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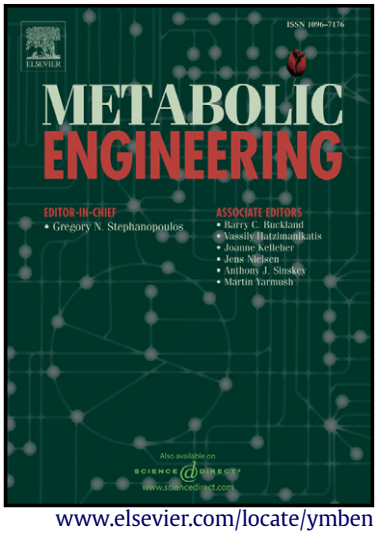

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Engineered production of fungal anticancer cyclooligomer depsipeptides in *Saccharomyces cerevisiae*

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ABSTRACT

Two fungal cyclooligomer depsipeptide synthetases (CODSs), BbBEAS (352 kDa) and BbBSLS (348 kDa) from *Beauveria bassiana* ATCC 7159, were reconstituted in *Saccharomyces cerevisiae* BJ5464-NpgA, leading to the production of the corresponding anticancer natural products, beauvericins and bassianolide, respectively. The titers of beauvericins (33.82±1.41 mg/l) and bassianolide (21.74±0.08 mg/l) in the engineered *S. cerevisiae* BJ5464-NpgA strains were comparable to those in the native producer *B. bassiana*. Feeding D-hydroxyisovaleric acid

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(D-Hiv) and the corresponding L-amino acid precursors improved the production of beauvericins and bassianolide. However, the high price of D-Hiv limits its application in large-scale production of these cyclooligomer depsipeptides. Alternatively, we engineered another enzyme, ketoisovalerate reductase (KIVR) from *B. bassiana*, into *S. cerevisiae* BJ5464-NpgA for enhanced *in situ* synthesis of this expensive substrate. Co-expression of BbBEAS and KIVR in the yeast led to significant improvement of the production of beauvericins. The total titer of beauvericin and its congeners (beauvericins A, B and C) was increased to 61.73±2.96 mg/l and reached 2.6-fold of that in the native producer *B. bassiana* ATCC 7159. Supplement of L-Val at 10 mM improved the supply of ketoisovalerate, the substrate of KIVR, which consequently further increased the total titer of beauvericins to 105.76±2.12 mg/l. Using this yeast system, we functionally characterized an unknown CODS from *Fusarium venenatum* NRRL 26139 as a beauvericin synthetase, which was named as FvBEAS. Our work thus provides a useful approach for functional reconstitution and engineering of fungal CODSs for efficient production of this family of anticancer molecules.

Keywords:

Fungal cyclooligomer depsipeptide synthetases

Beauvericins

Bassianolide

Ketoisovalerate reductase

Heterologous expression

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1. Introduction

Nonribosomal peptides represent an important family of natural products, including antimicrobials, anticancer agents, immunosuppressants, siderophores, herbicides, antifungals, insecticides, and anthelminthics. They are biosynthesized by giant modular enzymes called nonribosomal peptide synthetases (NRPSs) (Fischbach and Walsh, 2006; Koglin and Walsh, 2009). The assembly of the peptides is well programmed and controlled by the dedicated enzymes. Cyclooligomer depsipeptides (CODs) are a particular group of nonribosomal peptides often found in fungi as mycotoxins, such as beauvercins (**1** and **3**-**5**, Fig. 1), bassianolide (**2**, Fig. 1) and enniatins. **1** is produced by a number of fungal strains such as *Fusarium* species, *Beauveria bassiana*, *Paecilomyces fumoso-roseus* (renamed *Isaria fumosorosea*), and *P. tenuipes* (renamed *I. tenuipes*) (Gupta et al., 1995; Molnar et al., 2010; Moretti et al., 2007; Nilanonta et al., 2002). It is a cyclic trimer of a dipeptidol monomer synthesized from *N*-methyl-L-phenylalanine and D-hydroxyisovaleric acid (D-Hiv). Its congeners, beauvericins A-C (**3**-**5**), were also observed as minor products in many producing strains. **2** was isolated from *B. bassiana* (Xu et al., 2008), *Lecanicillium sp.* (Molnar et al., 2010), and the wood-decaying *Xylaria* sp. BCC1067 (Jirakkakul et al., 2008). It is an octadepsipeptide that is assembled as the cyclic tetrameric ester of the dipeptidol monomer D-Hiv-*N*-Methyl-L-leucine. Fungal CODs have displayed a wide variety of bioactivities, including antibiotic, insecticidal, anthelminthic, herbicidal, antiretroviral, cytotoxic, anti-haptotactic, anti-cholesterol, and chemosensitizer activities, as well as repression of amyloid plaque formation in Alzheimer's disease (Suessmuth et al., 2011). In addition, recent studies have shown that some fungal CODs are novel anticancer

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agents (Ivanova et al., 2006; Lee et al., 2008). **1** has displayed potent anticancer activity against various cell lines by increasing cytoplasmic Ca^{2+} concentration, triggering ATP depletion, and activating calcium-sensitive cell apoptosis (Chen et al., 2006; Jow et al., 2004; Lin et al., 2005). Recently, we have found that **1** can also inhibit migration of the metastatic prostate cancer (PC-3M) and breast cancer (MDA-MB-231) cells, and has antiangiogenic activity in HUVEC-2 cells at sublethal concentrations (Zhan et al., 2007). Inhibition of angiogenesis is an effective cancer chemotherapy strategy, and thus **1** may be a useful anticancer agent that restrains new tumor formation or increases successful containment of solid tumors. **2** was also shown to have moderate cytotoxic activity against different cancer cell lines (Jirakkakul et al., 2008). Therefore, these fungal natural products represent promising anticancer drug leads.

Fungal CODs are assembled by COD synthetases (CODSs), a subclass of Type B NRPSs which use their modules iteratively for the biosynthesis of several copies of identical or nearly identical peptide/peptidol monomer units that remain covalently bound on the enzyme. The monomer units of CODs undergo recursive head-to-tail condensation, or oligomerization, followed by macrocyclization (Du and Lou, 2010). Typically, the monomers are formed from one unit of 2-hydroxycarboxylic acid and one molecule of amino acid. Two building blocks of **1** are D-Hiv and L-phenylalanine (L-Phe), while **2** is biosynthesized from D-Hiv and L-leucine (L-Leu). We have previously used a precursor-directed biosynthesis approach to generate a series of new beauvericin analogs **6**-**11** (Fig. 1) by feeding 2-hydroxycarboxylic acids and amino acids into the fermentation broth of *B. bassiana*. The fluorine-containing products showed increased cytotoxicity while maintaining comparable antihaptotactic activity (Xu et al., 2007). These

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results suggested that the dedicated CODS has relaxed substrate specificity and may represent an interesting target for engineered biosynthesis of new molecules.

Both the biosynthetic gene clusters of **1** and **2** have recently been characterized from *B. bassiana* ATCC 7159 (Xu et al., 2009a; Xu et al., 2008). Analysis of the gene clusters revealed that the beauvericin synthetase (BbBEAS) and the bassianolide synthetase (BbBSLS) have the same domain organization of C_1-A_1-T_1-C_2-A_2-MT-T_{2a}-T_{2b}-C_3 (Fig. 2). In addition to the CODS gene, a *kivr* gene is found in the beauvericin biosynthetic gene cluster, which is proposed to be involved in the biosynthesis of D-Hiv (Xu et al., 2008). The detailed mechanism of these CODSs is not fully understood and requires more studies to demonstrate.

Heterologous expression of CODSs in strains that are easier to manipulate genetically than the native fungal producers will facilitate enzymatic characterization of CODSs and their engineered variants, enable efficient production of these anticancer agents, and promote combinatorial biosynthesis of novel CODs. BbBEAS has been functionally expressed in *Escherichia coli*, but the best beauvericin titer was achieved at only 8 mg/l in *E. coli* when D-Hiv and L-Phe was supplemented (Xu et al., 2008). This represents less than 40% of the titer of the native producer *B. bassiana*, suggesting that *E. coli* is not an efficient host for the expression and functional reconstitution of the giant CODS. On the other hand, *Saccharomyces cerevisiae* is a fungus and may represent a better system for engineering of fungal CODSs. *S. cerevisiae* BJ5464-NpgA is an engineered yeast strain containing a chromosomal copy of the 4'-phosphopantetheinyl transferase (PPTase) gene *npgA* from *Aspergillus nidulans* (Mootza et al., 2002). PPTases catalyze the 4′-phosphopantetheinylation of serine residues within T domains

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using coenzyme A as the 4′-phosphopantetheine donor to activate NRPSs (Mootza et al., 2002; Müller et al., 2012). Thus, it is essential to have a PPTase such as NpgA for functional expression of NRPSs in the yeast. *S. cerevisiae* BJ5464-NpgA has been used for functional reconstitution of giant enzymes such as the lovastatin nonaketide synthase, a highly reducing iterative polyketide synthase (PKS) (Ma et al., 2009). In the present study, we successfully engineered BbBEAS and BbBSLS into *S. cerevisiae* BJ5464-NpgA to reconstitute the production of **1**-**5**. Supplement of the direct biosynthetic precursors (D-Hiv and L-Phe/L-Leu) improved the production of these CODs in the yeast. Furthermore, engineering of KIVR into the strain further enhanced the production of beauvericins, and improved supply of the substrate of KIVR by feeding L-Val led to an unprecedented high titer of beauvericins. Using this expression system, we also characterized a new beauvericin synthetase, named FvBEAS, from *Fusarium venenatum* NRRL 26139.

2. Materials and methods

2.1. Strains, plasmids, and culture conditions

B. bassiana ATCC 7159 was obtained from the American Type Culture Collection (ATCC), maintained on potato dextrose agar (PDA), and fermented in potato dextrose broth (PDB) for the production of **1** and **2**. Fermentation of *B. bassiana* ATCC 7159 was carried out in 250-ml flasks containing 100 ml of PDB on a rotary shaker at 250 rpm and 30°C for 4 days. *F. venenatum* NRRL 26139 was obtained from the Agricultural Research Service Culture Collection (NRRL), and fermented in DPY medium (20 g/l dextrin, 10 g/l multipeptone, 5 g/l yeast extract, 5 g/l monopotassium phosphate, 0.5 g/l magnesium sulfate) at 30°C for 6-8 days to produce **1**. *E. coli*

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XL1-Blue (Agilent) was used for routine cloning and pJET1.2 (Fermentas) was used as the cloning vector. *E. coli* cells were grown in Luria-Bertani (LB) medium. When necessary, 50 μg/ml of ampicillin was added. *S. cerevisiae* BJ5464-NpgA (*MATα ura3-52 his3-Δ200 leu2- Δ1 trp1 pep4::HIS3 prb1 Δ1.6R can1 GAL*) was obtained from Dr. Nancy Da Silva at the University of California, Irvine. The plasmids pXW55 and pXW06 were gifts from Dr. Yi Tang at the University of California, Los Angeles. *S. cerevisiae* BJ5464-NpgA was maintained on YPD (yeast extract/peptone/dextrose) agar plates. The plasmid pXW55 carries the ampicillin-resistance gene and *URA3* as the selection markers and was used as an *E. coli/S. cerevisiae* shuttle vector to express BbBEAS, BbBSLS and FvBEAS. The plasmid pXW06 carries the ampicillin-resistance gene and *TRP1* as the selection markers and was used as an *E. coli/S. cerevisiae* shuttle vector to express KIVR.

2.2. DNA manipulations

Genomic DNAs were extracted from *B. bassiana* ATCC 7159 and *F. venenatum* NRRL 26139 and used as the templates for gene amplification. Plasmids were isolated from *E. coli* using a GeneJET™ Plasmid Miniprep Kit (Fermentas). Gene ligations were performed with T4 DNA ligase (New England Biolabs).

2.3. PCR amplification and plasmid construction

The total size of *bbBeas* is 9,570 bp (GenBank accession number EU886196), and *bbBsls* contains 9,441 bp (GenBank accession number FJ439897). The gene fragments *bbBeas-a* (7.3 kb), *bbBeas-b* (2.3 kb), *bbBsls-a* (6.9 kb), *bbBsls-b* (2.6 kb), and *bbkivr* (1,389 bp, GenBank accession number EU886196) were amplified by PCR from the genome of *B. bassiana* ATCC

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7159 with Phusion® High-Fidelity DNA Polymerase (New England Biolabs) using specific primers (Table 1). The *fvBeas* gene contains 9,390 bp and was deposited in GenBank under accession number JX975482. Similarly, two fragments of this gene, *fvBeas-a* (3.4 kb) and *fvBeas-b* (6.0 kb), were amplified from the *F. venenatum* NRRL 26139 genome. These gene fragments were ligated into the cloning vector pJET1.2 to yield seven plasmids including pJCZ47, pJCZ48, pJCZ49, pJCZ50, pDY47, pDY55 and pDY56 (Table 2). These plasmids were confirmed by digestion checks and gene sequencing.

The *bbBeas-b* insert was excised from pJCZ48 with *Nhe*I and *Pml*I and ligated into pXW55 between the same sites to generate pDY35, and then *bbBeas-a* was excised from pJCZ49 with *Nhe*I and inserted into pDY35 at the same site to generate pDY37 (Table 2 and Fig. S1). The *bbBsls-b* insert was excised from pJCZ47 with *Nde*I and *Pml*I and ligated into pXW55 between the same sites to generate pDY36. The *bbBsls-a* insert was excised from pJCZ50 with *Nde*I and ligated into pDY36 at the same site to yield pDY42 (Table 2). The *fvBeas-b* insert was excised from pDY56 with *Nde*I and *Pml*I and ligated into pXW55 between the same sites to generate pDY57, and then the *fvBeas-a* insert was excised from pDY55 with *Nde*I and ligated into pDY57 at the same site to generate pDY59 (Table 2). The *bbkivr* insert was excised from pDY47 with *Nde*I and *Pme*I and ligated into pXW06 between the same sites to generate pDY48 (Table 2 and Fig. S2).

2.4. Transformation and fermentation of S. cerevisiae BJ5464-NpgA

Transformation of plasmid pDY37, pDY42 or pDY59 and co-transformation of pDY37 and pDY48 into *S. cerevisiae* BJ5464-NpgA competent cells, as well as the selection of correct

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transformants were conducted as previously described (Gietz and Schiestl, 2007). The engineered *S. cerevisiae* strain carrying pDY37, pDY42 or pDY59 was fermented in a SC-Ura dropout medium (6.7 g/l yeast nitrogen base; 20 g/l glucose; 0.77 g/l -Ura DO supplement) at 30°C with shaking at 250 rpm until the OD_{600} reached 0.6, and then equal volume of YP medium (10 g/l yeast extract; 20 g/l peptone) with or without extra substrates was added. The cultures were maintained under the same conditions for an additional 72 h to produce the CODs. *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48 was fermented in a SC-Ura-Trp dropout medium (6.7 g/l yeast nitrogen base; 20 g/l glucose; 0.72 g/l -Trp/-Ura DO supplement) at 30°C with shaking at 250 rpm until the OD_{600} reached 0.6, and then equal volume of YP medium with or without extra substrates was added. The fermentation broths were cultured under the same conditions for an additional 72 h to produce beauvericins. *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48 was also grown at 20°C to compare the titer with that at 30°C.

2.5. Extraction, isolation, and analysis of metabolites in the wild type fungal strains and engineered S. cerevisiae strains

The culture of *B. bassiana* ATCC 7159 was filtered through Whatman No. 1 filter paper. The filtrate was extracted three times with the same volume of ethyl acetate. The mycelium cake from 100 ml of fermentation broth was extracted twice with 50 ml of methanol. The extracts were concentrated under reduced pressure and dissolved in 500 µl of methanol for LC-MS analysis. Extraction of the culture of *F. venenatum* NRRL 26139 was performed using the same method.

To purify **1** and **2** for establishing the standard curves, fermentation of *B. bassiana* ATCC

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7159 was carried out in a 2-l flask containing 500 ml of PDB on a rotary shaker at 250 rpm and 30°C for 4 days. The fermentation broth was then extracted as described above. A small portion (10%) of the extract was separated on an Agilent 1200 HPLC using an Agilent Ecilpse XDB-C18 column (5 μm, 4.6 mm × 150 mm column), washed with a gradient of methanol-water (80% to 100% over 20 min) at a flow rate of 1 ml/min. The peaks at 9.6 min and 12.6 min were collected to yield 1.7 mg of **1** and 1.2 mg of **2**. These two samples were dissolved in 500 μl of methanol, which was serially diluted to obtain different concentrations. For each concentration, 100 μl of these samples were injected into the HPLC to obtain the peak areas to establish standard curves for **1** and **2**. Compounds **1** and **3**-**5** were purified using the same HPLC method from the fermentation broth of *S. cerevisiae* BJ5464-NpgA/pDY37 for structural characterization. Standard curves based on the linear relationship between the amounts and the peak areas were established for **3**-**5** for quantification of their production in different engineered strains. Similarly, **2** was isolated from *S. cerevisiae* BJ5464-NpgA/pDY42 for structural characterization. NMR spectra were acquired on a JEOL instrument (300 MHz).

To analyze the products in the engineered *S. cerevisiae* BJ5464-NpgA strains, 100 ml of fermentation broth was centrifuged at 2,737 ×g for 5 min to pellet the cells. The supernatant was extracted three times with the same volume of ethyl acetate, and the cells were extracted twice with 50 ml of methanol. The extracts were evaporated *in vacuo* to remove the solvents. The residues were then dissolved in 500 μl of methanol and analyzed on an Agilent 6130 Single Quad LC-MS, eluted with a gradient of methanol-water (80% to 100% over 20 min) at a flow rate of 1 ml/min and detected at 210 nm.

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2.6. Extraction and analysis of the production of D-Hiv in S. cerevisiae BJ5464-NpgA/pDY37 and S. cerevisiae BJ5464-NpgA/pDY37+pDY48

S. cerevisiae BJ5464-NpgA/pDY37 and *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48 were grown and harvested as described above. For each strain, the concentrations of D-Hiv in the cells and supernatants were measured separately 24 and 48 h after the addition of YP medium. Experiments were conducted in triplicate. The cells were extracted by 50 ml of methanol twice and the supernatant was extracted by 100 ml of ethyl acetate for three times. The extracts were dried *in vacuo* and re-dissolved in 500 μl of methanol for HPLC analysis. The samples were analyzed by HPLC at 210 nm, eluted with 15% methanol-water containing 0.1% formic acid for 15 min. The retention time of D-Hiv is 11.9 min. A standard curve of D-Hiv (VWR) was established to quantify the concentration of this compound in the cells and supernatants.

3. Results

3.1. Reconstitution of BbBEAS and BbBSLS in S. cerevisiae BJ5464-NpgA

The expression plasmids pDY37 and pDY42, which carry *bbBeas* and *bbBsls*, respectively, were introduced into *S. cerevisiae* BJ5464-NpgA and the engineered strains were fermented to reconstitute the biosynthesis of **1** and **2**, respectively. As shown in Fig. 3a, HPLC analysis of *S. cerevisiae* BJ5464-NpgA/pDY37 indicated that peak **1** at 9.6 min was produced as a major product. The retention time and UV spectrum of **1**were the same as those for beauvericin produced by the original producing strain, *B. bassiana* ATCC 7159. ESI-MS (Fig. 3b) showed a series of quasimolecular ion peaks, including $[M+H]^+$ at *m/z* 784.1, $[M+NH_4]^+$ at *m/z* 801.4, $[M+Na]^+$ at *m/z* 806.3 and $[M+K]^+$ at *m/z* 822.3, which is consistent with the molecular weight of

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beauvericin (Fig. S3a). Three analogs **3**-**5** were also produced. ESI-MS analysis of these compounds (Fig. 3d-f) showed that their molecular weights were 797, 811 and 825, respectively, same as those of beauvericins A, B and C produced by the original host *B. bassiana* (Fig. S3c-e). We purified these four products from *S. cerevisiae* BJ5464-NpgA/pDY37 for structural analysis (Fig. S4). The NMR data of these compounds (Supplementary Materials, Fig. S5 and S6) are in agreement with those reported for beauvericin(Gupta et al., 1995; Zhan et al., 2007), beauvericin A (Gupta et al., 1995), beauvericin B (Gupta et al., 1995), and beauvericin C (Nilanonta et al., 2002), which confirmed the successful reconstitution of beauvericins in the yeast. Similarly, peak **2** at 12.6 min in trace (iii) of Fig. 3a was found to be a major product in *S. cerevisiae* BJ5464-NpgA/pDY42. ESI-MS analysis (Fig. 3c) revealed that it has the same molecular weight as bassianolide produced by *B. bassiana* (Fig. S3b). This compound was then purified from *S. cerevisiae* BJ5464-NpgA/pDY42 (Fig. S4) and subjected to NMR analysis. A comparison of the NMR data of **2** (Supplementary Materials) with the literature (Jirakkakul et al., 2008; Suzuki et al., 1977) confirmed that this compound is bassianolide. The titers of these fungal CODs were determined and are shown in Table 3. The titers of **1** and **2** were close to those in the native producer *B. bassiana*, which were 22.26±1.48 and 18.19±0.62 mg/l, respectively.

3.2. Improvement of the production of fungal CODs in S. cerevisiae by supplement of the biosynthetic precursors

D-Hiv and L-amino acid are essential substrates for the synthesis of **1**-**5** (Suessmuth et al., 2011). It is apparent that *S. cerevisiae* can provide these biosynthetic precursors since we have been able to reconstitute the production of **1**-**5** in this yeast by only expressing the corresponding

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CODSs. However, the supply of these precursors may not be sufficient to allow these heterologous CODSs to work at their full catalytic capacity. Thus, it is possible that supplement of the direct biosynthetic precursors will improve the titers of these compounds. To test the effects of addition of D-Hiv and L-Phe on the production of beauvericins, the fermentation broth of *S. cerevisiae* BJ5464-NpgA/pDY37 was fed with these two precursors at two different final concentrations, 0.8 and 2.0 mM. As shown in Table 3, at these two concentrations, the titer of **1** was increased to 29.69±1.91 and 36.08±3.44 mg/l, respectively. In contrast, the production of **3**-**5** was negatively influenced. While the titer of **3** was slightly decreased, the biosynthesis of **4** and **5** was completely not detectable at 2 mM of D-Hiv and L-Phe. The overall titer of all these beauvericins was increased from 33.82±1.41 mg/l (without supplemented precursors) to 38.54±0.64 mg/l (0.8 mM precursors) and 42.25±1.35 mg/l (2.0 mM precursors). Similarly, the fermentation broth of *S. cerevisiae* BJ5464-NpgA/pDY42 was fed with D-Hiv and L-Leu at 0.8 and 2.0 mM, which led to the obvious increase in the production of **2** as shown in Table 3.

3.3. Effects of co-expression of KIVR on beauvericin biosynthesis in S. cerevisiae

Although supplement of D-Hiv and L-amino acids apparently increased the titers of **1**-**5** in the engineered *S. cerevisiae* strains, the high cost of D-Hiv may hamper its use in large-scale production of these anticancer compounds. Thus, an improved *in situ* synthesis of D-Hiv in the yeast represents an attractive approach for enhanced biosynthesis of fungal CODs. Fungi biosynthesize D-Hiv by reducing the appropriate free ketocarboxylic acids, derived from amino acid catabolic and anabolic pathways. The beauvericin biosynthetic gene cluster in *B. bassiana* was found to contain a *kivr* gene encoding a putative reductase with a GxGxxGxxxA

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NAD(P)H-binding signature, which is highly similar to COG1893 ketopantoate reductases (Xu et al., 2009b). No similar gene was clustered with the *bbBsls* gene in the same strain. Thus, KIVR was proposed to supply D-Hiv for the biosynthesis of both **1** and **2**. This was confirmed by disruption of *kivr* in the genome of *B. bassiana*, which abolished the production of both **1** and **2** in the fungus. Chemical complementation of the mutant by supplementing the fermentation medium with D-Hiv restored the production of both CODs (Xu et al., 2009b). Thus, KIVR is the dedicated enzyme that synthesizes D-Hiv in *B. bassiana* for COD biosynthesis.

We next examined whether co-expression of KIVR with CODSs in *S. cerevisiae* BJ5464-NpgA will increase the titers of these CODs. The *kivr* gene was cloned into pXW06, and the resulting plasmid pDY48 was co-expressed in *S. cerevisiae* BJ5464-NpgA with pDY37. The engineered strain, *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48, thus contains a two-enzyme system composed of BbBEAS and KIVR. LC-MS analysis of the products revealed the production of **1** and its congeners **3**-**5**. Compared to *S. cerevisiae* BJ5464-NpgA/pDY37, this double transformant produced the same compounds except that the ratio of the products was different (Fig. S7). While **1** was the major product in *S. cerevisiae* BJ5464-NpgA/pDY37, **3** was found to be the most abundant product in *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48.

The titers of **1** and **3**-**5** in *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48 were measured. As shown in Table 4, although the titer of **1** was slightly lower than that in *S. cerevisiae* BJ5464-NpgA/pDY37 (Table 3), co-expression of KIVR and BbBEAS enhanced the production of **3**-**5** more than 3-fold. The total titer of beauvericins in *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48 reached 61.73±2.96 mg/l, a 2-fold increase compared to that in *S.*

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cerevisiae BJ5464-NpgA/pDY37. We also measured the production of D-Hiv in the yeast strains with and without KIVR. As shown in Table 5, D-Hiv was detected in both cells and supernatants and the production of D-Hiv in *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48 was higher than in *S. cerevisiae* BJ5464-NpgA/pDY37, further confirming that the expression of KIVR has enhanced D-Hiv biosynthesis in the yeast.

A previous study on the production of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine in *S. cerevisiae* reported that a 30-fold enhancement of the product titer was achieved by lowering the cultivation temperature from 30°C to 20°C (Siewers et al., 2009). To test the effects of fermentation temperatures on beauvericin biosynthesis, we also cultured *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48 at 20°C and measured the titer of beauvericins at 3 and 5 days. We found that the production of these compounds at 20°C were approximately 50% of that at 30°C.

3.4. Effects of supplement of L-amino acids on beauvericin biosynthesis in S. cerevisiae

D-Hiv and D-2-hydroxy-3-methylvalerate (D-Hmv) are common biosynthetic precursors for COD biosynthesis. D-Hiv is used in the biosynthesis of **1** and D-Hmv is a precursor for the biosynthesis of **5**, while the biosynthesis of **3** and **4** requires both substrates. D-Hiv and D-Hmv can be synthesized from the intermediates from both amino acid catabolic and anabolic pathways. One of the possible biosynthetic pathways using the α-keto acids from amino acid catabolism was proposed in Fig. 4. L-Val is converted into α-ketoisovalerate (Kiv) by a branched-chain amino acid transaminase, which was subsequently transformed into D-Hiv by KIVR. Similarly, D-Hmv can be synthesized from L-Ile catabolism. To examine whether supplement of L-amino

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acids can improve beauvericin biosynthesis in the yeast, L-Val or L-Ile was fed to the fermentation broth of *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48 at different concentrations. The growth of the yeast strain was not influenced by addition of these amino acids at the tested concentrations, as the final OD_{600} values were approximately 2.0 for all the cultures. It was found that supplement of L-Val significantly improved the production of **1** in *S. cerevisiae*. As shown in trace (i) of Fig. S8, the peak of **1** became predominant and the production of **4** and **5** was almost undetectable. Four different concentrations (3, 10, 30 and 100 mM) of L-Val were added into the fermentation broth of *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48 and the titers of beauvericins were measured. As seen in Fig. 5a, the titer of **1** was increased to 47.49±0.55, 69.25±0.95, and 74.06±0.28 mg/l, respectively, when 3, 10 and 30 mM L-Val was supplemented. However, when the concentration of L-Val was increased to 100 mM, the titer of **1** slightly dropped. The titer of **3** was increased when 3 and 10 mM L-Val was supplied, while higher concentrations (30 and 100 mM) led to a decrease in the production of **3**. The titers of **4** and **5** were decreased at all four tested concentrations of L-Val. The overall titer of all beauvericins was increased to 93.14±1.82, 105.76±2.13, 92.99±0.71 and 67.32±1.21 mg/l, respectively, at the four tested concentrations.

Supplement of L-Ile had a different effect on the production of beauvericins. The ratio of the different beauvericins has changed. As shown in trace (ii) of Fig. S8, when 10 mM L-Ile was supplemented, **5** became the most abundant product, with **1** as a minor product. The titers of the products are shown in Fig. 5b. The titer of **1** was decreased to 11.51±0.36, 6.86±0.61, 2.24±0.17, and 0.64±0.18 mg/l, respectively, when L-Ile was supplemented at 1, 3, 10 and 30 mM. Similarly, the titer of **3** was also decreased. The titer of **4** was increased to 18.11±0.35 and 19.49±1.21 mg/l,

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respectively, at 1 and 3 mM, but was decreased to 14.68±0.53 and 4.15±0.35 mg/l when 10 mM and 30 mM L-Ile was supplied. The titer of **5** was increased at the first three concentrations of L-Ile, but dropped when 30 mM of L-Ile was supplemented. The overall titer of total beauvericins was decreased to 57.92±1.19, 52.68±1.53, 38.77±1.41, and 12.37±1.70 mg/l at the four concentrations.

3.5. Functional characterization of a new beauvericin synthetase from F. venenatum

To further examine whether the activity of CODSs from other fungi could also be reconstituted in the yeast using this expression system, a putative CODS gene, *fvBeas,* was amplified from the genome of *F. venenatum* NRRL 26139. This gene contains 9,390 bp and analysis of the deduced amino acid sequence indicated that it is a putative CODS with the same domain organization as BbBEAS and BbBSLS. BLAST analysis of the deduced protein revealed that FvBEAS shares 58% identity and 74% similarity with BbBEAS (GenBank accession number ACI30655), and 57% identity and 73% similarity with BbBSLS (GenBank accession number ACR78148). Using a similar approach for the cloning of *bbBeas* and *bbBsls*, this gene was ligated into pXW55 to yield the expression plasmid pDY59, which was subsequently expressed in *S. cerevisiae* BJ5464-NpgA. Both the wild type *F. venenatum* and *S. cerevisiae* BJ5464-NpgA/pDY59 were fermented for product analysis. As shown in trace (i) of Fig. S9, the major metabolite in *F. venenatum* NRRL 26139 was confirmed to be **1** by LC-MS analysis, suggesting that *fvBeas* encodes a beauvericin synthetase. This was confirmed by detection of **1** in *S. cerevisiae* BJ5464-NpgA/pDY59, as shown in trace (ii) of Fig. S9. Thus, FvBEAS was functionally reconstituted in *S. cerevisiae* BJ5464-NpgA and was identified as a beauvericin

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synthetase.

4. Discussion

Fungal CODSs represent an important group of natrally occurring molecules, which have shown a variety of biolgoical activities. The compounds studied in this work, **1**-**5**, were found to be promising anticancer molecules. In spite of formidable technical difficulties, heterologous production of secondary metabolites is increasingly feasible (Halo et al., 2008; Zirkle et al., 2004). **1** has been previously produced in *E. coli* by expressing BbBEAS in *E. coli* (Xu et al., 2008). However, the titer is low. When the engineered *E. coli* strain was fermented at 16°C, with supplement of 15 mM D-Hiv and L-Phe, the final titer of **1** only reached approximately 8 mg/l, which is far less than that in the native producer (Xu et al., 2008). Thus, a convenient, fast and low-cost biosynthetic way to produce these anticancer molecules is desireable. In this work, we expressed BbBEAS and BbBSLS in *S. cerevisiae* BJ5464-NpgA. *S. cerevisiae* BJ5464-NpgA is a genetically modified strain that contains a chromosomal copy of the PPTase NpgA that is necessary for activation of NRPSs. This strain has been previously used for heterologous expression of several giant modular enzymes such as LovB, a PKS involved in lovastatin biosynthesis (Ma et al., 2009). Unlike the native producer *B. bassiana* ATCC 7159, which produces **1** and **2** as the major products, expression of BbBEAS in *S. cerevisiae* BJ5464-NpgA led to the production of a series of beauvericins, including **1** and **3**-**5**. Production of these different beauvericins indicated that *S. cerevisiae* can supply two different 2-hydroxycarboxylate precursors for beauvericin biosynthesis. This also revealed that BbBEAS, or more specifically the A1 domain of BbBEAS, has relatively relaxed substrate specificity. In addition to the natural

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substrate D-Hiv, the A1 domain can select and load D-Hmv as an alternative substrate onto the CODS to yield **3**-**5**. BbBEAS takes three units of D-Hiv and L-Phe as the biosynthetic precursors to synthesize **1**. When one unit of D-Hmv is taken, **3** will be synthesized by BbBEAS. Incorporation of two units of D-Hmv led to the production of **4**. When the three units of D-Hiv are completely replaced by D-Hmv, **5** will be produced. The production of **3**-**5** in relatively larger amounts is likely due to more abundant supply of D-Hmv in the yeast than *B. bassiana*. Reconstitution of BbBSLS further confirmed that fungal CODSs can be reconstituted in *S. cerevisiae.* Although expression of catalytic fragments of the enniatin synthetase has been done (Hornbogen et al., 2007), reconstitution of entire fungal CODSs in *S. cerevisiae* has not previously been achieved. This work thus represents the first example of functional reconstitution of fungal CODSs in *S. cerevisiae*. Unlike *E. coli* which requires exogenous supply of D-Hiv for the production of **1**, *S. cerevisiae* can directly supply the biosynthetic precursors D-Hiv and D-Hmv for the production of CODs.

Supplement of D-Hiv and L-Phe into the fermentation broth of *S. cerevisiae* BJ5464-NpgA/pDY37 led to an increase in the production of **1**, while decreased the production of **3**-**5**. This may be attributed to the increased supply of the direct precursors of **1**. The supplemented D-Hiv, together with the synthesized D-Hiv by the host, might have dominated the binding site of BbBEAS. This makes it more difficult for D-Hmv to be incorporated into the products. Thus, the product type is dominated by the relative amount of the available substrates. The overall titer of total beauvericins was increased due to the addition of D-Hiv and L-Phe. The titer of **2** was also increased when D-Hiv and L-Leu were supplied, confirming that substrate

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feeding is useful in improving the biosynthesis of CODs in *S. cerevisiae*, which also indicated that the supply of these precursors in the yeast is relatively insufficient for the catalytic capacity of the expressed enzymes.

KIVR has been previously reported to supply D-Hiv for the biosynthesis of both **1** and **2** in *B. bassiana* ATCC 7159, as disruption of *kivr* in this fungus abolished the biosynthesis of these compounds (Xu et al., 2009b). Because the high cost of D-Hiv makes it an expensive material for large-scale production of these anticancer CODs, we further engineered KIVR from *B. bassiana* into *S. cerevisiae* BJ5464-NpgA. We found that expression of KIVR in *S. cerevisiae* BJ5464-NpgA increased the concentration of D-Hiv in the cells and fermentation broth. While measurements of the concentration of D-Hiv provide a snapshot of the dynamic synthesis process of this precurosr, the increased metabolic flux was reflected in the significantly increased titer of total beauvericins in *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48. It was expected that the expression of KIVR will lead to better supply of D-Hiv and higher titer of **1**. However, it is interesting that the titers of **3**-**5** were increased, while the production of **1** was slightly decreased. This result suggested that KIVR is not strictly substrate specific and can synthesize both D-Hiv and D-Hmv in the yeast. This is similar to a KIVR recently characterized from *Fusarium proliferatum* LF061, which takes 2-ketovalerate, pyruvate, 2-ketocapronate, 2-ketoglutarate and 2-ketobutyrate in addition to Kiv, although the enzymatic activity towards these unnatural substrates was low (Zhang et al., 2012). In this work, we found that KIVR can efficiently synthesize D-Hmv, resulting in a significant increase in the formation of **3**-**5**. This enzyme utilizes Kiv to synthesize D-Hiv, and reduces α-keto-β-methylvalerate (Kmv) into D-Hmv (Fig.

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4). Kiv is an important metabolite from both L-Val catabolic and anabolic pathways. In *S. cerevisiae*, a bidirectional reaction between L-Val and Kiv is catalyzed by the branched-chain amino-acid aminotransferases Bat1 and Bat2, which are present in the mitochondrial matrix and the cytosol, respectively (Kispal et al., 1996; Lilly et al., 2006). Similarly, Kmv can be obtained from both L-Ile catabolism and anabolism. The structural compositions, the decreased titer of **1** and the increased titers of **3-5** suggested that much more D-Hmv was synthesized by the heterologous KIVR than D-Hiv in the yeast. This might be due to the different supply of Kmv and Kiv in the host or a substrate preference of KIVR to Kmv. In contrast, **1** was found to be the predominant product in *B. bassiana*, while **3-5** were only produced in small amounts. It is likely that the supply of Kiv, and consequently D-Hiv, was dominant in *B. bassiana*. This indicated that production of the substrates for KIVR in the native host and the yeast was different, which led to different production profiles of beauvericins in these strains.

One of the major sources of 2-hydroxycarboxylate precursors is the catabolism of L-amino acids, as proposed in Fig. 4. Supplement of L-Val significantly increased the titer of beauvericins. More specifically, the ratio of the products has changed and **1** became the predominant metabolite in *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48. Because the only 2-hydroxycarboxylate precursor for **1** is D-Hiv, improved biosynthesis of D-Hiv from the supplemented L-Val directly facilitated the biosynthesis of **1**. In contrast, the production of **3** and **4** was limited by the supply of the second precursor D-Hmv. The presence of large amount of D-Hiv reduced or even abolished the production of **5**, likely due to the substrate competition between D-Hiv and D-Hmv. Our work enabled the production of beauvericins at an

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unprecedented high level of 105.76±2.12 mg/l in *S. cerevisiae* when 10 mM L-Val was supplemented. This titer is approximately 4-fold of that in the native producer, which was 24.49±1.52 mg/l in the presence of the same concentration of L-Val. Thus, this work provides an efficient production process for these anticancer natural products. Higher concentrations decreased the production of beauvericins, likely due to the over occupation of the active site of BbBEAS by excess D-Hiv, which may lower the efficiency of this giant modular enzyme.

Supplement of L-Ile was supposed to improve the supply of D-Hmv, which was confirmed by the predominant production of **5** (Fig. S8). However, it was unexpected the overall titer of beauvericins was decreased. Since the growth of the yeast cells was not affected, supplemented L-Ile might have interfered with the expression or function of BbBEAS in *S. cerevisiae*. Overall, our feeding experiments confirmed that supplement of L-Val is an effective approach to enhancing the *in situ* supply of D-Hiv and the production of beauvericins.

Overexpression of the pathway-specific regulator has been previously shown to significantly improve the titer of cytochalasin E from 25 to 175 mg/l in *Aspergillus clavatus* NRRL 1 (Qiao et al., 2011), suggesting that this is a useful approach to enhancing natural product biosynthesis. Similarly, overexpression of the dedicated ABC transporter AvtAB improved avermectin production about two-fold in *Streptomyces avermitilis* (Qiu et al., 2011). Thus, further improvement of the titer of beauvericins may be achieved by overexpression of the related regulator and transporter in the beauvericin biosynthetic pathway. No transporter gene exists in the reported beauvericin biosynthetic gene cluster, while a gene *orf1* encoding a putative Gal4-like transcriptional regulator with a Zn_2Cys_6 binuclear cluster DNA-binding domain was

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located in the flanking region of *bbBeas* on the genome of *B. bassiana* ATCC 7159 (Xu et al., 2008). Although it is unclear whether this regulator is associated with beauvericin biosynthesis, this regulator represents a potential target for further improvement of beauvericins. Other KIVR analogues, such as the one recently identified in *F. proliferatum* LF061 that has broad substrate specificity, can also be engineered into the system to generate novel beauvericins (Zhang et al., 2012). Additionally, further understanding of the catalytic properties of BbBEAS and KIVR will also provide useful information for future engineering of these enzymes for improved efficiency.

5. Conclusions

In this work, we examined the heterologous reconsititution of fungal CODSs in *S. cerevisiae* and production of the corresponding anticancer CODs. The activities of two fungal CODSs, BbBEAS and BbBSLS from *B. bassiana* ATCC 7159, were reconstituted efficiently in *S. cerevisiae* BJ5464-NpgA. The titers of the anticancer natural products, beauvericins and bassianolide, are comparable to those in the native producer. Supplement of the biosynthetic precursors, D-Hiv and L-amino acids (L-Phe or L-Leu) can significantly increase the production of these CODs in the yeast. Alternatively, we co-expressed BbBEAS and KIVR in *S. cerevisiae* BJ5464-NpgA, leading to a nearly 100% increase in the total titer of beauvericins (from 33.82 to 61.73 mg/l). Furthermore, we fed L-Val and L-Ile into the engineered strain *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48 to test the effects of these amino acids on the production of beauvericins at different concentrations. The titer of beauvercins reached 105.76±2.12 mg/l when 10 mM L-Val was supplemented. The best titer of **1** was achieved at 74.06±0.28 mg/l when 30 mM L-Val was supplemented. With this efficient expression system, we also functionally

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characterized a CODS from *F. venenatum* NRRL 26139 as a new beauvericin synthetase, which was named as FvBEAS. This work provides a useful platform for functional reconstitution of fungal CODSs and efficient production of the corresponding natural products.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:

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Figure captions

Fig. 1. Naturally occurring and biosynthesized fungal CODs (**1**-**11**). Beauvericin (**1**), bassianolide (**2**), beauvericins A-C (**3**-**5**) are natural products. Beauvericins G_1-G_3 (**6**-**8**) and beauvericins H_1-H_3 (**9**-**11**) were produced by precursor-directed biosynthesis.

Fig. 2. Domain organization of BbBEAS and BbBSLS.

Fig. 3. LC-MS analysis of the production of **1**-**5** in *B. bassiana* ATCC 7159 and engineered *S. cerevisiae* strains. (a) HPLC traces of the extracts of *B. bassiana* ATCC 7159 (i), *S. cerevisiae*

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BJ5464-NpgA/pDY37 (ii), and *S. cerevisiae* BJ5464-NpgA/pDY42 (iii). (b) ESI-MS spectrum of **1.** (c) ESI-MS spectrum of **2.** (d) ESI-MS spectrum of **3.** (e) ESI-MS spectrum of **4.** (f) ESI-MS spectrum of **5**. All experiments were performed in triplicate, and a representative result was shown.

Fig. 4. Proposed biosynthesis of D-Hmv and D-Hiv by KIVR in *S. cerevisiae*. The substrates of KIVR can be recruited from both amino acid catabolic and anabolic pathways. This proposed biosynthetic pathway only shows the KIVR substrates from the catabolic pathways.

Fig. 5. Effects of supplement of L-Val and L-Ile on the production of **1** and its congeners **3-5** in *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48. (a) Titers of beauvericins in *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48 when various concentrations of L-Val were supplemented. (b) Titers of beauvericins in *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48 when various concentrations of L-Ile were supplemented. Data are presented as means ± SD (n = 3).

Highlights

- First reconstitution of two CODSs, BbBEAS and BbBSLS, was achieved in *S. cerevisiae.*
- Co-expression of KIVR and BbBEAS significantly enhanced beauvericin biosynthesis.
- Supplement of L-Val increased the yield of beauvericins to 105.76 mg/l.
- A new beauvericin synthetase was functionally identified from *F. venenatum.*

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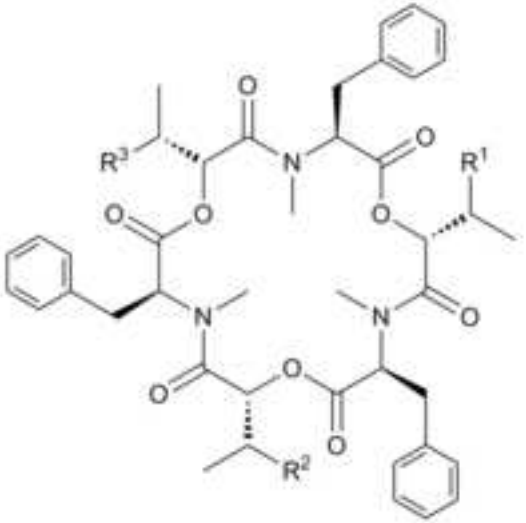
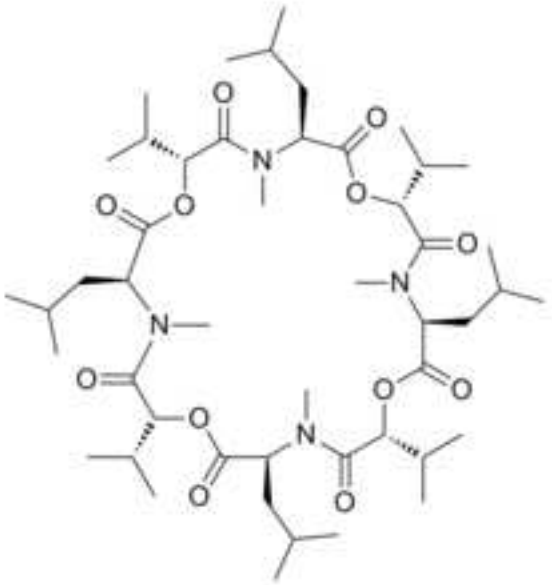
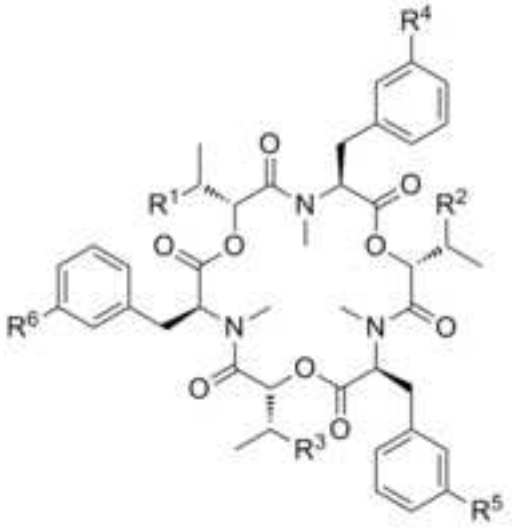
Figure 1



Beauvericin (**1**) $R^1=R^2=R^3=Me$
Beauvericin A (**3**) $R^1=R^2=Me$, $R^3=Et$
Beauvericin B (**4**) $R^1=Me$, $R^2=R^3=Et$
Beauvericin C (**5**) $R^1=R^2=R^3=Et$



Bassianolide (**2**)



Beauvericin G_1 (**6**) $R^1=R^4=R^5=R^6=H$, $R^2=R^3=Me$
Beauvericin G_2 (**7**) $R^1=R^2=R^4=R^5=R^6=H$, $R^3=Me$
Beauvericin G_3 (**8**) $R^1=R^2=R^3=R^4=R^5=R^6=H$
Beauvericin H_1 (**9**) $R^1=R^2=R^3=Me$, $R^4=F$, $R^5=R^6=H$
Beauvericin H_2 (**10**) $R^1=R^2=R^3=Me$, $R^4=R^5=F$, $R^6=H$
Beauvericin H_3 (**11**) $R^1=R^2=R^3=Me$, $R^4=R^5=R^6=F$

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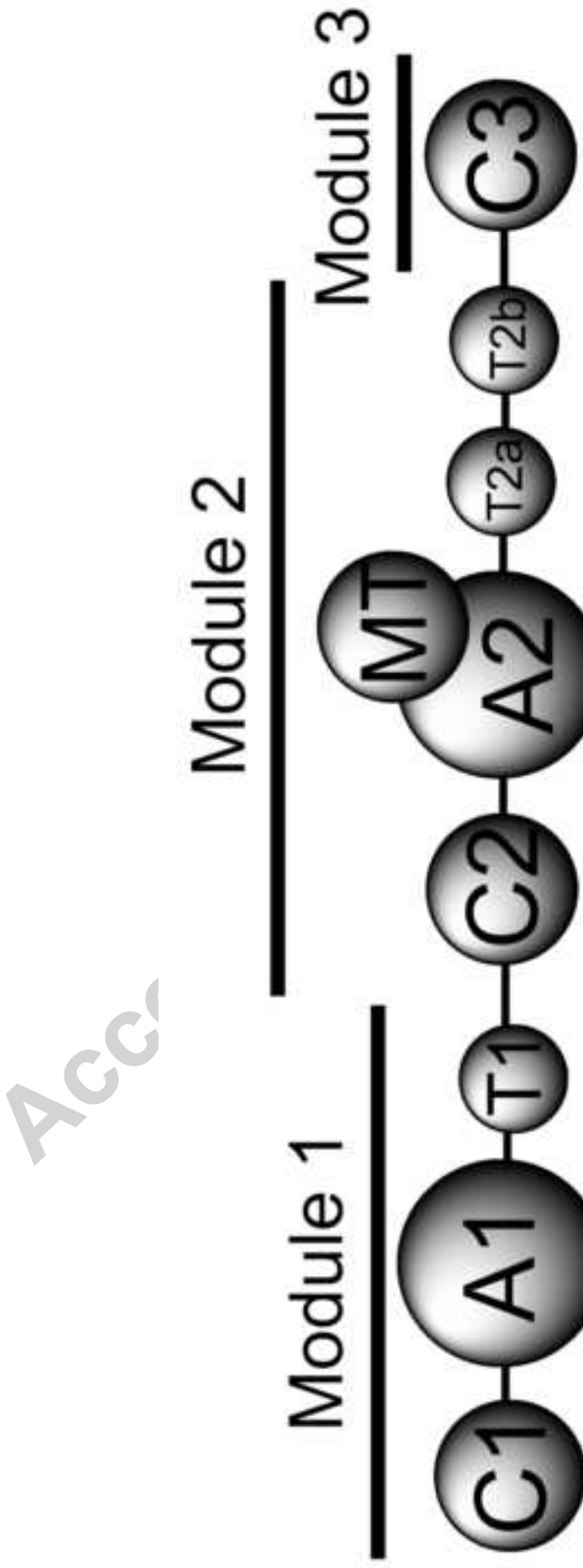

Figure 2

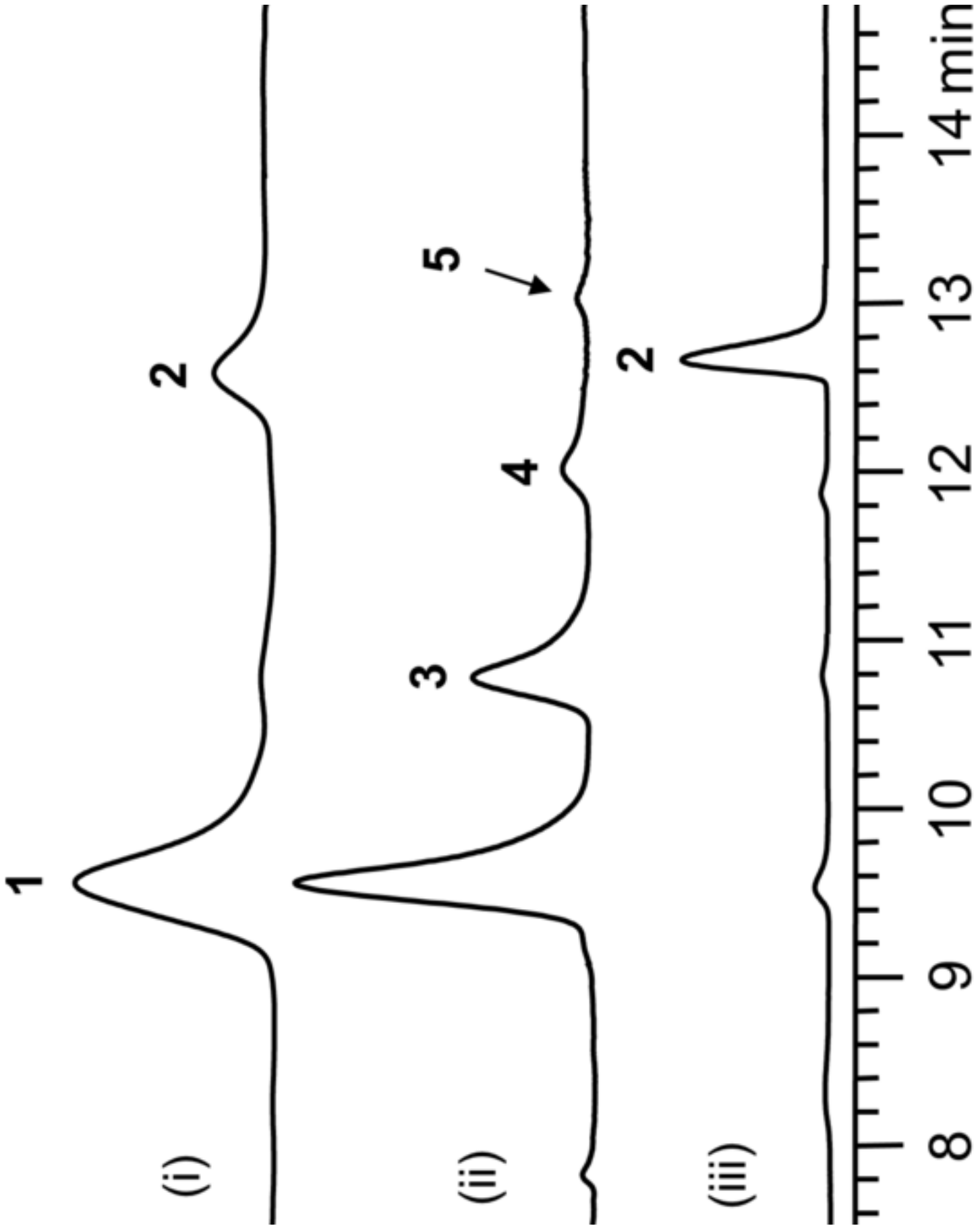

Figure 3a

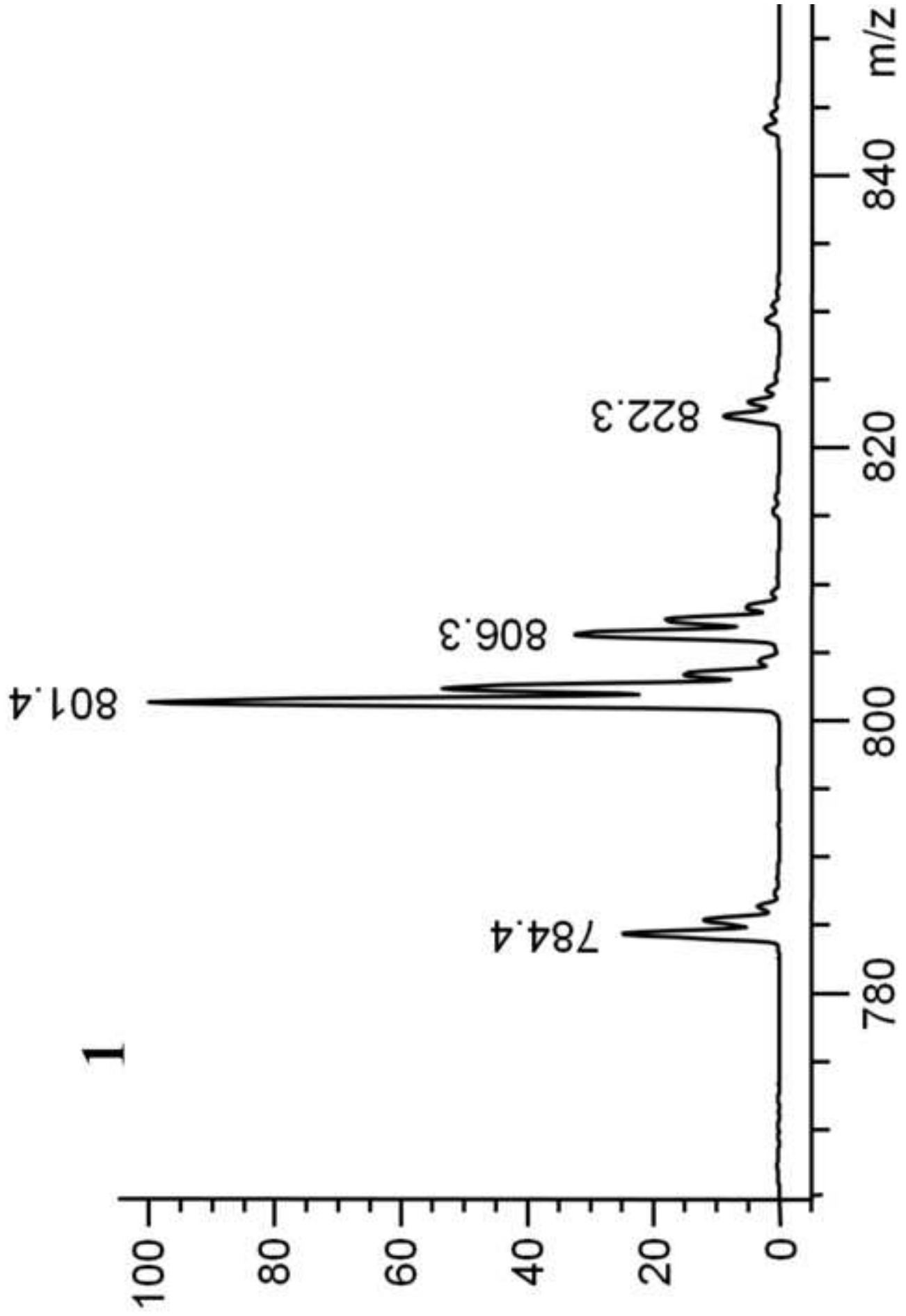

Figure 3b

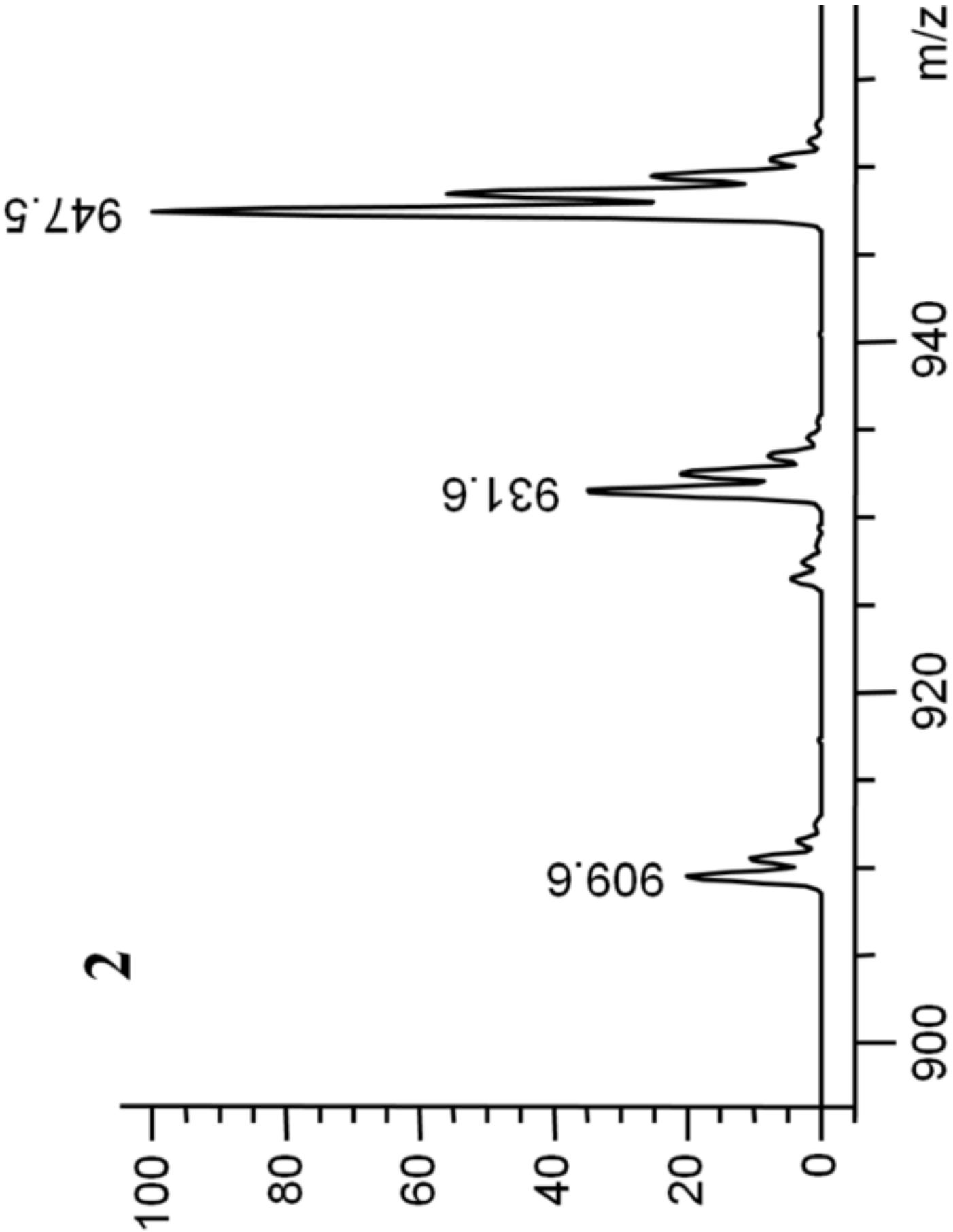

Figure 3c

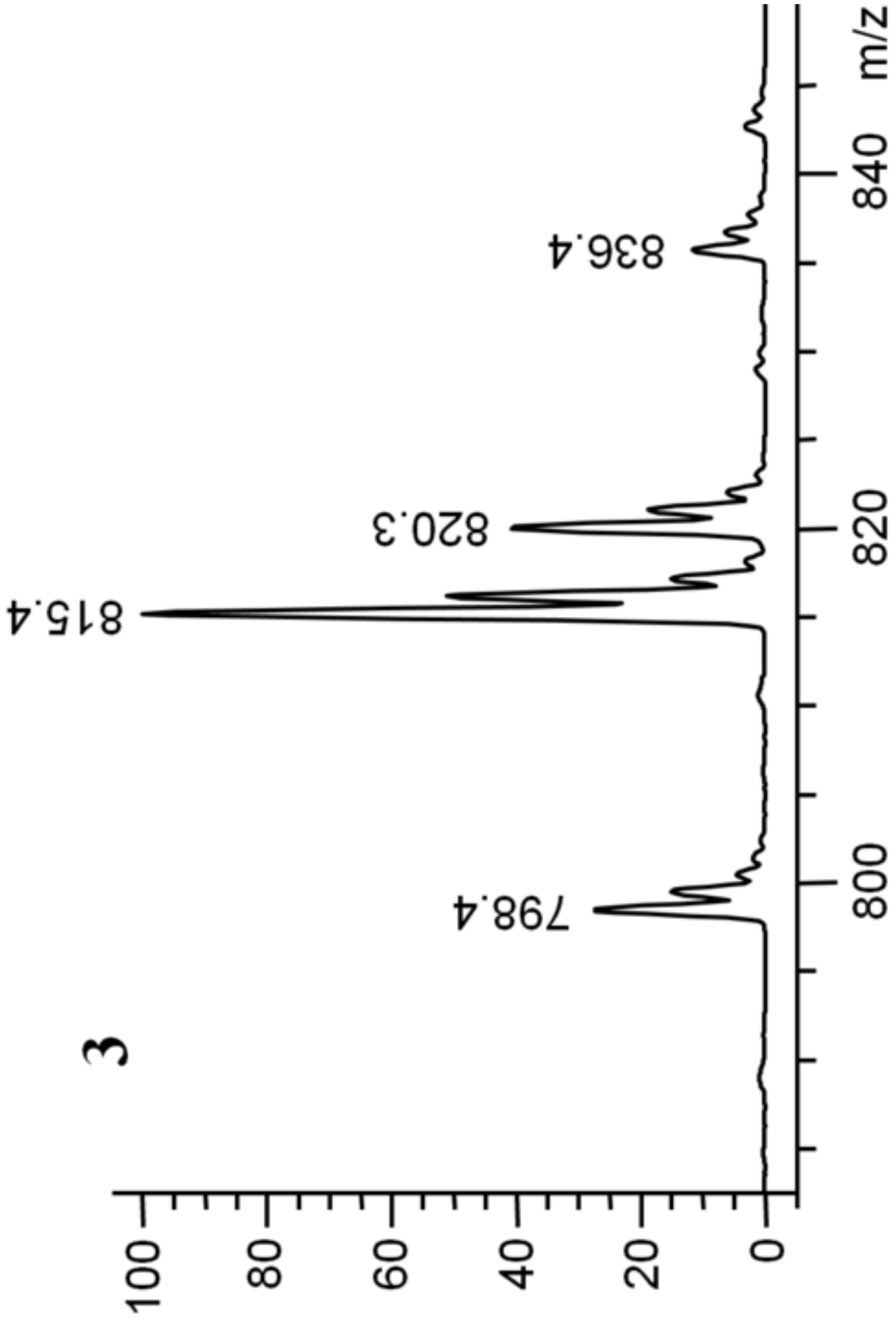

Figure 3d

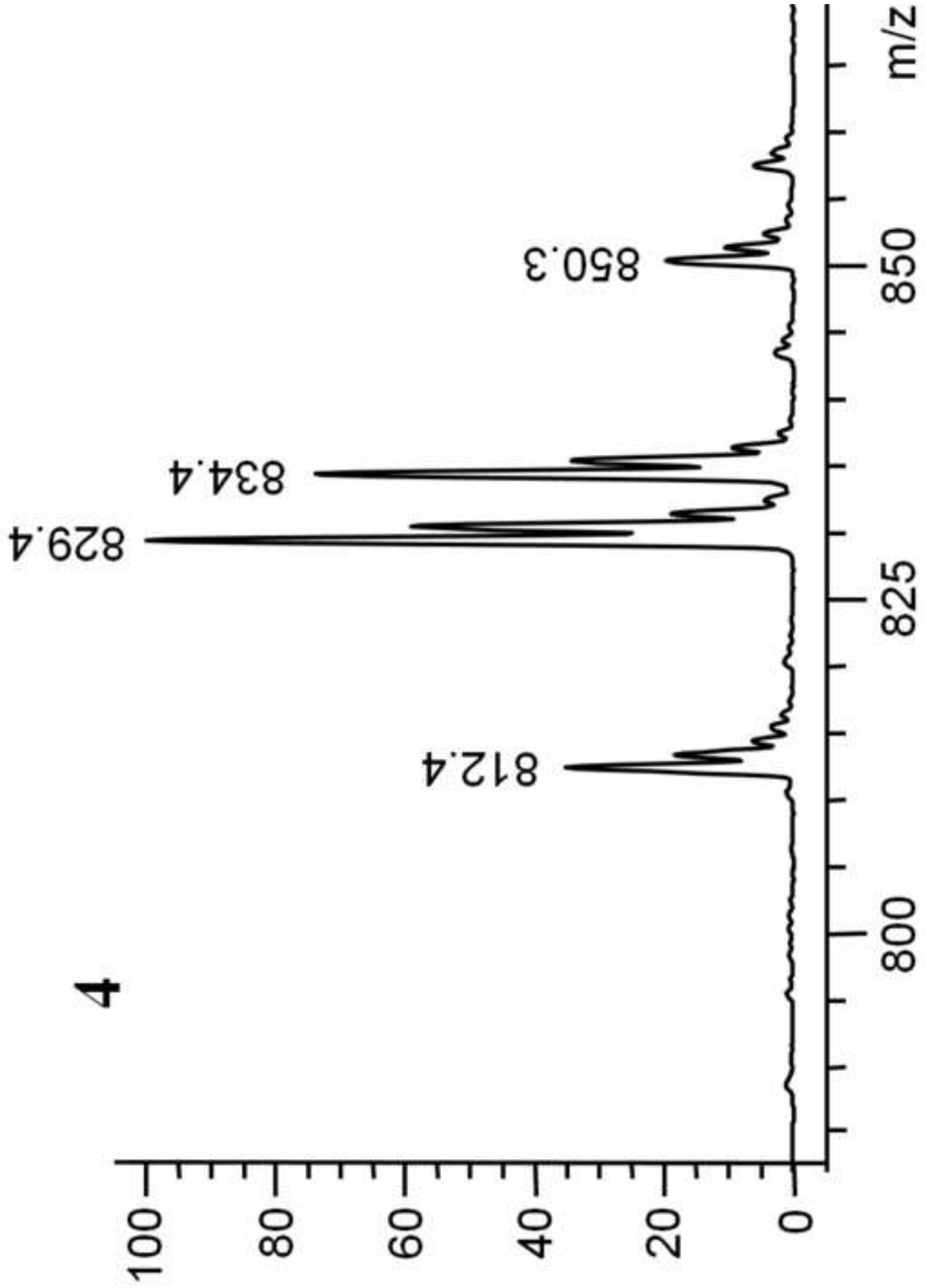

Figure 3e

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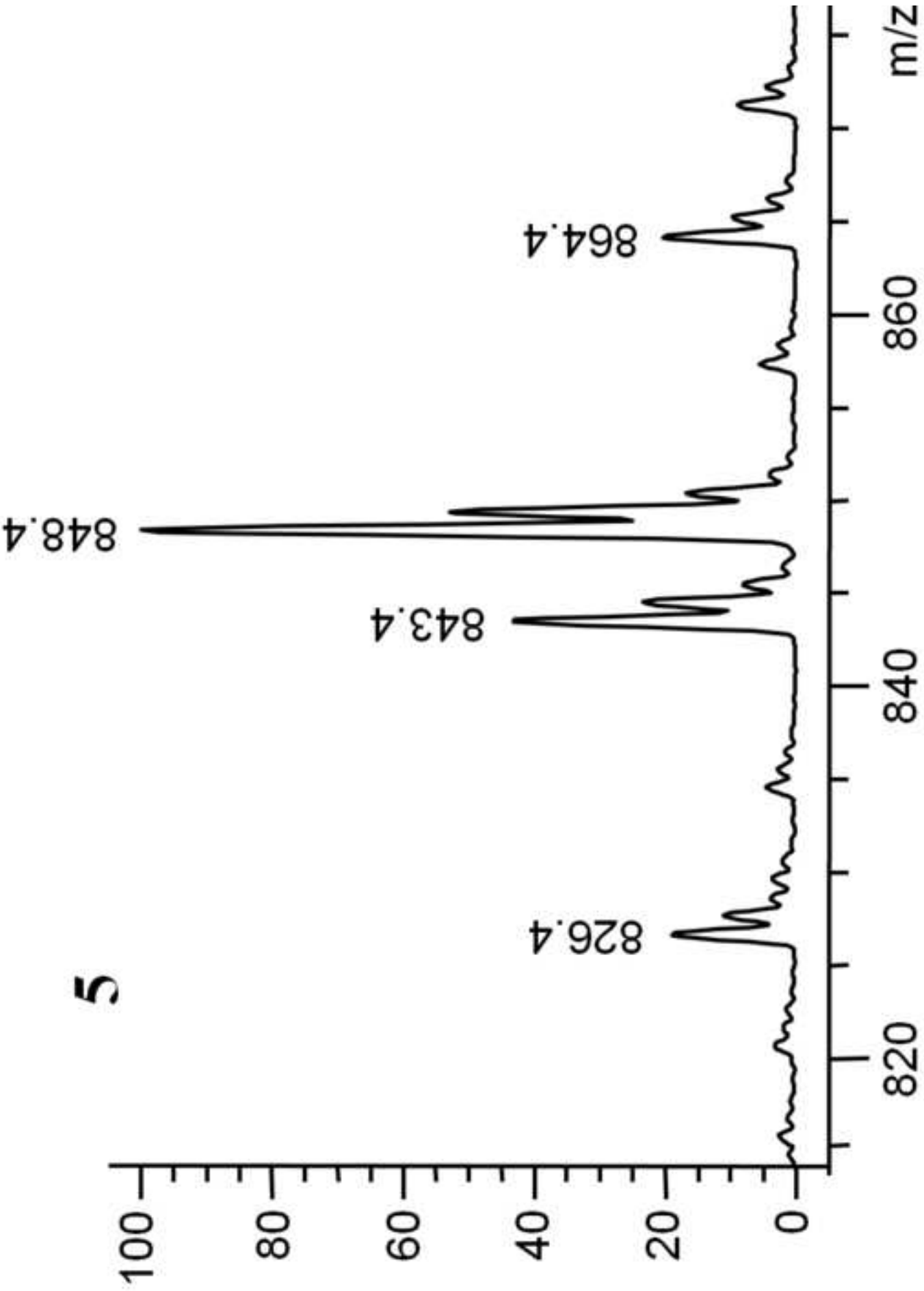
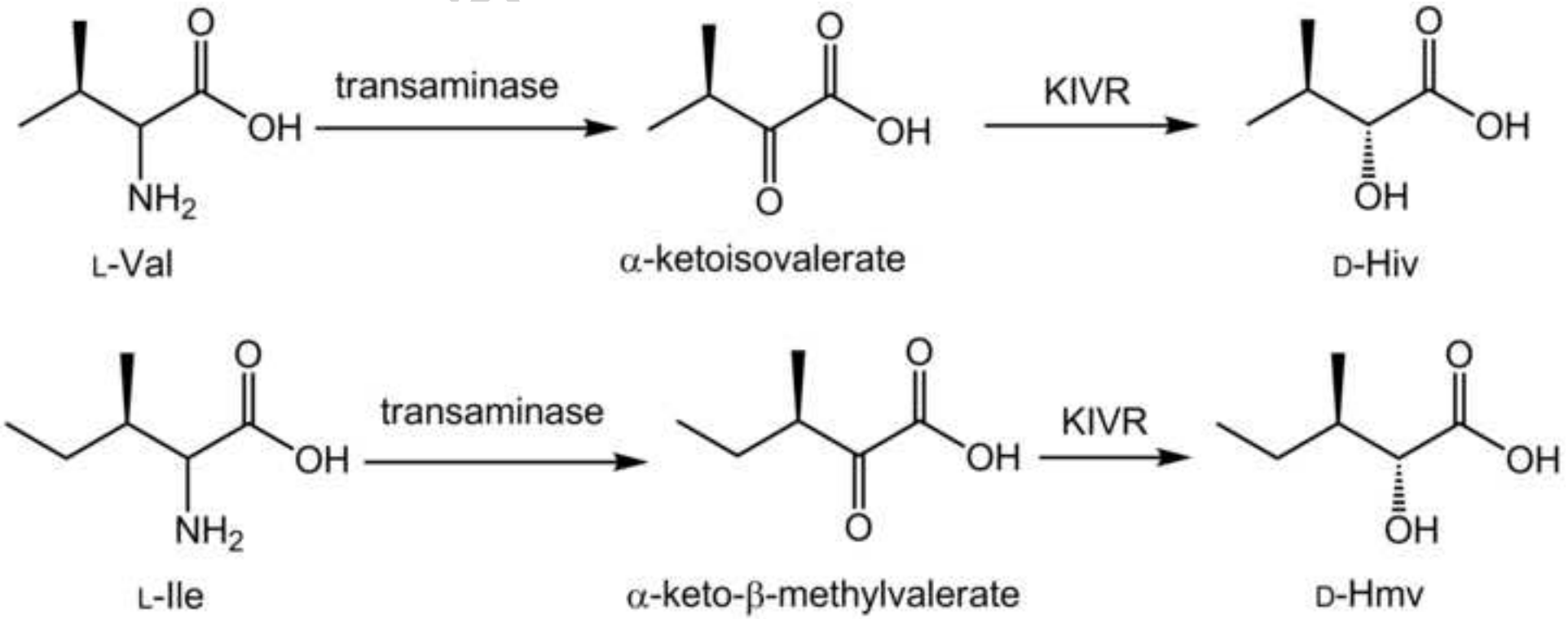


Figure 3f

Figure 4



ACCEPTED MANUSCRIPT

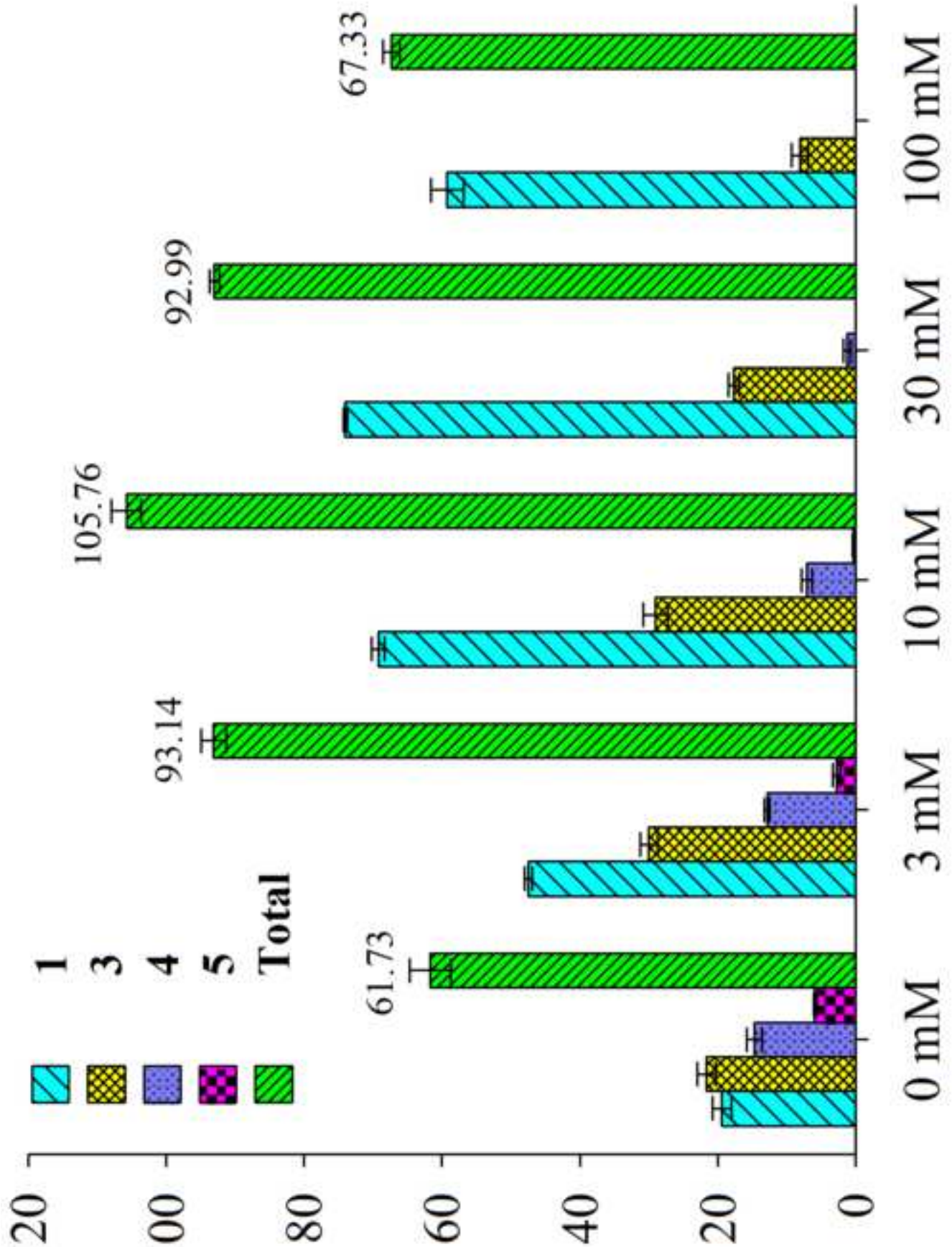

Yield (mg/l)
120
100
80
60
40
20
0
1
3
4
5
Total
61.73
93.14
105.76
92.99
67.33
0 mM
3 mM
10 mM
30 mM
100 mM

Figure 5a

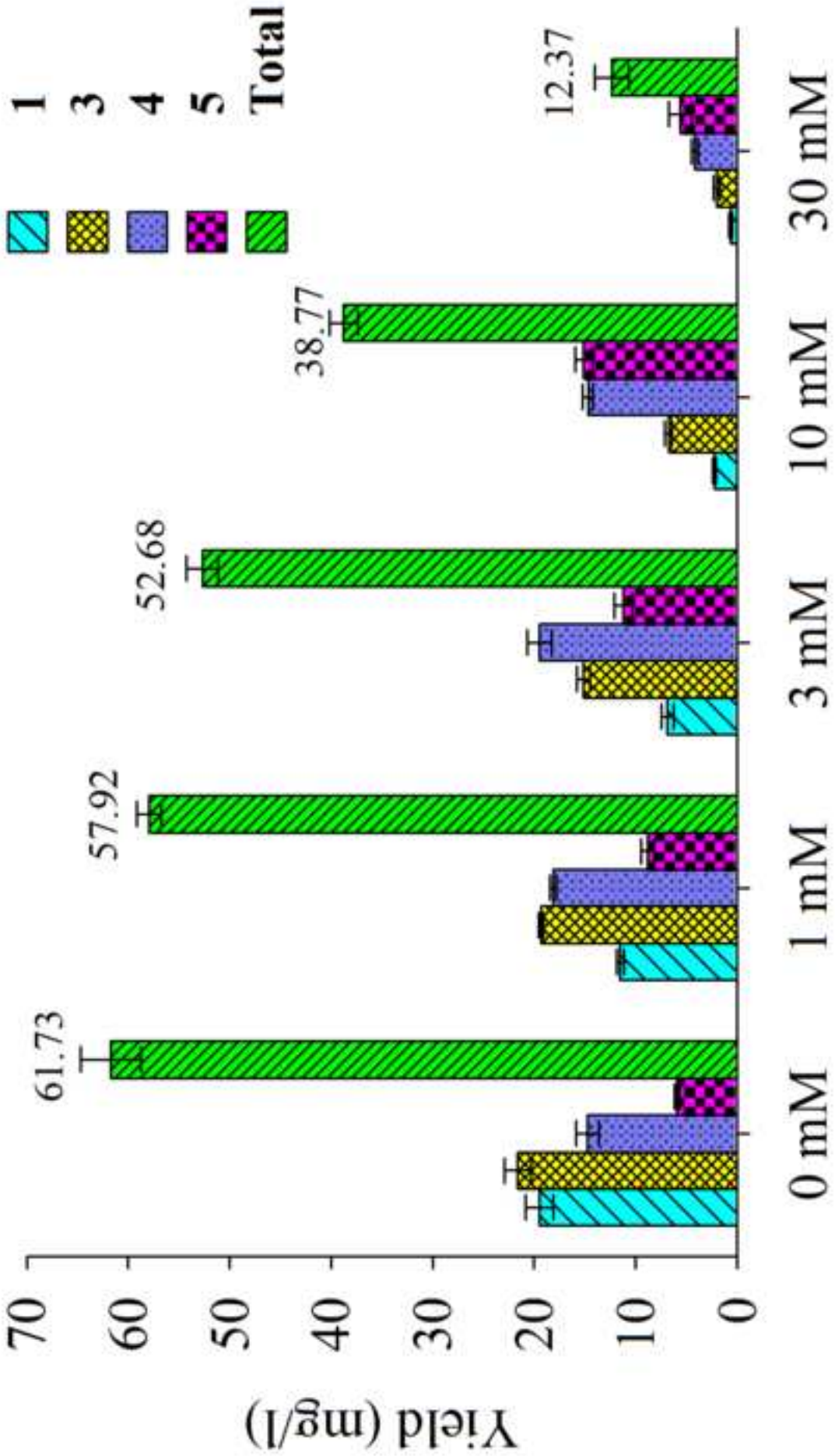

Yield (mg/l)
70
60
50
40
30
20
10
0
0 mM
1 mM
3 mM
10 mM
30 mM
61.73
57.92
52.68
38.77
12.37
1
3
4
5
Total

Figure 5b

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Table 1

Primers used in this study.

Primer	Sequence	Restriction site
bbBeas-a-F	5'-aaGCTAGCatggagccgctcaaaaatgt-3'	*Nhe*I
bbBeas-a-R	5'-tcggcgGCTAGCtgcacaag-3'	*Nhe*I
bbBeas-b-F	5'-ttgtgcaGCTAGCcgccgag-3'	*Nhe*I
bbBeas-b-R	5'-aaCACGTGcaaagccgagtttagactct-3'	*Pml*I
bbBsls-a-F	5'-aaCATATGgagccacccaacaacgc-3'	*Nde*I
bbBsls-a-R	5'-ttgtgCATATGggatgcttg-3'	*Nde*I
bbBsls-b-F	5'-aagcatccCATATGcacaag-3'	*Nde*I
bbBsls-b-R	5'-aaCACGTGtaaagacgcattcaaagcct-3'	*Pml*I
bbkivr-F	5'-aaCATATGccatctccggagcatcc-3'	*Nde*I
bbkivr-R	5'-aaGTTTAAACttacaacagccttctcgcca-3'	*Pme*I
fvBeas-a-F	5'-aaCATATGgaatatcttactgctgt-3'	*Nde*I
fvBeas-a-R	5'-tctcacagCATATGgaatca-3'	*Nde*I
fvBeas-b-F	5'-tctgattcCATATGctgtga-3'	*Nde*I
fvBeas-b-R	5'-aaCACGTGcaaagaagagttgagaccct-3'	*Pml*I

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Table 2

Plasmids constructed in this study.

Plasmid	Description	Plasmid	Description
pJCZ49	*bbBeas-a* in pJET1.2	pDY35	*bbBeas-b* in pXW55
pJCZ48	*bbBeas-b* in pJET1.2	pDY37	*bbBeas-a* and *bbBeas-b* in pXW55
pJCZ50	*bbBsls-a* in pJET1.2	pDY36	*bbBsls-b* in pXW55
pJCZ47	*bbBsls-b* in pJET1.2	pDY42	*bbBsls-a* and *bbBsls-b* in pXW55
pDY47	*bbkivr* in pJET1.2	pDY48	*bbkivr* in pXW06
pDY55	*fvBeas-a* in pJET1.2	pDY57	*fvBeas-b* in pXW55
pDY56	*fvBeas-b* in pJET1.2	pDY59	*fvBeas-a* and *fvBeas-b* in pXW55

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Table 3

Titers of **1**-**5** in engineered *S. cerevisiae* BJ5464-NpgA strains with or without supplemented biosynthetic precursors (D-Hiv and L-Phe for *S. cerevisiae* BJ5464-NpgA/pDY37; D-Hiv and L-Leu for *S. cerevisiae* BJ5464-NpgA/pDY42). Data are presented as means ± SD (n = 3).

		Titer (mg/l)		
Precursor concentration		0 mM	0.8 mM	2.0 mM
pDY37	**1**	21.89±0.77	29.69±1.91	36.08±3.44
	3	7.12±0.15	6.65±0.11	6.15±0.20
	4	3.41±0.25	1.68±0.46	ND*
	5	1.40±0.24	0.52±0.22	ND*
	Total beauvericins	33.82±1.41	38.54±0.64	42.23±1.35
pDY42	**2**	21.74±0.08	23.90±2.22	26.74±2.75

* ND: not detected.

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Table 4

Titers of beauvercins in *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48. Data are presented as means ± SD (n = 3).

	Titers (mg/l)
1	19.44±1.38
3	21.62±1.31
4	14.74±1.11
5	5.93±0.23
Total beauvericins	61.73±2.96

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Table 5

Production of D-Hiv in *S. cerevisiae* BJ5464-NpgA/pDY37 and *S. cerevisiae* BJ5464-NpgA/pDY37+pDY48. Data are presented as means ± SD (n = 3).

		pDY37	pDY37+pDY48
24 h	supernatant	5.15±0.08 mg/l	6.36±0.07 mg/l
	cells	2.99±0.07 mg/l	4.89±0.17 mg/l
	Total	8.12±0.15 mg/l	11.26±0.25 mg/l
48 h	supernatant	6.61±0.45 mg/l	8.71±1.04 mg/l
	cells	3.68±0.05 mg/l	6.63±0.09 mg/l
	Total	10.28±0.39 mg/l	15.34±1.14 mg/l

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