Discovery of new natural products and biosynthetic gene clusters encoded in the genome of *Streptomyces albus* subsp. *chlorinus*

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List of publications

Rodríguez Estévez M., Myronovskyi M., Gummerlich N., Nadmid S., Luzhetskyy A., Heterologous expression of the nybomycin gene cluster from the marine strain *Streptomyces albus* subsp. *chlorinus* NRRL B-24108, *Mar. Drugs*, 2018, 16(11):435.

Rodríguez Estévez M., Gummerlich N., Myronovskyi M., Zapp J., Luzhetskyy A., Benzanthric acid, a novel metabolite from *Streptomyces albus* Del14 expressing the nybomycin gene cluster, *Front. Chem.*, 2020, 7:896.

Ahmed Y., Rebets Y., **Rodríguez Estévez M.**, Zapp J., Myronovskyi M., Luzhetskyy A., Engineering of *Streptomyces lividans* for heterologous expression of secondary metabolite gene clusters, *Microb. Cell Fact.*, 2020, 19(1):5.

Rodríguez Estévez M., Myronovskyi M., Rosenkränzer B., Paululat T., Petzke L., Ristau J., Luzhetskyy A., Novel fredericamycin variant overproduced by a streptomycin resistant *Streptomyces albus* subsp. *chlorinus* strain, *Mar. Drugs*, 2020, 18(6):284.

Lasch C., Stierhof M., **Rodríguez Estévez M.**, Myronovskyi M., Zapp J., Luzhetskyy A., Dudomycins: new secondary metabolites produced after heterologous expression of an NRPS cluster from *Streptomyces albus* ssp. *chlorinus* NRRL B-24108, *Microorganisms*, 2020, 8(11):E1800.

Conference contributions

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Abstract

Streptomyces genomes encode numerous gene clusters able to synthesize secondary metabolites with potential pharmaceutical applications. However, a great number of these genes remain unexpressed under standard laboratory growth conditions, therefore hiding a rich reservoir of natural products in their encoded metabolic pathways. In this work, we applied two strategies to induce the expression of silent gene clusters from *Streptomyces albus* subsp. *chlorinus*: heterologous expression and *in situ* ribosome engineering.

In the first approach, a cryptic gene cluster encoded by *S. albus* subsp. *chlorinus* genome was heterologously expressed in the chassis strain *Streptomyces albus* Del14. The resulting strain produced the previously known antibiotic nybomycin, which is potently active against quinolone-resistant *Staphylococcus aureus*. Expression of the nybomycin cluster in *S. albus* Del14 also resulted in the biosynthesis of the novel compound benzanthric acid, which might be the product of interaction between the nybomycin pathway and the host´s metabolism.

In the second approach, we selected a highly streptomycin-resistant mutant derived from *S. albus* subsp. *chlorinus*. This strain exhibited enhanced expression of a type II PKS gene cluster compared to the wild type strain, leading to overproduction of the novel compound fredericamycin C_2. A point mutation leading to lack of RsmG protein is likely to be responsible for the mutant phenotype.

Zusammenfassung

Das Chromosom von Streptomyceten enthält zahlreiche Gencluster, welche die Baupläne für die Synthese von Naturstoffen und potentiellen Therapeutika beinhalten. Dennoch kann unter Standard-Laborbedingungen oftmals nur eine unzureichende Expression dieser Gene erzielt werden. Eine bedeutende Anzahl von Naturstoffen bleibt somit unentdeckt. In dieser Arbeit haben wir zwei unterschiedliche Strategien angewandt, um die Expression von stillen Genclustern des Stammes *Streptomyces albus* subsp. *chlorinus* zu aktivieren: Heterologe Expression und *in situ* Ribosom-Engineering.

Im ersten Ansatz wurde ein kryptisches Gencluster aus *S. albus subsp. chlorinus* im Wirt *Streptomyces albus* Del14 exprimiert. Die Mutante produzierte Nybomycin, ein bereits bekanntes Antibiotikum mit hoher Aktivität gegen Chinolon-resistenten *Staphylococcus aureus*. Die Expression des Nybomycin-Genclusters in *S. albus* Del14 stimulierte außerdem die Synthese des neuen Naturstoffs Benzanthric Acid - einem Produkt aus der Interaktion des Nybomycin-Stoffwechsels mit dem Wirtsmetabolismus.

Für den zweiten Ansatz wurde eine stark Streptomycin-resistente Mutante von *S. albus* subsp. *chlorinus* gewählt, die im Vergleich zum Wildtyp eine erhöhte Expression eines Type II PKS Genclusters aufwies. Dies führte zur Produktionssteigerung der neuen Substanz Fredericamycin C_2. Ursächlich für diesen Phänotyp ist das Fehlen des RsmG-Proteins ausgelöst durch eine Punktmutation.

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1. Introduction

1.1. Importance of natural products in the pharmaceutical industry

Natural products (NPs) are substances derived from natural sources such as plants, animals, and microbes. It is estimated that about 25% of all known natural products –over one million– display biological activities [1] that make them applicable in different industrial fields: pharmacy, agriculture, food preservation, and cosmetics, among others. More specifically in the pharmaceutical industry, NPs have found multiple uses, e.g. antibacterial, antiviral and antitumor drugs, antiparasitics, immunosuppressants, and cholesterol-reducing agents. According to the US Food and Drug Administration (FDA), more than 50% of all the new drugs approved between 1981 and 2019 were of natural origin or NP-based compounds, indicating the significance of NPs in the current therapeutic scenario [2]. The major category of all approved substances is represented by the anti-infective agents, of which 78% are NPs or their derivatives, showing the value of natural compounds in human health.

In the 1940s, the development of large scale production of penicillin from *Penicillium* fungi encouraged big pharmaceutical companies to focus their research on natural sources for the discovery of other antibiotics. Most of the antibiotic classes known up to date were discovered in the period between the 1940s and the 1970s, the "golden era" of antibiotics [3]. For instance, streptomycin and other aminoglycosides, a group of antibiotics potently active against Gram-negative bacteria, were first isolated by the Waksman group in the late 1940s [4]. Later on, other classes of antibiotics were characterised, such as penicillins, cephalosporins and tetracyclines [5-7]. Nevertheless, the high rate of bioactive microbial drugs discovery (200-300 per year in the late 1970s) drastically decreased in the last decades of the 20th century [1]. Some of the reasons for this decline were the high degree of rediscovery of known compounds, the difficulties in finding new potential producers due to extensive screening of terrestrial environments, and the small production yields [8]. Additionally, strict safety requirements, long duration time of clinical trials, short patent legitimacy periods and great market pressure to produce rapidly and profitably made NPs a less attractive source for drug development [9]. As a consequence, the pharmaceutical companies lost interest in NPs and redirected their drug

discovery programs towards the emerging high-throughput screening (HTS) of combinatorial synthetic compound libraries [10].

Despite the increasing interest in synthetic libraries, they present a series of disadvantages in relation to those based on NPs. First, the chemical diversity of synthetic libraries is limited by the oral bioavailability of the drugs, resulting in redundant collections of compounds that share similar structural cores [11]. In this regard, NPs and their derivatives offer a greater range of pharmacophores, more complex three-dimensional configurations, and higher number of stereocenters than purely synthetic molecules [12]. Second, despite the excellent toxicological and pharmacological properties of synthetic drugs, their range of targets is considerably narrower than that of NPs [13,14]. Third, they are generally inefficient in penetrating the complex cell wall of Gram-negative bacteria, in contrast to NP drugs and their analogues [15]. NPs have evolved for billion years to enter the cells and specifically bind proteins and other metabolites, becoming very efficient bioactive weapons of natural producers against their competitors [14]. Briefly, NPs still remain a highly valuable source of drug leads for the development of therapeutic agents that needs to be further explored.

During the 1990s and early 2000s, the attempts of the pharmacy industry to develop new drugs based on combinatorial chemistry approaches were rather disappointing. HTS of synthetic libraries has an average hit rate of <0.001%, much lower than the 0.3% hit rate resulting from the screening of NPs [16]. As a consequence of the moderate success of the chemistry-based discovery methods, the overall number of new active drugs approved by the FDA per year has significantly decreased between the 1990s and the 2000s [2]. This has especially affected the field of antibiotics. According to the World Health Organisation (WHO), in 2019 only 50 antibiotics in the world were in clinical development [17], which is not sufficient to combat the increasing appearance of antibacterial-resistant organisms. Antibiotic resistance was already observed by Fleming in 1929, when he identified some bacterial strains whose growth was not inhibited by penicillin [18]. In the mid-1940s, when streptomycin was clinically used for the treatment of tuberculosis, resistant strains of *Mycobacterium tuberculosis* were also identified [19,20]. Today, we encounter a bigger problem: many pathogens causing serious nosocomial (hospital-linked) infections are becoming multidrug resistant, giving rise to high mortality and morbidity rates, as no

effective therapeutic options are available for the treatment of these diseases [21]. Both Gram-positive and Gram-negative bacteria are included in this dangerous group, the so-called ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) [22]. Therefore, in the last years, some projects have been launched to propel the discovery of new classes of antibiotics and to confront the worldwide health threat of antimicrobial resistance [23,24]. The main focus of these initiatives is directed towards NPs, which still remain the preferred source for the search of anti-infective agents.

In summary, regarding the rapidly developing antibacterial-resistant pathogens, it is clear that there is an urgent need for new antibiotics to replace the current ineffective ones. NPs have demonstrated a broader structural and bioactive diversity and proven to be more efficient antimicrobials than chemically synthesized compounds. Therefore, promotion of NP discovery programs and exploitation of underexplored niches is mandatory to efficiently fight the incipient rise of resistant pathogens.

1.2. Novel approaches for natural product discovery

Traditionally, the preferred source of natural compounds has been soil bacteria, more specifically the filamentous bacteria actinomycetes, whose most promising genus is *Streptomyces*. In fact, 80% of the antibiotics used nowadays are derive from NPs synthesized by *Streptomyces* bacteria, including aminoglycosides (neomycin), carbapenem (doripenem), tetracyclines (chlortetracycline), and modified peptides (vancomycin, daptomycin), among others [25,26]. Since soil sampling is easy and inexpensive, terrestrial actinomycetes as well as their secondary metabolism products have been well characterised, frequently leading to the rediscovery of common species and already known compounds. Moreover, horizontal gene transfer occurring among actinomycetes contributes to the production of similar metabolic profiles in different species [27], limiting the structural diversity of NPs found in a specific area. Therefore, researchers have recently focused on alternative sources of NPs, primarily marine environments such as sea depths, coral reefs, salt lakes, polar regions, or fjord sediments [28-31]. The extreme conditions of these habitats force bacteria to adapt their metabolism by expressing genes that are usually silent under the milder conditions of terrestrial ecosystems. In many cases, the activation of these genes leads to the production

of novel bioactive secondary metabolites that represent interesting drug candidates. Many promising compounds have been isolated from marine bacteria, e.g. haloduracin, a broad-spectrum two-peptide lantibiotic isolated from *Bacillus halodurans* growing at an alkaline pH of >9.0 [32]; marinopyrroles A and B from a *Streptomyces* sp. collected at the coast of La Jolla, California, harbouring significant antimicrobial activity against methicillin-resistant *Staphylococcus aureus* [33], or kiamycin, an angucyclinone showing cytotoxic activity, isolated from *Streptomyces griseus* M268 in a marine sediment [34]. It is estimated that marine environments contain around 1.2 x 10^{29} prokaryotic cells/ml, suggesting the enormous potential of these niches to provide interesting natural structures [35].

Nevertheless, only up to 0.1% of the marine microbes and about 1% of all bacteria (both terrestrial and aquatic) is believed to be cultivable in the laboratory [36,37]. To overcome this problem, metagenomic approaches were introduced in the late 1990s, allowing the direct isolation of DNA from the environment, which can be further cloned and analysed in other bacterial species [38]. At the same time, DNA sequencing techniques started to become faster and more economic, turning metagenomics into a successful tool for the discovery of NP biosynthetic genes and their corresponding products [39].

Among the bacteria that are easily cultivable in the laboratory are actinomycetes. The genome sequencing of several actinomycete strains showed that these microorganisms harbour a great amount of secondary metabolite biosynthetic genes [40-43]. This finding demonstrates that the capacity of actinomycetes to produce bioactive compounds is even bigger than estimated from their expressed metabolome, and that the majority of secondary metabolites encoded in these bacteria remain undiscovered.

The genes responsible for the biosynthesis of secondary metabolites in bacteria are arranged in clusters. The expression of these clusters is not always achieved in the laboratory, since the applied growth conditions generally cannot mimic the complex environment and often extreme changes occurring in nature. Therefore, many of the secondary metabolite biosynthetic gene clusters (BGCs) encoded in the genomes of actinobacteria are cryptic (silent) under conventional laboratory conditions. Several strategies for the activation of silent secondary metabolite BGCs have been developed. They are classified in multiple-effect approaches and cluster-specific approaches. The methods with multiple effects involve the expression of many genes and generate global metabolic

changes in the cell. They include the OSMAC method (One Strain Many Compounds), where the production of several compounds can be activated by altering the cultivation conditions [44-46]; co-cultivation of different microorganisms [47]; manipulation of pleiotropic regulators [48,49], and upregulation of the transcription and translation machineries [50,51]. In contrast, the cluster-specific strategies provide a tighter control of gene expression and are focused on a particular BGC for which the gene organisation and transcriptional regulatory mechanisms are known. Among these methods are engineering of pathway-specific regulators, i.e., inducing the expression of transcriptional activators or deleting repressor genes [52,53], and refactoring of BGCs, which consists in exchanging the native inactive promoters for constitutive promoters that lead to upregulation of specific biosynthetic genes [54,55]. Refactoring of BGCs is often coupled with expression in a heterologous host suitable for genetic manipulation, especially if the original producer is not genetically amenable. Heterologous expression has become a useful tool for revealing the NPs encoded by many cryptic gene clusters.

1.3. Heterologous expression of secondary metabolite BGCs

Since the Hopwood group characterised the complete genome sequence of *Streptomyces coelicolor* and discovered that it encodes more secondary metabolite BGCs than previously expected [41], the idea of genome mining started to germinate. Genome mining consists in screening the genome sequence of microorganisms in the search for NP biosynthetic gene clusters. The increasing availability of genome sequence information, triggered by the new inexpensive next-generation sequencing techniques [56], has promoted the development of softwares able to predict gene clusters potentially involved in the synthesis of secondary metabolites, such as PRISM, antiSMASH, and BiG-SCAPE [57-59]. Heterologous expression facilitates the discovery of NPs produced by the BGCs identified through genome mining.

The expression of BGCs in a heterologous strain has numerous advantages. First of all, it facilitates the deciphering of orphan clusters, since the new compounds produced by a host microorganism can be attributed to the introduced genes [60,61]. Second, the commonly reduced metabolic background of modified host strains increases the availability of precursors, which are more efficiently channelled into the heterologous biosynthetic pathway. Moreover, the relatively simple metabolic profile accelerates the identification and

purification process of new secondary metabolites [62-64]. Third, the use of genetically amenable hosts enables the refactoring of the introduced gene clusters to activate their expression (e.g., promoter replacement, upregulation of transcriptional activators, deletion of repressors) [55,65,66] or to generate derivatives that might display new biological activities (combinatorial biosynthesis) [67-69]. Heterologous expression also allows for mobilisation of metagenomic DNA into a cultured surrogate strain, broadening the discovery of secondary metabolites to underexplored niches like unculturable bacteria [70,71]. Finally, unconventional BGCs that could not be directly detected by commonly used genome mining tools can be eventually identified through heterologous expression of DNA fragments from a genomic library, revealing the so far unknown biosynthetic routes of previously discovered NPs [72,73]. Briefly, heterologous expression of secondary metabolite BGCs represents a promising strategy for the discovery of new microbial NPs, the characterization of biosynthetic pathways, and the expansion of diversity of chemical structures and bioactivities.

Nonetheless, some challenges must be addressed when performing heterologous expression. Secondary metabolite gene clusters present a wide range of sizes: from a few kb to more than 100 kb. The vectors used for mobilisation of gene clusters into heterologous hosts can carry a limited size of DNA insert [74]. For small clusters, such as those of lasso peptides (2-5 kb), plasmid vectors may be utilised [75]. In cases where the BGCs of interest are bigger than 10 kb, genomic libraries based on cosmid vectors can be constructed, with the subsequent screening for clones that harbour the desired BGCs [76,77]. However, these vectors are limited to 40-50 kb inserts, which might not cover a complete gene cluster. A strategy to solve this limitation is the assembly of inserts cloned in different vectors into a single cosmid, resulting in reconstitution of the full gene cluster [78,79]. A significant drawback of this approach is that cosmid vectors become unstable with such large inserts. An alternative to the reassembly of BGCs is the use of bacterial artificial chromosomes (BACs) for the creation of genomic libraries with a capacity of >100 kb per clone. Both BAC and cosmid vectors are amenable to genetic manipulation in *E. coli* before being transferred to the final heterologous host for BGC expression [80].

Although heterologous expression often leads to acceptable production levels of the desired compound, in many occasions the yield is lower than that of the original producer

[81,82]. Some of the reasons for such low production levels might be the incompatibility of transcriptional regulators between the native and heterologous hosts, differences in precursor supply, toxicity of compounds expressed by the introduced gene cluster, or the absence of essential cofactors in the host strain. Therefore, genetic engineering of the heterologous gene cluster and/or the host strain is commonly performed to increase the yield of the final product and to facilitate its analysis [83-85].

1.4. Heterologous hosts for natural product discovery

Traditionally, well-established laboratory strains such as *E. coli* and *B. subtilis* have been used in biotechnology for the production of recombinant proteins and other primary metabolites [86,87]. However, the nature of these strains makes them unsuitable for the efficient production of secondary metabolites. They generally do not produce sufficient amounts of the precursors necessary for the biosynthesis of NPs, they lack resistance genes against antibiotics, their regulatory systems are different than those of NP original producers, and very often they cannot properly fold the biosynthetic enzymes of secondary metabolites. In contrast, other bacterial strains such as those of the genus *Streptomyces* are well known to produce a varied collection of NPs, thus representing a more convenient host for heterologous expression of these compounds. An optimal host organism for the expression of secondary metabolite BGCs and the discovery of new NPs should exhibit the following features: (a) rapid growth rate; (b) genetic tractability; (c) varied and abundant production of precursors; (d) great capability to express heterologous BGCs; (e) clean metabolic background with low production of native secondary metabolites; and (f) high production yields of the desired compound.

Currently, many efforts are being directed to develop optimal *Streptomyces* host strains that can efficiently express foreign BGCs and produce sufficient amounts of the compounds of interest to facilitate their purification and characterisation. One of the main obstacles of using streptomycetes as hosts is that they encode a high number of native secondary metabolite biosynthetic pathways, which compete for resources with the heterologous gene clusters, resulting in low yields of the desired product. To overcome this problem, many optimisation strategies have focused on diminishing the native secondary metabolism of heterologous hosts, creating cluster-free chassis strains [88]. It has been discovered that the

subtelomeric regions of three *Streptomyces* genomes, *S. avermitilis*, *S. coelicolor* A3(2) and *S. griseus*, contain unconserved non-essential genes, mostly secondary metabolite genes that could be removed without affecting the growth of the bacterium [42]. Genome reduction, often combined with other genetic manipulations, has been performed in several *Streptomyces* hosts, resulting in optimised heterologous strains.

Streptomyces coelicolor has been for decades the model organism of streptomycetes. Its genome was the first one of the genus to be sequenced [41] and it is the best genetically characterised *Streptomyces* strain [89]. A wide range of tools are available to genetically manipulate the organism, including protocols for transformation and conjugation, well-established replicative and integrative vectors, several promoters for gene expression, terminators, and reporter genes [90]. *S. coelicolor* encodes over 40 secondary metabolite BGCs that produce actinorhodin (*act*), undecylprodigiosin (*red*), calcium-dependant antibiotic (*cda*), and coelimycin (*cpk*), among other compounds. Many heterologous NP gene clusters have been successfully expressed in *S. coelicolor* or in engineered strains derived from the wild type [90-92].

The first modification made on *S. coelicolor* was the deletion of the *act* cluster and mutation of the *red* cluster, resulting in the strain *S. coelicolor* CH999, which is unable to produce both actinorhodin and undecylprodigiosin [93]. This strain was developed for the heterologous production of polyketides, such as the antitumor compound neocarzilin and the antiparasitic drug frenolicin B [94,95]. Another modified strain also deficient in producing actinorhodin and undecylprodigiosin is *S. coelicolor* M512, which was initially created to characterise the regulation of the biosynthesis of these compounds [96]. For that, the pathway-specific transcriptional activator genes, *actII-ORF4* and *red*, were deleted. Heterologous expression of the antibiotic clorobiocin gene cluster in *S. coelicolor* M512, together with genetic engineering of its biosynthetic pathway led to the production of some new analogues of the compound [97]. The generation of a chassis strain, *S. coelicolor* M1146, was achieved by deletion of the secondary metabolite gene clusters *act*, *red*, *cda*, and *cpk* from the wild type *S. coelicolor* M145 [98]. This strain was designed for optimising the heterologous expression of BGCs and facilitating the identification of the synthesised products. For instance, the gene cluster of the antibiotic coumermycin A1 was successfully expressed in *S. coelicolor* M1146, producing 7.5 times more compound than *S. coelicolor*

M512 [99]. Additionally, mutated *rpoB* and *rpsL* genes were introduced in *S. coelicolor* M1146, giving rise to the strains *S. coelicolor* M1152 and M1154 [98]. These mutated genes have been previously reported to improve the production levels of secondary metabolites [100]. The heterologous production of roseoflavin, desotamides, streptocollin, chroramphenicol, and congocidin, among others, was successfully achieved in *S. coelicolor* M1152 and M1154, which in most of the cases produced higher levels of the compounds than the parental strain *S. coelicolor* M1146 [73,98,101,102]. Subsequently, the three native type III polyketide synthase (PKS) genes of *S. coelicolor* M1152 were removed to generate the cluster-free strain *S. coelicolor* M1317 [103]. The type III PKS genes responsible for the production of germicidins were heterologously expressed in this strain, yielding 30% more production of the compounds than the parental strain *S. coelicolor* M1152 [103].

Streptomyces avermitilis is a well-studied microorganism used for industrial production the antiparasitic compound avermectin [104]. It is genetically stable and amenable and genetic manipulation toolkits are well established in *S. avermitilis* [105,106]. Therefore, it represents a promising candidate for the heterologous production of secondary metabolites. The Ikeda group constructed several genome-minimised strains derived from *S. avermitilis*. For instance, they created the large-deletion mutants SUKA2 and SUKA3, both lacking a 1.5-Mb region containing the gene clusters for avermectin and filipin, two of the main secondary metabolites of the organism. Additionally, they removed the biosynthetic genes for oligomycin, another polyketide related to avermectin, resulting in the chassis strains *S. avermitilis* SUKA4 and SUKA5 [104]. The SUKA5 strain was further modified by deletion of the BGCs of the terpenes geosmin, neopentalenolactone, and carotenoid, generating the strains *S. avermitilis* SUKA17 and SUKA22 [107]. The biosynthetic genes for the compounds oxytetracycline, resistomycin, nemadectin, and cephamycin C, among others, have been heterologously expressed in these strains, obtaining increased levels of these NPs in comparison to the wild type *S. avermitilis* or the original producer [107].

Streptomyces lividans is genetically related to *S. coelicolor* and has an exceptional feature of producing low levels of internal proteases, which makes it an ideal host for the expression of recombinant proteins [108]. Additionally, *S. lividans* has been successfully used as a host for the production of peptide-derived secondary metabolites, such as daptomycin, viomycin, labyrinthopeptins, and polycyclic tetramate macrolactams (PTMs) [109-112]. Several strains

based on the wild type *S. lividans* 66 have been engineered to improve the expression of heterologous NP gene clusters.

The most commonly used *S. lividans*-derived strain is *S. lividans* TK24, which lacks the endogenous plasmids SLP2 and SLP3 and contains the point mutation [K88E] in the *rpsL* gene, which is known to enhance the production of secondary metabolites [113]. *S. lividans* TK24 has been used as a heterologous host for identification of the antibiotic berninamycin BGC, synthesis of analogues of the insecticide spinosyn, and investigation of the metabolic pathway leading from the aminoglycoside antibiotic paromamine to kanamycin derivatives [114-116]. Deletion of the actinorhodin biosynthetic genes (*act*) from *S. lividans* TK24 generated the isogenic strains *S. lividans* K4-114 and K4-155. A temperature-sensitive vector was used for substitution of the *act* genes with a resistance gene in *S. lividans* K4-114, while a suicidal vector was introduced in K4-155 [117]. Heterologous expression and characterisation of the gene clusters of several compounds have been performed in the hosts *S. lividans* K4-114 and K4-155, such as the macrolide antibiotic oleandomycin and the aromatic polyketide enterocin gene cluster [62,118]. Moreover, a whole set of genome-reduced strains has been developed for the efficient heterologous expression of the mithramycin A BGC. The so called RedStrep host strains are deficient in the production of several native secondary metabolite gene clusters, and an increase of 3-fold in the production levels of mithramycin A was reported in one of the strains, compared to the parental strain *S. lividans* TK24 [85]. Recently, three new chassis strains, *S. lividans* ΔYA9, *S. lividans* ΔYA10 and *S. lividans* ΔYA11, were generated by deletion of up to 11 endogenous secondary metabolite gene clusters and the subsequent introduction of additional *attB* sites for integration of heterologous clusters [119]. These strains showed a better yield of the heterologously produced tunicamycin, deoxycinnamycin, and griseorhodin than *S. lividans* TK24 [119].

Streptomyces albus has been used in the past for studying the restriction endonuclease system and its ability to cleave bacteriophage DNA [120,121]. It has a fast growth rate (4 days in agar medium) and successful heterologous expression of many secondary metabolite BGCs has been reported in this strain [122]. Genetic manipulation toolkits for cloning and expression of genes are well established in *S. albus*. The strain *S. albus* J1074 is the most commonly used *S. albus*-based heterologous host. It was derived from the wild type *S. albus*

G by removal of the SalI restriction modification system [123] and it shows better conjugation efficiency than the parental strain. *S. albus* J1074 also synthesizes diverse precursors that enable the successful heterologous production of numerous secondary metabolites, e.g., landomycin, fredericamycin, grecocycline, lysolipin, rebeccamycin, and paulomycin [78,124-128]. In many cases, *S. albus* J1074 synthesizes higher levels of the heterologously produced compound than the original producer or other heterologous hosts. For instance, the yield of the herbicidal compounds thaxtomins was improved by 12 times in *S. albus* J1074 in comparison to the parental strain, and the antitumor agent iso-migrastatin was produced in titres 2-fold higher in *S. albus* J1074 than in *S. coelicolor* M-512 and *S. lividans* K4-114 [129,130]. Additionally, *S. albus* J1074 could successfully express BGCs from metagenomic DNA, a feature that is not commonly found in other heterologous hosts [131-134].

The strain *S. albus* J1074 has been further engineered by overexpression of the *crp* gene that codes for the pleiotropic regulator CRP (cAMP receptor protein) which promotes the synthesis of secondary metabolites [135]. Moreover, it has been reported that deletion of the phosphofructokinase gene (*pfk*) in *S. coelicolor* A3(2) leads to increased production of actinorhodin through redirection of the carbon flux towards the production of NADPH, which is necessary for the biosynthesis of secondary metabolites (Borodina, 2008). Therefore, the gene *pfk* was deleted in the *S. albus* strain overexpressing *crp*, resulting in *S. albus Δpfk+crp*. The heterologous production of actinorhodin was tested in both strains, *S. albus+crp* and *S. albus Δpfk+crp*, leading to an increased yield of 1.5 and 2-fold, respectively, when compared to *S. albus* J1074 [135].

The creation of a chassis strain derived from *S. albus* J1074 has been recently reported. A total of 15 native secondary metabolite BGCs, including all the PKS, NRPS, lanthipeptide, and glycoside antibiotic gene clusters, have been removed, resulting in the strain *S. albus* Del14 [64]. This strain displays a metabolic profile remarkably simpler than that of *S. albus* J1074, which is a huge advantage for the identification and purification of heterologously produced compounds. The new host was tested by expressing the gene clusters of various natural products: the polyketides didesmethylmensacarcin, aloesaponarin II, and pyridinopyrone A, the nucleoside antibiotic tunicamycin, and the ribosomally synthesised and post-translational modified peptide (RiPP) cinnamycin. The production yield of all of them was

significantly higher in the cluster-free strain *S. albus* Del14 than in the parental *S. albus* J1074. *S. albus* Del14 was further modified by introduction of additional *attB* sites, allowing the insertion of a higher copy number of gene clusters. The strains *S. albus* B2P1 and *S. albus* B4, carrying 3 and 4 *attB* sites, respectively, were created. Most of the compounds produced in these strains through heterologous expression showed improved yields in comparison to those of the parental strain *S. albus* J1074 [64].

NPs are highly diverse molecules synthesized by complex biosynthetic pathways. Many factors influence the successful activation of their biosynthetic genes and their production. It has been demonstrated that the transfer of secondary metabolite gene clusters to optimized heterologous hosts very frequently results in production of the corresponding compounds. A wide variety of heterologous hosts derived from several *Streptomyces* species has been developed for the expression of foreign gene clusters. Due to the varied nature of BGCs and their specific requirements for activation of transcription, the expression of a particular gene cluster might be triggered in a heterologous strain but not in others. The creation of a universal host strain for the expression of any type of BGC remains rather utopic and efforts in optimizing heterologous hosts need to focus on adapt to the specific features of each gene cluster.

1.5. Outline of the work

The main goal of this thesis was the characterization of cryptic gene clusters and identification of new NPs encoded in the genome of the marine actinomycete *Streptomyces albus* subsp. *chlorinus*. Heterologous expression of a BGC in a host strain allowed the identification of the nybomycin biosynthetic genes and led to the production of a new structure. Additionally, selection of streptomycin-resistant mutants of *S. albus* ssp. *chlorinus* resulted in activation of a type II PKS gene cluster and overproduction of the novel polyketide fredericamycin C_2.

As described in Publication I (Chapter 2), a cryptic BGC from *S. albus* ssp. *chlorinus* was heterologously expressed in the chassis strain *S. albus* Del14, leading to the production of the previously described antibiotic nybomycin. The homology between the heterologously expressed genes and the structurally related compound streptonigrin BGC led us to propose a biosynthesis pathway for nybomcyin. The identification of nybomycin BGC (*nyb* genes)

provides a better understanding of its biosynthesis and enables the potential generation of analogues with new bioactivities.

Besides nybomycin, heterologous expression of the *nyb* genes also yielded a novel compound, benzanthric acid (Publication II, Chapter 2). The new metabolite was found neither in the extract of the nybomycin original producer *S. albus* ssp. *chlorinus* nor in that of the host strain *S. albus* Del14. This suggests that benzanthric acid might be the product of interplay between the introduced *nyb* pathway and the host´s metabolism.

Finally, a streptomycin-resistant mutant was obtained from *S. albus* subsp. *chlorinus* (Publication III, Chapter 2). The mutant strain, *S. albus* subsp. *chlorinus* JR1, showed high production levels of the novel polyketide fredericamycin C_2, which expands the structural diversity of the fredericamycin family. A type II PKS gene cluster was confirmed to be responsible for the biosynthesis of this compound after heterologous expression in the host strain *S. albus* Del14. We propose that a frameshift point mutation causing a deficiency of the RsmG protein is responsible for both the increased production of fredericamycin C_2 and the higher streptomycin resistance in *S. albus* subsp. *chlorinus* JR1, compared to the wild type strain.

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2. Results

Publications

I

Heterologous expression of the nybomycin gene cluster from the marine strain *Streptomyces albus* subsp. *chlorinus* NRRL B-24108

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Communication

Heterologous Expression of the Nybomycin Gene Cluster from the Marine Strain *Streptomyces albus* subsp. *chlorinus* NRRL B-24108

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Abstract: Streptomycetes represent an important reservoir of active secondary metabolites with potential applications in the pharmaceutical industry. The gene clusters responsible for their production are often cryptic under laboratory growth conditions. Characterization of these clusters is therefore essential for the discovery of new microbial pharmaceutical drugs. Here, we report the identification of the previously uncharacterized nybomycin gene cluster from the marine actinomycete *Streptomyces albus* subsp. *chlorinus* through its heterologous expression. Nybomycin has previously been reported to act against quinolone-resistant *Staphylococcus aureus* strains harboring a mutated *gyrA* gene but not against those with intact *gyrA*. The nybomycin-resistant mutants generated from quinolone-resistant mutants have been reported to be caused by a back-mutation in the *gyrA* gene that restores susceptibility to quinolones. On the basis of gene function assignment from bioinformatics analysis, we suggest a model for nybomycin biosynthesis.

Keywords: streptomycetes; secondary metabolites; nybomycin gene cluster; heterologous expression; nybomycin biosynthesis

1. Introduction

Actinobacteria represent a prominent source of natural products with potential industrial applications. The genus *Streptomyces* is especially well known to produce a diverse spectrum of compounds with antibacterial, antifungal, antitumor and even insecticide and herbicide activity [1–3]. The increasing amount of sequenced microbial genomes has provided insight into the unprecedented potential of actinobacteria to biosynthesize natural products [4,5]. Generally, dozens of various secondary metabolite clusters are encoded in their genomes. However, these clusters are often poorly expressed under standard cultivation conditions or even remain silent, thus preventing the isolation and characterization of the encoded compounds. Such uncharacterized clusters with unknown biosynthetic products are usually regarded as cryptic. Different approaches can be used to characterize cryptic clusters, including changing cultivation parameters (OSMAC approach), expression of pleiotropic regulatory genes, introduction of antibiotic-resistant mutations, and refactoring of the biosynthetic pathways [6–9]. Currently, characterization of the cryptic gene clusters encoding natural products often relies on expression of their biosynthetic pathways in the optimized surrogate strains called heterologous hosts or chassis strains. The heterologous expression approach has a number of advantages compared to other cluster characterization methods. The simplified metabolic background of the chassis strains facilitates the identification of natural

products; fast DNA-recombineering methods in *E. coli* and DNA transfer methods into streptomycetes simplify biosynthetic studies, and high production yields enable product supply for structure elucidation and biological activity studies.

In this study, we report the identification and characterization of the previously uncharacterized nybomycin gene cluster from the marine strain *Streptomyces albus* subsp. *chlorinus* NRRL B-24108 through heterologous expression in *Streptomyces albus* Del14. Nybomycin was first isolated in 1955; however, the unique biological activity of the antibiotic was discovered only recently [10]. Nybomycin inhibits growth of quinolone-resistant *Staphylococcus aureus* by targeting the mutated enzyme gyrase. Interestingly, the intact gyrase encoded by the *gyrA* gene without the resistance mutation is not inhibited by the antibiotic. The rare nybomycin-resistant mutants derived from quinolone-resistant *S. aureus* have all been reported to contain the reverse mutation in the *gyrA* gene, causing loss of quinolone resistance. Despite this interesting mode of action, the biosynthetic gene cluster leading to nybomycin production remains unknown. Based on the cluster analysis, we also propose the biosynthetic route leading to the production of nybomycin.

2. Results

Identification of the Nybomycin Gene Cluster through Its Heterologous Expression

In the course of systematic activation of cryptic secondary metabolite clusters from *Streptomyces albus* subsp. *chlorinus* NRRL B-24108 [3], a cluster annotated by the antiSMASH genome mining software [11] as "fatty acid metabolism cluster" was expressed in the heterologous host strains. For this purpose, a BAC 4N24 containing the cluster was isolated from the previously constructed genomic library of *S. albus* subsp. *chlorinus* and transferred into *Streptomyces albus* Del14 and *Streptomyces lividans* TK24 [12,13]. The obtained exconjugant strains *Streptomyces albus* 4N24 and *Streptomyces lividans* 4N24 as well as the corresponding control strains without the BAC *S. albus* Del14 and *S. lividans* TK24 were fermented in the production medium. LC-MS analysis of the exconjugant strains containing the heterologous cluster confirmed its successful expression in *S. albus* 4N24, as indicated by a new peak that was observed in the extract of the strain (Figure 1A,B and Figure S1). Expression of the cluster in *S. lividans* 4N24 did not lead to the production of any new compounds compared with the control strain.

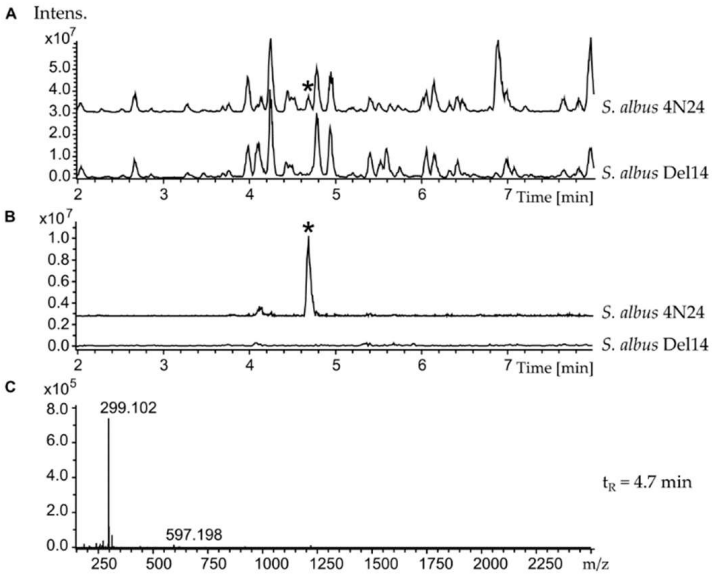

Figure 1. LC-MS chromatograms of crude extracts from *S. albus* 4N24 and *S. albus* Del14. The new peak found in *S. albus* 4N24 crude extract is indicated with an asterisk (*). (**A**) Base peak chromatograms; (**B**) extracted ion chromatograms (299.10 ± 0.1 Da); (**C**) mass spectrum associated to t_R = 4.7 min from *S. albus* 4N24 LC-MS chromatogram.

Analysis of the *S. albus* 4N24 extract by high-resolution MS analysis revealed that the identified peak corresponded to the compound with an $[M + H]^+$ of 299.102 m/z and the deduced molecular formula $C_{16}H_{15}N_2O_4$ (Figure 1C). A search in a natural product database revealed that the identified compound might correspond to the antibiotic nybomycin (Figure 2). To verify this, a nybomycin standard (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used. LC-MS analysis of the pure nybomycin, the *S. albus* 4N24 extract, and the *S. albus* 4N24 extract spiked with pure nybomycin confirmed that the new compound corresponded to nybomycin; the retention time and the mass spectrum of the new compound were identical to those of the pure standard (Figure S2). Additionally, to validate the identity of the detected compound as nybomycin, the former was purified. For this purpose, *S. albus* 4N24 was inoculated into 10 L of DNPM medium, and the culture broth of the strain was extracted with ethyl acetate. The compound was purified from the extract using normal-phase, size-exclusion, and reverse-phase chromatography. The purified compound, as well as the pure nybomycin standard, was used for NMR measurements. The recorded ^{1}H-NMR spectra of the purified compound and of nybomycin were identical (Table S1; Figure S3), which unambiguously proved the identity of the former as nybomycin.

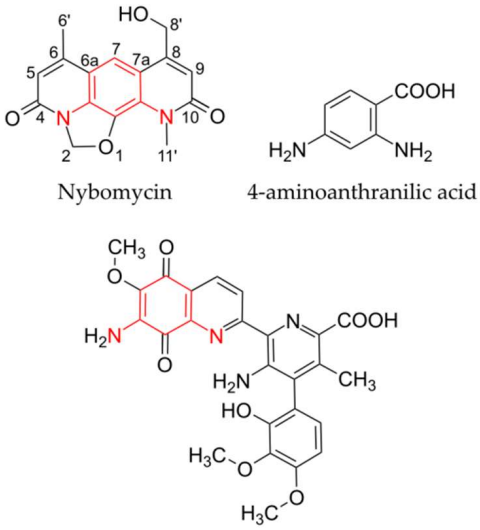

Figure 2. Chemical structures of nybomycin, streptonigrin, and 4-aminoanthranilic acid. The common core structure for nybomycin and streptonigrin are highlighted in red.

BAC 4N24, which contains the nybomycin biosynthetic genes, comprises a 36 kb genomic DNA region with 33 open reading frames (Figure 3, Table 1). A sequence similarity search revealed that nine open reading frames within this region shared homology at the protein level with the genes involved in the biosynthesis of streptonigrin, which is structurally related to nybomycin (Figure 2). Sequence analysis of the DNA fragment cloned in BAC 4N24 revealed the putative streptomycin-resistant gene, a hypothetical gene, a gene encoding an ATP-binding protein, and four additional hypothetical genes followed by the *nybA* gene encoding a putative 3-carboxy-cis,cis-muconate cycloisomerase at its 5′ end (Table 1), which implied that the *nybA* gene might constitute the 5′ end of the nybomycin cluster. The 3′ end of the cloned region comprised the genes encoding a putative methyltransferase, two isopenicillin N synthases, a transporter, three transcriptional regulators, and a hypothetical protein (*nybS*, *nybT*, *nybU*, *nybV*, *nybW*, *nybX*, *nybZ*, and *nybY*, respectively). To clarify whether these genes were part of the nybomycin biosynthetic cluster, two BACs 4M14 and 6M11 that partially overlap with BAC 4N24 were isolated from the genomic library of *S. albus* subsp. *chlorinus* and expressed in *S. albus* Del14. Both the isolated 4M14 and 6M11 BACs completely covered the 5′ end of the fragment cloned in the original BAC 4N24, which led to nybomycin production. Compared with BAC 4N24, BAC 4M14 lacked the region downstream of the *nybR* gene, while BAC 6M11 lacked the region downstream of the *nybL* gene (Figure 3). The obtained strains *S. albus* 4M14 and *S. albus* 6M11 were analyzed

together with *S. albus* 4N24 for nybomycin production. During the LC-MS analysis, no nybomycin was detected in the extracts of either the *S. albus* 4M14 or *S. albus* 6M11 strains (Figure S4). Nybomycin was readily detectable in the extract of *S. albus* 4N24. These results give evidence that the 3'-terminal region of 4N24, which contains the genes downstream of *nybR* (*nybS* to *nybZ*), is essential for nybomycin production. Taken together, our results suggest that the genes from *nybA* to *nybZ* might constitute the nybomycin gene cluster, which is further supported by the fact that the genes from *nybA* to *nybF* and *nybN* to *nybP* share high levels of homology with genes in the biosynthetic cluster of streptonigrin (Table 1), a compound that is structurally similar to nybomycin (Figure 2).

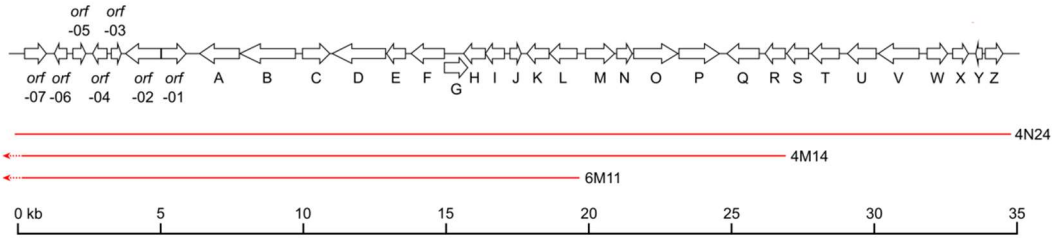

Figure 3. Nybomycin biosynthetic gene cluster. Genetic organization is shown. Below, maps of three different BAC clones are displayed: BAC 4N24 contains the full sequence of the nybomycin gene cluster. BAC 4M14 lacks the genes from *nybS* to *nybZ*; and BAC 6M11 lacks the genes from *nybM* to *nybZ*.

Table 1. Proposed functions of genes in nybomycin biosynthetic gene cluster and homology with streptonigrin biosynthetic genes.

Gene	Size (aa)	Proposed Function	GenBank homologue [1]	Identity/ Similarity (%)	Streptonigrin Gene Cluster Homologue [2]	Identity/ Similarity (%)
orf-07	268	Streptomycin 3″-adenylyltransferase	WP_037865927.1	71/77	-	-
orf-06	159	Hypothetical protein	WP_027736486.1	86/93	-	-
orf-05	163	ATP-binding protein	WP_052413949.1	76/83	-	-
orf-04	332	Hypothetical protein	WP_055499466.1	74/82	-	-
orf-03	341	Hypothetical protein	WP_030379123.1	71/80	-	-
orf-02	494	Hypothetical protein	WP_078869279.1	70/81	-	-
orf-01	242	Hypothetical protein	-	-	-	-
nybA	475	3-carboxy-cis,cis-muconate cycloisomerase	WP_066029238.1	66/85	*stnL* (AFW04563.1)	71/76
nybB	669	FAD-binding protein	WP_066029239.1	77/84	*stnK1* (AFW04562.1)	66/75
nybC	325	NADPH:quinone reductase	WP_066029240.1	81/87	*stnH1* (AFW04558.1)	63/71
nybD	638	Anthranilate synthase	WP_079145437.1	81/87	*stnM1* (AFW04564.1)	65/75
nybE	227	Isochorismatase	WP_066029243.1	84/89	*stnM2* (AFW04565.1)	66/76
nybF	402	DAHP synthase	WP_066029245.1	81/87	*stnM3* (AFW04567.1)	62/69
nybG	279	Hypothetical protein	-	-	-	-
nybH	257	Vicinal oxygen chelate protein	WP_066029246.1	64/72	-	-
nybI	222	NAD(P)H:dehydrogenase	WP_066029248.1	90/95	-	-
nybJ	135	Hypothetical protein	WP_066029250.1	76/86	-	-
nybK	266	*N*-acetyltransferase	WP_066029251.1	80/88	-	-
nybL	333	Amidohydrolase	WP_066029254.1	84/90	-	-
nybM	354	Acetoacetyl-CoA synthase	WP_066029256.1	82/87	-	-
nybN	182	Aromatase/cyclase	WP_066029258.1	78/85	*stnI* (AFW04559.1)	54/67
nybO	550	Long-chain acyl-CoA synthetase	WP_066029260.1	85/90	*stnJ* (AFW04560.1)	68/81
nybP	476	Salicylate hydroxylase	WP_079145438.0	74/79	*stnH2* (AFW04561.1)	62/72
nybQ	376	Hypothetical protein	WP_030685222.1	57/69	-	-
nybR	238	NAD-dependent epimerase	WP_066029265.1	82/89	-	-
nybS	253	SAM-dependent methyltransferase	WP_079145439.1	89/94	-	-
nybT	333	Isopenicillin N synthase family oxygenase	WP_078974705.1	80/89	-	-
nybU	342	Isopenicillin N synthase family oxygenase	WP_078974705.1	72/83	-	-
nybV	495	MFS transporter	WP_079145411.1	81/87	-	-
nybW	243	Transcriptional regulator	WP_079145410.1	82/93	-	-
nybX	197	Transcriptional regulator	WP_025356654.1	89/93	-	-
nybY	87	Hypothetical protein	WP_086560781.1	68/81	-	-
nybZ	219	Transcriptional regulator	WP_057613815.1	79/86	-	-

[1] NCBI accession numbers are given. [2] NCBI accession numbers are shown in parentheses.

3. Discussion

The antibiotic nybomycin was discovered in 1955 in the culture broth of streptomycetes A 717 [14]. The structural features of nybomycin as a fused pyridoquinolone ring system and an angularly fused oxazoline ring are of particular biosynthetic interest as they have not been reported for other natural products [15]. Despite the unique structure of nybomycin, its biosynthetic cluster and biosynthetic route

have remained elusive. Only the results of feeding studies imply that acetate, methionine, and some non-identified shikimate-type intermediates serve as biosynthetic precursors for nybomycin [15,16]. In this article, we have described the identification of the nybomycin biosynthetic gene cluster from the marine streptomycete *S. albus* subsp. *chlorinus* NRRL B-24108 through its expression in the cluster-free heterologous host *S. albus* Del14.

Sequence analysis of the DNA fragment cloned in BAC 4N24 has revealed that a number of genes within this fragment are highly homologous to the genes involved in biosynthesis of the antibiotic streptonigrin (Table 1) [17]. Direct comparison of nybomycin and streptonigrin has shown distinct structural similarity, with both structures containing a diamino-substituted, six-membered ring (Figure 2). The structural similarity and the partial homology of the gene clusters suggest that nybomycin and streptonigrin biosynthetic routes can have some similar biosynthetic intermediates and enzymatic reactions.

Based on the sequence analysis and the results of BAC expression, we propose that the genes *nybA* to *nybZ* constitute the nybomycin gene cluster. The *nybA* gene, encoding a putative 3-carboxy-cis, cis-muconate cycloisomerase, is homologous to the streptonigrin biosynthetic gene *stnL*. The *nybZ* gene encodes a putative transcriptional regulator that might also participate in nybomycin biosynthesis. Similar to the streptonigrin pathway, the 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase encoded by *nybF* is likely to catalyze the first reaction in the nybomycin biosynthetic route. DAHP synthase is responsible for the first reaction of the shikimate pathway—biosynthesis of 3-deoxy-D-arabino-hept-2-ulosonate 7-phosphate (DAHP) from phosphoenolpyruvate and D-erythrose 4-phosphate (Figure 4) which is supported by the results of feeding experiments that have demonstrated that the carbons of the central ring of nybomycin are derived from a shikimate-type intermediate [15]. Catalyzing the first reaction, DAHP synthase regulates the amount of carbon entering the shikimate pathway and therefore can be responsible for its upregulation to provide sufficient amounts of biosynthetic precursors for nybomycin. The genes encoding enzymes responsible for the conversion of DAHP into chorismate are absent in the nybomycin cluster and in the streptonigrin pathway [17]. Therefore, the host's primary metabolism enzymes most likely overtake these biosynthetic steps. We propose that the second reaction catalyzed by the enzymes encoded in the biosynthetic cluster is the conversion of chorismate into 4-aminoanthranilic acid (Figure 4), which is a key intermediate in both nybomycin and streptonigrin biosynthesis (Figure 2). Isolation of 4-aminoanthranilic acid from the culture broth of the streptonigrin producer *Streptomyces flocculus* supports this suggestion [18]. Furthermore, 4-aminoanthranilic acid has also been shown to incorporate into streptonigrin [18]. We propose that the products of the *nybC*, *nybD*, *nybE*, and *nybL* genes might be responsible for the conversion of chorismate into 4-aminoanthranilic acid. The *nybL* gene, encoding putative amidohydrolase [19], has no homologue in the streptonigrin biosynthetic pathway. We suggest that the protein product of *nybL* may provide enough supply of ammonia for the amination of chorismate by anthranilate synthase [20], encoded by *nybD*, during the biosynthesis of 4-aminoanthranilic acid. In the streptonigrin biosynthesis, the function of the *nybL* gene may be overtaken by the one of the host's primary metabolism enzymes.

After formation of 4-aminoanthranilic acid, it is then hydroxylated in the third position and decarboxylated, generating 2,6-diaminophenol (Figure 4). The *nybP* gene encoding putative salicylate hydroxylase might be responsible for the hydroxylation reaction. Then, two acetoacetate units are attached to the amino groups of 2,6-diaminophenol through the action of the *N*-acetyltransferase encoded by the *nybK* gene (Figure 4). The putative acetoacetyl-CoA synthase encoded by the *nybM* gene catalyses the formation of acetoacetyl-CoA from acetyl-CoA and malonyl-CoA. The NybM enzyme is likely responsible for the production of sufficient amounts of acetoacetate, which is used as a precursor in nybomycin biosynthesis. The precursor role of acetoacetate in nybomycin biosynthesis is supported by the results of the feeding experiments, which have unequivocally defined acetate as the source of the exterior carbons of the pyridone rings [16]. We speculate that after attachment of the acetoacetate units, the putative cyclase encoded by the *nybN* gene catalyses the closure of the pyridone rings, leading to

formation of intermediate **1** (Figure 4). The methylation of nitrogen within the pyridone rings is likely to be catalyzed by the SAM-dependent methyltransferase encoded by *nybS*. We hypothesize that the next reaction in nybomycin biosynthesis is a closure of the oxazoline ring (Figure 4). This step might be catalyzed by the product of *nybT* or *nybU*, which code for isopenicillin N synthases (IPNS). IPNS is responsible for the biosynthesis of isopenicillin N through a bicyclization reaction—first, the formation of a C-N bond generates a β-lactam ring; then, the closure of a five-membered thiazolidine ring is accomplished by the formation of a C-S bond [21]. Sulfur and oxygen have similar properties [22]. Therefore, in a similar way to thiazolidine ring closure, IPNS could be able to catalyze the formation of a C-O bond between the carbon atom of the *N*-methyl group and the oxygen atom of the OH group present in intermediate **2**, generating an oxazoline ring (Figure 4). Finally, a hydroxylation reaction takes place at C-8′, possibly catalyzed by the oxidoreductase NybB, giving rise to nybomycin final structure. The compound is then secreted to the extracellular space, most likely by the membrane transporter encoded by *nybV*. Expression of secondary metabolite biosynthesis genes is commonly regulated by activators and repressors coded by genes that are located within the same cluster. We hypothesize that the products of *nybW*, *nybX*, and *nybZ*, which encode putative transcriptional regulators, might control the expression of the genes involved in nybomycin biosynthesis.

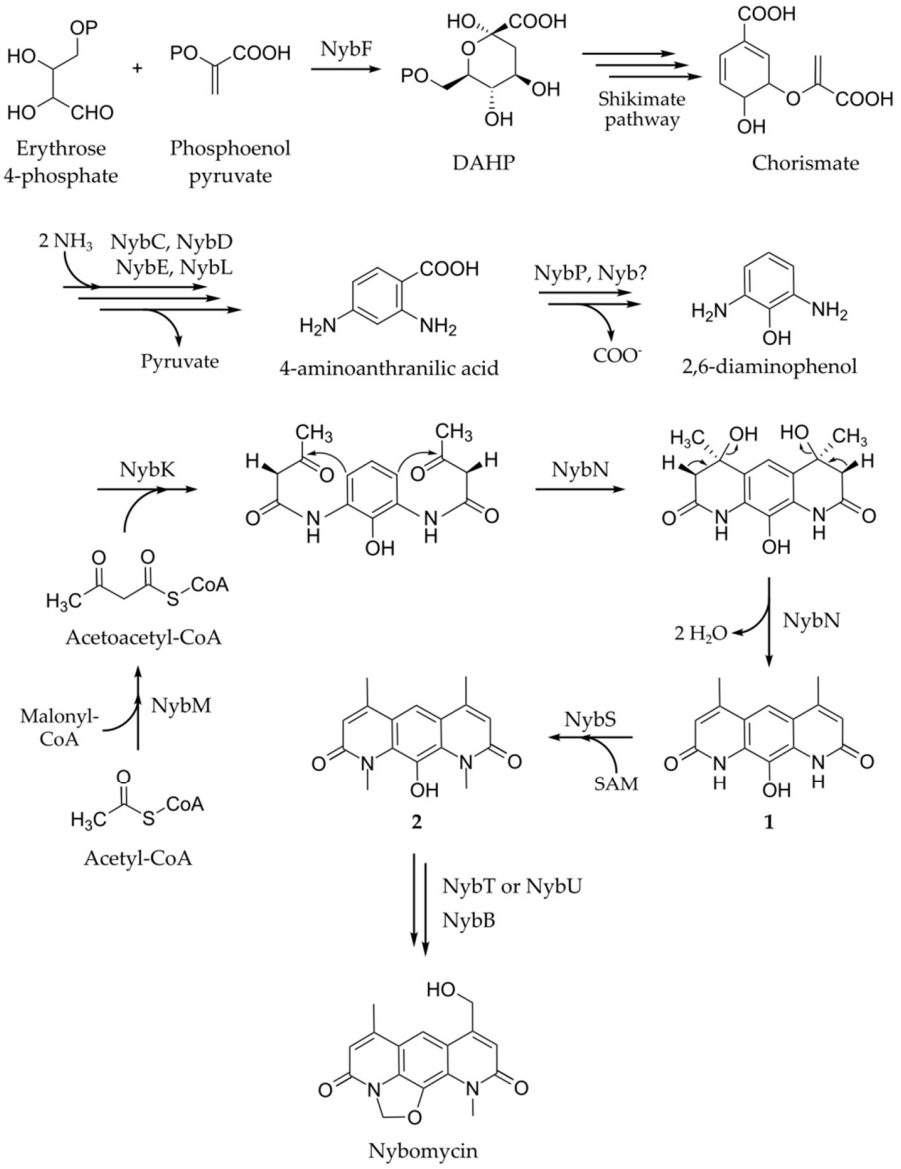

Figure 4. Proposed biosynthetic pathway of nybomycin.

In this paper we report the identification of the gene cluster encoding production of the structurally unique antibiotic nybomycin. Biological activity of nybomycin is also of particular interest as it inhibits growth of quinolone-resistant *Staphylococcus aureus*, dormant *Mycobacterium tuberculosis*, and other Gram-positive and Gram-negative bacteria [10,14,23]. Interestingly, nybomycin targets solely the mutant, quinolone-resistant DNA gyrase with a Ser84Leu substitution, while it is inactive against the wild-type, quinolone-sensitive form of the enzyme [10]. The mutation described thus far to cause nybomycin resistance is a reverse Leu84Ser mutation within the *gyrA* gene that restores quinolone sensitivity. The identification of the nybomycin cluster presented in this paper enables biosynthetic studies of nybomycin production, generation of new nybomycin derivatives, and optimization of its production as well as a nybomycin supply for further biological studies. Together, these works might help fight the development of quinolone resistance and revive quinolones as an effective class of antibiotics.

4. Materials and Methods

4.1. General Experimental Procedures

All strains and BACs used in this work are listed in Table S2. *Escherichia coli* strains were cultured in LB medium [24]. *Streptomyces* strains were grown on soya flour mannitol agar (MS agar) [25] for sporulation and conjugation and in liquid tryptic soy broth (TSB; Sigma-Aldrich, St. Louis, MO, USA). For secondary metabolite expression, liquid DNPM medium (40 g/L dextrin, 7.5 g/L soytone, 5 g/L baking yeast, and 21 g/L MOPS, pH 6.8) was used. The antibiotics kanamycin, apramycin and nalidixic acid were supplemented when required.

4.2. Isolation and Manipulation of DNA

BAC extraction from a *Streptomyces albus* subsp. *chlorinus* constructed genomic library (Intact Genomics, USA), DNA manipulation, *E. coli* transformation, and *E. coli*/*Streptomyces* intergeneric conjugation were performed according to standard protocols [24–26]. Plasmid DNA was purified with the BACMAX™ DNA purification kit (Lucigen, Middleton, WI, USA). Restriction endonucleases were used according to manufacturer's recommendations (New England Biolabs, Ipswich, MA, USA).

4.3. Metabolite Extraction and Analysis

Streptomyces strains were grown in 10 mL of TSB for 1 day, and 1 mL of each culture was used to inoculate 50 mL of production medium. Cultures were grown for 7 days at 28 °C. Metabolites were extracted with ethyl acetate from the supernatant, evaporated, and dissolved in methanol. One µL of each sample was separated using a Dionex Ultimate 3000 UPLC (Thermo Fisher Scientific, Waltham, MA, USA), a 10-cm ACQUITY UPLC® BEH C18 column, 1.7 µm (Waters, Milford, MA, USA) and a linear gradient of 0.1% formic acid solution in acetonitrile against 0.1% formic acid solution in water from 5% to 95% in 18 min at a flow rate of 0.6 mL/min. Samples were analyzed using an amaZon speed mass spectrometer or maXis high-resolution LC-QTOF system (Bruker, USA). Data were collected and analyzed with the Bruker Compass Data Analysis software, version 4.1 (Bruker, Billerica, MA, USA). Monoisotopic mass was searched in the natural product database DNP (Dictionary of Natural Products [27]).

4.4. Nybomycin Isolation and ^{1}H-NMR Spectroscopy

Streptomyces albus 4N24 was grown in 30 mL of TSB for 1 day, and 1 mL of the preculture was used to inoculate 100 flasks containing 100 mL of DNPM medium. Cultures were incubated at 28 °C for 7 days. Metabolites from the supernatant were extracted as described above. The crude extract was fractionated by normal phase chromatography on a prepacked silica cartridge (Biotage, Uppsala, Sweden) using hexane, dichloromethane, ethyl acetate, and methanol as the mobile phase. Fractions

containing nybomycin were detected by LC-MS analysis. They were pooled together, evaporated, and dissolved in methanol. The sample was further separated by size-exclusion chromatography on an LH 20 Sephadex column (Sigma-Aldrich, USA) using methanol as the solvent. Finally, the sample was separated by semipreparative HPLC (Dionex UltiMate 3000, Thermo Fisher Scientific, USA) using a C18 column (Synergi 10 µm, 250 × 10 mm; Phenomenex, Aschaffenburg, Germany) and a 0.1% formic acid solution in acetonitrile as the mobile phase to obtain nybomycin (0.1 mg). Individual peaks were collected and analyzed by LC-MS as described above. The ^{1}H-NMR spectra were recorded on a Bruker Avance 500 spectrometer (Bruker, BioSpin GmbH, Rheinstetten, Germany) at 300 K equipped with a 5 mm BBO probe using deuterated trifluoroacetic acid (Deutero, Kastellaun, Germany) as solvent containing tetramethylsilane (TMS) as a reference. The chemical shifts were reported in parts per million (ppm) relative to TMS. All spectra were recorded with the standard ^{1}H pulse program using 128 scans.

4.5. Genome Mining and Bioinformatics Analysis

The *S. albus* subsp. *chlorinus* genome was screened for secondary metabolite biosynthetic gene clusters using the antiSMASH [11] online tool (https://antismash.secondarymetabolites.org/#!/start) and the software Geneious 11.0.3 [28]. The DNA sequence of the nybomycin gene cluster was deposited into GenBank under accession number MH924838.

Supplementary Materials: The following are available online at http://www.mdpi.com/1660-3397/16/11/435/s1, Table S1: ^{1}H-NMR data for isolated and standard nybomycin; Table S2: Bacterial strains and BACs used in this work; Figure S1: UV chromatograms of crude extracts from *S. albus* 4N24 and *S. albus* Del14; Figure S2: Comparison of LC-MS chromatograms of *Streptomyces albus* 4N24 crude extract and a nybomycin standard; Figure S3: ^{1}H-NMR spectra of a nybomycin standard and nybomycin isolated from *S. albus* 4N24; Figure S4: Extracted ion chromatograms of *S. albus* 4M14, *S. albus* 6M11, and *S. albus* 4N24 crude extracts.

Author Contributions: M.R.E., M.M., and A.L. designed experiments; M.R.E. performed experiments; N.G. performed and evaluated the NMR analysis; S.N. contributed to compound identification; M.R.E., M.M., and A.L. analyzed the data and wrote the manuscript; and all authors reviewed the manuscript.

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Supplementary Information

Identification and Characterisation of the Nybomycin Gene Cluster from the Marine Strain Streptomyces albus subsp. chlorinus NRRL B-24108

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Tables

Table S1. ^{1}H-NMR (500 MHz, d-TFA) data for isolated and standard nybomycin.

Isolated nybomycin		Standard nybomycin	
Position	δ_H	Position	δ_H
2	6.77 s	2	6.77 s
5	7.09 s	5	7.09 s
6′	2.81 s	6′	2.81 s
7	7.67 s	7	7.67 s
8′	5.52 s	8′	5.52 s
9	8.10 s	9	8.10 s
11′	4.47 s	11′	4.47 s

Table S2. Bacterial strains and BACs used in this work.

Bacterial strain	Features	Reference/Source
Streptomyces albus subsp. *chlorinus* NRRL B-24108	*S. albus* subspecies strain harboring nybomycin biosynthetic gene cluster	[1]
Streptomyces albus Del14	Wild-type strain	[2]
Streptomyces albus 4N24	*S. albus* strain with BAC 4N24 insertion	This work
Streptomyces albus 4M14	*S. albus* strain with BAC 4M14 insertion	This work
Streptomyces albus 6M11	*S. albus* strain with BAC 6M11 insertion	This work
Streptomyces lividans TK24	Wild-type strain	[3]
Streptomyces lividans 4N24	*S. lividans* strain with BAC 4N24 insertion	This work
Escherichia coli ET12567 pUB307	Donor strain for intergeneric conjugation	[4]
Escherichia coli DH10β	General cloning strain	[5]
BACs		
pSMART	AmR; BAC vector	Lucigen (USA)
4N24/4M14/6M11	BACs containing full or partial nybomycin gene cluster	This work

Figures

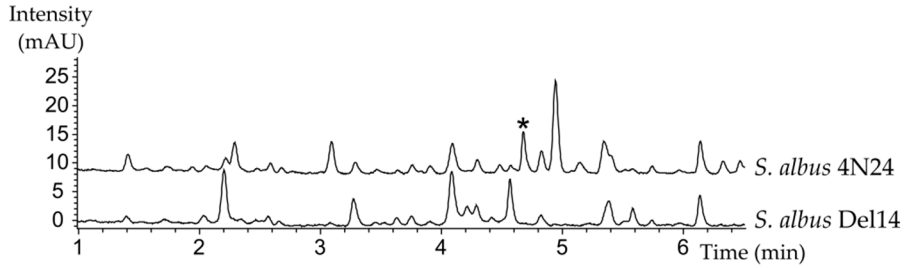

Figure S1. UV chromatograms of crude extracts from *S. albus* 4N24 and *S. albus* Del14. The new peak found in *S. albus* 4N24 crude extract is indicated with an asterisk (*). The profiles correspond to wavelength 285 nm.

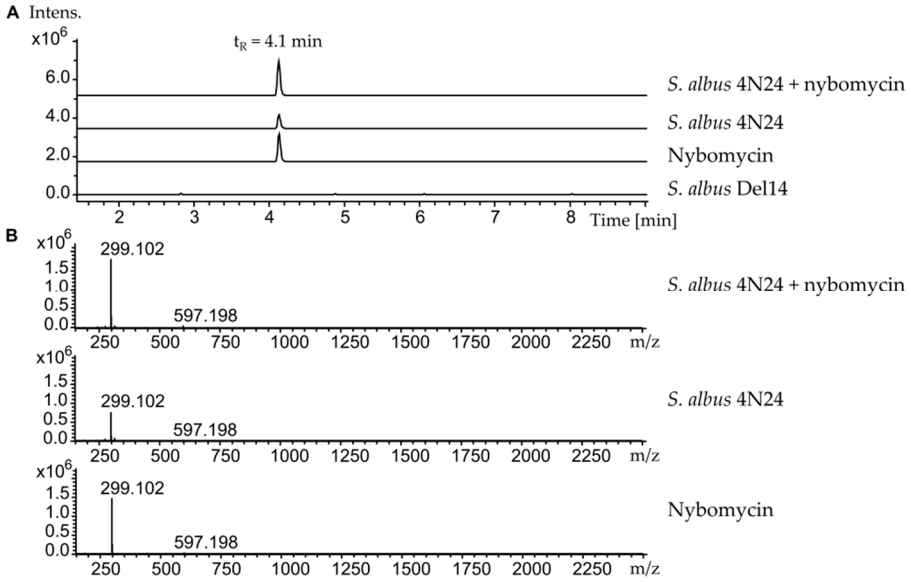

Figure S2. Comparison of LC-MS chromatograms at t_R = 4.1 min of *Streptomyces albus* 4N24 crude extract and a nybomycin standard. (**A**) Extracted ion chromatograms (299.10 ± 0.1 Da) of crude extract from *S. albus* 4N24 supplemented with 0.05 mg/ml of pure nybomycin, crude extract from *S. albus* 4N24 broth culture, a 0.05 mg/ml nybomycin solution in methanol, and crude extract from *S. albus* Del14 broth culture. (**B**) Mass spectra associated to the peak at t_R = 4.1 min from the three upper chromatograms displayed in (A).

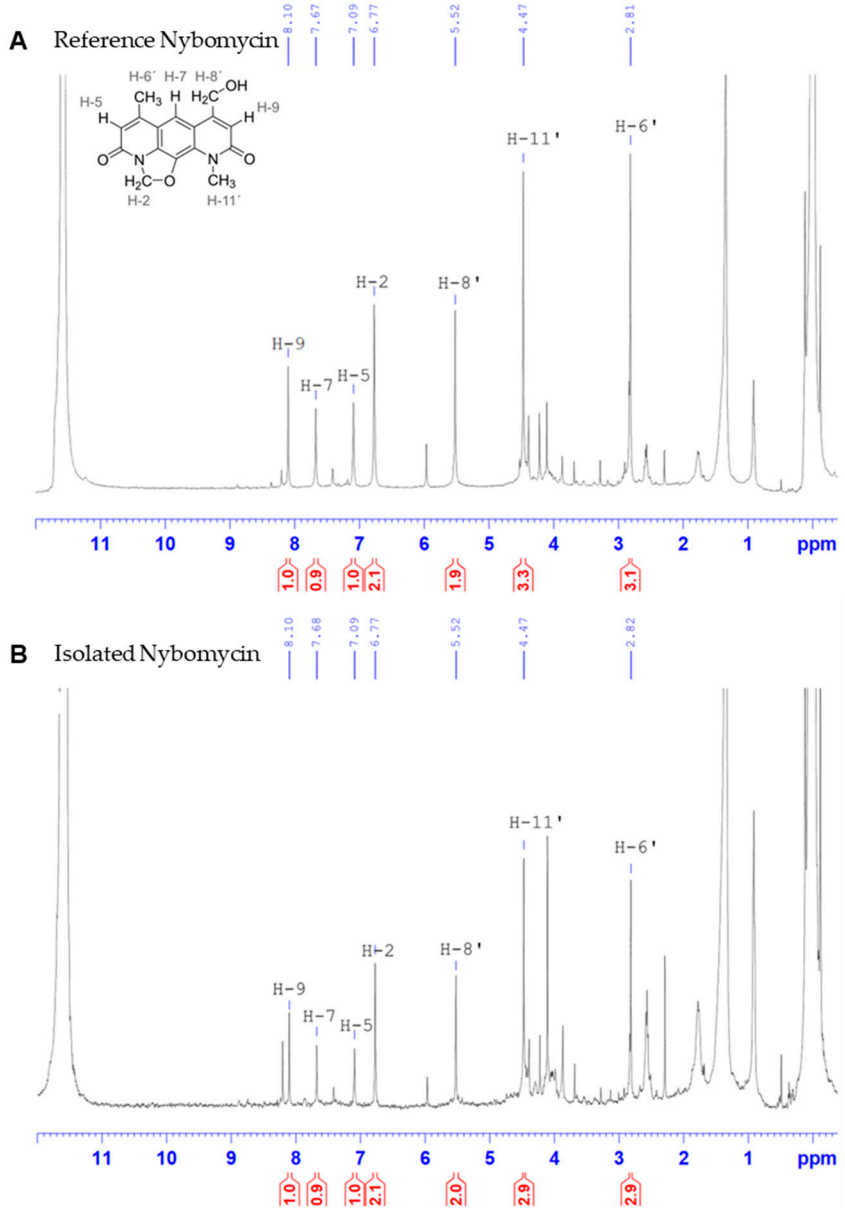

Figure S3. ^{1}H-NMR (500 MHz, d-TFA) spectra of a nybomycin standard and nybomycin isolated from *S. albus* 4N24. ^{1}H-NMR spectra of nybomycin standard (**A**) and the isolated nybomycin (**B**) are identical.

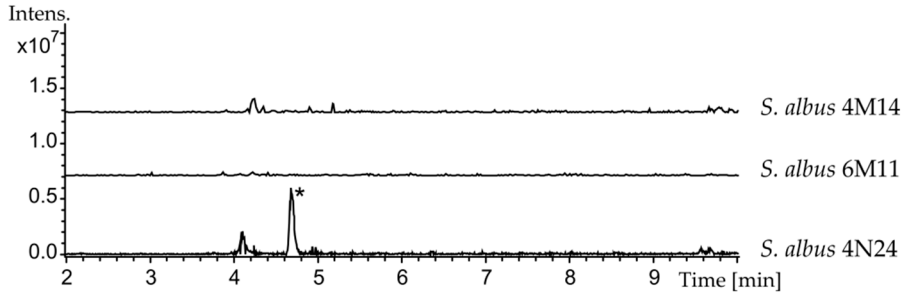

Figure S4. Extracted ion chromatograms of *S. albus* 4M14, *S. albus* 6M11, and *S. albus* 4N24 crude extracts. Nybomycin appears only in *S. albus* 4N24 crude extract, which shows a peak at t_R = 4.7 min (indicated with an asterisk) comprising an $[M+H]^+$ of 299.102 m/z (mass spectrum not shown).

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II

Benzanthric acid, a novel metabolite from *Streptomyces albus* Del14 expressing the nybomycin gene cluster

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Benzanthric Acid, a Novel Metabolite From *Streptomyces albus* Del14 Expressing the Nybomycin Gene Cluster

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Streptomycetes constitute a diverse bacterial group able to produce a wide variety of secondary metabolites with potential applications in the pharmacy industry. However, the genes responsible for the biosynthesis of these compounds are very frequently inactive or expressed at very low levels under standard laboratory cultivation conditions. Therefore, the activation or upregulation of secondary metabolite biosynthesis genes is a crucial step for the discovery of new bioactive natural products. We have recently reported the discovery of the biosynthetic genes for the antibiotic nybomycin (*nyb* genes) in *Streptomyces albus* subsp. *chlorinus*. The *nyb* genes were expressed in the heterologous host *Streptomyces albus* Del14, which produces not only nybomycin, but also a novel compound. In this study, we describe the isolation, purification, and structure elucidation of the new substance named benzanthric acid.

Keywords: benzanthric acid, biosynthetic gene cluster, heterologous expression, nybomycin, secondary metabolite, *Streptomyces*

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INTRODUCTION

Numerous species of the actinomycetal genus *Streptomyces* harbor metabolic pathways that produce secondary metabolites with a broad spectrum of bioactivities, which in many cases represent potential drug leads for the development of pharmaceuticals (Paulus et al., 2017; Protasov et al., 2017). The biosynthetic genes for these metabolites are generally arranged in clusters whose expression is often very low under laboratory cultivation conditions. Therefore, diverse strategies have been developed in order to induce the biosynthesis of natural products in *Streptomyces* strains (McKenzie et al., 2010; Olano et al., 2014; English et al., 2017). A widely used approach consists in the heterologous expression of a specific gene cluster in an optimized host strain where some of the native secondary metabolite genes have been removed (Myronovskyi et al., 2018; Bu et al., 2019). The simplified metabolic background promotes the channeling of biosynthetic precursors toward the production of the heterologous metabolites and facilitates their identification.

The marine strain *Streptomyces albus* subsp. *chlorinus* NRRL B-24108 is responsible for the production of bioactive metabolites such as the herbicide albucidin and the antibiotic nybomycin (Hahn et al., 2009; Rodriguez Estevez et al., 2018). We have recently discovered the biosynthetic genes for nybomycin (*nyb* genes) in *S. albus* subsp. *chlorinus* through a heterologous expression approach using the chassis strain *Streptomyces albus* Del14 as a host (Rodriguez Estevez et al., 2018).

Besides nybomycin, we detected a new metabolite in the extract of *S. albus* 4N24 expressing the *nyb* genes. In this study, we report the isolation, purification and structure elucidation of the novel compound, benzanthric acid, from the culture of *Streptomyces albus* 4N24.

MATERIALS AND METHODS

General Experimental Procedures

Streptomyces and *Escherichia coli* strains used in this work are listed in **Supplementary Table 1**. LB medium was used for cultivation of *E. coli* strains according to standard protocols (Green and Sambrook, 2012). *Streptomyces* strains were cultivated in standard media (Kieser et al., 2000): soy flour mannitol agar (MS agar) and liquid tryptic soy broth (TSB; Sigma-Aldrich, St. Louis, MO, USA). Additionally, liquid DNPM medium (40 g/L dextrin, 7.5 g/L soytone, 5 g/L baking yeast, and 21 g/L MOPS, pH 6.8) was used for secondary metabolite expression. The following antibiotics were supplemented when required at concentrations of 50 μg/μl (solid medium) or 25 μg/μl (liquid medium): kanamycin, apramycin, and nalidixic acid (Carl Roth, Germany; Sigma-Aldrich, USA).

Isolation and Manipulation of DNA

Bacterial artificial chromosome (BAC) 4N24 was isolated from a library comprising the genome of *Streptomyces albus* subsp. *chlorinus* (Intact Genomics, St. Louis, MO, USA). DNA manipulations and cloning procedures including *E. coli* transformation and intergeneric conjugation between *E. coli*/*Streptomyces* were performed following standard protocols (Kieser et al., 2000; Green and Sambrook, 2012; Rebets et al., 2017). Plasmid DNA was purified with the BACMAX™ DNA purification kit (Lucigen, Middleton, WI, USA). Restriction endonucleases were used for plasmid diagnostic test (New England Biolabs, Ipswich, MA, USA).

Metabolite Extraction and Analysis

Streptomyces albus 4N24 as well as the control strains *Streptomyces albus* De14 and *Streptomyces albus* subsp. *chlorinus* were cultivated in 15 mL TSB medium for 24 h at 28°C. Main cultures containing 50 mL of DNPM were inoculated with 1 mL of pre-culture. After 7 days of cultivation at 28°C, the secreted metabolites were extracted with ethyl acetate and butanol, followed by solvent evaporation. The dry extracts were solved in 1 mL methanol and 1 μL of the solved sample was separated using a Dionex Ultimate 3000 UPLC (Thermo Fisher Scientific, Waltham, MA, USA), and a 10-cm ACQUITY UPLC® BEH C18 column, 1.7 μm (Waters, Milford, MA, USA). The mobile phase was comprised of two solvents: formic acid solved in acetonitrile (0.1%) and formic acid solved in water (0.1%). Solvent concentrations varied in a linear gradient from 5 to 95% in 18 min at a flow rate of 0.6 mL/min. The UPLC system was coupled either to amaZon speed mass spectrometer or maXis high-resolution LC-QTOF system (Bruker, USA), allowing the mass spectrometry analysis of the extracts. The software Bruker Compass Data Analysis version 4.1 (Bruker, Billerica, MA, USA) was used for data analysis. Monoisotopic mass was searched in the natural product database DNP (Dictionary of Natural Products; Buckingham, 1993).

Benzanthric Acid Isolation and Nuclear Magnetic Resonance (NMR) Spectroscopy

A flask containing 30 mL TSB medium was inoculated with *S. albus* 4N24 and incubated at 28°C for 24 h. The production culture consisted of 10 L divided into 100 flasks, each containing 100 mL DNPM medium and inoculated with 1 mL of pre-culture. After 7 days of cultivation at 28°C, metabolite extraction was performed as described above. The crude extract was fractionated by size-exclusion chromatography on an LH 20 Sephadex column (Sigma-Aldrich, USA) using methanol as the mobile phase. Resulting fractions were analyzed by LC-MS and those containing benzanthric acid were further separated by preparative HPLC (Waters 2545 Binary Gradient module, Waters, Milford, MA, USA) using a Nucleodur® C18 HTec column (5 μm, 250 × 21 mm, Macherey-Nagel, Düren, Germany) with a linear gradient of 0.1% formic acid solution in methanol against 0.1% formic acid solution in water, yielding 5 mg of benzanthric acid. UV spectra were recorded with a PAD detector (Photodiode Array Detector, Waters 2998, Waters, Milford, MA, USA). All reported NMR spectra were recorded at 298 K on a Bruker Avance 500 with a 5 mm BBO probe (Bruker, BioSpin GmbH, Rheinstetten, Germany) using DMSO-d_6 (deuterated dimethyl sulfoxide) as solvent. The solvent peak was used as an internal standard and set to δ_H 2.49 for the ^{1}H-NMR and δ_C 39.50 for the ^{13}C-NMR, respectively. For the structural elucidation the following spectra were recorded with standard pulse programs: ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H-correlated spectroscopy (COZY), heteronuclear single quantum spectroscopy (HSQC), heteronuclear multiple bond correlation (HMBC), and rotating frame Overhauser enhancement spectroscopy (ROESY, spin-lock: 300 ms).

Feeding Experiment With Anthranilic Acid (Phenyl-$^{13}C_6$)

Two flasks containing 25 mL of DNPM medium were inoculated with *S. albus* 4N24 as described above (Section Metabolite Extraction and Analysis). One of the cultures was supplemented with 5 mg/mL of anthranilic acid (phenyl-$^{13}C_6$) (Cambridge Isotope Laboratories, Andover, MA, USA) at intervals of 12 h for 4 days, while the second one was used as a control. After further 24 h cultivation, the metabolites were extracted from the supernatant as described in Section Metabolite Extraction and Analysis.

Antimicrobial Susceptibility Test

Susceptibility tests were performed by the disk diffusion method described in Bauer et al. (1966). Ten mL of LB soft agar (10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract, 7 g/l agar) were inoculated with the strains *Escherichia coli* GB2005, *Bacillus subtilis* ATCC 6633 or *Pseudomonas putida* KT2440 and poured on LB agar plates. Four paper disks

(Macherey and Nagel, Düren, Germany) were coated with 100, 50, 10, and 0.5 μg of benzanthric acid solved in DMSO, respectively, and placed onto the solidified soft agar. DMSO was used as a negative control and the antibiotics ampicillin, chloramphenicol, and nalidixic acid (50 mg/mL, respectively) as positive controls. The plates were incubated at 28°C overnight.

Herbicidal Pre-emergence Test

Seeds of *Agrostis stolonifera* (Juliwa HESA, Heidelberg, Germany) were placed into the wells of a 96-well microtiter plate (Sarstedt, Nümbrecht, Germany). A solution containing 2.2 g/l Murashige & Skoog plant salts (Serva, Heidelberg, Germany) and 1.6 g/l Gamborg's B5 plant medium (Serva, Heidelberg, Germany) was added to the wells. Decreasing concentrations of benzanthric acid solved in DMSO were added (2 mM, 1 mM, 0.5 mM, 0.25 mM, 125 μM, and 62.5 μM). Identical volumes of DMSO without benzanthric acid were used as a toxicity test of the organic solvent. The solution containing the plant medium was used as a negative control. The plate was closed and incubated at room temperature under constant light (Osram Fluora lamp) in a humidity chamber. After 3 days of incubation, the plate lid was removed and a container with tap water was placed inside the chamber for increasing the air humidity. The plate was incubated up to 6 days. Three technical replicates were performed.

Genome Mining and Bioinformatics Analysis

The online tool antiSMASH (https://antismash.secondarymetabolites.org/#!/start) was used for the identification of secondary metabolite biosynthetic gene clusters in the genome of *S. albus* subsp. *chlorinus* (Weber et al., 2015). Gene cluster analysis was performed with the help of the software Geneious 11.0.3 (Kearse et al., 2012).

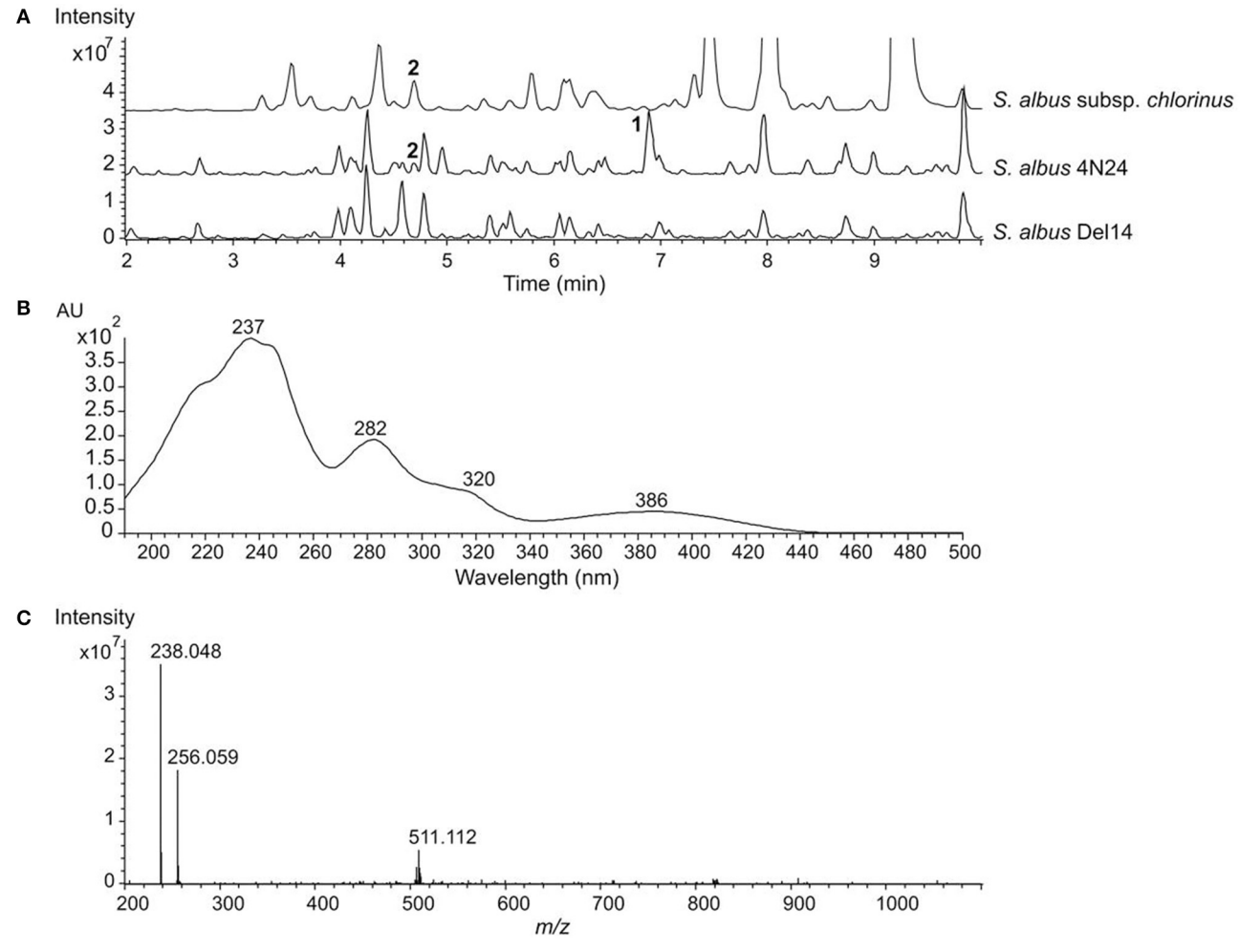

FIGURE 1 | HPLC-MS analysis of benzanthric acid production. **(A)** Base peak chromatograms of crude extracts from the parent strain *S. albus* subsp. *chlorinus*, the recombinant strain *S. albus* 4N24 harboring the nybomycin gene cluster and the host strain *S. albus* Del14. Peaks corresponding to benzanthric acid and nybomycin are indicated by **1** and **2**, respectively. **(B)** UV spectrum of benzanthric acid. **(C)** Mass spectrum of benzanthric acid (*m/z* 256.0 $[M+H]^+$). The signal at *m/z* 238.0 corresponds to the $[M+H-H_2O]^+$ ion and the signal at *m/z* 511.1 corresponds to the $[2M+H]^+$ ion.

RESULTS

We have previously reported the identification of the nybomycin gene cluster from *S. albus* subsp. *chlorinus* NRRL B-24108 through heterologous expression of BAC 4N24 harboring the *nyb* genes in the host *S. albus* Del14 (Rodriguez Estevez et al., 2018). In addition to nybomycin, the HPLC-MS analysis of an extract from the recombinant strain *S. albus* 4N24 expressing the *nyb* genes revealed the presence of a peak at $t_R = 6.9$ min and *m/z* 256.059 $[M + H]^+$ (**Figure 1**), exhibiting UV absorption signals at λ_{max} 237, 282, 320, and 386 nm (**Figure 1B**). Unlike nybomycin, which is produced by both *S. albus* 4N24 and the parent strain *S. albus* subsp. *chlorinus*, the peak at *m/z* 256.059 was solely detected in the extract of *S. albus* 4N24 (**Figure 1**). The search of the monoisotopic mass 255.051 in a natural product database yielded no coincidences, which suggested a putatively new compound and encouraged us to purify it for structure elucidation.

After seven days of cultivation of the strain *S. albus* 4N24 in 10 L of DNPM liquid medium, the broth was centrifuged and the metabolites were extracted from the supernatant. The extract was fractionated by size-exclusion chromatography through a Sephadex column followed by preparative high performance liquid chromatography (HPLC) to yield 5 mg of the novel metabolite benzanthric acid (**Figure 2A**). The molecular formula of benzanthric acid was determined as $C_{14}H_9NO_4$ by high-resolution electrospray ionization mass spectrometry (HRESMS) and NMR (*m/z* 256.05949, −3.688 ppm). The analysis of the ^{13}C-NMR (125 MHz, DMSO-d_6) revealed the presence of fourteen carbons. Two carbons were assigned as carbonyl groups (δ_C 168.67 C-1, 160.22 C-9). The twelve remaining carbons were assigned as olefinic carbons (δ_C 135.28 C-6, 133.40 C-5a, 129.95[2x] C7 & C8, 122.58 C-5, 122.34 C-4a, 121.52 C-8a, 118.57 C-10, 113.29 C-2, 108.84 C-4) including one oxygenated carbon (δ_C 140.59 C-9a) and one aminated carbon (δ_C 147.81 C-3; **Supplementary Figures 1–3**). The ^{1}H-NMR (500 MHz, DMSO-d_6) showed six aromatic signals: δ_H 8.24 (1H, dd; $J_{1,2} = 7.9$, 1.1 Hz; H-8), δ_H 8.16 (1H, d, J = 8.1 Hz, H-5), δ_H 7.96 (1H, dt, $J_{1,2}$ =7.7, 1.1 Hz, H-6), δ_H 7.70 (1H, dt, $J_{1,2}$ = 7.6, 0.6 Hz, H-7), δ_H 7.64 (1H, s, H-10), and δ_H 7.57 (1H, s, H-4; **Supplementary Figures 1, 4–6**). The ^{1}H-^{1}H-COZY spectrum showed correlations between H-5 and H-6, H-6 and H-7, H-7, and H-8 (**Supplementary Figures 7, 8**). The phase sensitive HSQC spectrum revealed eight quaternary carbons (C-1, C-2, C-3, C-4a, C-5a, C-8a, C-9, and C-9a; **Supplementary Figures 9, 10**). Through correlation in the heteronuclear multiple bond correlation (HMBC) experiment connections between the spin systems were

TABLE 1 | NMR chemical shifts and 2D correlations of benzanthric acid.

Position	δ^{13}C[ppm][a] type	δ^1H[ppm] [b] (J in Hz)	COZY	HMBC[c]
1	168.67 C	–	–	–
2	113.29 C	–	–	–
3	147.81 C	–	–	–
4	108.84 CH	7.57 s	–	C-1, C-2, C-3, C-5a, C-9a, C-10
4a	122.34 C	–	–	–
5a	133.40 C	–	–	–
5	122.58 CH	8.16 d (8.1)	6	C-4a, C-5a, C-6, C-8, C-8a, C-9
6	135.28 CH	7.96 dt (7.7, 1.1)	5; 7	C-5a, C-7, C-8
7	129.95 CH	7.70 dt (7.6, 0.6)	6; 8	C-5, C-5a, C-6, C-8, C-8a, C-9
8	129.95 CH	8.24 dd (7.9, 1.1)	7	C-5a, C-6, C-9
8a	121.52 C	–	–	–
9	160.22 C	–	–	–
9a	140.59 C	–	–	–
10	118.57 CH	7.64 s	–	C-1, C-2, C-4, C-4a, C-5a, C-9a, C-10
COOH[d]	–	7.00–9.50 s br	–	–
NH_2^d	–	7.00–9.50 s br	–	–

[a] *125 MHz for ^{13}C-NMR.*
[b] *500 MHz for ^{1}H-NMR.*
[c] *HMBC correlations from protons to the indicated carbons.*
[d] *Exchangeable protons.*

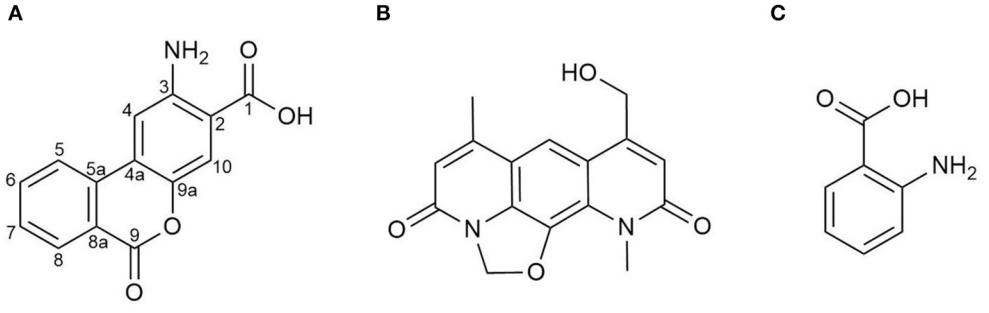

FIGURE 2 | Chemical structures of **(A)** benzanthric acid, **(B)** nybomycin and **(C)** anthranilic acid.

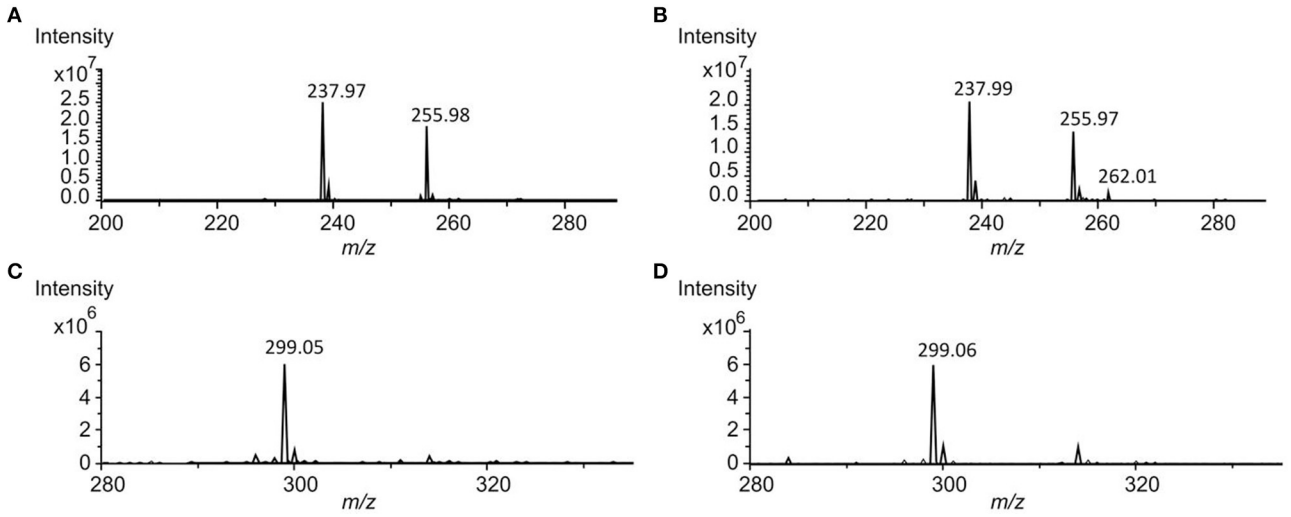

FIGURE 3 | Analysis of anthranilic acid (phenyl-$^{13}C_6$) incorporation. **(A)** Mass spectrum of benzanthric acid (*m/z* 255.9 $[M+H]^+$) extracted from the culture of *S. albus* 4N24. The signal at *m/z* 237.9 corresponds to the $[M+H-H_2O]^+$ ion. **(B)** Mass spectrum of benzanthric acid extracted from the culture of *S. albus* 4N24 fed with anthranilic acid (phenyl-$^{13}C_6$). The signal at *m/z* 262.0 corresponds to the $[M+H]^+$ ion after incorporation of the labeled anthranilic acid. **(C)** Mass spectrum of nybomycin (*m/z* 299.0 $[M+H]^+$) extracted from the culture of *S. albus* 4N24. **(D)** Mass spectrum of nybomycin extracted from the culture of *S. albus* 4N24 fed with anthranilic acid (phenyl-$^{13}C_6$). No incorporation of anthranilic acid can be detected.

identified (**Supplementary Figures 11, 12**). The complete list including all correlations can be found in **Table 1**. Important correlations to establish connections between the spin systems were H-4 to C-5a, H5 to C-4a as well as H-10 to C-4a and C-4. A ^{15}N-HMBC experiment revealed the correlation between H-4 and the nitrogen of the primary aromatic amine (**Supplementary Figure 13**). The ROESY spectrum showed a correlation through space between H-4 and H-5 (**Supplementary Figure 14**).

Benzanthric acid's structure has been determined as a benzoate part bound to an anthranilate moiety. This arrangement considerably differs from the core structure of nybomycin (**Figures 2A,B**), which is also produced by the strain *S. albus* 4N24 harboring the *nyb* genes. In order to test whether anthranilic acid functions as a precursor in the biosynthesis of benzanthric acid and nybomycin, a culture of *S. albus* 4N24 was fed with anthranilic acid (phenyl-$^{13}C_6$) (**Figure 2C**). HPLC-MS analysis of the resulting extract revealed the incorporation of the labeled anthranilate into the structure of benzanthric acid. The mass spectrum of benzanthric acid shows two signals: a signal at *m/z* 256 corresponding to the $[M+H]^+$ ion of the compound and a signal at *m/z* 238 corresponding to its derivative after water loss (**Figure 3A**). After feeding with labeled anthranilic acid an additional signal at *m/z* 262 is observed (**Figure 3B**), implying the incorporation of the six ^{13}C atoms of the labeled anthranilate into benzanthric acid. No incorporation of anthranilic acid into the structure of nybomycin was detected (**Figures 3C,D**).

The antimicrobial activity of benzanthric acid against the Gram-negative bacteria *Escherichia coli* and *Pseudomonas putida*, as well as the Gram-positive bacterium *Bacillus subtilis* was assayed through disk diffusion test, revealing no growth inhibitory activity (**Supplementary Figure 15A**). The herbicidal activity of the new metabolite was tested against the monocot grass species *Agrostis stolonifera*. The purified benzanthric acid was added to the plant seeds and incubated in a minimal medium. Seed germination was observed after 3 days, indicating no inhibitory effect against the tested plant (**Supplementary Figure 15B**).

DISCUSSION

In this paper, we describe the isolation and chemical structure elucidation of the novel metabolite benzanthric acid, produced by *S. albus* 4N24 containing the nybomycin biosynthetic gene cluster. The strain simultaneously produces nybomycin and benzanthric acid, suggesting that both compounds might share common biosynthetic steps. The structure of benzanthric acid (**Figure 2A**) suggests anthranilate and benzoate as possible biosynthetic precursors. The precursor role of anthranilic acid has been proved by feeding studies. Interestingly, no incorporation of anthranilic acid into nybomycin's structure could be detected, indicating substantial differences in the biosynthetic routes leading to nybomycin and benzanthric acid.

In living organisms, anthranilic acid is mainly synthesized either through the shikimate pathway or through the tryptophan degradation pathway (Haslam, 1974; Kurnasov et al., 2003). One of the nybomycin biosynthetic genes, *nybF*, encodes a putative 3-Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase, which catalyzes the first reaction of the shikimate pathway. Since the DAHP synthase controls the amount of carbon entering the pathway, the expression of the *nybF* gene can lead to

its upregulation and increased intracellular concentrations of benzanthric acid's precursor—anthranilic acid. Additionally, the product of the *nybD* gene shows homology at the protein level with anthranilate synthase and might be also responsible for the additional supply of anthranilic acid. In this case, anthranilic acid is most probably only a by-product of the enzyme's reaction, since it was shown not to be a precursor of nybomycin biosynthesis.

We propose that benzoic acid serves as the second direct precursor for benzanthric acid production. No genes which could lead to the biosynthesis of benzoic acid were identified within the DNA fragment containing the nybomycin cluster. Therefore, it is likely that benzoic acid is provided by the metabolism of the host strain *S. albus* Del14. The phenylalanine degradation pathway could be responsible for the supply of this precursor (Tabor and Tabor, 1970).

The attachment of benzoic acid to the anthranilate moiety is necessary for the formation of benzanthric acid. The enzyme catalyzing this biosynthetic step could not be identified within the nybomycin gene cluster. We propose that the required enzyme is encoded by the genome of the host strain *S. albus* Del14. This is further supported by the data revealing no production of benzanthric acid by the original nybomycin producer (**Figure 1A**).

The fact that the new compound, benzanthric acid, could be generated through the expression of the characterized nybomycin gene cluster in the well-studied heterologous host *S. albus* Del14 is intriguing. The isolated compound can be found neither in the extracts of the natural nybomycin producer nor in that of the heterologous host *S. albus* Del14. Benzanthric acid is also not a degradation product of nybomycin. The most plausible explanation for the origin of the isolated compound is the interplay between the host's metabolism and the introduced biosynthetic pathway. The isolation of benzanthric acid raises the question of whether this is rather an exception or the integration of foreign metabolic pathways or their parts into the host's metabolism can be used as a tool for the generation of new natural products.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GenBank under accession number MH924838.

AUTHOR CONTRIBUTIONS

MR, MM, and AL designed the experiments. MR performed the experiments. NG and JZ performed and evaluated the NMR analysis. MR, NG, and MM wrote the manuscript. All the authors reviewed the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fchem.2019.00896/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

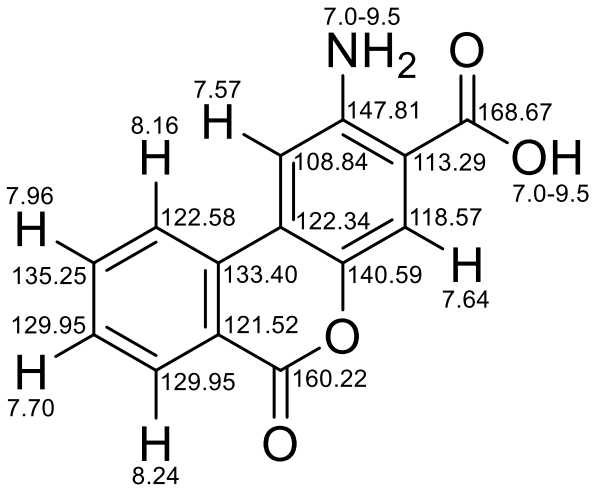
Supplementary Material

1 Supplementary Tables

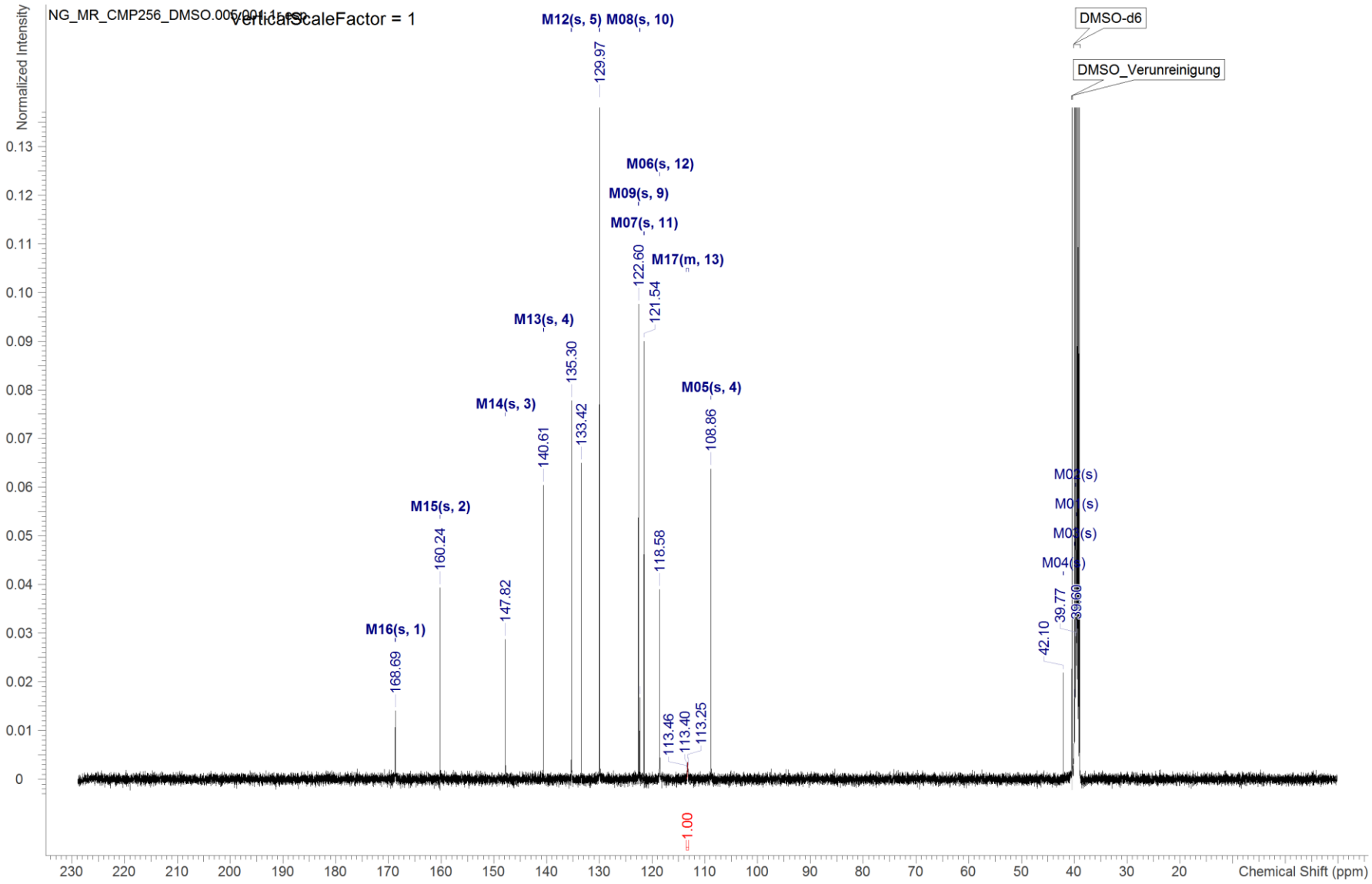
Supplementary Table 1. Bacterial strains and BAC (bacterial artificial chromosome) vectors used in this work.

Bacterial strain	Features	Reference/ Source
Streptomyces albus subsp. *chlorinus* NRRL B-24108	*S. albus* subspecies strain harboring nybomycin biosynthetic gene cluster	(Hahn et al. 2009)
Streptomyces albus Del14	Wild-type strain	(Myronovskyi et al. 2018)
Streptomyces albus 4N24	*S. albus* strain with BAC 4N24 insertion	(Rodriguez Estevez et al. 2018)
Escherichia coli ET12567 pUB307	Donor strain for intergeneric conjugation	(Flett et al. 1997)
Escherichia coli DH10β	General cloning strain	(Grant et al. 1990)
BACs		
pSMART	AmR; BAC vector	Lucigen (USA)
4N24	BAC containing nybomycin gene cluster	Intact Genomics (USA)

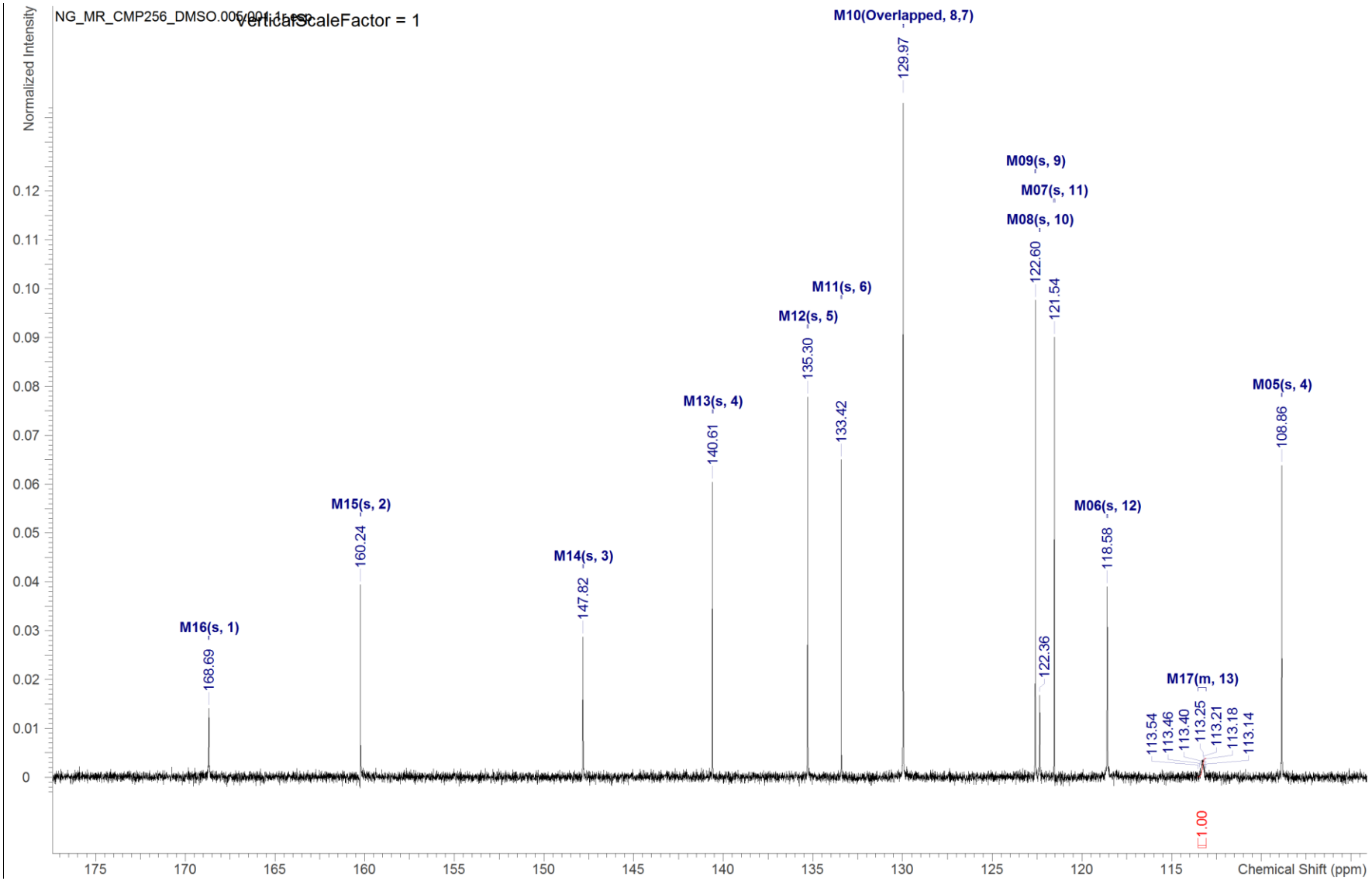
2 Supplementary Figures



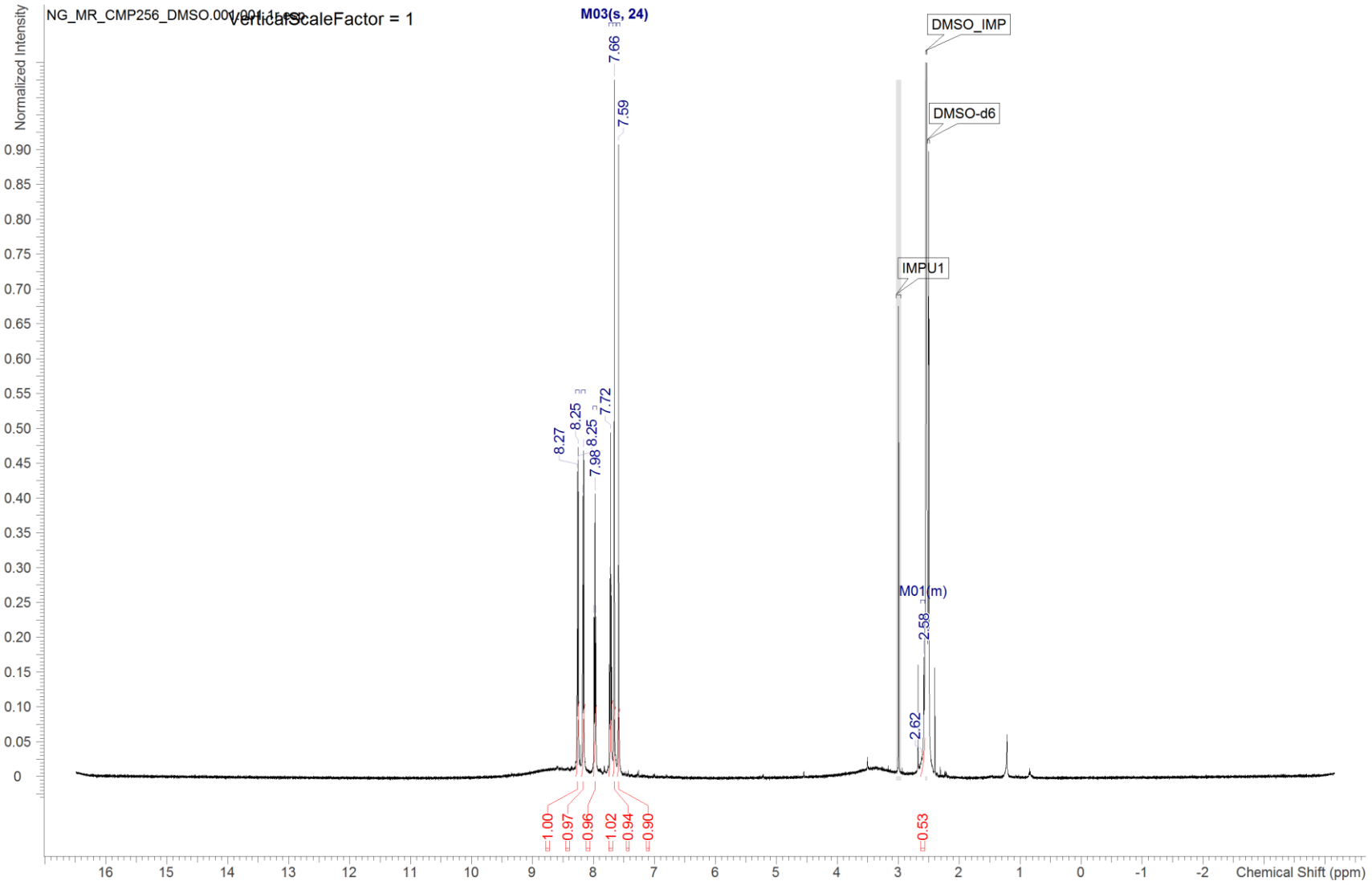
Supplementary Figure 1. Structure of benzanthric acid including chemical shifts of protons and carbons.



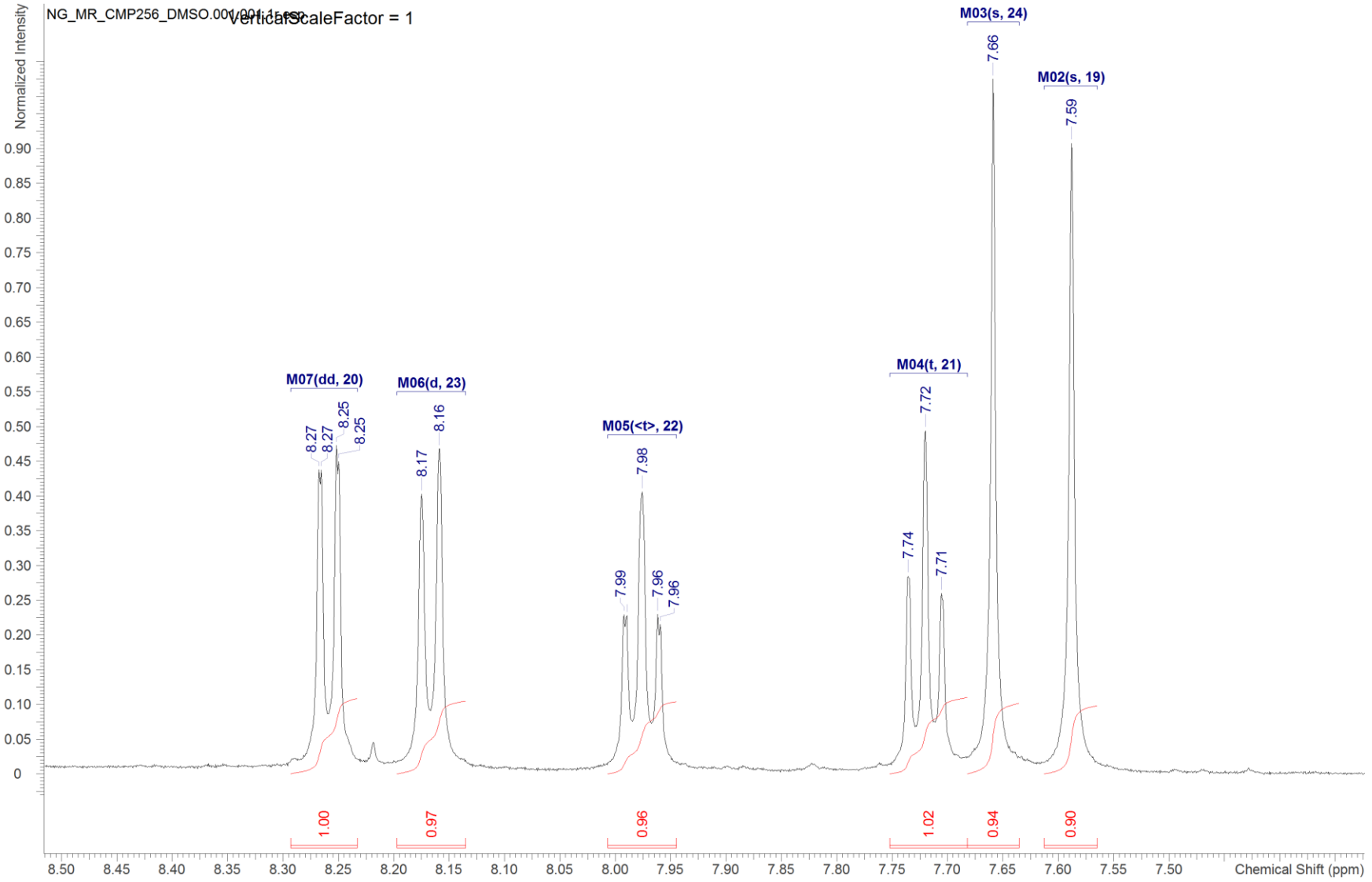
Supplementary Figure 2. ^{13}C-NMR spectrum (125 MHz, DMSO-d_6) of benzanthric acid.



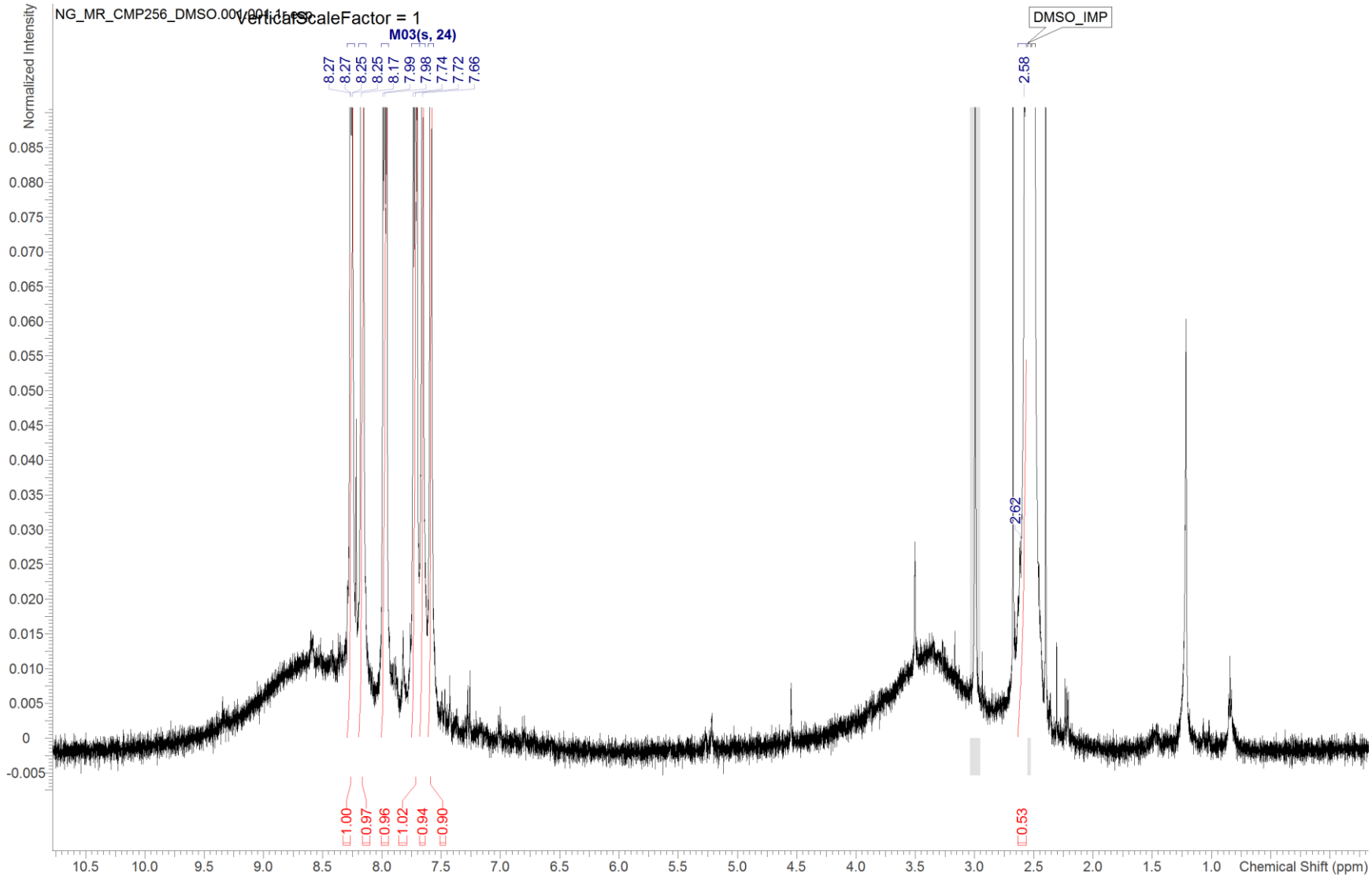
Supplementary Figure 3. ^{13}C-NMR spectrum (125 MHz, DMSO-d_6) of benzanthric acid (100 ppm – 175 ppm).



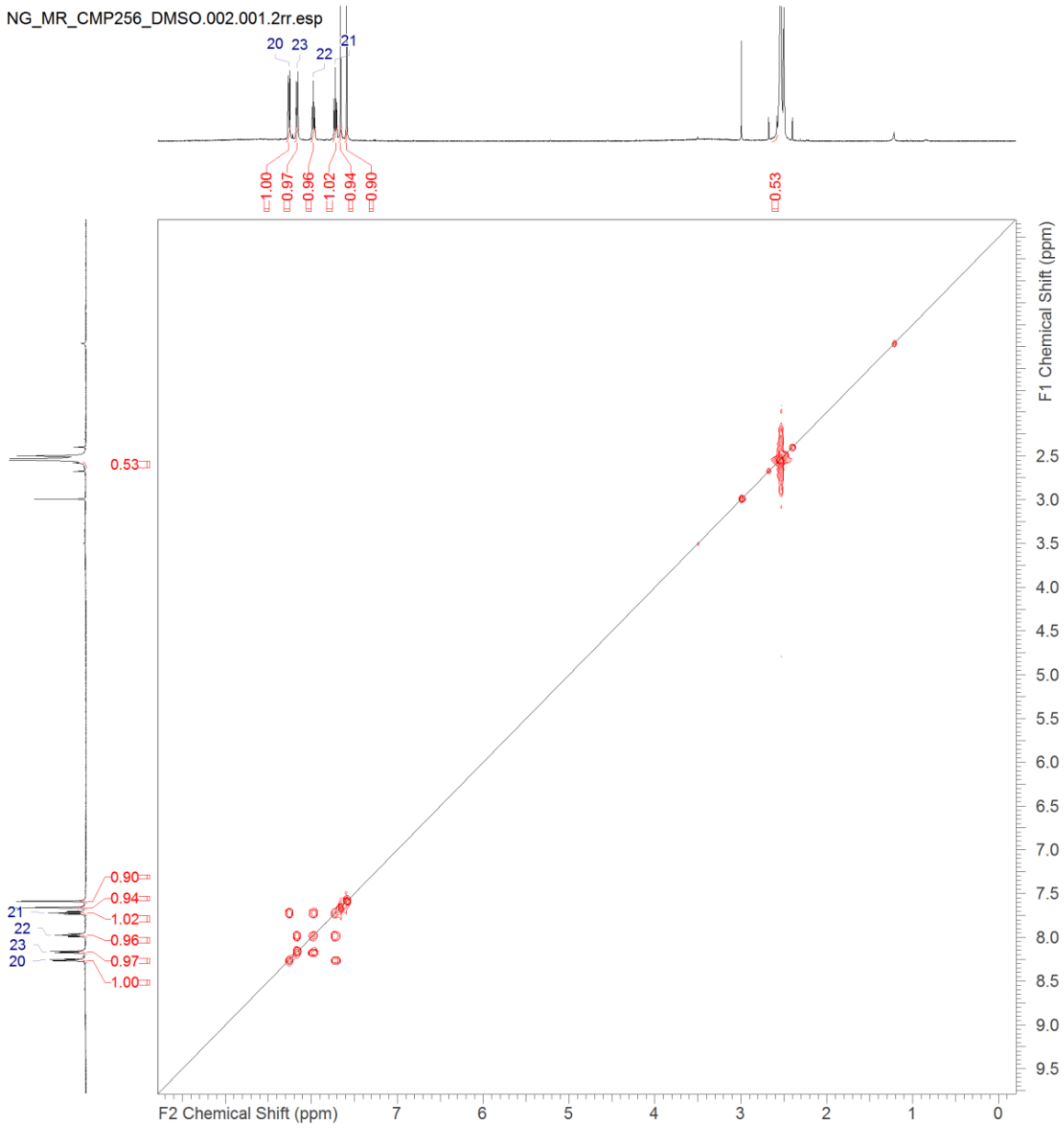
Supplementary Figure 4. ^{1}H-NMR spectrum (500 MHz, DMSO-d_6) of benzanthric acid.



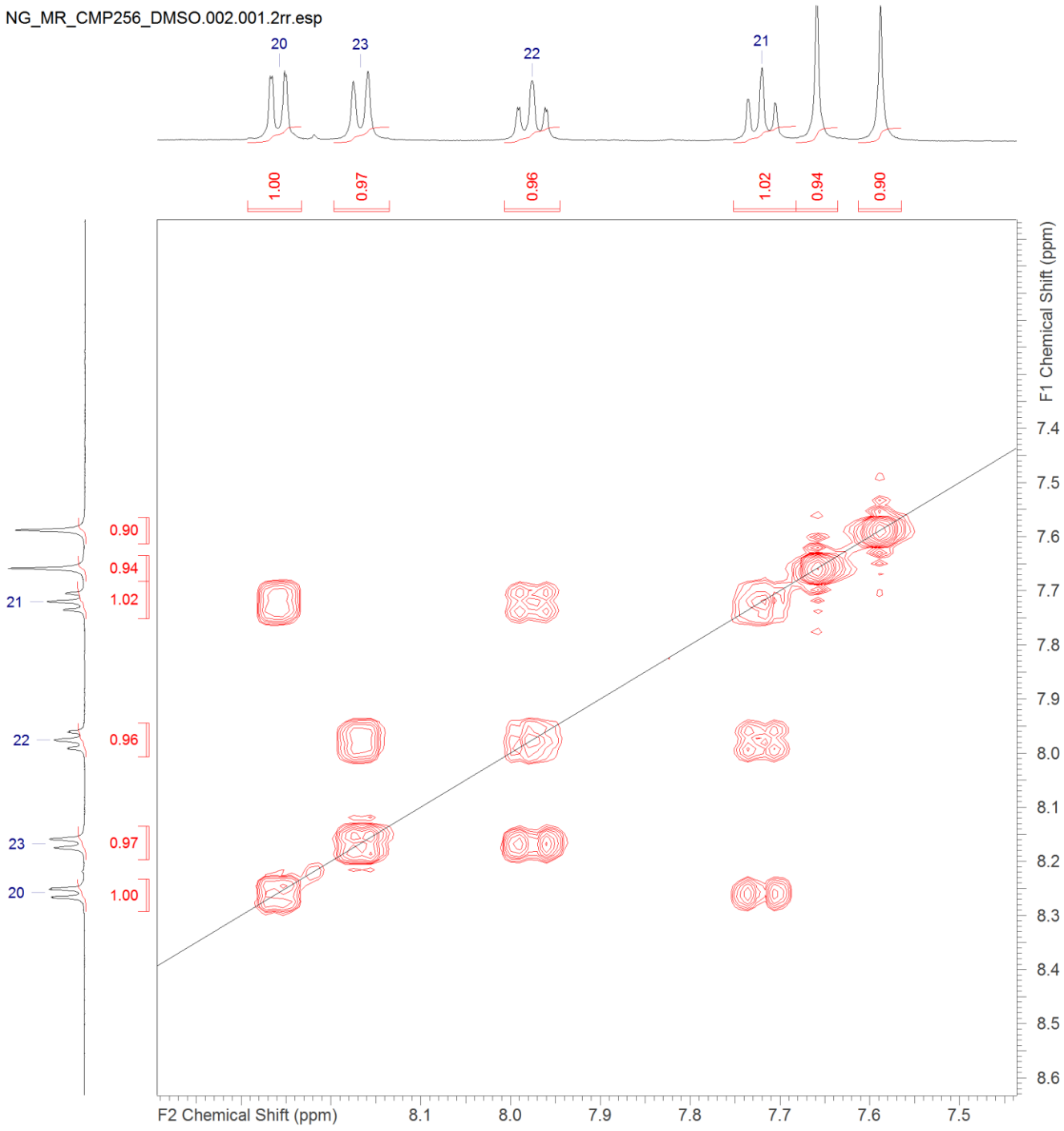
Supplementary Figure 5. ^{1}H-NMR spectrum (500 MHz, DMSO-d_6) of benzanthric acid (7.5 ppm – 8.5 ppm).



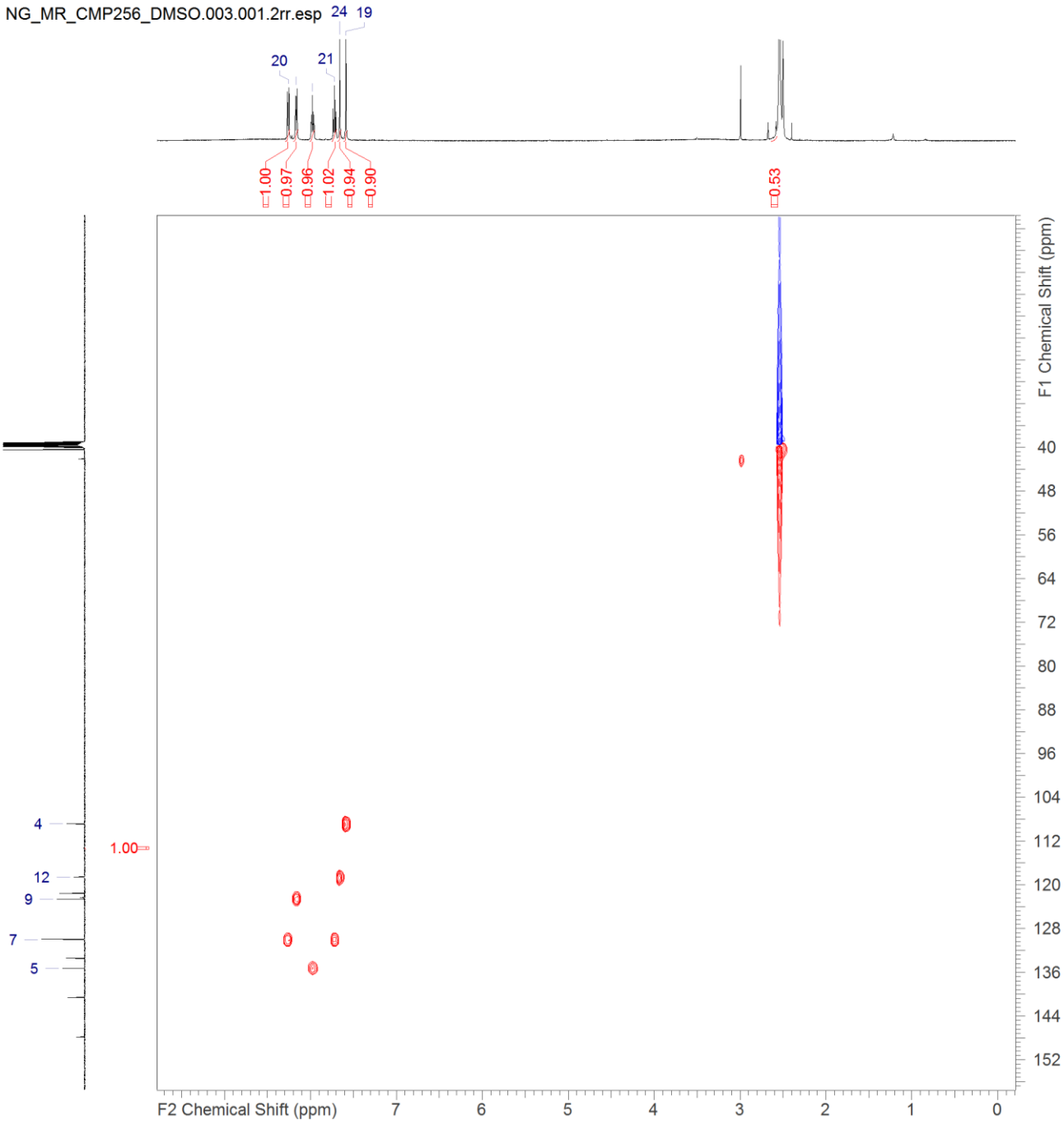
Supplementary Figure 6. ^{1}H-NMR spectrum (500 MHz, DMSO-d_6) of benzanthric acid with increased intensity.



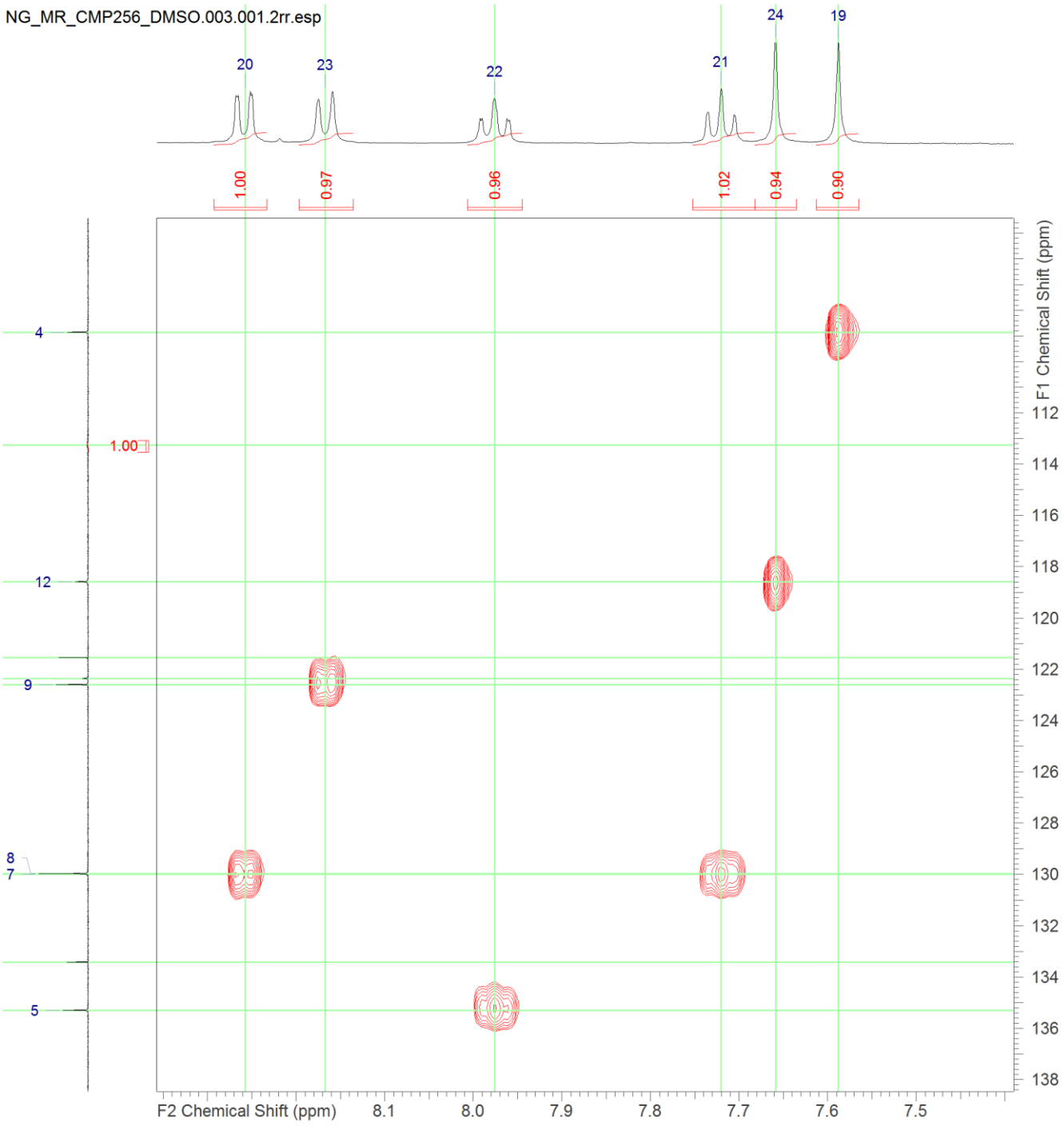
Supplementary Figure 7. ^{1}H-^{1}H-COSY spectrum (DMSO-d_6) of benzanthric acid.



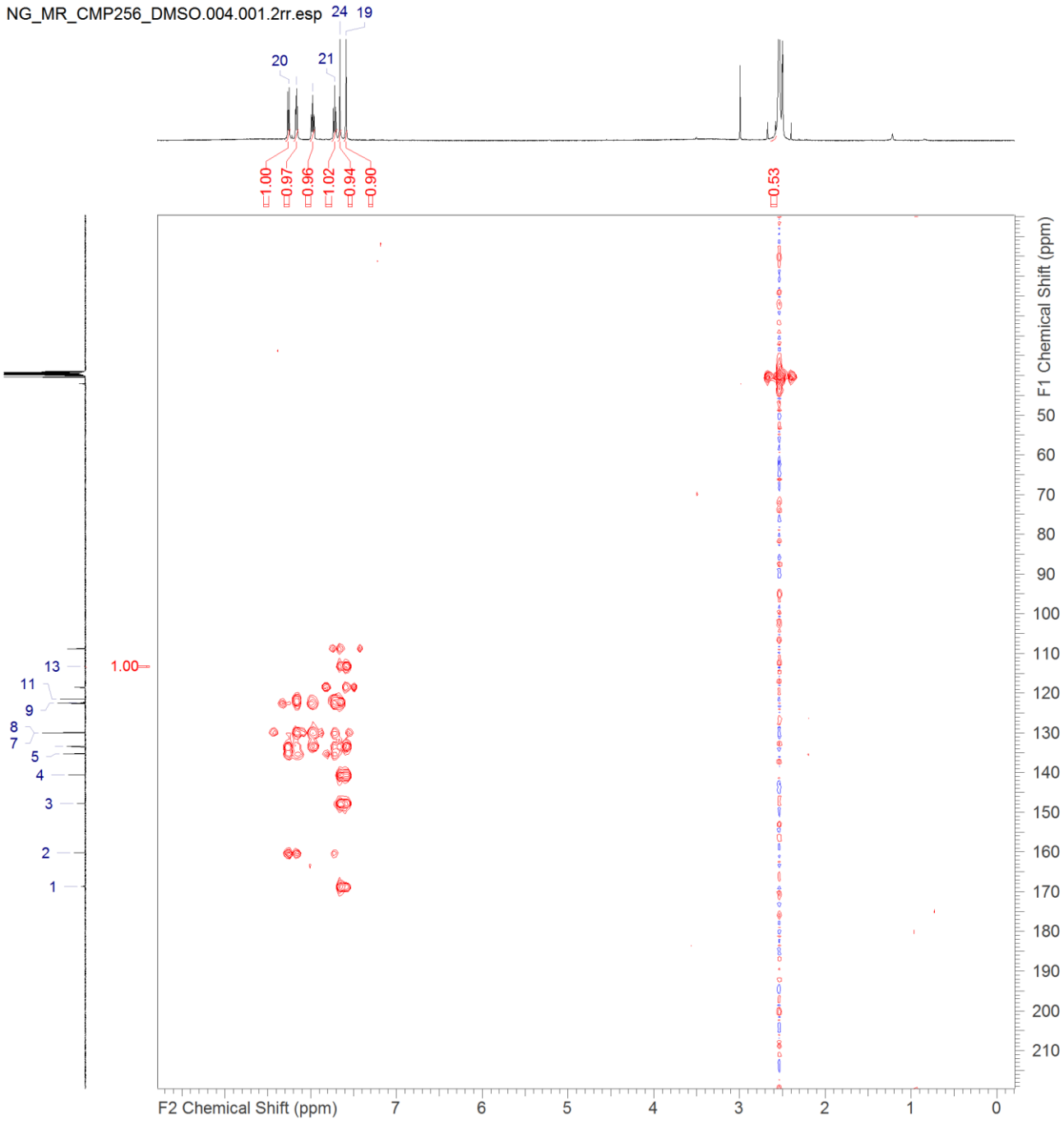
Supplementary Figure 2 ^{1}H-^{1}H-COSY spectrum (DMSO-d_6) of benzanthric acid (7.2 ppm – 8.6 ppm).



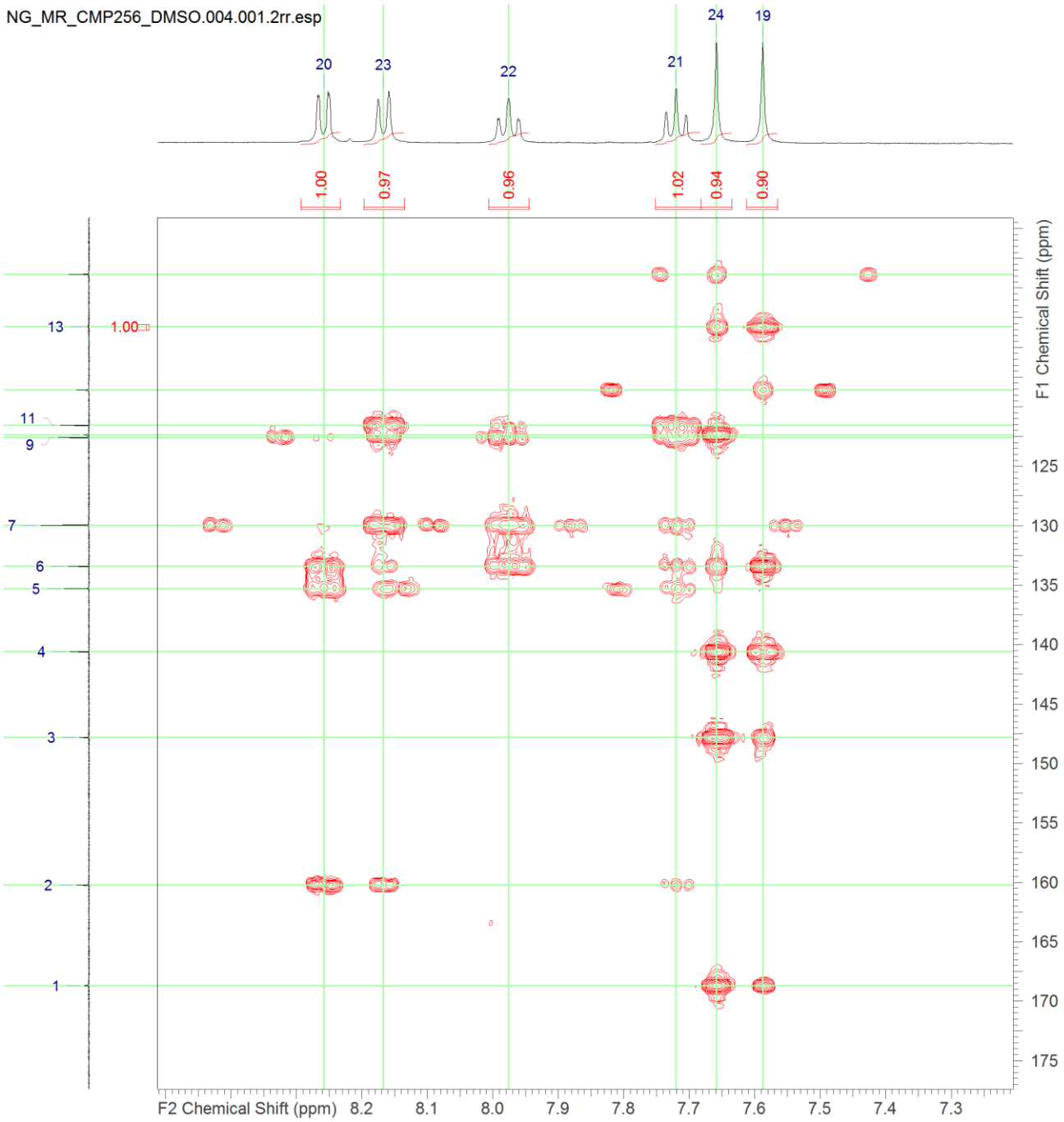
Supplementary Figure 3. HSQC spectrum (DMSO-d_6) of benzanthric acid.



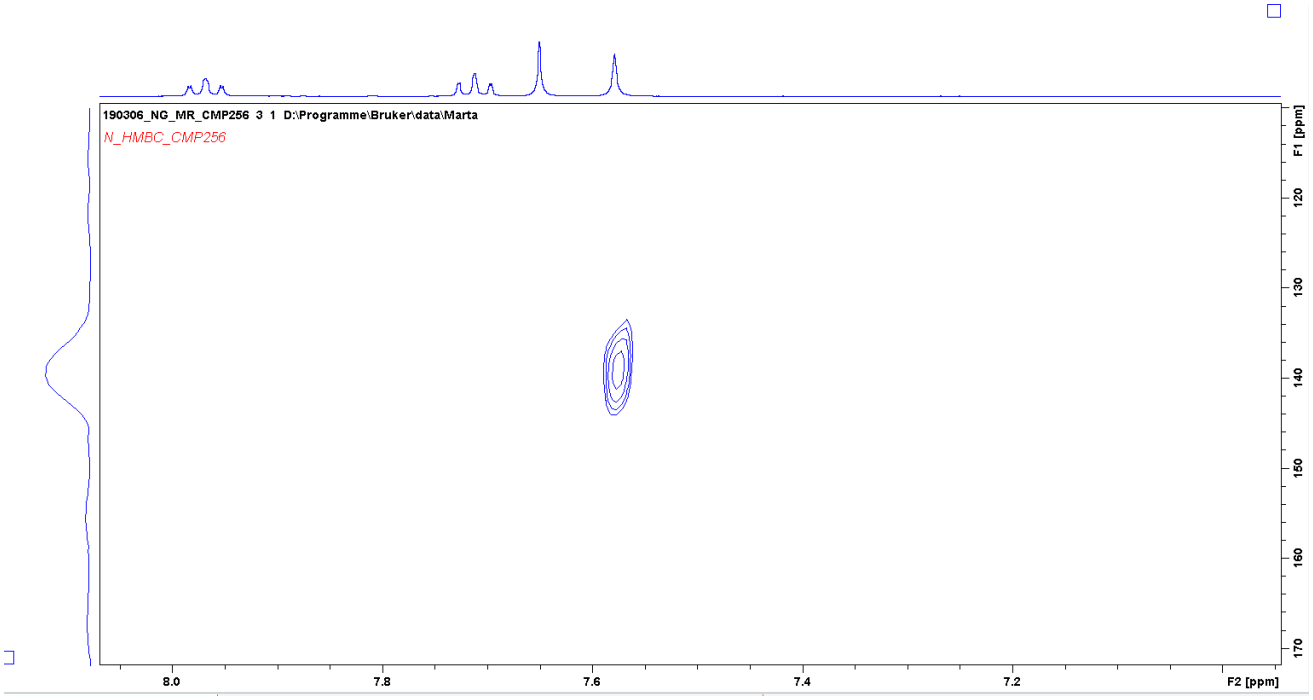
Supplementary Figure 4. HSQC spectrum (DMSO-d_6) of benzanthric acid (7.5 ppm – 8.3 ppm & 105 ppm – 138 ppm).



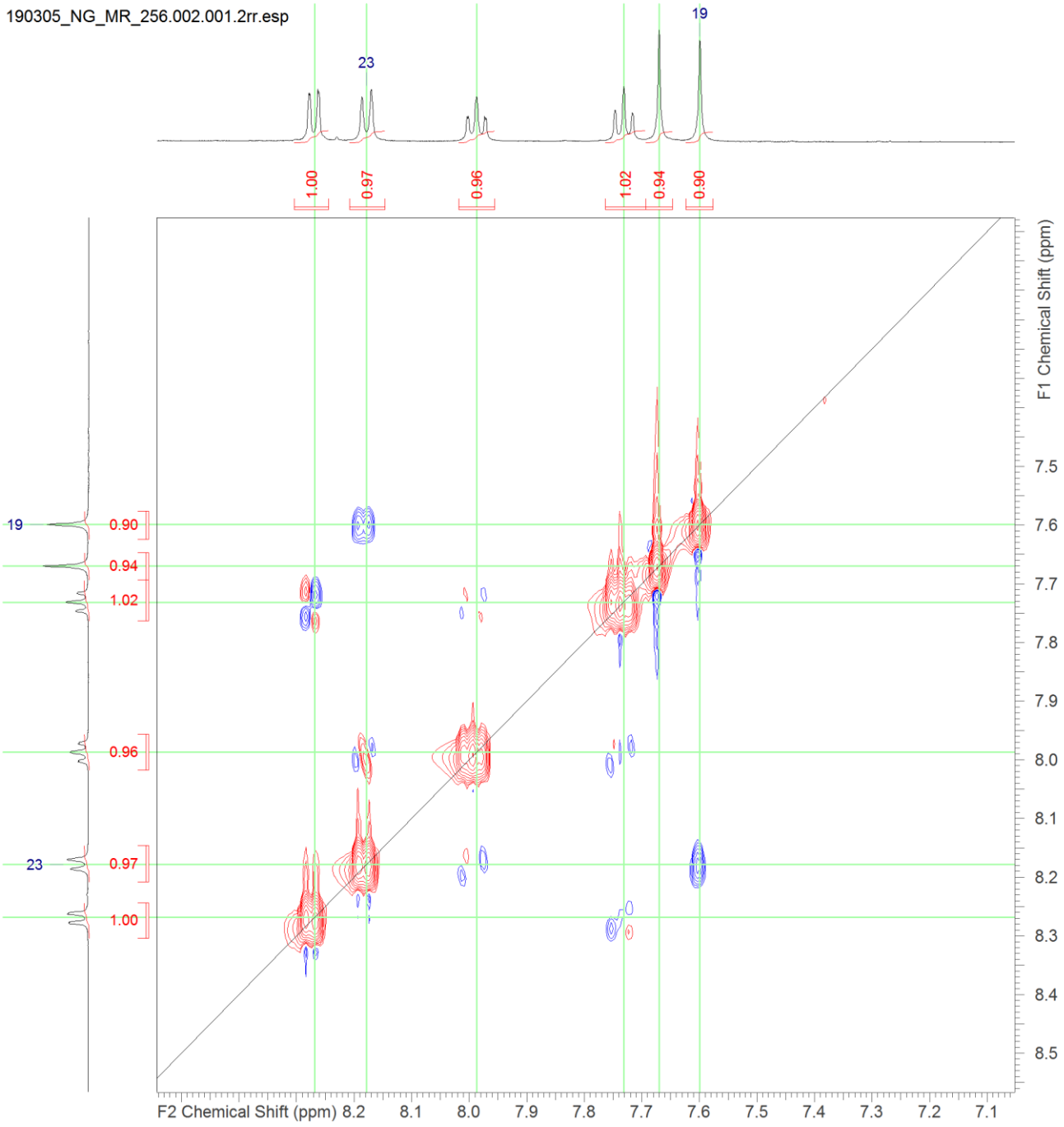
Supplementary Figure 5. HMBC spectrum (DMSO-d_6) of benzanthric acid (7.5 ppm – 8.3 ppm & 105 ppm – 138 ppm).



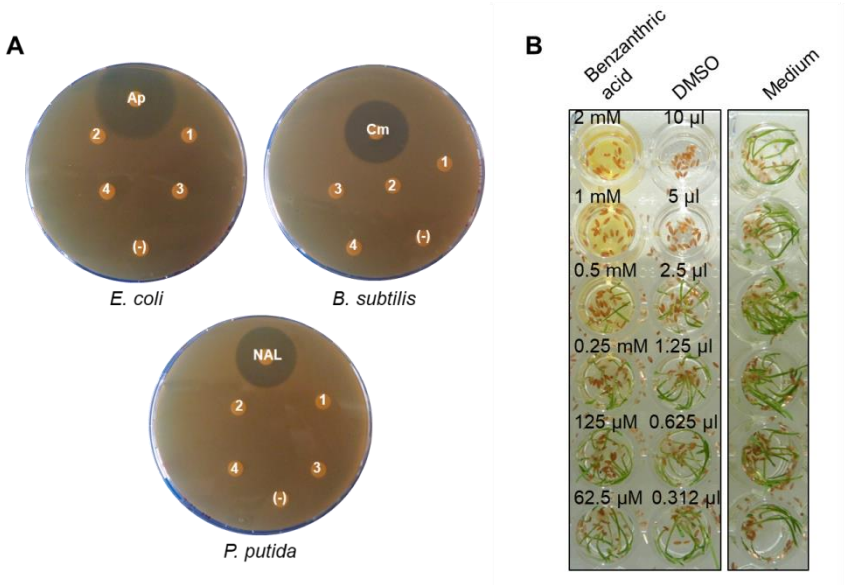
Supplementary Figure 6. HMBC spectrum (DMSO-d_6) of benzanthric acid (7.3 ppm – 8.5 ppm & 105 ppm – 175 ppm).



Supplementary Figure 13. ^{15}N-HMBC spectrum (DMSO-d_6) of benzanthric acid.



Supplementary Figure 14. ROESY spectrum (DMSO-d_6) of benzanthric acid.



Supplementary Figure 15. Antimicrobial and herbicidal bioactivity tests. **(A)** Disk diffusion assay against growth of *Escherichia coli*, *Bacillus subtilis*, and *Pseudomonas putida*. Paper disks 1, 2, 3, and 4 were coated with 100 µg, 50 µg, 10 µg, and 0.5 µg, respectively, of benzanthric acid solved in DMSO. Ap: ampicillin; Cm: chloramphenicol; NAL: nalidixic acid. (-): negative control (DMSO). **(B)** Herbicidal test against seed germination of the monocot grass plant *Agrostis stolonifera*. Decreasing concentrations of benzanthric acid solved in DMSO were applied (first column). Identical volumes of solvent without benzanthric acid were applied (second column). Growth inhibitory effect of DMSO is observed at volumes of 10 µl and 5 µl. Minimal medium for plant growth (see section 2.7) was used as a negative control (third column).

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III

Novel fredericamycin variant overproduced by a streptomycin-resistant *Streptomyces albus* subsp. *chlorinus* strain

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Article

Novel Fredericamycin Variant Overproduced by a Streptomycin-Resistant *Streptomyces albus* subsp. *chlorinus* Strain

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Abstract: Streptomycetes are an important source of natural products potentially applicable in the pharmaceutical industry. Many of these drugs are secondary metabolites whose biosynthetic genes are very often poorly expressed under laboratory cultivation conditions. In many cases, antibiotic-resistant mutants exhibit increased production of natural drugs, which facilitates the identification and isolation of new substances. In this study, we report the induction of a type II polyketide synthase gene cluster in the marine strain *Streptomyces albus* subsp. *chlorinus* through the selection of streptomycin-resistant mutants, resulting in overproduction of the novel compound fredericamycin C_2 (**1**). Fredericamycin C_2 (**1**) is structurally related to the potent antitumor drug lead fredericamycin A.

Keywords: antitumor; fredericamycin; overproduction; secondary metabolites; streptomycetes; streptomycin-resistant; type II PKS

1. Introduction

The bacterial genus *Streptomyces* is well-known for producing a huge variety of bioactive secondary metabolites with potential pharmaceutical applications [1,2]. The genes responsible for their biosynthesis are generally clustered together. However, many of these biosynthetic genes are poorly or not expressed (silent) under laboratory cultivation conditions. Thus, the activation of silent secondary metabolite gene clusters is an essential step for the discovery of new natural drugs. A simple strategy to activate or upregulate the expression of these genes consists in screening for antibiotic-resistant mutants [3,4]. This approach is based on the work of Ochi and his team. They discovered that certain mutations in the *rpsL* and *rpoB* genes, which code for the ribosomal protein S12 and the β-subunit of RNA polymerase, respectively, lead to an altered gene product that confers resistance to streptomycin (*str* mutants) or to rifampicin (*rif* mutants), respectively. The mutations in these genes also increase the production of secondary metabolites in several *Streptomyces* strains [3–7]. Presumably, some of the *str* and *rpoB* mutations give rise to diverse metabolic changes which typically occur during the stringent response. The stringent response is triggered in *E. coli* [8] and other prokaryotic microorganisms [9,10] under amino acid starvation conditions by the signaling molecule, guanosine tetraphosphate (ppGpp). This molecule generates a series of physiological changes, including a significant decrease of protein synthesis [11], downregulation of rRNA transcription [12], and activation of stationary-phase metabolic

processes, such as the expression of secondary metabolite biosynthetic genes [9]. Thus, it is suggested that the mutant gene products of *rpsL* and *rpoB* may be responsible for antibiotic overproduction, mimicking the role of ppGpp in the stringent response [3–6].

In previous studies, the marine strain *Streptomyces albus* subsp. *chlorinus* NRRL B-24108 has been shown to harbor genes for the production of several bioactive secondary metabolites, such as the herbicide albucidin or the antibiotic nybomycin [13,14]. Here, we report the induction of a type II polyketide synthase (PKS) gene cluster in *S. albus* subsp. *chlorinus*, leading to overproduction of the novel compound fredericamycin C_2 (**1**). This substance is structurally related to fredericamycin C (**2**), a secondary metabolite first isolated in 1981, together with the compounds fredericamycin A and fredericamycin B, from the culture broth of *Streptomyces griseus* ATCC 49344 [15]. All fredericamycin variants known to date (A, B, C, C_1, and E) share a similar structure that involves two aromatic moieties, which in the case of fredericamycins A and E are linked by a rare stereogenic spiro carbon center [16–18]. Fredericamycin A displays strong in vivo anticancer activity against several mammal tumor cell lines [19], and it has been patented as an effective treatment for certain types of cancer in humans [20]. Additionally, fredericamycins A, B, C, and C_1 exhibit moderate antibacterial and antifungal activities [19]. In this study, we present the generation of the high-level streptomycin-resistant strain, *Streptomyces albus* subsp. *chlorinus* JR1, which overproduces the novel compound fredericamycin C_2 (**1**). We also describe the mutation likely causing this phenotype and propose the initial biosynthetic steps of fredericamycin C_2 (**1**), based on the gene cluster homology with that of fredericamycin A.

2. Results

2.1. High-Level Streptomycin-Resistant Mutant S. albus subsp. chlorinus Overproduces the Novel Compound Fredericamycin C_2

The strain *S. albus* subsp. *chlorinus* NRRL B-24108 was cultured in MS agar medium containing increasing concentrations of streptomycin. After several rounds of selection, we isolated a streptomycin-resistant colony that displayed a distinctive violet color when cultured on a solid medium, in contrast to the characteristic white color of the parental strain (Figure 1). We named the mutant strain *S. albus* subsp. *chlorinus* JR1. While the growth of the wild type of *S. albus* subsp. *chlorinus* was inhibited by a streptomycin concentration of 50 µg/mL, the mutant strain was able to grow in the presence of up to 200 µg/mL of antibiotic (Table S1). The metabolic profiles of both strains were analyzed using liquid chromatography and mass spectrometry, revealing the presence of a peak at t_R = 12.7 min that corresponds to an $[M + H]^+$ ion of *m/z* 521.107 and displays UV absorption signals at λ_{max} 195, 248, 274, 345, and 490 nm (Figure 2). The peak area revealed an over 50-fold greater production of the corresponding compound by *S. albus* subsp. *chlorinus* JR1 compared to the parental strain's yield (Table S2, Figure S1). The search of this mass in a natural product database yielded no coincidences, suggesting a potentially undescribed metabolite overproduced by *S. albus* subsp. *chlorinus* JR1. The compound was extracted from a 2 l solid culture of *S. albus* subsp. *chlorinus* JR1 and purified by normal phase chromatography through a silica column followed by reverse phase chromatography.

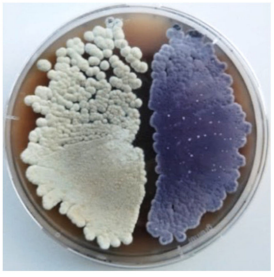

Figure 1. *Streptomyces albus* subsp. *chlorinus* NRRL B-24108 (left) and *Streptomyces albus* subsp. *chlorinus* JR1 (right) spores on MS agar medium.

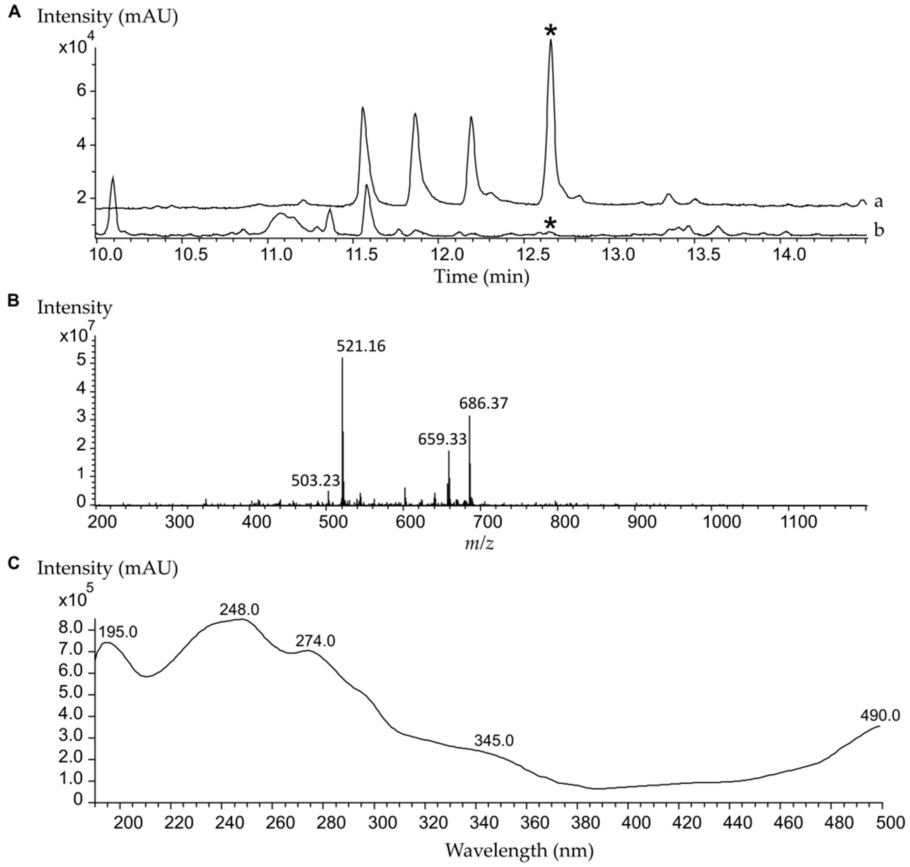

Figure 2. HPLC-MS analysis of crude extract from solid cultures of *S. albus* subsp. *chlorinus* JR1 (**a**) and its parental strain *S. albus* subsp. *chlorinus* NRRL B-24108 (**b**). (**A**) UV chromatogram. The asterisk (*) indicates the peak corresponding to fredericamycin C_2 (**1**) at t_R = 12.7 min. (**B**) Mass spectrum associated with t_R = 12.7 min from the UV chromatogram displayed in (**A**). (**C**) UV spectrum of purified fredericamycin C_2 (**1**).

The molecular formula of the substance was determined to be $C_{27}H_{20}O_{11}$ based on high-resolution MS (ESI) showing the quasi molecular ion *m/z* 521.107 ($[M + H]^+$, calculated for $C_{27}H_{21}O_{11}$). In the proton NMR spectrum, two broad multiplets are visible at δ_H 2.63 (t, 6′-H_2) and 2.79 (t, 7′-H_2) ppm, which are two neighbored methylene groups. All other signals are singlets: One methoxy group at δ_H 3.94 (6-OCH_3), one additional methylene group at δ_H 4.06 (4′-H_2), one methyl group at δ_H 2.17 (1″-H_3), and two methines at δ_H 6.52 (5′-H) and 6.88 (7-H). Moreover, broad singlets at δ_H 5.5, 12.8, and 13.2 ppm indicate hydroxyl groups to be present in the molecule (Table 1). Typical quinone carbonyl signals at δ_C 186.9 (C-4) and 187.6 (C-9) are visible in the ^{13}C-NMR spectrum. A signal at δ_C 169.3 ppm (C-1′) shows an acid functionality which is attached at C-8′ proven from an HMBC (Heteronuclear Multiple Bond Coherence) correlation C-1′/ 5′-H. A very weak signal at δ_H 204.1 ppm (C-3′) shows a ketone supported by an HMBC cross-peak C-3′/1″-H_3 (Table 1). This ketone is part of a propan-2-on sidechain which is attached at C-6′ based on HMBC signals C-6′/4′-H_2, C-6′/1″-H_3, C-5′/4′-H_2, and C-7′/4′-H_2. The HMBC signal C-6/6-OCH_3 indicates the methoxy group to be attached at C-6 next to the aromatic proton 7-H. One of the rings contains a CH_2-CH_2 moiety (C-6′-C7′) which is in ring D proven from HMBC signals C-3/7′-H_2, C-7a′/7′-H_2, C-8′/7′-H_2, C-7a′/6′-H_2, and C-5a′/6′-H_2 (Figure S2). Comparison to literature shows high similarity of the new compound named fredericamycin C_2 (**1**) to fredericamycin C (**2**) [17], which differs only in the sidechain attached at C-6′ (fredericamycin C) and to KS-619-1 (**3**) [21] with a different substitution pattern of the pentacyclic ring system (KS-619-1) (Figure 3).

Table 1. NMR data of fredericamycin C_2 (**1**) (600/150 MHz, DMSO-d6/Pyridine-d5 95:5, 35 °C, solvent (DMSO) as internal reference).

Pos. [a]	δ_C	δ_H (*J* Hz)	COSY [b]	HMBC [b]	ROESY [b]
9′	164.3			5′-H	
9a′	115.3			4′-H_2, 5′-H	
3′	140.7			4′-H_2, (5′-H)	
5′	120.4	6.52 s	(4′-H_2)	^1J, 4′-H_2, (5′-H), 6′-H_2	4′-H_2, 6′-H_2, (7′-H_2)
5a′	144.3			5′-H, 6′-H_2, 7′-H_2	
6′	28.8	2.63 br t (7Hz)	7′-H_2	5′-H, 7′-H_2	5′-H, 7′-H_2
7′	21.2	2.79 br t (7Hz)	6′-H_2	6′-H_2	
7a′	137.3			6′-H_2, 7′-H_2	
3	152.5			7′-H_2	
3a	112.2				
4	186.9				
4a	113.2			(7-H)	
5	148.8 [c]			7-H	
6	157.6			6-OCH_3, 7-H	
6-OCH_3	56.4	3.94 s	6-H	^1J	7-H
7	105.8	6.88 s	6-OCH_3	6-OCH_3	6-OCH_3
8	158.6			7-H	
8a	105.8			7-H	
9	187.6			7-H	
9a	114.4				
1	156.3				
2 = 8′	136.3			(5′-H), 7′-H_2	
8a′	121.0			5′-H	
1′	169.3			5′-H	
4′	49.5	4.06 s	(5′-H, 1″-H_3)	5′-H, (1″-H_3)	5′-H, 1″-H_3
3′	(204.1) [d]				
1″	29.7	2.17 s			4′-H_2
		13.22 br s			
OH		12.80 br s			
		5.5 br s			

[a] Numbering according to fredericamycin C (**2**); [b] weak signals in brackets; [c] from HMBC; [d] very weak signal in carbon NMR spectrum.

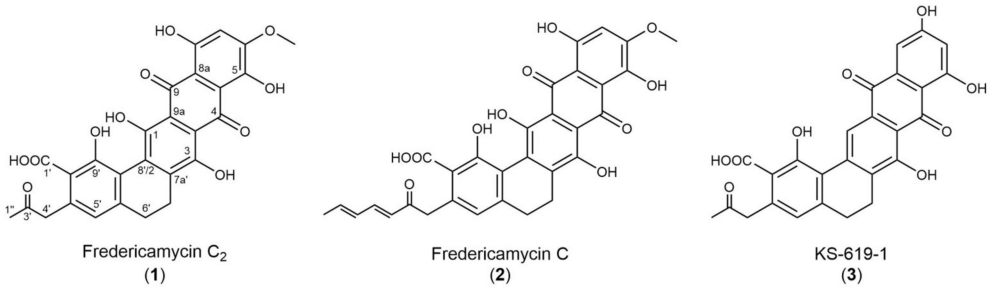

Figure 3. Structures of fredericamycin C_2 (**1**), fredericamycin C (**2**) and KS-619-1 (**3**).

2.2. *Fredericamycin C_2 Is Biosynthesized by a Type II PKS Gene Cluster*

Fredericamycin C_2 (**1**) is structurally related to other fredericamycin variants, such as C and A, which are biosynthesized by a type II PKS gene cluster [22]. This suggests the involvement of a type II PKS system in the production of fredericamycin C_2 (**1**). Although a subclass of type I PKSs (iterative type I PKSs) have also been reported to synthesize aromatic compounds [23–26], their products are structurally smaller and simpler than the complex multicyclic metabolites produced by type II PKSs [27]. The pentacyclic aromatic polyketide structure of fredericamycin C_2 (**1**) (Figure 3) further supports the assumption of type II PKS genes involved in its biosynthesis. The genome of *S. albus*

subsp. *chlorinus* was screened for secondary metabolite genes, revealing the presence of one type II PKS gene cluster. Based on protein BLAST analysis, we assigned the genes putative functions, which are summarized in Table 2. To test whether the expression of these genes leads to fredericamycin C_2 (**1**) production, BAC 2P5 containing the identified type II PKS cluster was isolated from a genomic library of *S. albus* subsp. *chlorinus* NRRL B-24108 and transferred via intergeneric conjugation into the heterologous host *Streptomyces albus* Del14. HPLC-MS analysis of the extract from the resulting ex-conjugant *S. albus* 2P5 revealed the presence of a peak with identical retention time and *m/z* to those of fredericamycin C_2 (**1**), demonstrating that the type II PKS cluster from *S. albus* subsp *chlorinus* is responsible for fredericamycin C_2 (**1**) biosynthesis (Figure S3, Figure 2). BAC 2P5 comprises a 35 kb genomic region containing a total of 37 open reading frames (ORFs), 10 of which share homology at protein level with the fredericamycin A gene cluster from *S. griseus* (Table 2, Figure 4), which is also responsible for fredericamycin C (**2**) biosynthesis [22]. The homologue genes include those coding for the minimal PKS ketosynthase subunits (KS_α and KS_β), two polyketide cyclases, four tailoring enzymes, a transcriptional regulator, and a protein of unknown function. The gene similarity with fredericamycin A cluster, together with the heterologous expression results (Figure S3), indicates the relevance of these genes in fredericamycin C_2 (**1**) biosynthesis.

Table 2. Proposed functions of genes present in the type II polyketide synthase (PKS) cluster of *S. albus* subsp. *chlorinus* and homology with fredericamycin A gene cluster.

Gene	Size (aa)	Proposed Function	GenBank Homologue [1]	Identity/ Similarity (%)	Fredericamycin A Gene Cluster Homologue	Identity/ Similarity (%)
c2fdmA	406	Cytochrome P450 oxygenase	WP_017596471.1	66/73	-	-
c2fdmB	594	Monooxygenase	WP_017596470.1	67/75	-	-
c2fdmC	337	O-methyltransferase	WP_043504920.1	42/55	-	-
c2fdmD	620	Asparagine synthase	WP_017596467.1	79/88	-	-
c2fdmE	497	Monooxygenase	WP_081620749.1	59/70	-	-
c2fdmF	216	Polyketide cyclase	REH43750.1	43/54	-	-
c2fdmG	107	Monooxygenase	WP_027732672.1	44/64	-	-
c2fdmH	237	3-ketoacyl-ACP reductase	WP_017596481.1	72/83	-	-
c2fdmI	134	Polyketide cyclase	WP_109361109.1	72/83	-	-
c2fdmJ	389	O-methyltransferase	WP_061257536.1	67/79	*fdmN*	56/70
c2fdmK	138	Oxidoreductase	WP_020573863.1	56/68	-	-
c2fdmL	263	Unknown	WP_017596478.1	56/71	-	-
c2fdmM	138	Oxidoreductase	WP_017596477.1	70/85	-	-
c2fdmN	150	Unknown	WP_017596476.1	76/89	-	-
c2fdmO	358	O-methyltransferase	WP_043504920.1	40/54	-	-
c2fdmP	169	Unknown	WP_017596474.1	62/75	-	-
c2fdmQ	113	Monooxygenase	WP_017596454.1	65/76	-	-
c2fdmR	454	Biotin carboxylase	WP_017596455.1	80/87	-	-
c2fdmS	175	Biotin carboxyl carrier protein	WP_026120848.1	60/71	-	-
c2fdmT	585	Carboxyl transferase	WP_017596457.1	75/80	-	-
c2fdmU	111	Monooxygenase	WP_017596458.1	70/78	*fdmQ*	50/66
c2fdmV	113	Monooxygenase	WP_017596459.1	76/83	*fdmP*	51/70
c2fdmW	248	3-ketoacyl-ACP reductase	WP_017596460.1	83/92	*fdmO*	55/71
c2fdmX	153	Unknown	WP_015621174.1	61/74	-	-
c2fdmY	156	Polyketide cyclase	WP_075740187.1	67/82	*fdmI*	56/74
c2fdmZ	87	ACP	WP_017596463.1	46/71	-	-
c2fdmA1	409	KS_β	WP_017596464.1	77/86	*fdmG*	61/72
c2fdmB1	422	KS_α	WP_017596465.1	83/90	*fdmF*	63/77
c2fdmC1	112	Polyketide cyclase	WP_017596466.1	83/90	*fdmD*	64/76
c2fdmD1	254	Transcriptional regulator	WP_116247593.1	58/78	*fdmR1*	46/64
c2fdmE1	256	Transcriptional regulator	WP_081620746.1	67/80	-	-
c2fdmF1	144	Unknown	WP_017596472.1	78/89	*fdmE*	59/72
c2fdmG1	179	Transcriptional regulator	KPC87453.1	80/84	-	-
c2fdmH1	271	Serine hydrolase	WP_099880484.1	90/93	-	-
c2fdmI1	225	Unknown	WP_099880487.1	81/86	-	-
c2fdmJ1	394	Transcriptional regulator	WP_055497612.1	98/98	-	-
c2fdmK1	315	ACP S-malonyltransferase	WP_099880491.1	87/92	-	-

[1] NCBI accession numbers are given.

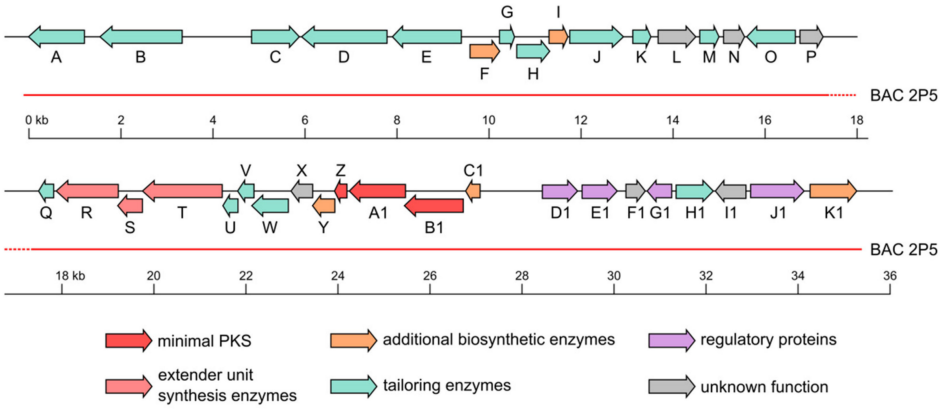

Figure 4. Map of the genes encoded in BAC 2P5 isolated from a genomic library of *S. albus* subsp. *chlorinus*. Characters from A to K1 indicate the corresponding *c2fdm* gene described in Table 2.

2.3. Screening for the Mutation Causing Fredericamycin C_2 Overproduction and Streptomycin Resistance in S. albus subsp. chlorinus JR1

Frequently, streptomycin resistance results from a point mutation in the *rpsL* gene, which codes for the ribosomal protein S12 [3,4,28–30]. Following genome sequencing of *S. albus* subsp. *chlorinus* JR1, the resulting reads were mapped to the genome of the wild type strain and single nucleotide mutations were searched in the sequence corresponding to the *rpsL* gene. No point mutations were found in this sequence, indicating that the mutation responsible for streptomycin resistance in *S. albus* subsp. *chlorinus* JR1 is located elsewhere in the genome. A total of fifteen point mutations were detected in the genome of *S. albus* subsp. *chlorinus* JR1 (Table S3). A nucleotide insertion was detected within the coding sequence of the *jag* gene (SACHL2_00217; position 3714884) that codes for a single-stranded DNA binding protein. This gene partially overlaps with the adjacent downstream gene *rsmG* (SACHL2_00216), indicating their co-transcription in a bicistronic operon. The insertion of a cytosine nucleotide in the sequence of *jag* creates a premature stop codon that truncates the protein translation, also affecting the expression of the co-translating *rsmG* gene. *rsmG* codes for a 16S rRNA methyltransferase, and it has been reported that point-nonsense mutations in its sequence, as well as the deletion of this gene, lead to increased resistance to streptomycin and enhanced production of secondary metabolites in different bacterial strains [31,32]. Therefore, we believe that a point mutation in the *jag* gene is responsible for high-level streptomycin resistance and upregulation of fredericamycin C_2 (**1**) biosynthetic gene expression in *S. albus* subsp. *chlorinus* JR1 by preventing the translation of the *rsmG* gene.

2.4. Biological Activity of Fredericamycin C_2

Fredericamycin C_2 (**1**) was tested for antibacterial activity against a Gram-positive (*Bacillus subtilis*) and two Gram-negative strains (*Escherichia coli* and *Pseudomonas putida*) through disk diffusion test. The new fredericamycin variant displays a growth inhibition zone against *P. putida* at a minimal concentration of 2.5 mg/mL (Figure S4). Fredericamycin C_2 (**1**) shows no inhibitory activity against the growth of *B. subtilis* and *E. coli*.

3. Discussion

Fredericamycin variants constitute a family of aromatic polyketides with significant toxicity against tumor cells as well as moderate antibiotic and antifungal activity. Here, we present the novel variant fredericamycin C_2 (**1**), which is overproduced by the strain *S. albus* subsp. *chlorinus* JR1, a spontaneous streptomycin-resistant mutant derived from *S. albus* subsp. *chlorinus* NRRL B-24108. We suggest that a mutation in the *jag* gene affecting the translation of the adjacent gene *rsmG* is responsible for the phenotype of the mutant strain. The frame shift originated by the point insertion

putatively generates a truncated non-functional Jag protein. In previous studies, deletion of a *jag* homologue in *Streptococcus pneumoniae* led to retarded growth and smaller cell size compared to the wild type strain, indicating that Jag is likely involved in cell division [33]. However, no association of *jag* deletion with increased antibiotic resistance or induction of secondary metabolite production has been reported before. The point mutation in the *jag* gene has a polar effect on the overlapping gene *rsmG*, preventing its transcription. The enhanced production of fredericamycin C_2 (**1**), as well as the increased streptomycin resistance observed in *S. albus* subsp. *chlorinus* JR1, is most likely derived from the lack of RsmG function. This is consistent with previous studies where *rsmG* deletion mutants showed higher resistance to streptomycin and improved yields of secondary metabolites at a late-growth phase [34,35]. *rsmG* encodes a methyltransferase that catalyzes the methylation of 16S rRNA at the residue G527 (*E. coli* numbering). This residue, together with C526 and the S12 protein, interacts with the antibiotic streptomycin [36]. These interactions tend to stabilize the tRNA-mRNA tandem, which affects the proof-reading process and results in misreading of the genetic code [37]. The absence of RsmG would generate 16S rRNA molecules non-methylated at residue G527, causing a weaker binding to streptomycin and making the strain resistant to the antibiotic. Although the mechanism by which the lack of 16S rRNA methyltransferase may induce the expression of fredericamycin C_2 (**1**) biosynthetic genes in *S. albus* subsp. *chlorinus* JR1 remains unknown, we hypothesize that the mutant experiences an increased protein synthesis rate at a stationary phase, as it has been previously observed [34,35]. The increased protein synthetic activity leads to expression of both pleiotropic and pathway-specific regulatory proteins, which eventually enhance the transcription of poorly expressed secondary metabolite gene clusters. Several attempts to complement the *jag* and *rsmG* gene functions in *S. albus* subsp. *chlorinus* JR1 resulted in no recombinant colonies, suggesting the genetic intractability of the strain (data not shown).

The structure of fredericamycin C_2 (**1**) presented in this paper only differs from that of fredericamycin C (**2**) in the polyketide chain length. While fredericamycin C (**2**) backbone consists of 30 carbon atoms, fredericamycin C_2 (**1**) contains a C_{26} polyketide chain (Figure 3). Fredericamycin C (**2**) biosynthesis begins with the generation of a C_6 primer unit (hexadienyl-ACP) by the PKS initiation module. This starter unit is then transferred to the elongation module, which presumably catalyzes the sequential decarboxylative condensation of 12 malonyl-CoA molecules, delivering a C_{30} polyketide chain [22,38]. In the case of fredericamycin C_2 (**1**), we propose that acetyl-CoA functions as the starter unit, which is elongated by the polyketide synthase through successive incorporation of malonyl-CoA extender units (Figure 5). This process is most likely catalyzed by the minimal PKS enzymes encoded by the genes *c2fdmB1*, *c2fdmA1*, and *c2fdmZ*, and the ACP S-malonyltransferase encoded by *c2fdmK1* (Table 2). The resulting C_{26} polyketide chain is subsequently modified by tailoring enzymes to eventually yield the product fredericamycin C_2 (**1**) (Figure 5).

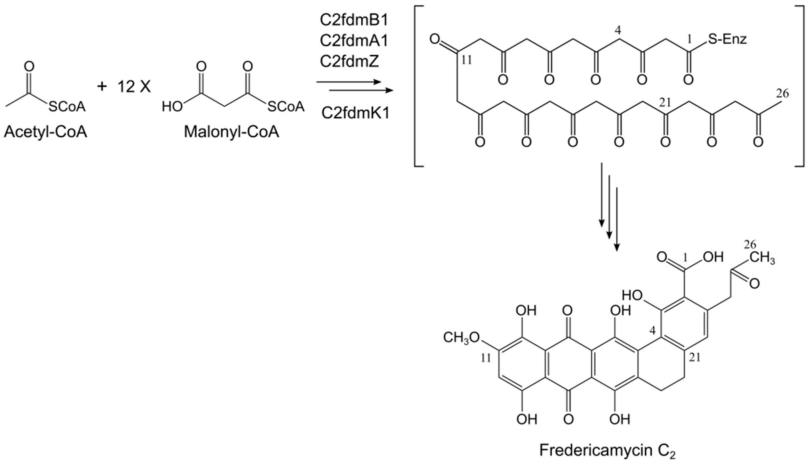

Figure 5. Proposed early biosynthesis steps of fredericamycin C_2 (**1**) in *S. albus* subsp. *chlorinus*.

4. Materials and Methods

4.1. General Experimental Procedures

All strains and BACs (bacterial artificial chromosomes) used in this work are listed in Table S4. *Escherichia coli* strains were cultured in LB medium [39]. *Streptomyces* strains were grown on soy flour mannitol agar (MS agar) [40] for sporulation and conjugation and in liquid tryptic soy broth (TSB; Sigma-Aldrich, St. Louis, MO, USA). For metabolite expression, liquid DNPM medium (40 g/L dextrin, 7.5 g/L soytone, 5 g/L baking yeast, and 21 g/L MOPS, pH 6.8) or MS agar were used. The antibiotics kanamycin, apramycin, and nalidixic acid were supplemented when required.

4.2. Isolation and Manipulation of DNA

BAC extraction from a *Streptomyces albus* subsp. *chlorinus*-constructed genomic library (Intact Genomics, St. Louis, MO, USA), DNA manipulation, *E. coli* transformation, and *E. coli*/*Streptomyces* intergeneric conjugation were performed according to standard protocols [39–41]. Plasmid DNA was purified with the BACMAX™ DNA purification kit (Lucigen, Middleton, WI, USA). Restriction endonucleases were used according to manufacturer's recommendations (New England Biolabs, Ipswich, MA, USA).

4.3. Strain Generation, Metabolite Extraction, and Analysis

The spontaneous streptomycin-resistant mutant *Streptomyces albus* subsp. *chlorinus* JR1 was obtained after several rounds of selection of streptomycin-resistant colonies growing on MS agar medium containing increasing concentrations of the antibiotic. MICs of streptomycin were determined by spreading spores on MS agar plates containing 50, 100, and 200 µg/mL of streptomycin. Metabolites were extracted from the agar with ethyl acetate acidified with 100% acetic acid up to pH = 2.0, evaporated and dissolved in methanol. One µL of extract was separated using a Dionex Ultimate 3000 UPLC (Thermo Fisher Scientific, Waltham, MA, USA), a 10-cm ACQUITY UPLC®BEH C18 column, 1.7 µm (Waters, Milford, MA, USA), and a linear gradient of 0.1% formic acid solution in acetonitrile against 0.1% formic acid solution in water from 5% to 95% in 18 min at a flow rate of 0.6 mL/min. Samples were analyzed using an Orbitrap speed mass spectrometer (Thermo Scientific, Waltham, MA, USA). Data were collected and analyzed with the Thermo Xcalibur software, version 3.0 (Thermo Scientific, Waltham, MA, USA). The monoisotopic mass was searched in a natural product database.

4.4. Fredericamycin C_2 Purification and Quantification

S. albus subsp. *chlorinus* JR1 was grown for 8 days at 28 °C on 50 Petri dishes, each containing 40 mL of DNPM agar. Fredericamycin C_2 (**1**) was extracted from the solid agar using ethyl acetate acidified with 100% acetic acid up to pH = 2.0. The crude extract was first separated by normal phase chromatography on a prepacked silica cartridge (Biotage, Uppsala, Sweden) using hexane (solvent A), chloroform (solvent B), ethyl acetate (solvent C), and methanol (solvent D) (1:1:1:1) as the mobile phase, in a linear gradient from 0% to 100% of each pair of solvents (A-B, B-C, and C-D). Fractions containing fredericamycin C_2 (**1**) were detected by LC-MS analysis, pooled together, and further fractionated by semi-preparative HPLC (Dionex UltiMate 3000, Thermo Fisher Scientific, Waltham, MA, USA) using a C18 column (Synergi 10 µm, 250 × 10 mm; Phenomenex, Aschaffenburg, Germany) and a 0.1% formic acid solution in acetonitrile as the mobile phase in a linear gradient. UV spectra were recorded with a DAD detector at 274 nm and 525 nm. Finally, 0.8 g of fredericamycin C_2 (**1**) was collected in a single fraction. For quantification, a calibration curve with different fredericamycin C_2 (**1**) concentrations was constructed (Figure S1).

Fredericamycin C_2 (**1**)*:* Violet, amorphous solid; *m/z* 521.1077 $[M + H]^+$ (calculated for $C_{27}H_{21}O_{11}$, 521.1084); UV λ_{max} (MeOH) 195, 248, 274, 345, 490 nm; ^1H and ^{13}C-NMR data, Table 1 and Supplementary Information Figures S2, S5–S10.

4.5. H-NMR Spectroscopy

NMR data were measured using a Varian VNMR-S600 spectrometer equipped with 3 mm triple resonance inverse and 3 mm dual broadband probes. Fredericamycin C_2 (**1**) samples were dissolved in 150 µL DMSO-d6/Pyridine-d5 95:5 and measured at 35 °C. The residual solvent signal of DMSO was used as an internal reference.

4.6. Antimicrobial Susceptibility Test

Disk diffusion tests were performed according to [42]. Ten mL of LB soft agar (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, 7 g/L agar) was inoculated with the strains *Escherichia coli* GB2005, *Bacillus subtilis* ATCC 6633, or *Pseudomonas putida* KT2440, and poured on LB agar plates. Five paper disks (Macherey and Nagel, Düren, Germany) were coated with 10, 5, 2.5, 0.5, or 0.25 mg/mL of fredericamycin C_2 (**1**) solved in methanol and placed onto the solidified soft agar. An additional disk loaded with methanol was used as a negative control and the antibiotics nalidixic acid, ampicillin, and chloramphenicol (50 µg/mL, respectively) were used as positive controls. The plates were incubated at 28 °C overnight.

4.7. Genome Sequencing, Genome Assembly, and Analysis

S. albus subsp. *chlorinus* JR1 was sequenced using an Illumina MiSEQ library with 301-bp inserts (Illumina, San Diego, CA, USA). *S. albus* subsp. *chlorinus* JR1 strain genome assembly has a total of 57 contigs, and 5 final scaffolds—7,539,766 bp; 63,833 bp; 4,962 bp; 2,694 bp; and 2,548 bp (assembled with Newbler version 2, Roche, Basel, Switzerland). Sequencing reads coverage against the *S. albus* subsp. *chlorinus* B-24108 genome (Genbank accession number VJOK00000000) was examined with Geneious, version 11.0.3 (Biomatters Ltd., Auckland, New Zealand).

4.8. Genome Mining and Bioinformatics Analysis

The genome of *S. albus* subsp. *chlorinus* was screened for secondary metabolite biosynthetic gene clusters using the antiSMASH [43] online tool (https://antismash.secondarymetabolites.org/#!/start). Analysis of genetic data was performed using the Geneious software, version 11.0.3 (Biomatters Ltd., Auckland, New Zealand) [44].

5. Conclusions

Here, we demonstrate the significance of inducing poorly expressed secondary metabolite gene clusters for the identification of new microbial natural products. A mutation in the genome of the strain *S. albus* subsp. *chlorinus* JR1 obtained by selection of streptomycin-resistant colonies has led to overproduction and the discovery of the so far undescribed compound fredericamycin C_2 (**1**). This secondary metabolite expands the structural variability of the fredericamycin family, whose most prominent member, fredericamycin A, is a potent antitumor drug. Screening for antibiotic-resistant strains represents a simple and inexpensive approach that has enabled the improvement of secondary metabolite production in the genetically intractable bacterial strain *S. albus* subsp. *chlorinus*.

Supplementary Materials: The following are available online at http://www.mdpi.com/1660-3397/18/6/284/s1; Table S1: Streptomycin MICs for *S. albus* subsp. *chlorinus* and *S. albus* subsp. *chlorinus* JR1; Table S2: Quantification of fredericamycin C_2; Table S3: Type and location of point mutations in the genome of *S. albus* subsp. *chlorinus* JR1; Table S4: Bacterial strains and BACs used in this work; Figure S1: Calibration curve for fredericamycin C_2 quantification; Figure S2: Important 2D NMR correlations in fredericamycin C_2; Figure S3: HPLC-MS chromatograms of crude extracts from *S. albus* subsp. *chlorinus* JR1, *S. albus* 2P5, and its parental strain *S. albus* Del14; Figure S4: Antibacterial evaluation of fredericamycin C_2. Figure S5: ^{1}H NMR; Figure S6: ^{13}C NMR; Figure S7: COSY NMR; Figure S8: HSQC NMR; Figure S9: HMBC NMR; Figure S10: ROESY NMR.

Author Contributions: M.R.E., M.M., and A.L. designed experiments; M.R.E., M.M., and B.R. performed experiments; T.P. performed and evaluated the NMR analysis; L.P. and J.R. developed and provided the mutant strain; M.R.E., M.M., and A.L. analyzed the data and wrote the manuscript; all authors reviewed the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Information

Novel Fredericamycin Variant Overproduced by a Streptomycin-Resistant *Streptomyces albus* subsp. *chlorinus* Strain

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Tables

Table S1. Streptomycin MICs for *S. albus* subsp. *chlorinus* and *S. albus* subsp. *chlorinus* JR1.

Strain	MIC (μg/ml)
Streptomyces albus subsp. *chlorinus* NRRL B-24108	< 50
Streptomyces albus subsp. *chlorinus* JR1	200

Table S2. Quantification of fredericamycin C_2 produced by *S. albus* subsp. *chlorinus* JR1 and the parental strain *S. albus* subsp. *chlorinus* NRRL B-24108.

Strain	Peak area (AU)[a]	Fredericamycin C_2 concentration (μM)[a b]
Streptomyces albus subsp. *chlorinus* JR1	571,003,904	773.25 (0.1)
Streptomyces albus subsp. *chlorinus* NRRL B-24108	10,157,194	14.08 (0.0)

[a]Values indicate the average of three independent measurements

[b]Standard deviation values are shown in parentheses

Table S3. Type and location of point mutations in the genome of *S. albus* subsp. *chlorinus* JR1.

Position (bp)	Strain	Sequence (5′ → 3′)	Type of mutation
30077	WT	30068-CGGGGGGGGGA-30079	G insertion
	mutant	30068-CGGGGGGGGG**G**A-30080	
636229	WT	363224-CCCTC**C**TCGA-636233	C deletion
	mutant	363224-CCCTCTCGA-636232	
685900	WT	685893-GGCGGGGCGG-685902	G insertion
	mutant	685893-GGCGGGG**G**CGG-685903	
1120949	WT	1120943-GGCCTG**G**TAC-1120952	G deletion
	mutant	1120943-GGCCTGTAC-1120951	
1922752	WT	1922746-CCCGCG**A**GCG-1922755	A deletion
	mutant	1922746-CCCGCGGCG-1922754	
2222897	WT	2222890-GCCCCCC**C**GG-2222899	C deletion
	mutant	2222890-GCCCCCCGG-2222898	
2548236	WT	2548230-CTCCGC**G**AGC-2548239	G deletion
	mutant	2548230-CTCCGCAGC-2548238	
3714884	WT	3714886-CGACCGTGCC-3714877	C insertion
	mutant	3714886-CGA**C**CCGTGCC-3714876	
5766153	WT	5766149-GAGT**G**CGCGC-5766158	G → C substitution
	mutant	5766149-GAGT**C**CGCGC-5766158	
5766154	WT	5766149-GAGTG**C**GCGC-5766158	C → G substitution
	mutant	5766149-GAGTC**G**GCGC-5766158	
5766161	WT	5766156-CGCCG**G**CCGG-5766165	G deletion
	mutant	5766156-CGCCGCCGG-5766164	
5790341	WT	5790331-CGGGGGGGGGGT-5790342	G insertion
	mutant	5790331-CGGGGGGGGGG**G**T-5790343	
6179245	WT	6179240-GGGCC**G**CACC-6179249	G → C substitution
	mutant	6179240-GGGCC**C**CACC-6179249	
6213269	WT	6213264-TCCGC**C**GCTG-6213273	C deletion

	mutant	6213264-TCCGCGCTG-6213272	
7015221	WT	7015216-CCTTCCACCC-7015225	C deletion
	mutant	7015216-CCTTCACCC-7015224	

Table S4. Bacterial strains and BACs used in this work.

Bacterial strain	Features	Reference/Source
Streptomyces albus subsp. *chlorinus* NRRL B-24108	Strain harboring fredericamycin biosynthetic gene cluster	[2]
Streptomyces albus subsp. *chlorinus* JR1	Streptomycin-resistant mutant strain overproducing fredericamycin C_2	This work
Streptomyces albus Del14	Heterologous expression host	[3]
Streptomyces albus 2P5	*S. albus* Del14 strain harboring BAC 2P5	This work
Escherichia coli ET12567 pUB307	Donor strain for intergeneric conjugation	[4]
Escherichia coli DH10β	General cloning strain	[5]
BACs		
pSMART	AmR; BAC vector	Lucigen (USA)
2P5	BAC containing fredericamycin gene cluster	Intact Genomics (USA)

Figures

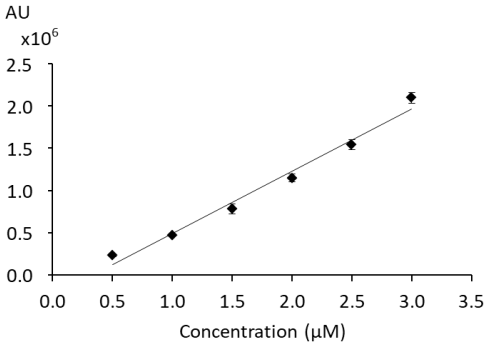

Figure S1. Calibration curve for fredericamycin C_2 quantification. Peak area is represented against fredericamycin C_2 concentration (μM). The values represent the average of three independent measurements. The black line shows the trendline that leads to the equation: $\boldsymbol{y = 7.38 * 10^5 x - 2.49 * 10^5}$.

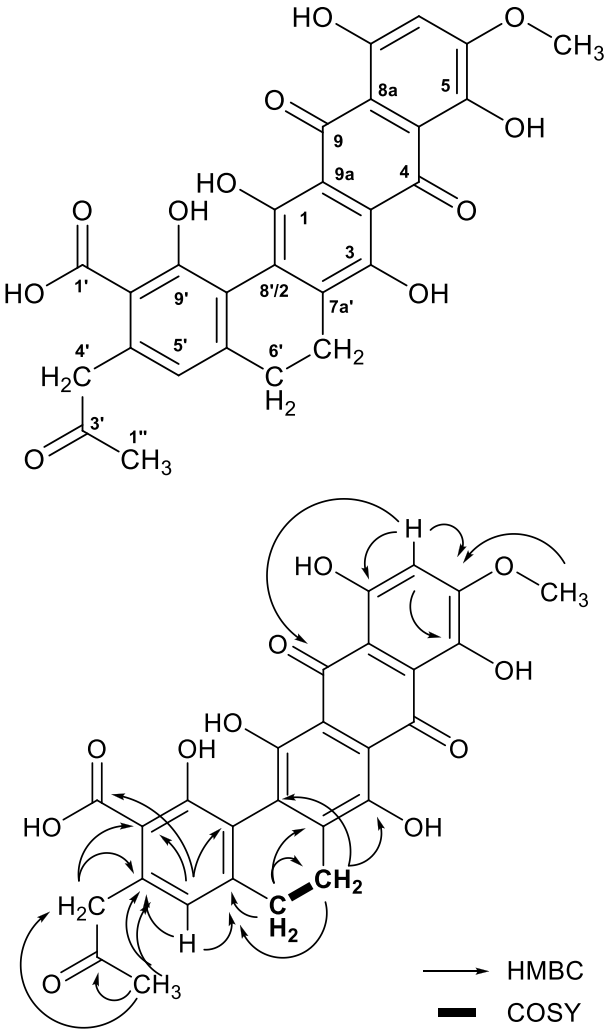

Figure S2. Important 2D NMR correlations in fredericamycin C2.

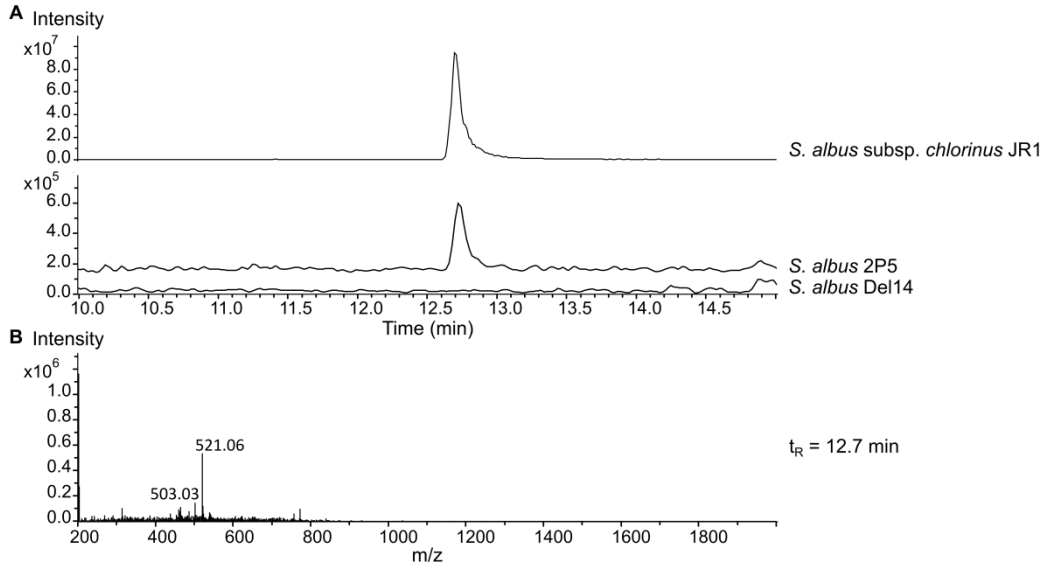

Figure S3. HPLC-MS chromatograms of crude extracts from *S. albus* subsp. *chlorinus* JR1, *S. albus* 2P5, and its parental strain *S. albus* Del14. (**A**) Extracted ion chromatograms (521.12 ± 0.1 Da). (**B**) Mass spectrum corresponding to t_R = 12.7 min from *S. albus* 2P5 chromatogram displayed in A.

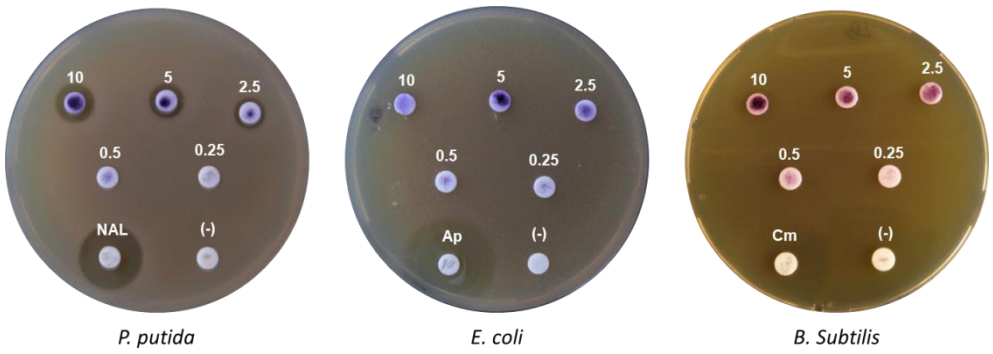

Figure S4. Antibacterial evaluation of fredericamycin C_2. Disk diffusion tests against *Pseudomonas putida*, *Escherichia coli*, and *Bacillus subtilis* are shown. Fredericamycin C_2 solved in methanol was loaded onto paper disks at concentrations of 10 mg/ml, 5, 2.5, 0.5, and 0.25 mg/ml. NAL: nalidixic acid; Ap: ampicillin; Cm: chloramphenicol; (-): negative control (methanol).

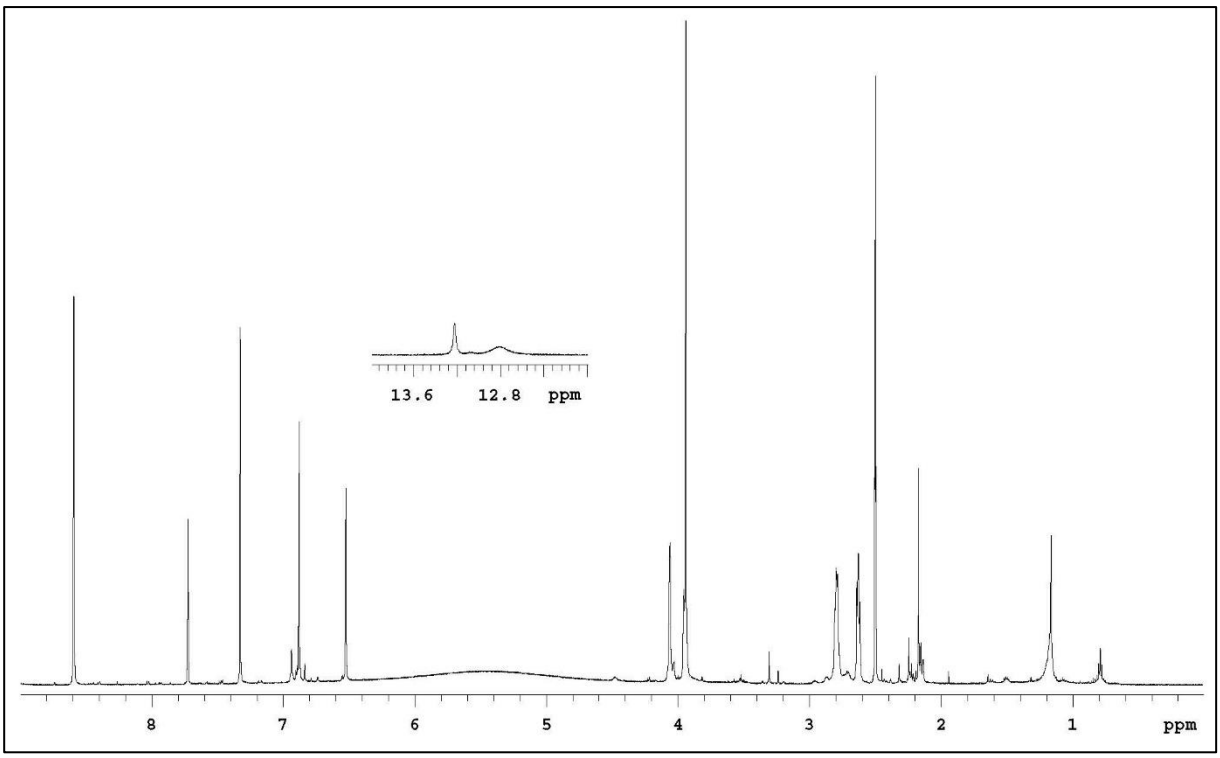

Figure S5. ^{1}H NMR (600 MHz, DMSO-d6/Pyridine-d5 95:5, 35 °C).

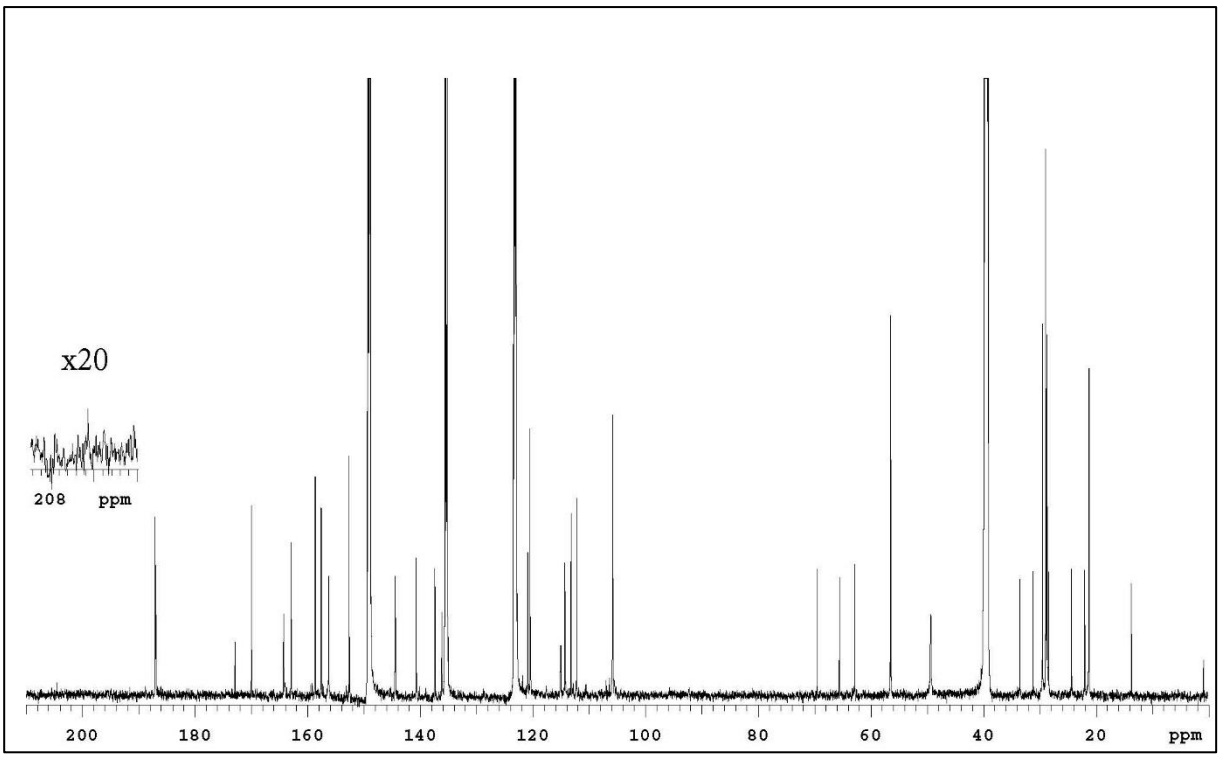

Figure S6. ^{13}C NMR (150 MHz, DMSO-d6/Pyridine-d5 95:5, 35 °C).

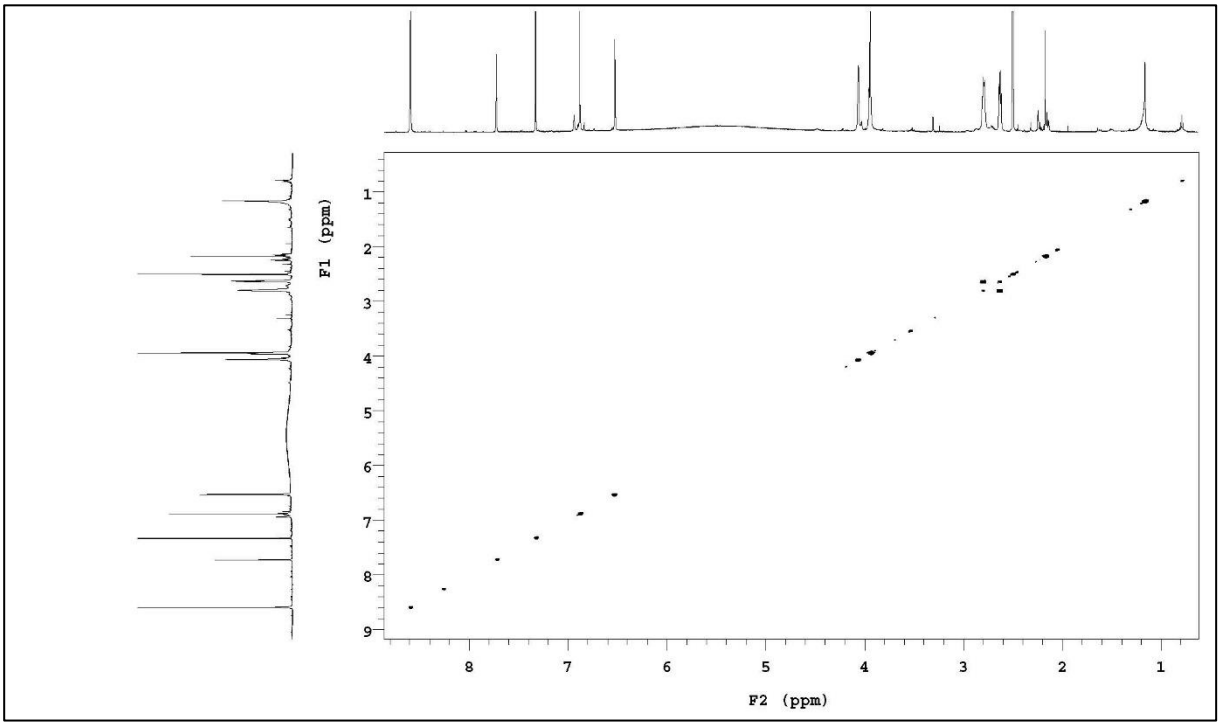

Figure S7. COSY NMR (600 MHz, DMSO-d6/Pyridine-d5 95:5, 35 °C).

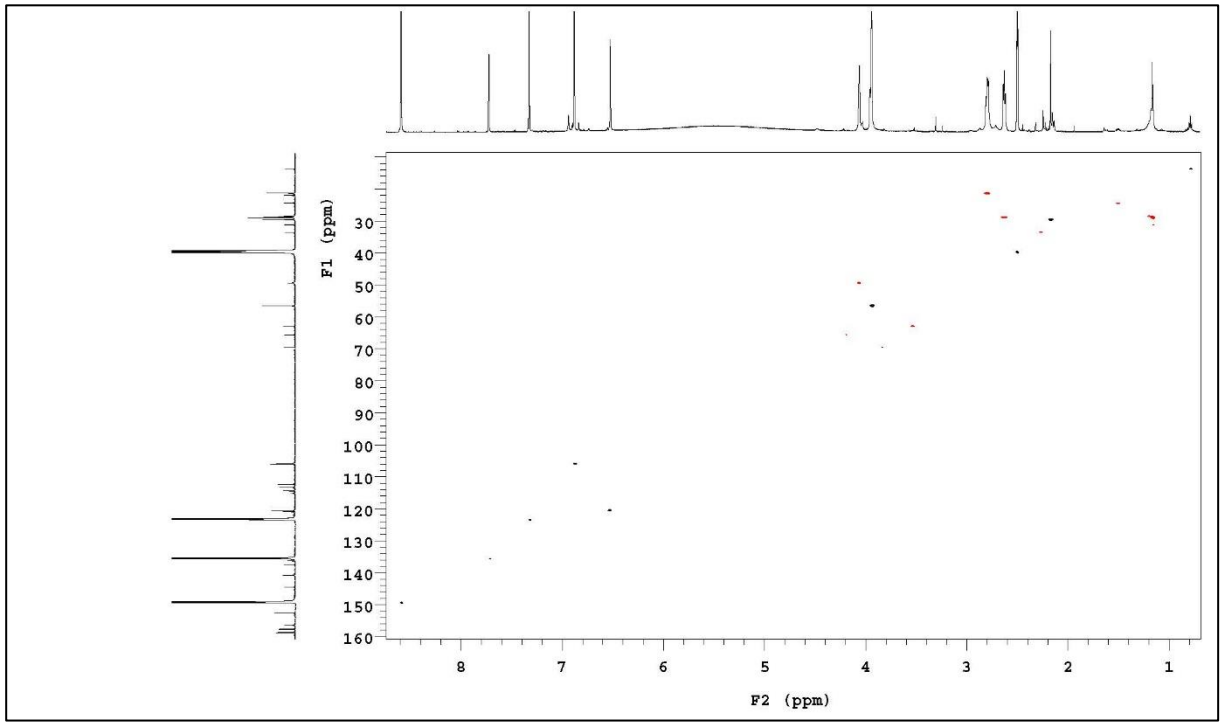

Figure S8. HSQC NMR (600 MHz, DMSO-d6/Pyridine-d5 95:5, 35 °C).

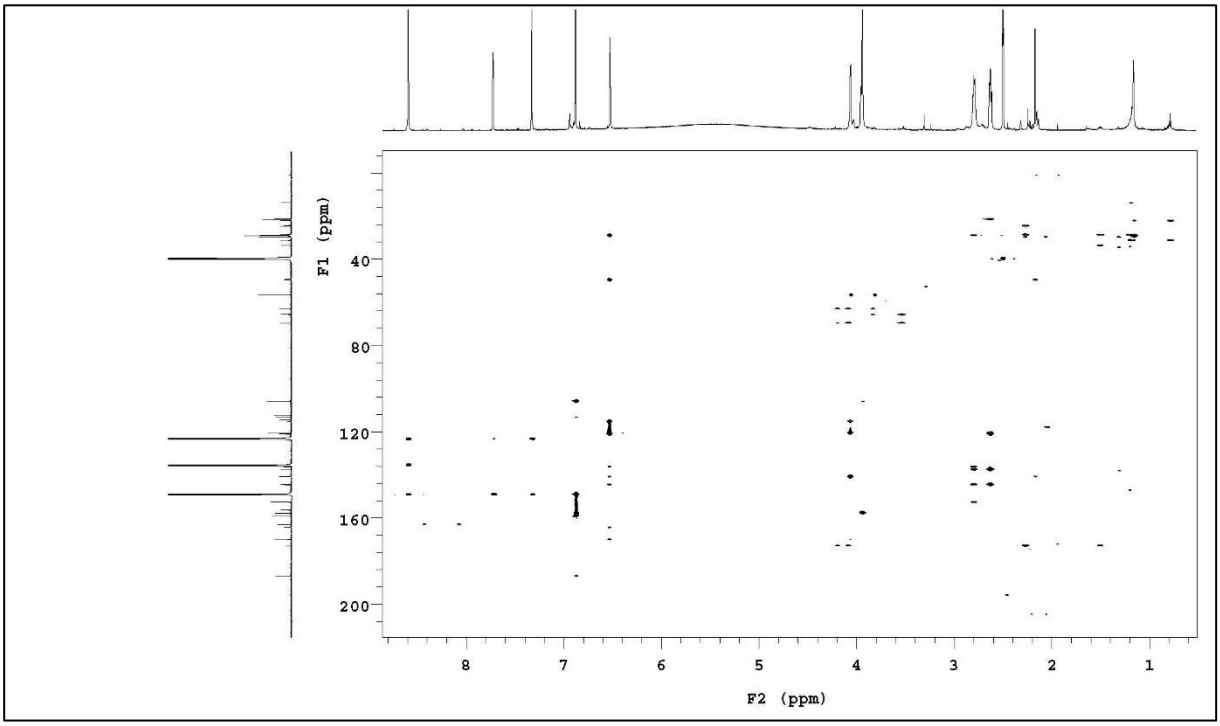

Figure S9. HMBC NMR (600 MHz, DMSO-d6/Pyridine-d5 95:5, 35 °C).

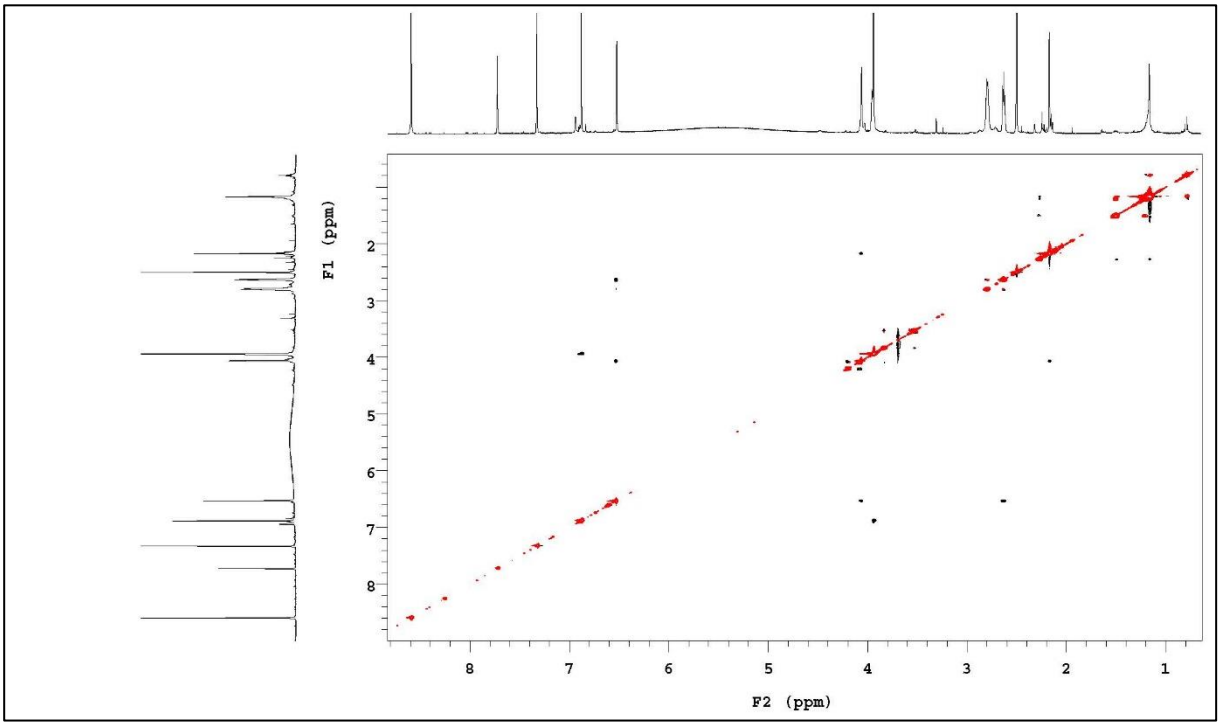

Figure S10. ROESY NMR (600 MHz, DMSO-d6/Pyridine-d5 95:5, 35 °C).

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Conclusions

Considering the significant amount of approved drugs based on NPs, it is evident that these compounds are still a valuable source for the development of new drug leads. The application of bioinformatics and genome sequencing techniques in bacterial genomes, especially Actinobacteria, has revealed the presence of a vast number of NP gene clusters. However, these clusters are very often poorly expressed or inactive under standard laboratory cultivation conditions, hindering the detection of their corresponding products. In this work, we have applied ribosome engineering, i.e., selection of antibiotic-resistant mutants, and heterologous expression of secondary metabolite BGCs to induce the expression of silent gene clusters.

The generation of a high-level streptomycin-resistant mutant derived from *S. albus* subsp. *chlorinus* led to *in situ* activation of a gene cluster, resulting in overproduction of the new compound fredericamycin C_2. It has been previously reported that certain spontaneous mutations in the ribosome are responsible for high resistance to antibiotics and increased protein synthetic activity during the stationary phase. The enhanced protein synthesis can trigger the translation of several global regulatory proteins, which subsequently induce the transcription of secondary metabolite gene clusters. Ribosome engineering is a simple and cost-effective approach to accelerate the discovery of NPs. Nevertheless, one of its drawbacks is the unpredictable effect of the ribosomal mutations on the metabolic profile of the mutant strain, since the activation of gene expression is not targeted towards any specific gene cluster.

In contrast, heterologous expression allows the activation of selected clusters, and the new products synthesized by the host strain can simply be associated to the transferred introduced biosynthetic genes. In an attempt to activate the expression of secondary metabolite BGCs, 17 putative gene clusters encoding different classes of NPs, including polyketides, non-ribosomal peptides, lanthipeptides, and phenazines, were isolated from the genomic library of *S. albus* subsp. *chlorinus* and introduced into the heterologous hosts *S. albus* Del14 and *S. lividans* ΔYA9. As a result, seven clusters (40%) were successfully expressed either in *S. albus* Del14 or in *S. lividans* ΔYA9. This approach led to detection of nybomycin in the extract of one of the clones, enabling

the characterization of its biosynthetic genes, which remained unknown so far. Thus, heterologous expression clearly represents a versatile cluster-oriented strategy that efficiently unravels the natural structures encoded in bacterial cryptic gene clusters.

Due to the varied nature of NPs, their successful biosynthesis is influenced by many factors, including carbon and nitrogen sources, availability of precursors, and expression of their biosynthetic genes, among others. For this reason, the activation of silent BGCs is not always achieved either by heterologous expression or by ribosome engineering. To overcome these limitations, alternative strategies could be applied. For instance, it would be convenient to use several hosts for heterologous expression of gene clusters. In the last decade, a panel of diverse heterologous hosts has been developed. They harbour different genetic and metabolic backgrounds that might provide an optimal environment for the activation of certain BGCs, such as cluster-free genomes and absence of competitive metabolic routes, diverse precursors, overexpressed global transcription regulators, or absence of transcription repressors, among others. Alternatively, the expression of gene clusters can be induced by refactoring strategies, which consist in engineering of transcriptional regulatory elements of the biosynthetic genes such as promoters, terminators, ribosome binding sites, etc. This approach enables the biosynthetic pathways to be uncoupled from their native regulation systems.

In conclusion, the diverse strategies available to induce the expression of cryptic secondary metabolite BGCs have successfully untapped novel NPs produced by *S. albus* subsp. *chlorinus*. Given the structural complexity of NPs of streptomycetes, the development of a universal approach that allows the activation of any biosynthetic pathway seems very unlikely. Therefore, the establishment of different methods remains a crucial starting point to achieve the biosynthesis of the broad array of NPs encoded in the genomes of streptomycetes.