.1, orf11 of nucleotides 47244 to 46504 of Seq.-ID No. 1, orf12 of nucleotides 47547 to 47975 of Seq.-ID No.1, orf13 of nucleotides 48288 to 49268 of Seq.-ID No.1, orf14 of nucleotides 49483 to 55209 of Seq.-ID No.1, and orf15 of nucleotides 55212 to 55565 of Seq.-ID No. 1.

4. A nucleic acid molecule encoding an amino acid sequence having enzymatic activity of a synthetic pathway enzyme of any one of claims 1-3, wherein the nucleic acid molecule comprises the nucleotide sequence arg2 of Seq.-ID No. 3 and arg3 of Seq.-ID No. 4.

5. The nucleic acid molecule according to claim 4, having a nucleic acid sequence having at least 90% sequence identity to the nucleotide sequence arg2 of Seq.-ID No. 3 and arg3 of Seq.-ID No. 4, and/or by having a nucleotide sequence of at least 90% sequence identity to a nucleotide sequence which is reverse complementary to the nucleotide sequence arg2 of Seq.-ID No. 3 and arg3 of Seq.-ID No. 4, the nucleic acid sequence encoding proteins having the same biological activity as arg2 of Seq.-ID No. 3 and arg3 of Seq.-ID No. 4.

6. The nucleic acid molecule according to claim 4 or 5, further comprising arg1 of Seq.-ID No. 2, arg4 of Seq.-ID No. 5 and/or arg5 of Seq.-ID No. 6.

7. The nucleic acid molecule according to any one of claims 4 to 6, further comprising at least one nucleic acid sequence from the group comprising orf1 of nucleotides 1608 to 4 of Seq.-ID No. 1, orf2 of nucleotides 3615 to 1687 of Seq.-ID No. 1, orf3 of nucleotides 5139 to 3661 of Seq.-ID No. 1, orf4 of nucleotides 7388 to 5274 of Seq.-ID No.1, orf5 of nucleotides 7710 to 8048 of Seq.-ID No.1, orf6 of nucleotides 8870 to 8043 of Seq.-ID No.1, orf7 of nucleotides 9293 to 10282 of Seq.-ID No.1, orf8 of nucleotides 11057 to 10320 of Seq.-ID No.1, orf9 of nucleotides 45620 to 44706 of Seq.-ID No.1, orf10 of nucleotides 46507 to 45617 of Seq.-ID No.1, orf11 of nucleotides 47244 to 46504 of Seq.-ID No. 1, orf12 of nucleotides 47547 to 47975 of Seq.-ID No.1, orf13 of nucleotides 48288 to 49268 of Seq.-ID No.1, orf14 of nucleotides 49483 to 55209 of Seq.-ID No.1, and orf15 of nucleotides 55212 to 55565 of Seq.-ID No. 1.

8. A micro-organism, which is genetically manipulated to contain a nucleic acid molecule of any one of claims 4 to 7.

9. The micro-organism according to claim 8, wherein the micro-organism is the product of a genetic manipulation which includes the step of providing the micro-organism with nucleic acid sequences encoding pathway enzymes Arg1 of Seq.-ID No. 7, Arg2 of Seq.-ID No. 8, Arg3 of Seq.-ID No. 9, and Arg4 of Seq.-ID No. 10 for use in the synthesis of Argyrins.

10. A process for the production of Argyrins, using synthetic pathway enzymes, wherein the enzyme is contained in a micro-organism that has been genetically altered to contain the synthetic pathway enzymes of any one of claims 1 to 3.

11. The process according to claim 10, wherein the enzyme is used in a cell - free form for conversion of a precursor compound to a compound selected from the group comprising pre-Argyrin and Argyrins A to H.

Figures

Figure 1:

Figure 3:

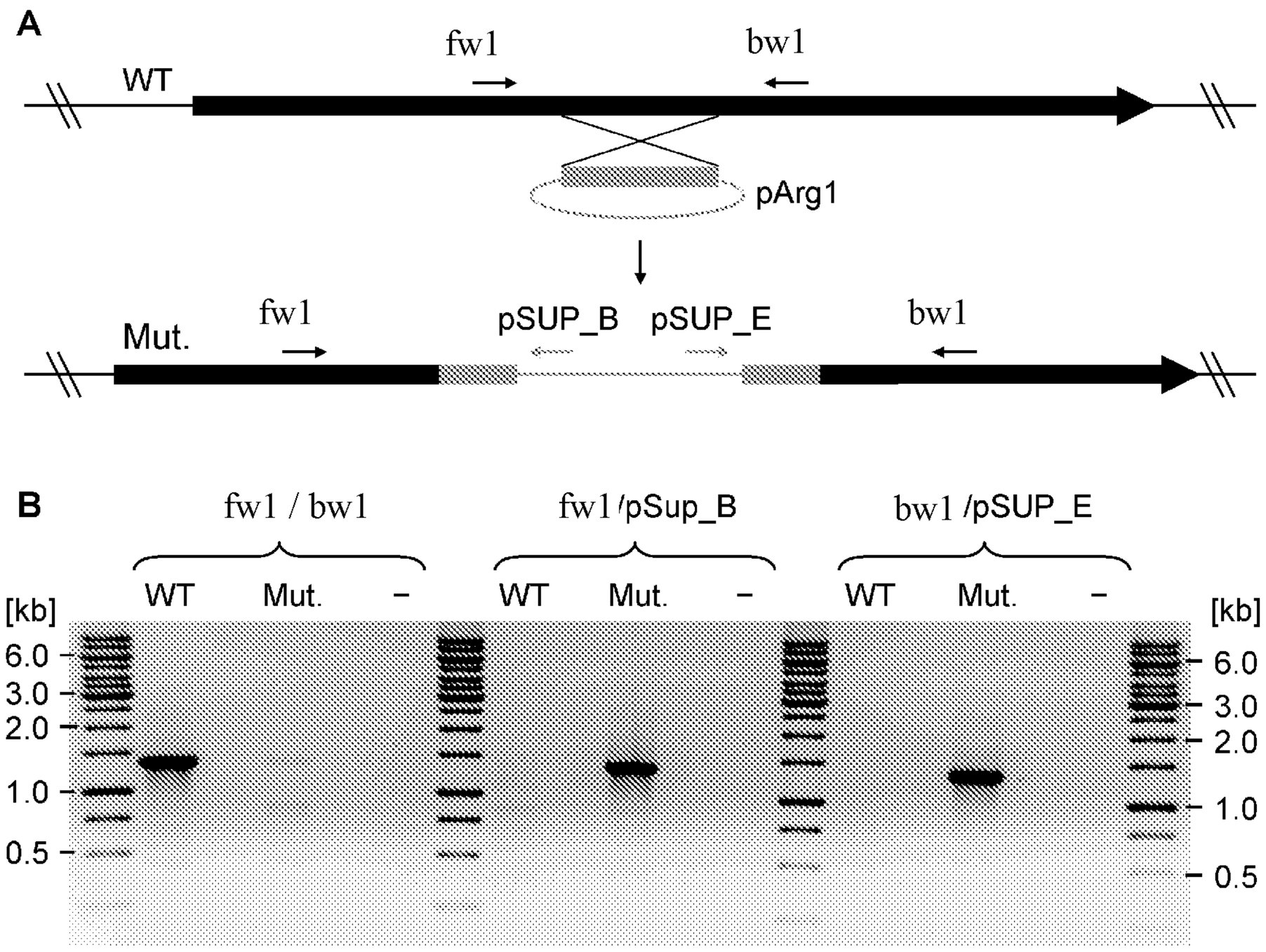


Figure 2:

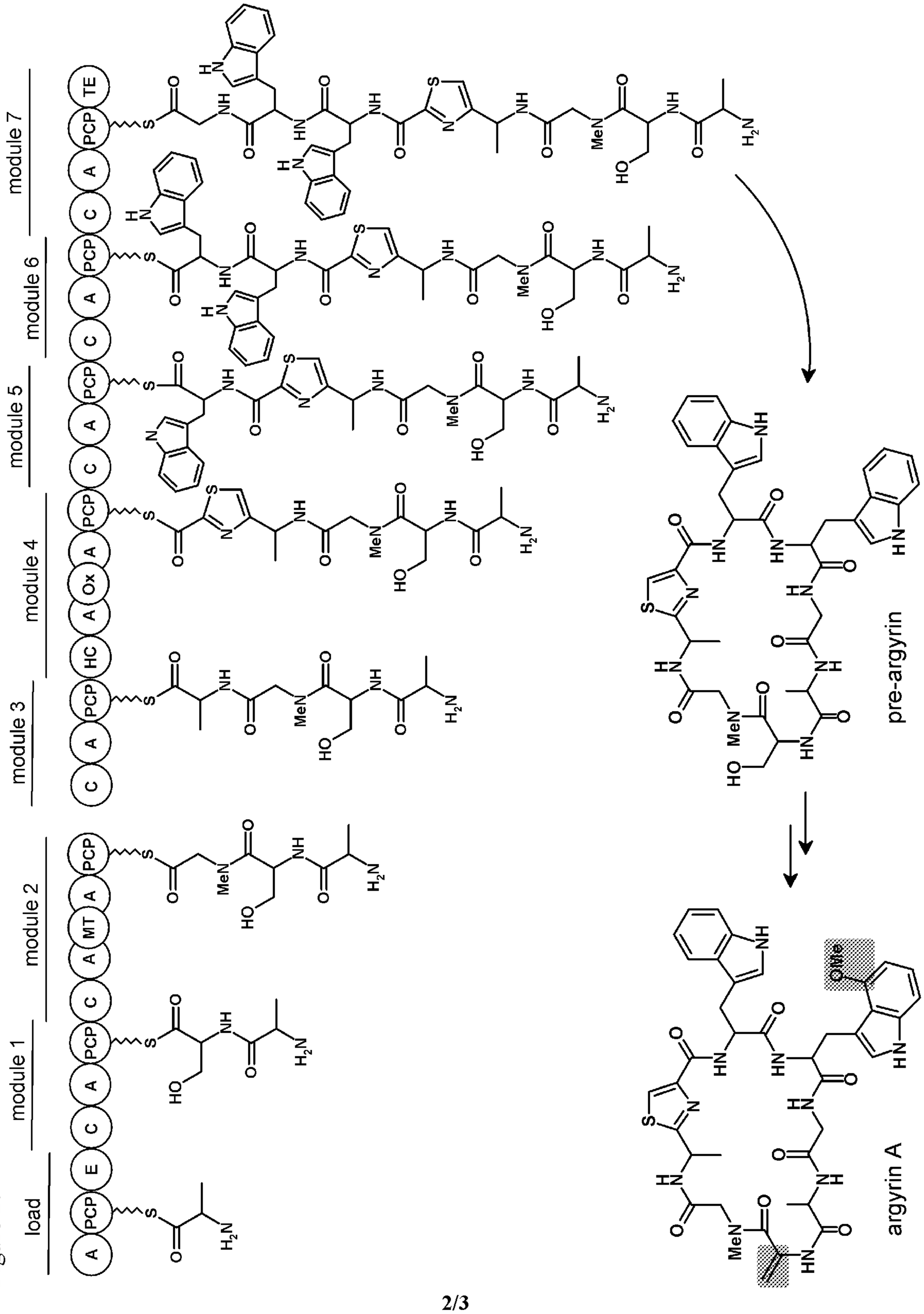
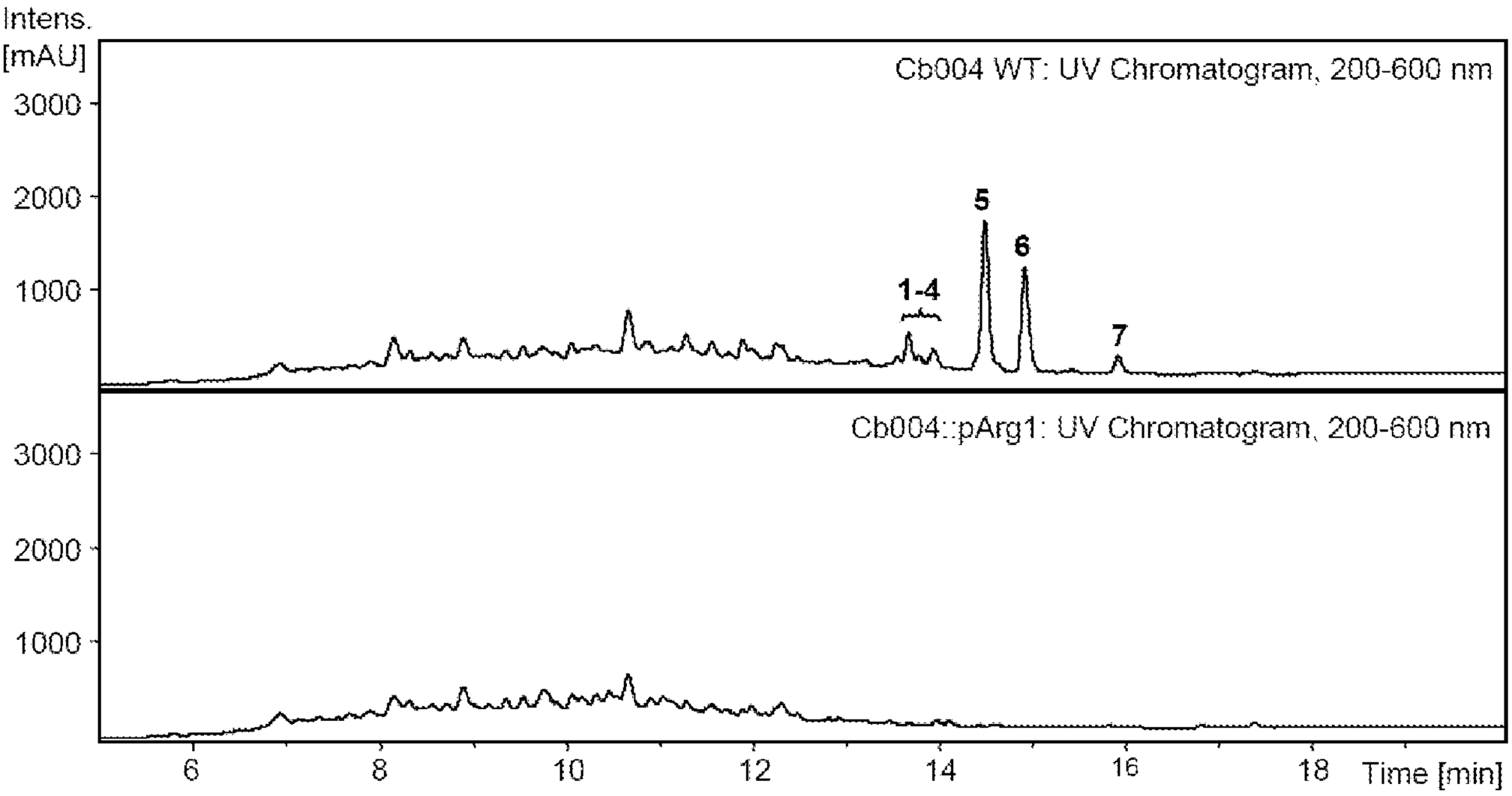

load
module 1
module 2
module 3
module 4
module 5
module 6
module 7
A PCP E C
A PCP MT
C A PCP HC
A Ox PCP C
C A PCP C
A PCP C
A PCP TE
MeN
HO
H_2N
pre-argyrin
argyrin A
OMe

Figure 4:


Intens.
[mAU]
Cb004 WT: UV Chromatogram, 200-600 nm
3000
2000
1000
1-4
5
6
7
Cb004::pArg1: UV Chromatogram, 200-600 nm
3000
2000
1000
6
8
10
12
14
16
18
Time [min]